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Research article

Alginate-derived oligosaccharides promote water stress tolerance in cucumber (*Cucumis sativus* L.)

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ABSTRACT

Marine alginate-derived oligosaccharides (ADOs) are prepared from degraded alginate. Our experiments were carried out to determine the mechanism of ADOs to improve resistance to water stress in cucumber (*Cucumis sativus* L.). We evaluated the effects of ADOs on physiological indices, photosynthesis, reactive oxygen species (ROS) levels, antioxidant enzyme activities, and relative expression levels of drought resistance genes. The growth of drought stressed cucumber decreased markedly. However, treatment with ADOs significantly improved the diameter, fresh weight, photosynthetic rate, transpiration rate, stomatal conductance, maximum quantum yield of photosystem II (*Fv/Fm*) and chlorophyll degradation; thus, reversing the effects of drought stress. Moreover, the antioxidant levels and ROS scavenging enzyme activities also increased in response to the ADOs. Additionally, the genes involved in abscisic aid (ABA) signaling and the drought stress response, such as superoxide dismutase [Cu-Zn] *(CsSOD(Cu-Zn)),* the peroxidase superfamily protein (*CsPOD3*), ABA deficient 2 (*CsABA2*), responsive to ABA 18 (*CsRAB18*), abscisic acid insensitive 5 (*CsABI5*), responsive to dehydration 22 (*CsRD22*), and responsive to dehydration 29A (*CsRD29*A) were upregulated by ADOs. The ABA content was also improved by ADOs. Our results suggest that ADOs induced the expression of some antioxidant enzyme synthetic genes involved in the ABA signaling pathway by stimulating ABA synthesis to improve the drought resistance capacity in cucumber.

1. Introduction

Cucumber (*Cucumis sativus* L.) is an important vegetable crop grown worldwide. Drought occurs every year in many regions of the world (Ludlow and Muchow, 1990), and cucumbers have shallow root systems and large leaf areas, hence, are sensitive to an inadequate supply of water (Liu et al., 2010). This continuous drought stress leads to various biochemical and physiological responses, which cause adverse effects on cucumber growth, including inhibited elongation of roots and aerial parts, cell oxidative damage, and inhibited photosynthesis (Smirnoff, 1993).

Photosynthetic parameters, such as photosynthetic rate, transpiration rate, stomatal conductance, internal CO_2 concentration, and chlorophyll content, decrease in plants exposed to abiotic stress (Flexas et al., 2002). Chlorophyll is the main component of photosynthesis in higher plants, and damage to chlorophyll seriously affects plant growth and development. The change in chlorophyll content reflects plant health status to some extent (Yamori et al., 2006).

Water stress also induces increases in reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet-}$), hydroxyl free radicals (•OH), and hydrogen peroxide (H_2O_2) (Mittler, 2002). The accumulation of ROS leads to lipid peroxidation and death of cells (Imlay, 2003). Plants have evolved enzymatic antioxidants to scavenge ROS, including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) (Xu et al., 2008). These antioxidant enzymes directly react with ROS in plants under abiotic stress conditions. Lipid peroxidation, as measured by malondialdehyde (MDA) content, reflects oxidative damage to cell components. Cell membranes are susceptible to various stressors accompanied by increased MDA content, which is a typical symptom of membrane lipid damage. Hence, MDA content has been measured as an indicator of drought stress damage (Chaoui et al., 1997).

Along with discoveries of drought-resistant related genes, more

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studies have focused on the abscisic acid (ABA) signaling pathway in plants. It is well known that the ABA-dependent signaling pathway is involved in the drought response (Shinozaki et al., 2003). Under water deficit conditions, the ABA content is improved, the ABA synthetic genes *ABA2* and *ABA3*, and some ABA-mediated stress-responsive genes are induced, including the typical ABA-dependent genes *CsRAB18* and *CsABI5* (Yoshida et al., 2015), and drought stress genes *CsRD29*A and *CsRD22* (Matus et al., 2014). Some osmotic protective, carbohydrate, and secondary metabolic products are also regulated by ABA in plant tissues (Fujita et al., 2011; Yamaguchi-Shinozaki and Shinozaki, 2006).

The main method to improve drought resistance in plants is to apply chemical drought resistant reagents, but these may leave residues and cause environmental pollution. Genetic modification is a potential solution, but has yet to achieve worldwide acceptance (Griffiths et al., 2016). Hence, it is necessary to find an environmentally friendly biological drought resistant agent to enhance tolerance to drought and replace chemical reagents. Some oligosaccharides have been developed, such as chitosan oligosaccharides (Nguyen et al., 2011) and raffinose family oligosaccharides (Minorsky, 2003).

Alginates are quite abundant in nature because they are a structural component of marine brown algae, comprising up to 40% of dry matter. Their commercial production started in 1927 and has expanded to about 30,000 tons per year worldwide. About 30% of this tonnage is devoted to the food industry and the rest is used in industrial, pharmaceutical, and dental applications (Ertesvag et al., 2009).

ADOs are marine oligosaccharides produced from alginate, composed of α-L-glucuronic acid, β-D-mannuronic acid, or both heterogeneous segments. ADOs have several positive effects on plants due to their unique physiological activities (Akimoto et al., 1999). It has been reported that ADOs promote root growth (Hien et al., 2000; Iwasaki and Matsubara, 2000; Natsume et al., 1994) and crop yield of plants (Hu et al., 2004). Therefore, they could be used as a new type of plant growth regulator in agriculture. Further studies have demonstrated that ADOs alleviate plant damage caused by abiotic stressors, such as high salt (Tang et al., 2011) drought (Liu et al., 2013), and heavy metals (Ma et al., 2010), ADOs pretreatment significantly increases antioxidant enzyme activities and reduced MDA content in leaves and roots, but the resistant mechanism of ADOs is uncertain, and few studies have investigated the effects of different ADO polymers on drought stress resistance in vegetable crops.

2. Materials and methods

2.1. Materials

The ADOs were gifted by Beijing Leili Marine Bioindustry Inc. (Beijing, China). ADO1 was the ADOs with a molecular weight of about 4,000, and ADO2 was the ADOs with a molecular weight of about 8000. The following experiments were conducted at China Agricultural University, Beijing (39.9°N 116.3°E). Cucumber (*Cucumis sativus* L. Jinyan 4) seeds were provided by the Beijing Academy of Agriculture and Forestry Sciences.

2.2. Stress and reagents treatments

The seeds were sown in plastic pots (8-cm diameter) with peat soil in a sunlit greenhouse in middle of October, in Beijing (39.9°N 116.3°E). The seedlings were divided into four groups when the third leaf had expanded. Watering was stopped in the CK2, ADO1, and ADO2 groups, whereas the CK1 group was watered normally. Distilled water or the ADO solutions were sprayed onto the seedling leaf surfaces of the corresponding treatments after 7 days of dehydration. CK1 was treated with distilled water, CK2 was treated with water after the drought stress, and the ADO groups were treated with 10 mL of 0.2% ADO solution per plant after the drought stress. A preliminary study showed that a 0.2% ADO solution was optimum for enhancing drought tolerance in tomato seedlings (Liu et al., 2009). Samples were harvested at different time points after spraying (2 and 4 days). Every treatment group contained three parallel samples.

2.3. Evaluation of growth parameters

Diameter and fresh weight were estimated 4 days after ADO treatments. Fresh plants were weighed immediately to obtain fresh weight (FW).

2.4. Evaluation of photosynthesis and chlorophyll fluorescence measurements

Photosynthetic traits were measured *in situ* on the third or fourth mature leaves from three plants per treatment during the late morning (09:00–11:00 am) using the Li-6400 portable photosynthesis system (Li-COR Inc., Lincoln, NE, USA). Light intensity was set at 800 Lx during the measurement periods. Measurements of net photosynthesis rate, transpiration rate, internal CO_2 concentration, stomatal conductance, and chlorophyll fluorescence (*Fv/Fm*) were carried out on the same leaf. Chlorophyll was extracted in 80% chilled acetone and quantified with a spectrophotometer.

2.5. Evaluation of lipid peroxidation

The levels of lipid peroxidation in cucumber leaves were evaluated by determining their peroxidation products. MDA and endoperoxides were determined according to the method described by Hodges et al. (1999). A 0.5 g leaf sample was homogenized in 5 mL 0.1% TCA. The homogenate was centrifuged at 10,000 × g for 5 min. Four mL of 20% TCA containing 0.5% TBA was added to 1 mL of the supernatant, and the solution was incubated in boiling water for 20 min. The reaction was stopped by placing the reaction tubes on ice. MDA absorption was measured spectrophotometrically at 450, 532, and 600 nm. The concentration of lipid peroxides was quantified in terms of the MDA concentration and expressed as nmol/g fresh weight.

2.6. Evaluation of ROS

Cucumber leaves (0.5 g FW) were homogenized with 5 mL cold acetone. •OH production was estimated as described by Halliwell and Grootveld (1988). Formation of the MDA breakdown products was determined by mixing 0.5 mL of centrifuged incubation medium with 1 mL of 2-thiobarbituric acid and 1 mL of ethylic acid. The reaction mixture was boiled for exactly 30 min, cooled in water, clarified by centrifugation, and measured spectrophotometrically at 532 nm against reagent blanks.

2.7. Antioxidant enzyme extraction and assays

Samples of leaves (0.5 g FW) were homogenized in 50 mM phosphate buffer (pH 7.8) in an ice bath. The mixture was centrifuged at 10,000 × g for 10 min. SOD activity was assayed as described by Giannopolitis and Ries (1977) [photochemical nitroblue tetrazolium (NBT) method]. The reaction mixture (6 mL) contained 50 mM phosphate buffer (pH 7.8), 20 mM riboflavin, 130 mM methionine, 100 mM EDTA, 750 mM NBT, and 100 mL of protein extract. A reaction mixture containing no protein extract was used as the control. One unit of SOD was defined as the quantity of enzyme necessary to inhibit the NBT reaction by 50%/min, and the results are expressed as U mg^{-1} FW.

Samples of leaves (0.5 g FW for each seedling) were homogenized in 50 mM phosphate buffer (pH 7.8 including 1 mM EDTA) in an ice bath. The mixture was centrifuged at 4 °C and 10,000 × g 20 min. The supernatant was the crude POD extract. POD activity was measured as described by Zhang and Kirkham (1994) using guaiacol as the substrate. The reaction mixture (5 mL) contained 50 mM phosphate buffer (pH