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# Size effects of chitooligomers with certain degrees of polymerization on the chilling tolerance of wheat seedlings

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ABSTRACT

In this study, six fully deacetylated single chitooligomers (COSs, chitobiose to chitoheptaose) and one fully deacetylated COS mixture were applied to wheat seedlings to investigate their effects on the plants' defence response under chilling stress. The results showed that exogenous COS with different degrees of polymerization (DPs) could promote the growth of plants, decrease the concentration of malondialdehyde (MDA), increase the content of chlorophyll and modulate the activities of antioxidant enzymes. The analysis of genes expression suggested that COS could alleviate the damage of chilling stress by effectively regulating the expression of antioxidant enzyme genes. Furthermore, chitohexaose and chitoheptaose had the most effective activities of alleviating chilling stress to wheat seedlings, which suggested the DP played important roles in the activities of COS. The close relationship between the activities of COSs and their DPs reveals that the activities of COSs are dependent upon a specific structure to interact with elicitor receptors on plant cell membrane.

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## 1. Introduction

Chilling is one of the severe environmental stresses that limits the geographical distribution and productivity of crops. Chilling stress disrupts the metabolic balance of cells resulting in excessive production of reactive oxygen species (ROS), including superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^-$), and hydrogen peroxide ($H_2O_2$) (Imlay, 2003). Fortunately, plants have evolved very complex and efficient antioxidant systems comprised of antioxidant enzymes and compounds to protect cellular membranes and organelles from the harmful effects of ROS (Li, Du, Yang, Feng, Li, & Chen, 2005). Enhancement of the antioxidant enzyme activities and regulation of the antioxidant compounds are helpful to alleviate the negative effects of exposure to various stresses.

Chitosan are a wide group of molecules deriving from treatment of shellfish and crustaceous industry's wastes. COSs are homo- or heterooligomers of *N*-acetylglucosamine and D-glucosamine, and can be produced from either chitin or chitosan. COS has been reported to possess diverse biological activities such as antioxidative activity (Li, Liu, Xing, Qin, & Li, 2013), antibacterial activity (Li, Liu, Shan et al., 2013) and antiviral activity (Kulikov, Chirkov, Il'ina, Lopatin, & Varlamov, 2006). It also has been recognized as a product to improve plant growth and enhance the tolerance to abiotic stress of plants. Previous studies showed that COS could increase the chlorophyll content and enhance the photosynthesis of the *Dendrobium orchid* (Limpanavech et al., 2008), promote mineral nutrient uptake of *Phaseolus vulgaris* (Chatelain, Pintado, & Vasconcelos, 2014). Therefore, COS has broad application prospects and has attracted increasing interest in the field of agriculture.

The bioactivities of COS depend closely on its structure and physicochemical properties. The DP (also referred to as molecular weight (Mw)), degree of deacetylation (DD), charge distribution and the oligomer structure pattern has important influence on its bioactivities (Cabrera & Van Cutsem, 2005; Davydova et al., 2011). In particular, the DP of COS is a very important factor in the study of structure–function relationship of COS. When compared with chitosan, COS has lower molecular weight and higher water solubility. It was reported to exhibit better antibacterial (Li, Liu, Shan et al., 2013) and antiviral (Kulikov et al., 2006) activity and better ability to promote plant growth (Khan, Prithiviraj, & Smith, 2002). Li et al. further demonstrated that the antibacterial activity of COS required

*Abbreviations:* APX, ascorbate peroxidase; CAT, catalase; COS, chitooligomer; DD, degree of deacetylation; DP, degree of polymerization; FW, fresh weight; $H_2O_2$, hydrogen peroxide; MDA, malondialdehyde; $O_2^{\bullet -}$, superoxide radical; $OH^-$, hydroxyl radical; POD, peroxide enzyme; PSII, photosystem II; qRT-PCR, quantitative real time reverse transcriptase-PCR; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

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structural essential with a DP of at least 5 and the inhibitory effect increased with increasing DP (Li et al., 2014).

Though many application experiments were conducted, the mechanisms of COS on plant immunity regulation remain to be elucidated. The main reason is that COS mixtures were used in experiments in most of these reports. Therefore, this work was conducted to use COSs with certain DP including six single COSs (chitobiose to chitooctaose) to investigate the size effects of COS on the chilling tolerance of plants. Our preliminary studies using various Mw of COSs (1300, 3300,5300, 9300 Da) showed that 1300 Da COS was close to optimal for enhancing salt tolerance of wheat seedlings. Thus, a fully deacetylated COS mixture (1300 Da) was also chose as an experimental group. Furthermore, we evaluated the expression of a series of antioxidant enzyme genes in wheat seedlings by quantitative RT-PCR to explore the physiological mechanisms of COS on plants' tolerance to chilling stress.

## 2. Materials and methods

### 2.1. Materials

Six fully deacetylated single COSs, chitobiose (≥95%), chitotriose (≥95%), chitotetraose (≥95%), chitopentaose (≥95%), chitohexaose (≥95%), chitoheptaose (≥95%) were purchased from Zhaoqing Dragon Biological Technology co. (China, Guangdong). The fully deacetylated COS fragment was prepared by the degradation of highly deacetylated chitosan as reported by Li, Xing, Liu, Qin et al. (2012). All other chemicals and reagents were of analytical grade.

### 2.2. Plant treatments

The following experiments were conducted with wheat (*Triticum aestivum* L. Jimai 22) seeds, which were surface sterilized with a 1% sodium hypochlorite solution for 10 min and thoroughly washed with distilled water. Seeds were soaked in distilled water for 6 h and then transferred to a petri dish with moist gauze for germination at 25 °C for 24 h in the dark. Germinated seeds were showed in petri dishes with nylon mesh and were grown in Hoagland solution in a growth incubator at a day/night cycle of 14 h/10 h, at 25 °C/20 °C, respectively, with a relative humidity of 65% and a light intensity of 800 μmol $m^{-2}$ $s^{-1}$. When the second leaf was fully expanded, the wheat seedlings were randomly divided into nine groups. There were 30 plants in every petri dishes and each group contained three petri dishes. The wheat seedlings were sprayed with 0.01% COS solution with 0.1% Tween-20, which served as the COS plus chilling-stressed groups. The other wheat seedlings were sprayed with distilled water along with 0.1% Tween-20, which served as the control and chilling-stressed groups. The volume of distilled water or COS solutions sprayed on each group was 15 ml. The wheat seedlings were placed in normal growth condition for 24 h and then were exposed to chilling stress. Chilling stress was performed at a temperature of 4 °C for 48 h. During the 48 h, every group was sprayed with COS solution or distilled water once every 24 h. The chilling-stressed treatment followed by a recovery growth period under the normal condition for another 48 h. The nutrient solution was renewed every other day.

### 2.3. Growth parameters

The growth of wheat seedlings were evaluated by shoot length, root length, shoot fresh weight and dry weight. After chilling stress treatment and recovery period, wheat seedlings of each group were harvested for determination of growth parameters, after which samples were dried at 105 °C for 2 h to obtain dry weight.

### 2.4. Measurement of MDA content

The level of lipid peroxidation in plants was determined based on MDA content. MDA, a product of lipid peroxidation, was determined using a thiobarbituric acid (TBA) reaction (Seckin, Sekmen, & Türkan, 2008). Leaf samples (0.5 g) were homogenized in 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 4000*g* for 10 min. The supernatant was used for estimating the MDA content. Then, 2 ml of 0.6% TBA was added to 2 ml supernatant, and the mixture was heated at 100 °C for 15 min and cooled in an ice bath immediately afterwards. Next, the mixture was centrifuged at 10,000*g* for 15 min. The absorbance of the supernatant was recorded at 450 nm, 532 nm and 600 nm, separately. The MDA content was expressed as μg MDA $g^{-1}$ fresh weight (FW).

### 2.5. Determination of soluble sugar content

Soluble sugar was measured by the following procedure (Zou et al., 2016): 0.5 g of leaf samples were cut up and heated at 100 °C for 30 min in 5 ml distilled water. The extract was diluted 5-fold for determination. 500 μl diluents, 1 ml 5% phenol and 5 ml sulfuric acid were mixed and after standing for 5 min, the absorbance was read at 485 nm. Soluble sugar concentration was quantified by comparison with a standard curve using the criterion of glucose.

### 2.6. Determination of proline content

Leaf samples (0.2 g) were ground in liquid nitrogen and homogenized in 5 ml of 3% sulfosalicylic acid (Huang, Wang, Sun, & Wei, 2015). After heated at 100 °C for 10 min, the homogenate was cooled to room temperature and centrifuged at 5000*g* for 4 min. Then the resulting supernatant was used to measure the proline content at 520 nm.

### 2.7. Determination of chlorophyll content

Chlorophyll was extracted with 95% ethanol, and the total chlorophyll (a + b) content was determined with spectrophotometer at 665 nm and 649 nm. All the processes, from extraction to spectral measurement, were performed in dim light to avoid degradation of the chlorophyll in the samples (Pongprayoon, Roytrakul, Pichayangkura, & Chadchawan, 2013).

### 2.8. Determination of antioxidant enzyme activities

After 24 h and 48 h of chilling stress treatment and 48 h of recovery, enzymes were extracted from the second fully expanded leaves (0.5 g). The samples were homogenized in liquid nitrogen and brought up to a volume of 5 ml by cold sodium phosphate buffer solution (pH 7.8). The homogenates were centrifuged at 12000*g* at 4 °C for 15 min, after which the supernatants were immediately used for determination of enzyme activities.

The total soluble protein was determined using the Bradford method (Mishra, Bhoomika, & Dubey, 2013). A total of 100 μl of supernatant and 5 ml of Coomassie brilliant blue G250 staining were mixed, and the absorbance was read at 595 nm. Protein concentration was quantified by comparison with a standard curve using bovine serum albumin.

Superoxide dismutase (SOD) activity was assayed by the inhibition of the photochemical reduction of β-nitro blue tetrazolium chloride (NBT) (Rasool, Ahmad, Siddiqi, & Ahmad, 2012). One unit of SOD was defined as the amount of enzyme needed to produce a 50% inhibition of NBT reduction at 560 nm. The activity of catalase (CAT) was calculated based on the rate of disappearance of $H_2O_2$, which was measured as a decline in the absorbance at 240 nm. The CAT activity was expressed as $H_2O_2$ reduced $min^{-1}$ $mg^{-1}$ protein (Nasir

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