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Oligochitosan induced *Brassica napus* L. production of NO and H_2O_2 and their physiological function

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ABSTRACT

NO (nitric oxide) and H_2O_2 (hydrogen peroxide) are important signaling molecule in plants. *Brassica napus* L. was used to understand oligochitosan inducing production of NO (nitric oxide) and H_2O_2 (hydrogen peroxide) and their physiological function. The result showed that the production of NO and H_2O_2 in epidermal cells of *B. napus* L. was induced with oligochitosan by fluorescence microscope. And it was proved that there was an interaction between NO and H_2O_2 with *L*-NAME (N^G-nitro-L-arg-methyl eater), which is an inhibitor of NOS (NO synthase) in mammalian cells that also inhibits plant NO synthesis, and CAT (catalase), which is an important H_2O_2 scavenger, respectively. It was found that NO and H_2O_2 induced by oligochitosan took part in inducing reduction in stomatal aperture and *LEA* protein gene expression of leaves of *B. napus* L. All these results showed that oligochitosan have potential activities of improving resistance to water stress.

1. Introduction

Oligosaccharide as an elictor, which can induce plant gene expression (Yin, Li, Zhao, Du, & Ma, 2006) and production of NO (nitric oxide) and H_2O_2 (hydrogen peroxide) (Zhao, She, Du, & Liang, 2007a,b). An oilseed rape (*Brassica napus* L.) cDNA microarray containing 8095 expressed sequence tags (ESTs) was used to analyze the *B. napus* L. gene expression changes elicited by oligochitosan (COS). Transcript levels for 393 genes were altered twofold or more in oligochitosan-treated seedlings compared to the control. These 393 genes were involved in different processes and had different functions including defense, primary metabolism, transcription, and signal transduction, etc. (Yin et al., 2006). The ccd-1 (CCD, C-terminal centrin like Domain) mRNA expression was rapidly induced by treatment with chitosan oligosaccharide elicitors (Takezawa, 2000). In rice, CIGR1 and CIGR2 (chitin-inducible gibberellin-responsive) were inducible by the potent elicitor *N*-acetylchitooligosaccharide (Day et al., 2004). EL5, a rice gene responsive to *N*-acetylchitooligosaccharide elicitor, encoded a RING-H2 finger protein with structural features common to the plant-specific ATL family (Salinas-Mondragón, Garcidueñas-Piña, & Guzmán, 1999; Takai, Hasegawa, Kaku, Shibuya, & Minami, 2001). Apart from these, oligochitosan induced increase in net photosynthesis of *B. napus* L. under drought stress (Li et al., 2008). But the mechanism regarding how oligochitosan induced the defense reaction in plant is still unclear.

NO is a bioactive signaling molecule first described in mammals (Schmidt & Walter 1994). After 4 years, it was found that NO as a signaling molecule took part in plants growth, development, respiratory metabolism, senescence and maturation as well as plant response to abiotic and biotic stressors (D'Silva et al., 1998; Dumer & Klessig, 1999; Garcia-Mata & Lamattina, 2001; Hatsugai et al., 2004; Hung & Kao, 2003; Lamattina, Garcia-Matta, Graziano, & Pagnussat, 2003; Leshem, Wills, & Ku, 1998; Pedroso, Magalhaes, & Durzan, 2000; Prado, Porterfield, & Feijó, 2004; Rojo et al., 2004; Zhao et al., 2007a,b; Zottini et al., 2002). Stomatal closure of *Vicia faba* was induced by NO (Garcia-Mata & Lamattina, 2007). And NO and H_2O_2 interaction was proved (Bright, Desikan, Hancock, Weir, & Neill, 2006; She, Song, & He, 2004). Under abiotic stresses, H_2O_2 acted as a signaling molecule in photo adaption, and ABA inducing stomatal closure (Neill, Desikan, Clarke, Hurst, & Hancock, 2002). H_2O_2 took part in ABA inducing accumulation NH_4^+, which is an important medium in nitrogen metabolism (Miflin & Lea, 1976; Hung & Kao, 2005). NO and H_2O_2 adjusted stoma movement separately or interactionally (She et al., 2004). NO and H_2O_2 as a signaling molecule regulated plants growth and development (Arasimowicz & Floryszak-Wieczorek, 2007; She et al., 2004), which implicits that NO and H_2O_2 can induce genes expression of plants. Late embryogenesis abundant (LEA) proteins accumulate during the late stages of seed development and associate with the acquisition of desiccation tolerance. LEA proteins are also found in vegetative tissues in response to exogenous abscisic acid (ABA) as well as dehydration.

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Under serious drought stress, group 3 *LEA* genes accumulation was related nearly to wheat resistance to drought. Moreover, dehydration resulted in increase in group 3 *LEA* mRNA level (Bray, 1993). Under soil water deficit conditions, transgenic wheat that expressed the group 3 *LEA*, barley *HVA1* gene had significantly greater mass and weight than the control, which further supports its role in the dehydration response. Transgenic Chinese cabbage (*B. campestris* ssp. pekinensis) expressing a *B. napus* group 3 *LEA* gene had been generated. Transgenic Chinese cabbage plants were demonstrated enhanced growth ability under drought stress conditions (Park, Liu, Kanno, & Kameya 2005). Examination above has proved that group 3 *LEA* genes play key roles in dehydration tolerance.

Our experiments based on fluorescent probe DAF-2DA and H_2DCF-DA showed that the level of endogenous NO and H_2O_2 in epidermal cells of *B. napus* L. treated with oligochitosan and their physiological function.

2. Materials and methods

2.1. Materials

Seeds of *B. napus* L. (Huyou 15, cv.) were from our lab. The seeds were sown in plastic containers filled with 100% soil without any fertilization, grown in the glasshouse and used at the 4- to 8-leaf stage after about 2 months in culture. The daily air temperature was 22–29 °C and the daily relative humidity ranged between 63% and 81% with 12 h light and 12 h dark cycle. Oligochitosan was prepared according to Zhao et al. (2007a,b).

2.2. Measurement of endogenous NO and H_2O_2 by fluorescence microscope

NO and H_2O_2 measurement was performed by using their fluorescent indicator dye 4,5-diaminofluorescein diacetate (DAF-2DA) and 2′,7′-dichloroluorescindiacetate (H_2DC-DA) as described previously (She et al.,2004; Zhao et al., 2007a,b) with slight modifications. The epidermis was peeled carefully from leaves and cut into 5 mm length. Epidermal strips were incubated in MS/KCl buffer including various compounds for 3 h in light. After this step, the strips were placed into Tris/KCl buffer (Tris 10 mmol/L and KCl 50 mmol/L, pH 7.20 containing DAF-2DA at a final concentration of 10 μmol/L for 30 min, or H_2DCF-DA at 50 μmol/L for 30 min at 24–26 °C. After washed of excess dye with fresh Tris/KCl buffer, the epidermal strips were placed in Tris/KCl buffer containing oligochitosan (50 mg/L, the same below) or oligochitosan and inhibitors. Examination of peels was performed using fluorescence microscopy until the following settings: excitation 428–485 nm, eyeable fluorescence origination spectrum 515 nm. Images acquired from the fluorescence microscope were analyzed by us. The experiments were repeated at least three times in each treatment, and obtained the same results.

2.3. Stomatal bioassays

Seedlings leaves of *B. napus* L. were sprayed with dH_2O (CK), oligochitosan of 50 mg/L, H_2O_2 of 100 mM, CAT (catalase) of 100 Um/L that contained oligochitosan of 50 mg/L, and Vc of 0.1 mM that contained oligochitosan of 50 mg/L, SNP (sodium nitroprusside, a donor of NO) of 1 mmol/L, *L*-NAME (N^G-nitro-L-arg-methyl eater) of 1 mmol/L that contained oligochitosan of 50 mg/L. After 30 min, stomatal apertures were determined by the way of fixation under a light microscope with a calibrated micrometer scale (Phsiology, 1987). Data were presented as the mean of three independent experiments. All analyses were carried out with Excel and SPSS.

Table 1
Primers used in RT-PCR

Primers	Sequences	References
BnActin-1	5′-GTGACAATGGAACTGGAATGG-3′	Yin et al., 2006
BnActin-2	5′-ACGGAGGATAGCGTGAGGAA-3′	Yin et al., 2006
BnLEA -1	5′-GGCAAGGACAAGACTTCCCA-3′	Park et al., 2005
BnLEA-2	5′-CGGATCAGTGCTCTGAGTAG-3′	Park et al., 2005

2.4. RNA isolation and RT-PCR

Total RNA was isolated from the frozen leaves using TRIZOL Reagent according to the manufacturer's protocol with slight modifications, and the RNA quality was validated using electrophoresis and spectrophotometer measurements. RNA isolated from oligochitosan treated (for 0, 15, 30 min, 1, 4, 8, 24, 48 and 168 h) *B. napus* L. seedlings was reverse transcribed to first strand cDNAs using oligo (dT) primer in a total volume of 10 μL according to the supplier's instruction (TaKaRa RNA PCR kit VER 3.0). Resulting cDNAs were then used as templates for PCR amplification of *LEA* gene (GenBank Accession No. AB083362) in a volume of 20 μL as follows: 94 °C for 5 min; then 30 cycles at 96 °C for 45 s, 55 °C for 45 s, 72 °C for 1.5 min; and finally with an extension at 72 °C for 10 min, preserved at 4 °C. The *B. napus actin* gene (GenBank Accession No. AF111812) was used as positive internal control in a volume of 20 μL as follows: 94 °C, 5 min; then 30 cycles at 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min; 72 °C, 10 min; preserved at 4 °C (Yin et al., 2006). Primers used for the selected clones in RT-PCR are listed in Table 1 (Park et al., 2005; Yin et al., 2006). Amplified PCR products (6 μL) were electrophoresed on a 2% (w/v) agarosegel and monitored using the FR-980 Bio-Electrophoresis Image Analysis System.

3. Result

3.1. Measurement of production of NO and H_2O_2 in epidermal cells of *B. napus* L. by fluorescence microscopy

To study the effects of oligochitosan on the production of NO and H_2O_2 in *B. napus* L. epidermal cells, the NO-sensitive fluorescent indicator DAF-2DA and H_2O_2-sensitive fluorescent indicator H_2DCF-DA were used. It was found that oligochitosan could enhance the level of intracellular DAF-2DA fluorescence in epidermal cells of *B. napus* L. leaves, indicating massive production of NO (Fig. 1b). However, the DAF-2DA fluorescence indicating production of NO was faint in the epidermal cells only loaded with DAF-2DA (Fig. 1a). The results also indicated that *L*-NAME could inhibit the level of DAF-2DA fluorescence in the epidermal cells of *B. napus* L. leaves treated with oligochitosan (Fig. 1c).

The results also showed that oligochitosan caused an increase of intracellular H_2DCF-DA fluorescence in epidermal cells of leaves of *B. napus* L., indicating the production of H_2O_2. Fluorescence became visible in the epidermal cells of *B. napus* L. leaves treated with oligochitosan (Fig. 1e), but the fluorescence was very faint in the epidermal cells only loaded with H_2DCF-DA (Fig. 1d). The Fig. 1f showed that CAT could inhibit the level of H_2DCF-DA fluorescence in epidermal cells of *B. napus* L. leaves treated with oligochitosan.

Oligochitosan-induced H_2DCF-DA fluorescence in epidermal cells was substantially prevented by *L*-NAME (Fig. 1g). As shown in Fig. 1h exogenous application of oligochitosan and CAT together inhibited the relative fluorescence intensity of DAF-2DA in epidermal cells.

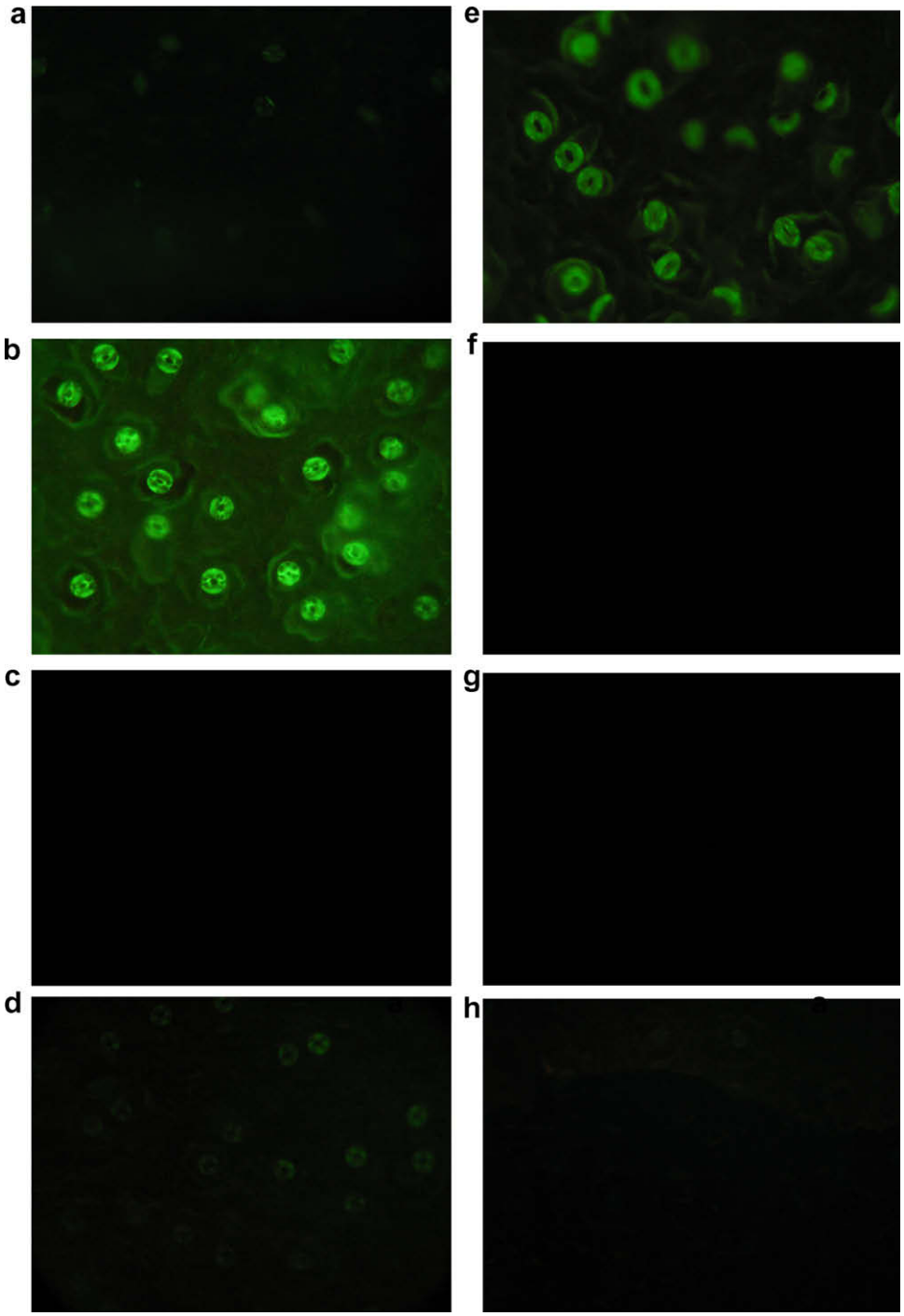

Fig. 1. Oligochitosan induced production of NO and H_2O_2 of *B. napus* L. (a) The cells loaded with DAF-2DA. (b) The cells loaded with DAF-2DA before treatment with oligochitosan. (c) The cells loaded with DAF-2DA before co-treatment with oligochitosan and *L*-NAME. (d) The cells loaded with H_2DCF-DA. (e) The cells loaded with H_2DCF-DA before treatment with oligochitosan. (f) The cells loaded with H_2DCF DA before co-treatment with oligochitosan and CAT. (g) The cells loaded with H_2DCF-DA before co-treatment with oligochitosan and *L*-NAME. (h) The cells loaded with DAF-2DA before co-treatment with oligochitosan and CAT.

3.2. Oligochitosan induced reduction in stomatal aperture of B. napus L. leaves by induced production of NO and H_2O_2

To find the effects of NO and oligochitosan on stomatal aperture of *B. napus* L. leaves, the leaves were sprayed dH_2O (control), SNP, oligochitosan, oligochitosan and *L*-NAME.

Significant reduction in stomatal aperture of *B. napus* L. leaves treated with oligochitosan and SNP for 30 min ($P < 0.05$). Co-treatment of oligochitosan and *L*-NAME blocked the reduction in stomatal aperture ($P < .05$) (Fig. 2a).

To understand the effects of H_2O_2 and oligochitosan on stomatal aperture *B. napus* L. leaves. The leaves were sprayed with dH_2O (control), H_2O_2, oligochitosan, oligochitosan and CAT or oligochitosan and Vc (one of the anti-oxidant to eliminate ROS). Significant reduction in stomatal aperture of *B. napus* L. leaves treated with oligochitosan and H_2O_2 for 30 min ($P < 0.05$).

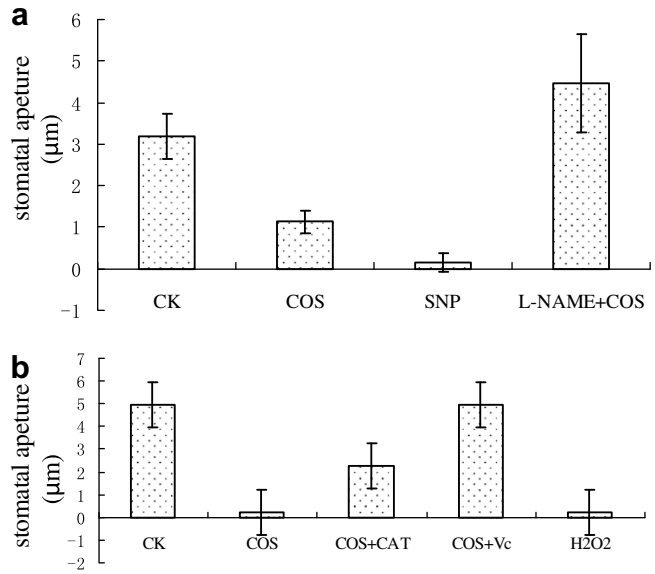

Fig. 2. Effects of oligochitosan and inhibitors on stomatal aperture *B. napus* L. (a) Effects of SNP, oligochitosan, co-treament of oligochitosan and *L*-NAME on stomatal aperture *B. napus* L. (b) Effects of H_2O_2, oligochitosan, co-treatment oligochitosan and CAT or oligochitosan and Vc on stomatal aperture *B. napus* L.

Co-treatment with oligochitosan and CAT or oligochitosan and Vc blocked the reduction in stomatal aperture ($P < 0.05$) (Fig. 2b).

3.3. Oligochitosan induced expression of LEA gene of B. napus L. by inducing production of NO and H_2O_2

To evaluate oligochitosan induced change of gene expression of *B. napus* L., group 3 *LEA* selected for semi-quantification RT-PCR. Three independent experiments were performed. In order to examine their expression levels after oligochitosan treatment, total RNAs were extracted from *B. napus* L. seedlings treated with oligochitosan for 0 (control), 15 and 30 min, 1, 4, 8, 24, 48 and 168 h, respectively. The gene expression levels changed differently (Fig. 3a). In detail, *LEA* gene was not expressed on control, but at 24 h, increased and reached the highest level. At 48 h, then declined.

As NO and H_2O_2 appear to be key factors associated with plants induced change of expression of plants genes, it was interest to test the effect of co-treatment of oligochitosan and *L*-NAME or co-treatment of oligochitosan and CAT. It was found that co-treatment with oligochitosan and *L*-NAME or co-treatment of oligochitosan and CAT both inhibited expression of group 3 *LEA* gene of *B. napus* L. (Fig. 3b and c).

4. Discussion

Plant elicitors can induce various defense reactions including accumulation of phytoalexins, changes in membrane permeability, membrane depolarization, production of reactive oxygen species (ROS), etc. (Hahn, 1996,). Many polysaccharides and oligosaccharides such as chitin, chitosan, oligogalacturonide and oligochitosan were recognized as elicitors (Darvill et al., 1992). Zhao et al. (2007a,b) examined the effects of oligochitosan on inducing defense reaction against tobacco mosaic and stimulating the production of NO and H_2O_2 in tobacco cells. Beside, oligochitosan induced increase in net photosynthetic rate of *B. napus* L. under water stress (Li et al., 2008). The results indicate that oligochitosan is an effective agent to induce defense response.

There were evidences showing that plant response to abiotic stressors as drought, high or low temperature, salinity, heavy metals, oxidative stress and biotic stressor, was regulated by NO (Arasimowicz & Floryszak-Wieczorek, 2007; Zhao et al., 2007a,b). Recently, many researchers have paid more attention to the functional aspects of H_2O_2. H_2O_2 is a constituent of oxidative metabolism and is itself a ROS. Because H_2O_2 is relatively stable and diffusible through membrane, it is generally thought to serve as a signal molecule under stresses (Neill et al., 2002; Pei et al.,

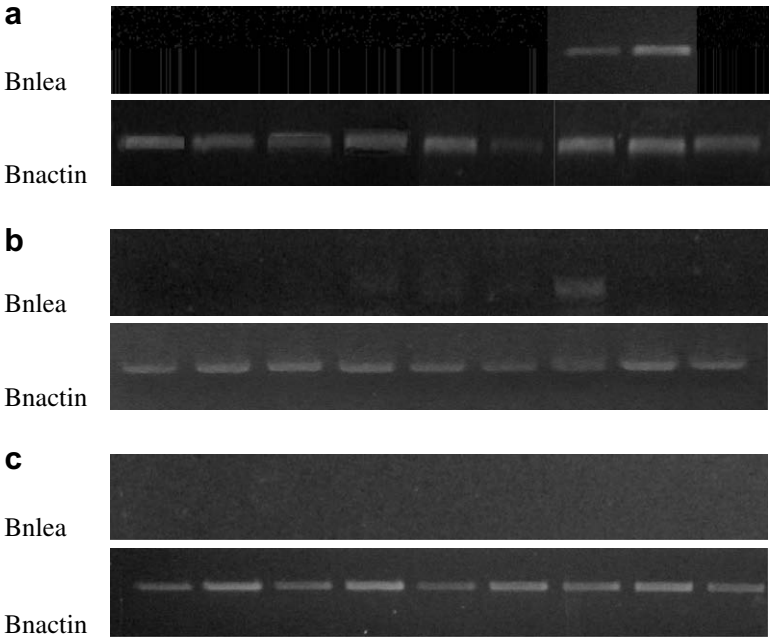

Fig. 3. RT-PCR analyses of group 3 *LEA* gene of *B. napus* L. leaves treated with oligochitosan. (a) RT-PCR analyses of group 3 *LEA* gene of *B. napus* L. leaves treated with oligochitosan for 0 (control), 15, 30 min, 1, 4, 8, 24, 48 and 168 h [(From left to right. The same below) (*B. napus* L. actin coding gene was included as internal control . The same below)]. (b) RT-PCR analyses of group 3 *LEA* gene of *B. napus* L. leaves co-treated with oligochitosan and *L*-NAME. (c) RT-PCR analyses of *LEA* genes of *B. napus* L. leaves co-treated with oligochitosan and CAT.

2000), in acclimation to photo-oxidative stress (Karpinski et al., 1999), in plant–pathogen interactions (Levine, Tenhaken, Dixon, & Lamb, 1994), and in ABA-induced stomatal closure (Zhang et al., 2001). Besides, H_2O_2 promoted germination of the seeds of *Panicum virgatum* L., *Andropogon gerardii* Vitman and *Sorghastrum nutans* L., and improved the growth of roots and stem (Sarath et al., 2006).

NO and H_2O_2 are important defense-related signaling molecules. In this paper, we showed that oligochitosan have the ability to induce production of NO and H_2O_2 within epidermal cells of *B. napus* L. We used the molecular probed DAF-2DA and H_2DCF-DA to measure the change of NO and H_2O_2 within epidermal cells of *B. napus* L. treated with oligochitosan, respectively. The two compounds were converted to the membrane-impermeant DAF-2 and H_2DCF by esterases when they were taken up by the cel1. The triazole-like substance generated by DAF-2 and NO can emit strongly a kind of green fluorescence (Kojima et al., 1998). Similarly, H_2DCF is rapidly oxidized to the highly green fluorescent DCF by intracellular H_2O_2 (Allan & Fluhr, 1997). By fluorescence microscopy, oligochitosan and inhibitors, *L*-NAME and CAT, we proved that the NO fluorescence and H_2O_2 fluorescence of *B. napus* L. leaves treated with oligochitosan were very striking (Fig. 1b and e) over the control (Fig. 1a and d), respectively. These results were in agreement with the results of production of NO and H_2O_2 in tobacco cells induced by oligochitosan (Zhao et al., 2007a,b). In addition, *L*-NAME inhibited H_2O_2 production accumulation induced by oligochitosan, and CAT inhibited NO production accumulation induced by oligochitosan (Fig. 1g and h), which showed that there was cross-talk in NO and H_2O_2.

To test oligochitosan functions on inducing reduction in stomatal aperture of *B. napus* L. via NO pathway, we examined the effects of oligochitosan, exogenous NO and co-treatment of oligochitosan and *L*-NAME on the change of stomatal aperture of *B. napus* L. We found that oligochitosan induced reduction in stomatal aperture of *B. napus* L., co-treatment of oligochitosan and *L*-NAME prevented the reduction induced by oligochitosan. So oligochitosan induced reduction in stomatal aperture of *B. napus* L. by NO pathway. The result was consistent with the result reported by Zhao et al. (2007a,b) who founded that the defense response induced by oligochitosan was connected with the NO pathway, too.

Furthermore, *L*-NAME could reverse NO generation accumulation, stomatal closure induced by oligochitosan, we presumed that the NO generation in *B. napus* L. likely related to NOS, which is agree with She et al., who found H_2O_2 and NO induced *Vicia faba* L. stomatal closure (She et al., 2004). Zhao et al. proved production of NO in tobacco was induced by oligochitosan (Zhao et al., 2007a,b), and Clark et al. reported that NO inhibited activity of CAT to result in production of H_2O_2 (Clark, Durner, Navarre, & Klessig, 2000).We also found that CAT inhibited oligochitosan to induce production of H_2O_2, so we suggested that oligochitosan induced production of NO, and then inhibited activity of CAT, at last resulted in production of H_2O_2.

To found oligochitosan functions on inducing reduction in stomatal aperture of *B. napus* L. via H_2O_2 pathway, we examined the effects of oligochitosan, exogenous H_2O_2, co-treatment of oligochitosan and CAT, and co-treatment of oligochitosan and Vc on the change of stomatal aperture of *B. napus* L. We found that oligochitosan induced reduction in stomatal aperture of *B. napus* L. CAT and Vc prevented the reduction induced by oligochitosan. So oligochitosan induced reduction in stomatal aperture of *B. napus* L. by H_2O_2 pathway. The result was consistent with the result reported by Kolla et al., who demonstrated that H_2O_2 is an essential secondary messenger during inducing stomatal closure in *Arabidopsis* (Kolla, Vavasseur, & Raghavendra, 2007). There is not any report on oligochitosan inducing reduction in stomatal aperture of plants.

In a word, stomatal aperture is related to plants against water stress. NO and H_2O_2 induced stomatal closure and enhances the plant adaptive responses against water stress (Mata & Lamattina, 2001).

Effects of oligochitosan on inducing gene expressing in *B. napus* L. were proved (Yin et al., 2006). The effect of oligochitosan on the transcripts of Phenylalanine ammonia-lyase (PAL) and chitinase in tobacco cells were investigated by RT-PCR, the results showed that the accumulation of PAL and chitinase mRNA in response to oligochitosan was markedly increased within 10 h after treating by oligochitosan (Zhao et al., 2007a,b).

There is evidence that NO affects the gene expression in plants. For more details on modulation of gene expression through NO see review paper by Grün, Lindermayr, Sell, and Durner (2006).

The effect of oligochitosan on the transcripts of *LEA* in *B. napus* L. was investigated by RT-PCR, LEA proteins are thought to be prominent in the stress response in various organisms including plants, algae, yeasts and bacteria. They are hydrophilic and accumulate in higher plants under conditions of extreme desiccation and exogenous ABA, during the last stage of seed formation, and during periods of water deficit in vegetative organs (Gala, Glazera, & Koltai, 2004; Moons, Keyser, & Montagu, 1997).

In our study, we also found that the effect of oligochitosan on inducing up regulation of expression of group 3 *LEA* gene mRNA by inducing production of H_2O_2 and NO with oligochitosan and inhibitors, CAT and *L*-NAME. There was no any report on oligochitosan, NO or H_2O_2 inducing *LEA* gene expression before.

In conclusion, oligochitosan have a potential function of inducing plants against water stress by inducing reduction in stomatal aperture as well as up regulation in *LEA* gene expression of plants via NO path and H_2O_2 path. In addition, oligochitosan also can induce plant disease resistance (Zhao et al., 2007a,b), so it will be promising as a new biological pesticide. But there are many questions that remain unanswered, including specify oligochitosan recognition and the participation of additional molecules in transmembrane signaling.

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