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Burdock fructooligosaccharide induces stomatal closure in *Pisum sativum*

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ABSTRACT

Burdock fructooligosaccharide (BFO) isolated from the root tissue of *Arctium lappa* is a reserve carbohydrate that can induce resistance against a number of plant diseases. Stomatal closure is a part of plant innate immune response to restrict bacterial invasion. In this study, the effects of BFO on stomata movement in *Pisum sativum* and the possible mechanisms were studied with abscisic acid (ABA) as a positive control. The results showed that BFO could induce stomatal closure accompanied by ROS and NO production, as is the case with ABA. BFO-induced stomatal closure was inhibited by pre-treatment with L-NAME (N^G-nitro-L-arginine methyl ester, hydrochloride; nitric oxide synthase inhibitor) and catalase (hydrogen peroxide scavenger). Exogenous catalase completely restricted BFO-induced production of ROS and NO in guard cells. In contrast, L-NAME prevented the rise in NO levels but only partially restricted the ROS production. These results indicate that BFO-induced stomatal closure is mediated by ROS and ROS-dependent NO production.

1. Introduction

Plants armaments against pathogens include preformed and inducible defenses that are displayed in the pest-colonised organs (Shah, 2009). The plant defense responses can also be activated, or primed by elicitors, which are chemical or biological molecules from various sources that induce marked physiological changes and trigger defense responses (Zhao et al., 2005). Oligosaccharides are one of the well-characterized elicitors, which have been considered as an alternative tool for disease control in agricultural fields because of their well-defined chemical structure and ability to trigger a large array of defense responses (Aziz et al., 2004; Meng et al., 2010; Shibuya & Minami, 2001; Yin et al., 2010). Most oligosaccharides with elicitor activity that have been characterized are formed by β-linked glucose units and derived from the cell walls of fungi (β-glucoside, chitin and chitosan oligosaccharide) or plants (oligogalacturonides) (Falasca et al., 2008; Radman et al., 2003).

Burdock fructooligosaccharide (BFO), a reserve carbohydrate, is a fructosan oligomer. It is first isolated from the root tissue of *Arctium lappa*, which composed of a linear chain of twelve β-2,1-linked fructofuranose residues with one α-1,2-linked glucopyrance (Hao et al., 2005). BFO can induce resistance against a number of plant diseases (Wang et al., 2009a, 2009b; Zhang et al., 2009). Previous studies showed that the mechanism of BFO might attribute to activate systemic acquired resistance (SAR) (Wang et al., 2009a, 2009b; Zhang et al., 2009). Solexa technology analysis showed the relationship of BFO with multiple signaling pathways and defence components to enhance host defence responses in plants (Guo et al., 2012).

Pathogens entry into host tissue is a critical step in causing infection in plants. It has been assumed that natural surface openings, such as stomata, are important entry sites of bacteria. Recent studies have shown that plant stomata is a checkpoint of host immunity and pathogen virulence (Zeng et al., 2010). Stomatal closure is a part of a plant innate immune response to restrict bacterial invasion (Melotto et al., 2006; Melotto et al., 2008). Bacterial PAMPs such as the flagellin peptide flg22 can induce stomatal closure (Melotto et al., 2008). Another elicitor such as chitosan, oligogalacturonic acid, and oligochitosan can also induce stomatal closure (Lee et al., 1999; Li et al., 2009).

Pisum sativum is widely used in the study of stomatal movements (Olszyk & Tibbitts, 1981; Puli & Raghavendra, 2012; Srivastava et al., 2009). Relatively, the stomatal size of *P. sativum* is larger, and the stomatal pattern is easily observed. In light of

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BFO could induce plant defense responses as a plant reserve carbohydrate (Wang et al., 2009a, 2009b; Zhang et al., 2009), we investigated the effect of BFO on stomatal closure of *P. sativum* and the potential mechanisms in this process.

2. Materials and methods

2.1. Plant materials

The seedlings of *P. sativum* (cv. Jinnong) were grown in flowerpots (with the diameter of 20 cm) in a greenhouse (average day/night temperature of about 25/18 °C, photoperiod of 12 h and 60% relative humidity). The second to fourth completely unfolded leaves from 2 to 3 week-old plants were sampled for the experiments.

Abscisic acid (ABA) and 2-(N-morpholino) ethanesulphonic acid (MES) were obtained from Sigma (USA); 3-amino, 4-aminomethyl-2′,7′-difluorescein, diacetate (DAF-FM DA), 2′7′-dichlorofluorescein diacetate (DCFH-DA), N^G-nitro-L-arginine methyl ester, hydrochloride (L-NAME) and catalase were from Beyotime Institute of Biotechnology (Shanghai, China).

2.2. BFO preparation

BFO was prepared according to the methods as described previously (Hao et al., 2005). BFO was isolated from the roots of *A. lappa* by water extraction, using DEAE-cellulose columns (Sigma, USA) and Sephadex G-50 (GE Healthcare Life Sciences, USA). When analyzed by high-performance gel permeation chromatography (HPGPC), BFO showed a symmetrical peak, indicating a homogenous fraction with molecular weight of 2134 Da.

2.3. Stomatal closure in epidermal strips

Stomatal bioassays were carried out essentially as described by Srivastava et al. (2009) with modification. The concentration of ABA was the same as used in a previous study (Bright et al., 2006). The concentration of inhibitors used in the experiments was according to the manufacturer's instructions. The abaxial (lower) epidermis of leaves was peeled off and first incubated in MES buffer (10 mM MES-KOH and 20 mM KCl, pH 6.25) for 3 h under conditions promoting stomatal opening (at 25 °C, under a photon flux density of 250 μmol/m^2/s) to open the stomata. The epidermis was then transferred to MES buffer in the presence of ABA (0, 10 μM) or BFO (0, 20, 50, 100, 200, 500, 1000 μg/mL) for another 3 h in the same conditions. When inhibitors used, leave epidermis were incubated in the presence of L-NAME (5 mM) or catalase (100 U/mL) for 30 min prior to treatment with BFO. Stomatal closure was observed under the microscope and pictures were taken of random regions. The width of the stomatal aperture was measured using the software ImageJ for Windows. The experiments were repeated at least three times, making each measurement of stomatal aperture an average of at least 90 stomata.

2.4. Detection of ROS or NO production in guard cells

ROS or NO production in guard cells of *P. sativum* was examined by using fluorescent probe DCFH-DA or DAF-FM DA, as described previously (Srivastava et al., 2009). The concentration of fluorescent probes used in the experiments was according to the manufacturer's instructions and the inhibitors used were similar to those in the above Section 2.3. We used BFO at 200 μg/mL because maximum stomatal closure occurred at the concentration. The concentration of ABA was the same as used in the previous study (Bright et al., 2006). Epidermal peels of *P. sativum* were pre incubated for 3 h in MES buffer (10 mM MES-KOH and 20 mM KCl, pH 6.25), under white light (250 μmol/m^2/s) condition at 25 °C. Then the epidermal strips were soaked in 10 μM DCFH-DA (for detection of ROS) or 5 μM DAF-FM DA (for detection of NO) diluted in MES buffer for 30 min under dark condition, washed three times in MES buffer, and then incubated with MES buffer in the presence of ABA (10 μM) or BFO (200 μg/mL) with and without L-NAME (5 mM) or catalase (100 U/mL). ROS or NO production was monitored in controls and 20 min after treatment with either ABA or BFO. Photographs of guard cells were taken with a digital camera attached to a laser scanning confocal microscope (LSM700, Zeiss) to observe the fluorescence of DCFH-DA (excitation 488 nm, emission 525 nm) or DAF-FM DA (excitation 495 nm, emission 515 nm). The captured images and the relative fluorescence emission of guard cells were analyzed by using ImageJ for Windows.

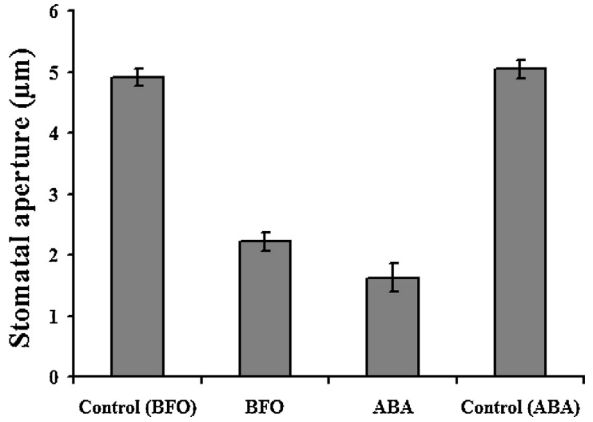

Fig. 1. Effect of BFO (1 mg/mL) and ABA (10 μM) on stomatal aperture in epidermal strips of *P. sativum*. Stomata with 5 μL/mL ethanol (ABA solvent) as control of ABA. Averages from three independent experiments are shown.

2.5. Solvent effects, replications and statistical analysis

The control sets were added with an equal volume of solvents used for their stocks. Ethanol was used as solvent for ABA, while milli-Q water was used for BFO. The data presented are the average values (±SD) of results from at least three experiments. Significant differences were evaluated by using Student's *t*-test and statistical significance was taken into consideration at $P < 0.05$.

3. Results and discussion

3.1. BFO-induced stomatal closure

In this study, BFO could induce stomatal closure in *P. sativum*, as is the case with ABA. BFO decreased the average width of stomatal apertures by 54.0% at a concentration of 1 mg/mL and 10 μM ABA could reduced stomatal apertures by 67.3% in the same conditions (Fig. 1). The effect of BFO on promotion of stomatal closure was significant ($P < 0.05$) at a concentration of 50 μg/mL. Application of 50 μg/mL, 100 μg/mL, 200 μg/mL BFO reduced stomatal apertures by 17.1%, 51.3%, and 54.5%, respectively, and showed a does-dependent manner (Fig. 2). Maximum stomatal closure occurred at 200 μg/mL (Fig. 2), under which conditions the stomatal apertures were 2.16 ± 0.24 μm, or 45.5% of the control value (5.01 ± 0.14 μm).

Stomata started to close after 30 min with the application of BFO (200 μg/mL) (Fig. 3). The average width of the stomatal aperture decreased markedly in epidermal peels at 1 h after incubation with BFO, whereas it did not decrease in epidermal peels that were incubated with water (Fig. 3). Maximum closure occurred by about 2 h

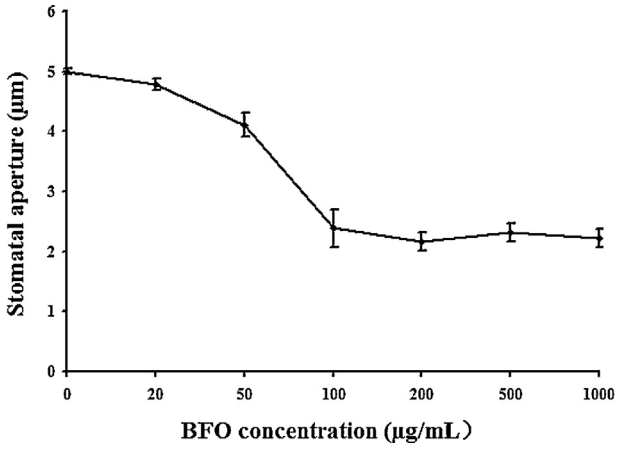

Fig. 2. Concentration-dependent stomatal closure by BFO treatment in *P. sativum*. Results are the average ± SD of three independent experiments.

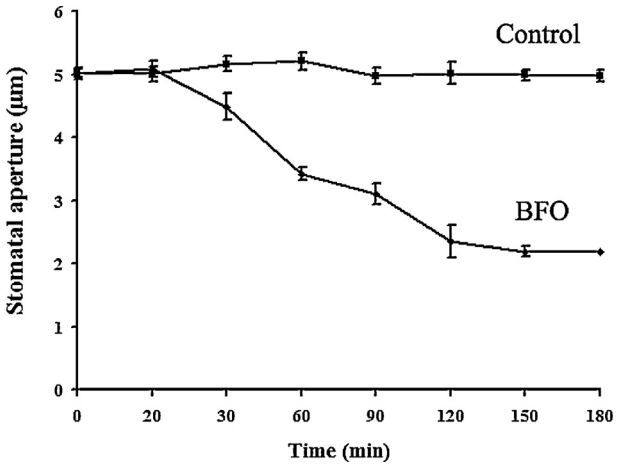

Fig. 3. Stomatal closure induced by 200 μg/mL BFO in abaxial epidermis of *P. sativum*. Results are the average ± SD of three independent experiments.

after exposure to BFO (Fig. 3). All together, BFO can quickly induce stomatal closure in a time- and concentration-dependent manner.

Stomatal closure contributes to plant adaptation during stresses and act as an effective barrier against pathogens (Khokon et al., 2011; Melotto et al., 2006). Stomatal closure is not only induced by phytohormones particularly ABA but also by bacteria and elicitors (Assmann & Shimazaki, 1999; Klüsener et al., 2002; Melotto et al., 2008). Chitosan induced a dose-dependent stomatal closure in abaxial epidermis of *P. sativum*, as is the case with ABA (Srivastava et al., 2009). Application of yeast elicitor (YEL) reduced stomatal aperture in *Arabidopsis* and also showed a dose-dependent effect (Khokon et al., 2010a). In this study, stomatal closure induced by BFO with dose-dependent was observed in *P. sativum*.

3.2. ROS and NO production in guard cells induced by BFO

In the present study, both ABA and BFO stimulated guard cells ROS and NO synthesis (Figs. 4 and 5). Application of 10 μM ABA effectively induced ROS and NO production in guard cells of *P. sativum* (Figs. 4 and 5). These observations are similar to the findings that ABA induced stomatal closure in *Arabidopsis* (Bright

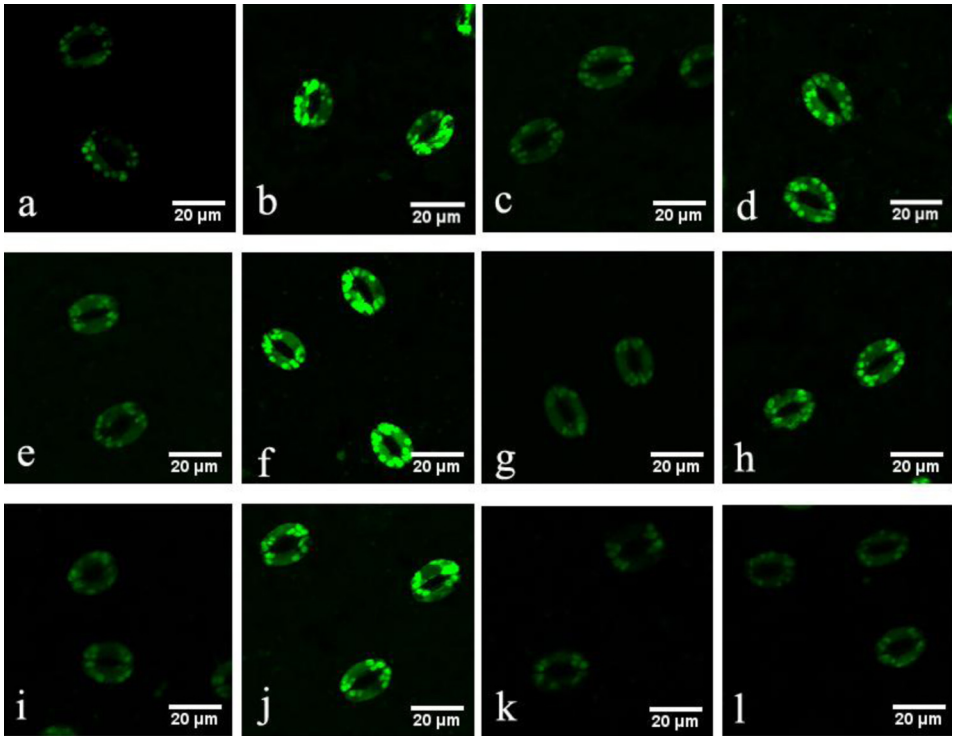

Fig. 4. Effect of ABA or BFO on ROS and NO production in guard cells of *P. sativum*.
(a-d). Confocal images demonstrating ABA induced ROS and NO synthesis in guard cells of *P. sativum*. (a–b) Fluorescence images of guard cells loaded with 10 μM DCFH-DA reflecting the levels of ROS. Bar = 20 μm. (a) Stomata with 5 μL/mL ethanol (ABA solvent) as control of ABA. (b) Stomata with 10 μM ABA. (c–d) Fluorescence images of guard cells loaded with 5 μM DAF-FM DA reflecting the levels of NO. Bar = 20 μm. (c) Stomata with 5 μL/mL ethanol (ABA solvent) as control of ABA. (d) Stomata with 10 μM ABA. (e–h) Fluorescence images of guard cells loaded with 10 μM DCFH-DA reflecting the levels of ROS induced by BFO. Bar = 20 μm. (e) Control, without BFO and modulator. (f–h) Guard cells exposed to 200 μg/mL BFO. (f) No modulators added. (g) 100 U/mL catalase added. (h) 5 mM L-NAME added. (i–l) Fluorescence images of guard cells loaded with 5 μM DAF-FM DA reflecting the levels of NO induced by BFO. Bar = 20 μm. (i) Control, without BFO and modulator. (j–l) Fluorescence images of guard cells exposed to 200 μg/mL BFO. (j) No modulators added. (k) 100 U/mL catalase added. (l) 5 mM L-NAME added.

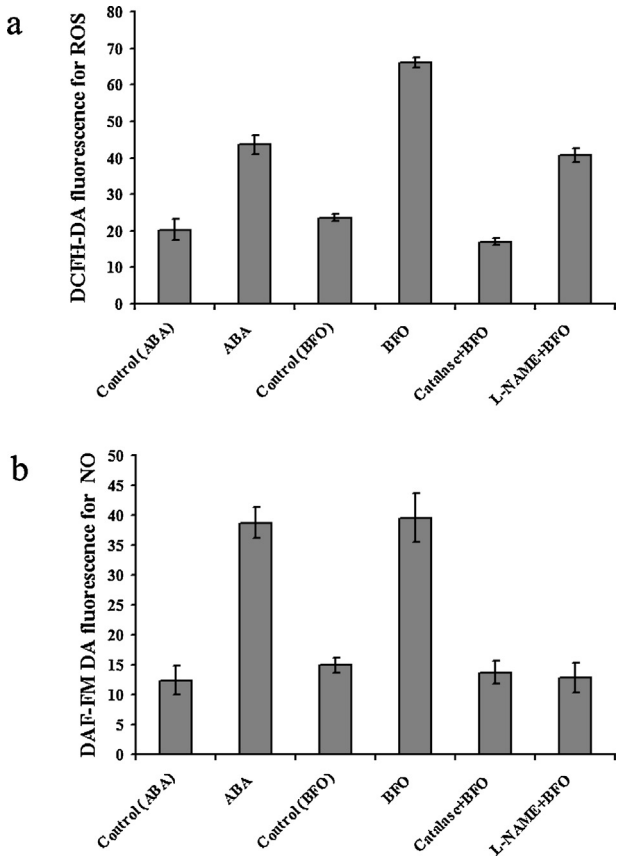

Fig. 5. Effect of ABA (10 μM) or BFO (200 μg/mL) on the extent of ROS and NO production in guard cells of *P. sativum*. (a) Effect of ABA (10 μM) or BFO (200 μg/mL) on the extent of ROS production in guard cells of *P. sativum*, as monitored by the fluorescent probe DCFH-DA. Results are the average ± SD of three independent experiments. (b) Effect of of ABA (10 μM) or BFO (200 μg/mL) on the extent of NO production in guard cells of *P. sativum*, as monitored by the fluorescent probe DAF-FM DA. Results are the average ± SD of three independent experiments.

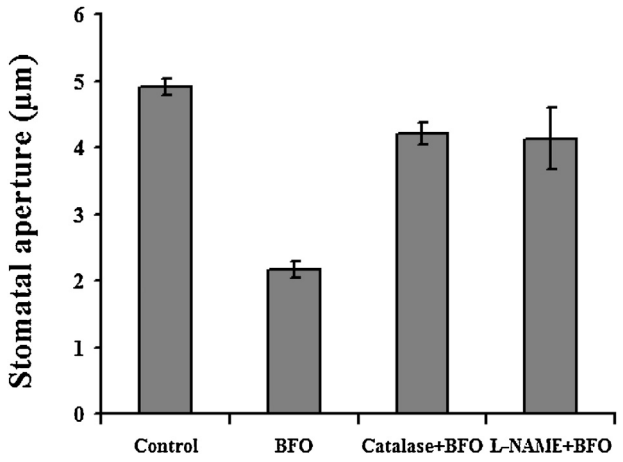

Fig. 6. Effect of ROS or NO modulators on stomatal closure induced by 200 μg/mL BFO in guard cells of *P. sativum*. Results are the average ± SD of three independent experiments.

et al., 2006). Application of 200 μg/mL BFO significantly induced ROS production in guard cells of *P. sativum* (Figs. 4 and 5). On the other hand, BFO at similar concentration induced a marked rise in production of NO in guard cells (Figs. 4 and 5). The current results indicate that BFO can significantly induce ROS and NO production in guard cells of *P. sativum*. Similar interactions of ROS and NO were also observed during chitosan effects on guard cells of *P. sativum* and oligochitosan effects on epidermal cells of *Brassica napus* (Srivastava et al., 2009; Li et al., 2009).

3.3. NO production induced by BFO is dependent on ROS production

The ROS production induced by BFO in guard cells was completely abolished by catalase (Figs. 4 and 5). Similarly, the NO production was completely abolished by L-NAME, a nitric oxide synthase (NOS) inhibitor (Figs. 4 and 5). It indicates that BFO-induced NO is from NOS pathway. The rise in NO induced by BFO was also completely abolished by catalase (Figs. 4 and 5). This shows that ROS is indispensable to BFO induce NO production. In contrast, L-NAME alone also had limited effect on the ROS accumulation (Figs. 4 and 5). These results indicate that there may be a cross-talk in BFO-induced NO and ROS production in guard cells of *P. sativum*.

Stomatal movements are modulated by guard cells, which can respond to environmental stresses, pathogen infection, and plant hormones through several secondary messengers including ROS, NO, calcium and protein kinases/protein phosphatases (Klüsener et al., 2002; Melotto et al., 2006; Pei et al., 2000). Salicylic acid (SA)-, YEL-, chitosan-induced stomatal closure is accompanied by ROS production (Khokon et al., 2010a, 2010b, 2011). NO production induced by ABA and chitosan is dependent on ROS synthesis, which is required for ABA and chitosan to close stomata (Bright et al., 2006; Srivastava et al., 2009). In the present study, the rise in NO induced by BFO was also completely abolished by catalase, but L-NAME alone also had limited effect on the ROS accumulation. Another, L-NAME and catalase completely inhibited H_2O_2 or NO production induced by oligochitosan in guard cells of *B. napus* (Li et al., 2009). However, NO inhibitors had no effect on ROS production induced by chitosan in guard cells of *P. sativum*, while ROS inhibitors restricted the chitosan-induced NO production (Srivastava et al., 2009). The contradictions in NO and ROS production during stomatal closure induced by elicitors still needs further studies.

3.4. BFO-induced stomatal closure mediated by ROS and NO

BFO-induced stomatal closure was suppressed by ROS and NO inhibitors (Fig. 6). L-NAME effectively prevented BFO-induced stomatal closure (Fig. 6). It indicates that NO is required for BFO to close stomata. Similarly, catalase also prevented the BFO induced stomatal closure (Fig. 6). Taken together, these results suggest that ROS and NO are required for BFO to induce stomatal closure.

ROS and NO are essential signaling components during stomatal closure induced by ABA and chitosan (Bright et al., 2006; Srivastava et al., 2009). Addition of exogenous NO to both monocot and dicotyledonous epidermal strips was sufficient to induce stomatal closure (García-Mata & Lamattina, 2001). Similarly, exogenous application of H_2O_2 promoted stomatal closure in *Vicia faba* (Zhang et al., 2001). Catalase could prevent stomatal closure induced by ABA, chitosan and YEL (Bright et al., 2006; Khokon et al., 2010a; Srivastava et al., 2009). N^G-nitro-L-arginine (L-NNA), an inhibitor of NOS, effectively prevents flg22-, LPS-, and *Escherichia coli* O157:H7-induced stomatal closure in *Arabidopsis* (Melotto et al., 2006). L-NAME effectively prevents chitosan-induced stomatal closure (Srivastava et al., 2009). In this study, catalase and L-NAME effectively prevented BFO-induced stomatal closure. All together, BFO-induced stomatal closure is mediated by ROS and ROS-dependent NO production, which is similar to ABA-, YEL-, and chitosan-induced stomatal closure (Bright et al., 2006; Khokon et al., 2010a; Srivastava et al., 2009).

4. Conclusion

In this study, the effect of BFO on stomata movement in *P. sativum* and the possible mechanism for stomatal closure was studied. BFO could induce stomatal closure like ABA. BFO-induced stomatal closure is mediated by ROS and ROS-dependent NO production. Our findings may contribute to a better understanding of BFO functions in plants against pathogens.

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