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Extracellular Polymeric Substances of *Pseudomonas chlororaphis* O6 Induce Systemic Drought Tolerance in Plants

*Corresponding author
Tel: +82-62-530-2071
Fax: +82-62-530-0208
E-mail: yckimyc@chonnam.ac.kr
ORCID
https://orcid.org/0000-0002-7661-7600

Song Mi Cho[1], Anne J. Anderson[2], and Young Cheol Kim ID[3]*

[1]*Department of Floriculture, Chunnam Techno University, Jeonnam 57500, Korea*
[2]*Department of Biological Engineering, Utah State University, Logan, UT 84322-5305, USA*
[3]*Institute of Environmentally-Friendly Agriculture, Department of Plant Biotechnology, Chonnam National University, Gwangju 61186, Korea*

Pseudomonas chlororaphis O6 induces systemic tolerance in plants against drought stress. A volatile, 2R, 3R-butanediol, produced by the bacterium causes partial stomatal closure, thus, limiting water loss from the plant. In this study, we report that applications of extracellular polymeric substances (EPS) from *P. chlororaphis* O6 to epidermal peels of leaves of *Arabidopsis thaliana* also reduce the size of stomatal openings. Growth of *A. thaliana* seedlings with applications of the EPS from *P. chlororaphis* O6 reduced the extent of wilting when water was withheld from the plants. Fluorescence measurements showed photosystem II was protected in the *A. thaliana* leaves in the water stressed EPS-exposed plants. These findings indicate that *P. chlororaphis* O6 has redundancy in traits associated with induction of mechanisms to limit water stress in plants.

Keywords: Abiotic stress, Biological control, Priming, Stomatal closure

Plant possess elegant and effective survival systems against abiotic stress and biotic invasion (Zhu, 2001). Under drought conditions, the level of the plant hormone, abscisic acid (ABA) increases, triggering adaptive responses that are essential for the survival of plants (Zhu, 2001). One effect of the enhanced ABA level is stomatal closure that minimizes water loss through transpiration (Leung and Giraudat, 1998). Consequently, because of the problems associated with the regulation of stomatal aperture, plants with defects in ABA-biosynthesis (*aba* mutants) and ABA-responses (i.e., ABA-insensitive *abi* mutants) are susceptible to drought stress (Leung and Giraudat, 1998; Schroeder et al., 2001). Another plant growth regulator, jasmonate (Evans, 2003; Suhita et al., 2004), also stimulates stomatal closure under drought conditions (Creelman and Mullet, 1997).

In plants, drought tolerance can be induced upon colonization by certain microbes. *Pseudomonas chlororaphis* O6, a plant probiotic microbe (Kim and Anderson, 2018), elicits both induced systemic resistance (ISR) to pathogens and induced systemic tolerance (IST) to environmental stresses (Kim et al., 2011; Yang et al., 2009, 2017). Induced tolerance to drought in plants also occurs with colonization by several Gram-positive *Bacillus* strains (Ryu et al., 2004; Timmusk and Wagner, 1999; Zhang et al., 2010), other Gram-negative bacterial isolates (Mayak et al., 2004), and fungi such as *Trichoderma harziarum* (Bae et al., 2008; Shukla et al., 2015). The volatile 2R,3R-butanediol is a metabolite produced by the plant-associated bacteria strains that causes stomatal closure and induces the expression of genes promoting drought tolerance (Kim et al., 2012). Butanediol-induced drought tolerance in *Arabidopsis* involves activation of salicylic acid (SA), jasmonic acid, and ethylene-dependent pathways (Cho et al., 2008).

Recent reviews indicate that there is an array of different

types of microbial determinants inducing systemic tolerance in plants against biotic and/or abiotic stresses (Bakker et al., 2013; Cheng et al., 2017; Kim and Anderson, 2018). Some of the determinants have been identified by examining mutants in root-colonizing beneficial microbes impaired in their abilities to cause IST (Cheng et al., 2017; Han et al., 2006; Kim et al., 2007). The active microbial structures include such secreted metabolites (Kim and Anderson, 2018) as antibiotics, siderophores, and organic volatiles (e.g. butanediol) and bacterial surface structures, the lipopolysaccharides and flagella proteins (Melotto et al., 2006; Underwood et al., 2007). These findings illustrate that a single microbe may produce several factors that will cause IST.

We observed that induced drought tolerance by *P. chlororaphis* O6 correlates with decreased stomatal aperture size on the leaf in plants with roots colonized by this microbe (Cho et al., 2008). In this study, we extend the knowledge of microbial products inducing tolerance to drought to include the extracellular polymeric substances (EPS) from *P. chlororaphis* O6. The potential of EPS to induce drought tolerance was studied by determining whether they caused stomatal closure in the epidermal peels. At the whole plant level, the extent of wilting upon withholding water was assessed by visual comparison between plants with and without EPS treatments. The extent of drought was also characterized by measuring the chlorophyll fluorescence in the leaf tissues (Woo et al., 2008). Drought causes changes in the photosynthetic potential of plants that can be detected by measuring the F_v/F_m ratio (Woo et al., 2008).

Isolation of EPS from *P. chlororaphis* O6. Cells from a single colony of *P. chlororaphis* O6, grown for 2 d on Luria-Broth (LB, Difco Inc., Detroit, MI, USA) agar plates, were inoculated into 500 ml of LB broth and incubated at room temperature for 4 d with agitation at 250 rpm. The EPS of *P. chlororaphis* O6 were isolated using a modified ethanol precipitation method as described previously (Hung et al., 2005). The bacterial cells were removed by centrifugation for 10 min, and 30 Anson unit protease (0.5 mg/l, Quiagen Inc., Valencia, CA, USA) was added to the supernatant and incubated at 37℃ for 12 h with agitation at 70 rpm. Ethanol was added to the supernatant to produce a 1:4 (v/v) mixture of the supernatant:ethanol and stirred at 4℃ for 14 h. The precipitate was removed by filtration through a 0.2 µm Millipore membrane. The precipitate was dissolved in sterile water and mixed with ethanol (1:4 ratio) at 4℃ for 14 h. The EPS were pelleted by centrifugation for 15,000×g at 4℃ for 30 min. The pellet was dried at room temperature for 5 h and the mass measured before dissolving in sterile distilled water to produce solutions with known mass/ml. The final EPS preparation was streaked on LB agar plate to confirm no contamination by any culturable microbes.

Data were analyzed by ANOVA ($p<0.01$), using SPSS (version 23, SPSS Inc., Chicago, IL, USA) and if the value of the *F* was significant, differences between each measurement were further elucidated through Duncan's multiple range test ($p<0.01$).

Examination of stomatal opening on epidermal peels. To determine the direct effects of EPS on stomatal opening, plants were grown in well plates for 4 weeks. *Arabidopsis thaliana* Col-O seeds were surfaced-sterilized with 0.3% sodium hypochlorite, followed by extensive washing with sterile water. Seeds were transferred to well plates (12-well microtiter plates; SPL Inc., Seoul, South Korea) that contained half-strength Murashige and Skoog salt (MS, Sigma-Aldrich, St. Louis, MO, USA) medium, 0.3% Phytagel and 3% sucrose (pH 5.7) (Cho et al., 2008, 2012). The plates were sealed with Parafilm to prevent drying and no additional water was added during their growth. To maximize stomatal opening, the plants were maintained under light for at least 3 h before excising the epidermal peels. The peels were transferred on to glass slides. The cuticle side was treated with water (a negative control), defined dilutions of the EPS solution (10, 100, and 1000 µg/ml, the test treatments), or a suspension of *P. chlororaphis* O6 cells (a positive control). Another positive control for stomatal closure was treatment with 100 µM ABA (Sigma-Aldrich). Two hours after incubation, the stomatal apertures were observed by light microscopy (Leica M165 FC, Leica Microsystems, Wetzlar, Germany). The size of the openings was assessed using the image acquisition software, Image-Pro (version 2.32 for Windows, Media Cybermetics, Rockville, MD, USA). At least 100 stomata/plants were counted, with five plants per treatment being used in each experimental replicate.

The treatment of leaf epidermal peels with ABA and with the suspension of intact *P. chlororaphis* O6 cells decreased the size of stomatal aperture relative to stomata treated exclusively with water (Fig. 1A). These findings were expected based on the previous findings (Cho et al., 2008). Stomatal
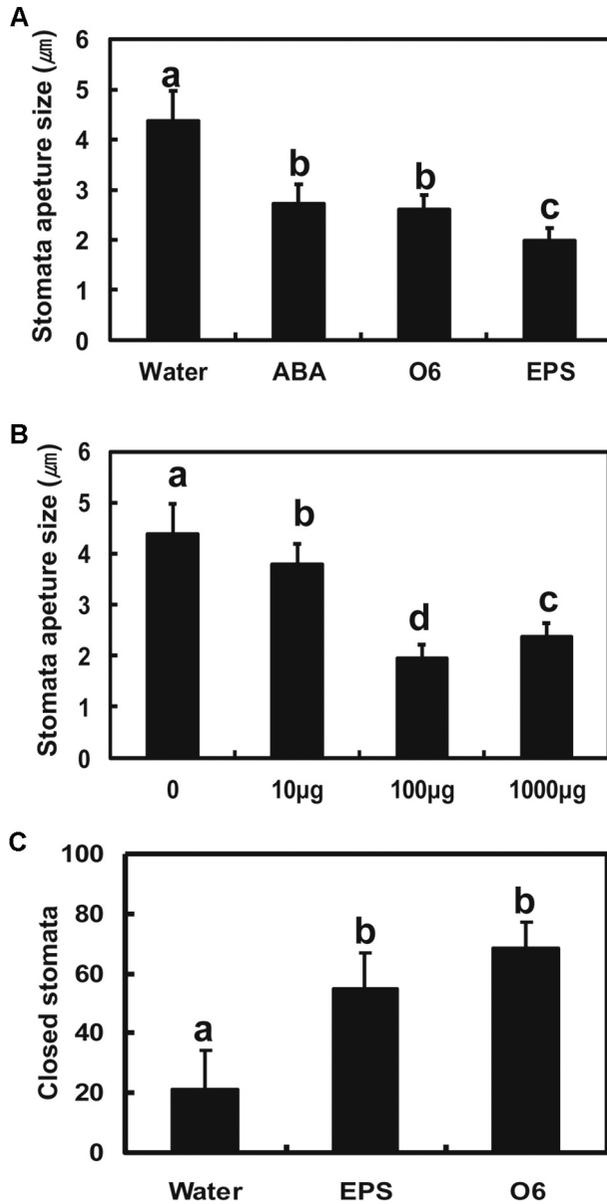
Fig. 1. Stomatal closure in epidermal peels by treatments with *Pseudomonas chlororaphis* O6 cells, its EPS and ABA. The treatments for 2 h were (A): water; 100 μM abscisic acid (ABA), or *P. chlororaphis* O6 cells (1×10^8 cfu/ml) applied to the peel in 10 μl aliquots and EPS (100 μg/ml) in (B) water or *P. chlororaphis* O6 EPS at defined concentrations. The width of the stomatal apertures was measured from light microscope images using the software Image-Pro C. (C) The % closed stomates with treatments of water, EPS (100 μg/ml) or *P. chlororaphis* O6 cells (1×10^8 cfu/ml). At least 100 stomata per plant were counted, with five plants per treatment used in each experimental replicate. Different letters indicate significant differences between treated and control samples based on Duncan's multiple range test at $p < 0.01$.

closure also was observed with treatment of 100 μg/ml EPS solution (Fig. 1A–C). The extent of closure was dose-dependent (Fig. 1B); when doses of 10, 100, and 1000 μg/ml were used, the 100 μg/ml treatment was optimal.

Plant wilting bioassay.　To determine whether the treatments that caused the narrowing of stomatal openings corresponded with drought tolerance, the degree of wilting was assessed visually using *A. thaliana* seedlings grown in the well plates as described previously (Cho et al., 2008). The surface-sterilized seeds were placed on Whatman # 1 paper and placed over the MS medium contained in the microtiter wells. The microtiter plates were sealed with parafilm to prevent drying. After two weeks of growth, the seedlings were treated with 10 μl/plant, water as a control, 10 μl per plant, *P. chlororaphis* O6 cell suspensions (1×10^8 cfu/ml), or 10 μl per plant, 100 μg/ml EPS solution. After 3 d, seedlings on the filter papers were transferred to open, empty Petri dishes. The extent of wilting of the plants was assessed visually after 2 h. The experiment was repeated three times with 12 plants per treatment.

When *A. thaliana* seedlings were exposed to the air, prior treatments for 3 d with EPS, or with live *P. chlororaphis* O6 cells, resulted in plants that exhibited less visible wilting than the plants treated with water as a control (Fig. 2A). Thus, *P. chlororaphis* O6 cells and extracted EPS reduced the wilt symptoms in intact plants deprived of water.

Quantitative assessment of drought survival using chlorophyll fluorescence.　The changes in photosynthetic potential with drought were assessed with *A. thaliana* seedlings grown in sterile soil mix. The solid growth matrix was a sterile nursery soil mixture of Bio-Sangto, vermiculite, perlite (125 g/vessel, 3:1:1; Seminis Korea, Seoul, Korea) contained in sterile Magenta boxes (10 cm×10 cm×9 cm, Sigma-Aldrich). Five milliliters of water were added daily to the boxes. The seedlings were grown under a 16 h/8 h light/dark cycle with 40-W fluorescent lights (3,000 lx, 80 μmol photon $m^{-2}s^{-1}$). The temperature was maintained at 23±2°C with a relative humidity of 50–60%.

The seedlings were grown for 3 weeks before treatment with 2 ml of sterile water as a negative control, or with equal volumes of 100 μM ABA as a positive control, or *P. chlororaphis* O6 EPS (100 μg/ml). A subset of the seedlings was drought-stressed by withholding water. After 12 d of drought stress, the chlorophyll fluorescence in the leaf tissues was measured using a Phyto-PAM (Heinz-Walz GmbH, Effeltrich, Germany) as described previously (Woo et al.,
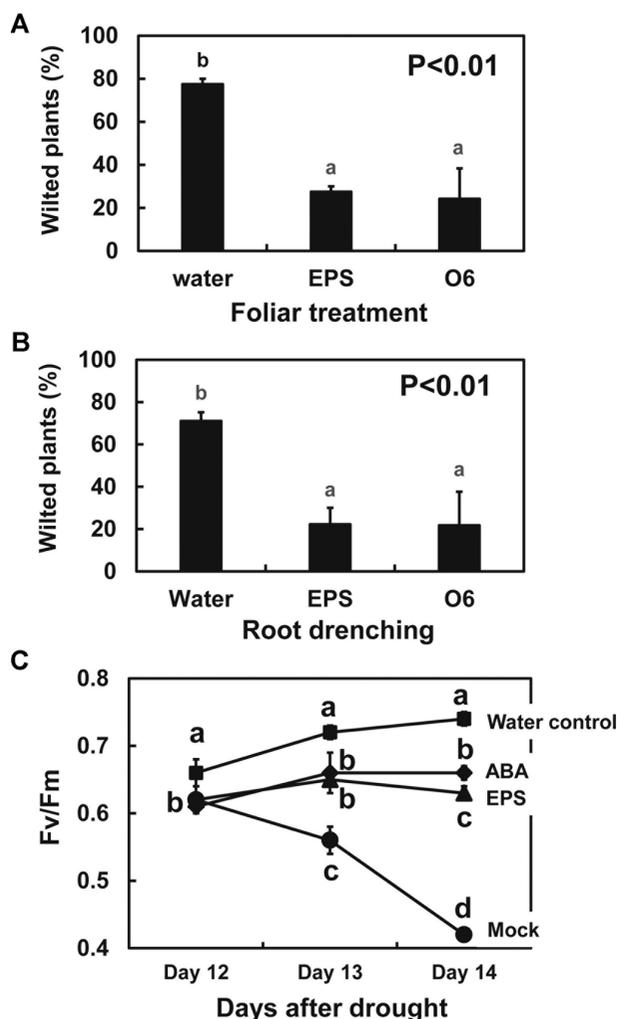
Fig. 2. Protective effects of *Pseudomonas chlororaphis* O6 and its EPS against drought stress in *Arabidopsis thaliana* Col-0 seedlings grown in MS agar. The seedlings leaves (A) and roots (B) were treated with water (Water) as a control, *P. chlororaphis* O6 cells (O6, 1×10^8 cfu/ml), or 100 µg/ml EPS. The extent of wilting of the plants deprived of water was assessed visually after 2 h. The study was performed three times with 12 plants per treatment. (C) Change in the Fv/Fm value of leaves of *A. thaliana* Col-0 plants raised in sterile soil mix. A subset of the plants had water withheld for 15 d whereas the control set had normal watering (water control). Treatments were water (mock), 100 µM ABA or EPS (100 µg/ml). The fluorescence measurements were recorded daily to compare the Fv/Fm values. The study was performed two times with 3 plants per treatment. The data presented are means and standard deviation from two studies. Different letters indicate significant differences between treated and control samples based on Duncan's multiple range test at $p<0.01$.

2008). The F_v/F_m was determined by the application of a single saturating pulse to dark-adapted leaves for 30 min and used as a quantitative measure of drought tolerance (Woo et al., 2008). The experiment was repeated twice with 3 plants

per treatment.

In plants grown in pot cultures that were maintained with normal hydration, the F_v/F_m value for the leaves increased daily, indicating healthy photosynthesizing leaves (Fig. 2B). In plants that were subjected to drought stress by withholding water, the F_v/F_m value steadily declined with the number of days of stress due to the impaired functioning of photosystem II. However, plants that were subjected to water withholding when treated with either ABA or EPS had leaves that showed no reduction in the F_m/F_v values (Fig. 2B). This response was expected, because the partial closure of the stomata maintained function of photosystem II. Thus, treatments of the intact plants with the EPS from *P. chlororaphis* was as effective as ABA in protecting the plant from reduced photosynthetic efficacy under drought stress.

The present study indicated that the ethanol-precipitated EPS from *P. chlororaphis* O6 were effective in inducing protection against drought, as measured by limited wilting and by the maintenance of photosynthetic efficacy upon water withholding in intact *Arabidopsis* plants. Leaf tissues exposed to the EPS showed stomatal closure eliciting the same plant response as 2R, 3R-butanediol (Cho et al., 2008, 2013). Consequently, we demonstrated that EPS from *P. chlororaphis* O6 is another plant immune effector activating drought tolerance.

The EPS are part of the matrix that embeds bacterial cells in biofilms (Anderson and Kim, 2018). The composition of EPS likely includes different polysaccharides and extracellular DNA (Hung et al., 2005; Wingender et al., 1999). *P. chlororaphis* O6 forms patchy but layered and complex biofilms on roots as part of the surface colonization process (Bonebrake et al., 2018). Timmusk et al. (2015), based on their work with root-colonizing Gram-positive bacteria that active IST, concluded that the biofilm formation is required in induction of drought tolerance. Thus, the EPS produced in the root surface biofilms may be part of the microbial "microbe-associated molecular patterns" (MAMPs) that activate plant immunity. Indeed, EPS from *Bacillus cereus* acts as a MAMP for stimulating plant disease resistance (Jiang et al., 2016). We are currently characterizing the components in the EPS from *P. chlororaphis* O6. An interesting question is whether alginate is present. Alginate present in the EPS from *P. aeruginosa*, xanthan from *Xanthomonas campestris*, and amylovoran from *Erwinia amylovora* suppress plant immunity by binding calcium, thus, preventing MAMP-induced signaling in the plant host (Aslam et al., 2008).

The EPS from *P. chlororaphis* O6 functions similarly to the EPS from other plant-associated bacteria, e.g., *Pantoea agglomerans*, *Burkholderia gladioli*, and *P. putida*, in the activation of defense mechanisms (Ortmann et al., 2006; Park et al., 2008; Sandhya et al., 2009; Santaella et al., 2008). These findings suggest that it is feasible to develop formulations containing EPS from certain plant-associated bacteria as "green formulations" to boost plant health during crop production. Such formulations would overcome the problem of requiring live microbial cells to induce stress tolerance. Applications of EPS-based formulations maybe especially valuable to aid in alleviating the stress imposed on crops by water shortage caused by higher temperatures and altered precipitation due to climate change.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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