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e-Xtra*

2R,3R-Butanediol, a Bacterial Volatile Produced by *Pseudomonas chlororaphis* O6, Is Involved in Induction of Systemic Tolerance to Drought in *Arabidopsis thaliana*

Song Mi Cho,[1] Beom Ryong Kang,[2] Song Hee Han,[3] Anne J. Anderson,[3] Ju-Young Park,[4] Yong-Hwan Lee,[4] Baik Ho Cho,[5] Kwang-Yeol Yang,[5] Choong-Min Ryu,[6] and Young Cheol Kim[5]

[1]Department of Floriculture, Chunnam Techno College, Jeonnam 516-911, Korea; [2]Jellanamdo Agricultural Research and Extension Services, Jeonnam 520-715, Korea; [3]Department of Biology, Utah State University, Logan 84322-5305, U.S.A.; [4]Department of Agricultural Biotechnology, Center for Fungal Genetic Resources, and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea; 5Department of Plant Biotechnology and Biotechnology Research Institute, Chonnam National University, Gwangju 500-757, Korea; [6]Systems Microbiology Research Center, KRIBB, Daejeon 305-600, Korea

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Root colonization of plants with certain rhizobacteria, such as *Pseudomonas chlororaphis* O6, induces tolerance to biotic and abiotic stresses. Tolerance to drought was correlated with reduced water loss in *P. chlororaphis* O6-colonized plants and with stomatal closure, indicated by size of stomatal aperture and percentage of closed stomata. Stomatal closure and drought resistance were mediated by production of 2R,3R-butanediol, a volatile metabolite of *P. chlororaphis* O6. Root colonization with bacteria deficient in 2R,3R-butanediol production showed no induction of drought tolerance. Studies with *Arabidopsis* mutant lines indicated that induced drought tolerance required the salicylic acid (SA)-, ethylene-, and jasmonic acid-signaling pathways. Both induced drought tolerance and stomatal closure were dependent on Aba-1 and OST-1 kinase. Increases in free SA after drought stress of *P. chlororaphis* O6-colonized plants and after 2R,3R-butanediol treatment suggested a primary role for SA signaling in induced drought tolerance. We conclude that the bacterial volatile 2R,3R-butanediol was a major determinant in inducing resistance to drought in *Arabidopsis* through an SA-dependent mechanism.

Additional keywords: abscisic acid, β-aminobutyric acid, guard cell, jasmonate, microbe-associated molecular patterns.

Plants possess elegant survival systems to protect themselves against environmental stresses, including drought. Adaptive responses include an increase in the plant hormone abscisic acid (ABA) (Schroeder et al. 2001; Zhu 2001), causing stomatal closure to minimize water loss through transpiration (Leung and Giraudat 1998). ABA mediates stomatal closure by coordinating events involving production of activated oxygen species, alkalization, and changes in Ca^{2+} and K^+ fluxes within the guard cells (Bright et al. 2006; Mori et al. 2001; Pandey et al. 2007). However, ABA does not act alone; other plant-signaling compounds, including methyl jasmonate (JA) (Evans 2003; Gomi et al. 2005; Liu et al. 2005; Suhita et al. 2004), salicylic acid (SA) (Manthe et al. 1992; Mori et al. 2001), and ethylene (Desikan et al. 2006), also are involved in regulating stomatal closure. For instance, SA modulates stomatal closure by interfering with ABA signaling (Rait et al. 1986). This may explain the role of SA in inducing tolerance to water stress in wheat (Bezrukova et al. 2001; Sharkirova and Bezrukova 1997) and muskmelon (Korkmaz et al. 2007).

Induced systemic tolerance to drought stress is observed in plants with roots colonized by bacteria and fungi. Root colonization by *Paenibacillus polymyxa* (Timmusk and Wagner 1999); by rhizobacteria expressing an ACC-deaminase, which lowers ethylene levels (Mayak et al. 2004); or by mycorrhizal fungi (Augé et al. 2007) enhances drought tolerance. Induction of drought tolerance also was observed with pretreatment with β-aminobutyric acid (BABA) (Ton et al. 2005). However, details of the mechanisms involved in inducing drought tolerance by microorganisms have not been determined.

In this article, we assessed whether root colonization by *Pseudomonas chlororaphis* O6 elicited tolerance to drought. Systemic affects from root colonization with this bacterium are already demonstrated through induction of resistance to bacterial leaf pathogens (Spencer et al. 2003). We determined whether the induced drought tolerance observed was related to a decrease in stomatal aperture. We examined whether treatments with the microbe-associated molecular patterns, such as flg22 and LPS (Melotto et al. 2006), and the priming agent, BABA, that induce stomatal closure also caused drought tolerance. Because induced disease resistance correlated with production of 2R,3R-butanediol by *P. chlororaphis* O6 (Han et al. 2006), we determined whether this volatile also induced stomatal closure and drought tolerance. To explore the roles of cell-signaling pathways in the plant in induced drought tolerance, we used *Arabidopsis* mutants impaired in appropriate signaling pathways. In addition, we investigated how root colonization by *P. chlororaphis* O6 and the direct application of 2R,3R-butanediol affected ABA and SA levels in *Arabidopsis*.

RESULTS

Root colonization with *P. chlororaphis* O6 induced systemic tolerance against drought stress.

Water withholding of *Arabidopsis* seedlings for 13 days showed that relative water content decreased to a greater ex-

Corresponding author: Y. C. Kim; Telephone: ++82-62-530-2071; Fax: ++82-62-530-2079; E-mail: yckimyc@chonnam.ac.kr

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tent in control plants compared with plants with roots colonized by *P. chlororaphis* O6 (Fig. 1A). When water was withheld for 16 days followed by a 2-day rehydration period, we observed that root colonization of *Arabidopsis* by *P. chlororaphis* O6 in creased their survival (Fig. 1B). Recovery rate upon rehydra tion of plants colonized by *P. chlororaphis* O6 was 95 ± 1% in contrast to the lower recovery of 9 ± 1% for the controls (Fig. 1B). Additionally, when leaflets were cut from plants grown with and without root colonization by *P. chlororaphis* O6 and were exposed to the air, weight loss in 2 h was 39 ± 3% for the colonized plants compared with 85 ± 5% of weight loss in noninoculated plants (data not shown). These studies demon strated that the plants with roots colonized by *P. chlororaphis* O6 were more tolerant to water withholding.

Root colonization by *P. chlororaphis* O6 reduced stomatal aperture.

The stomatal apertures for leaves of plants with roots colonized by *P. chlororaphis* O6 were compared with stomata from noncolonized (control) plants under conditions of adequate watering. Stomatal apertures of wild-type Col-0 on the leaves of plants grown without root colonization by *P. chlororaphis* O6 were 4.8 μm, whereas stomatal apertures of the strain O6-colonized plants were less than 3 μm (Fig. 2A). Increased stomatal closure was apparent 3 days after inoculation with *P. chlororaphis* O6 without drought stress. At 3, 5, 7, and 9 days post inoculation of *P. chlororaphis* O6, the stomates were 60 to 80% closed compared with 20 to 30% in the control plants (Fig. 2B). When water was withheld for 7, 8, and 11 days, a level of 60% closed stomates was observed in control plants

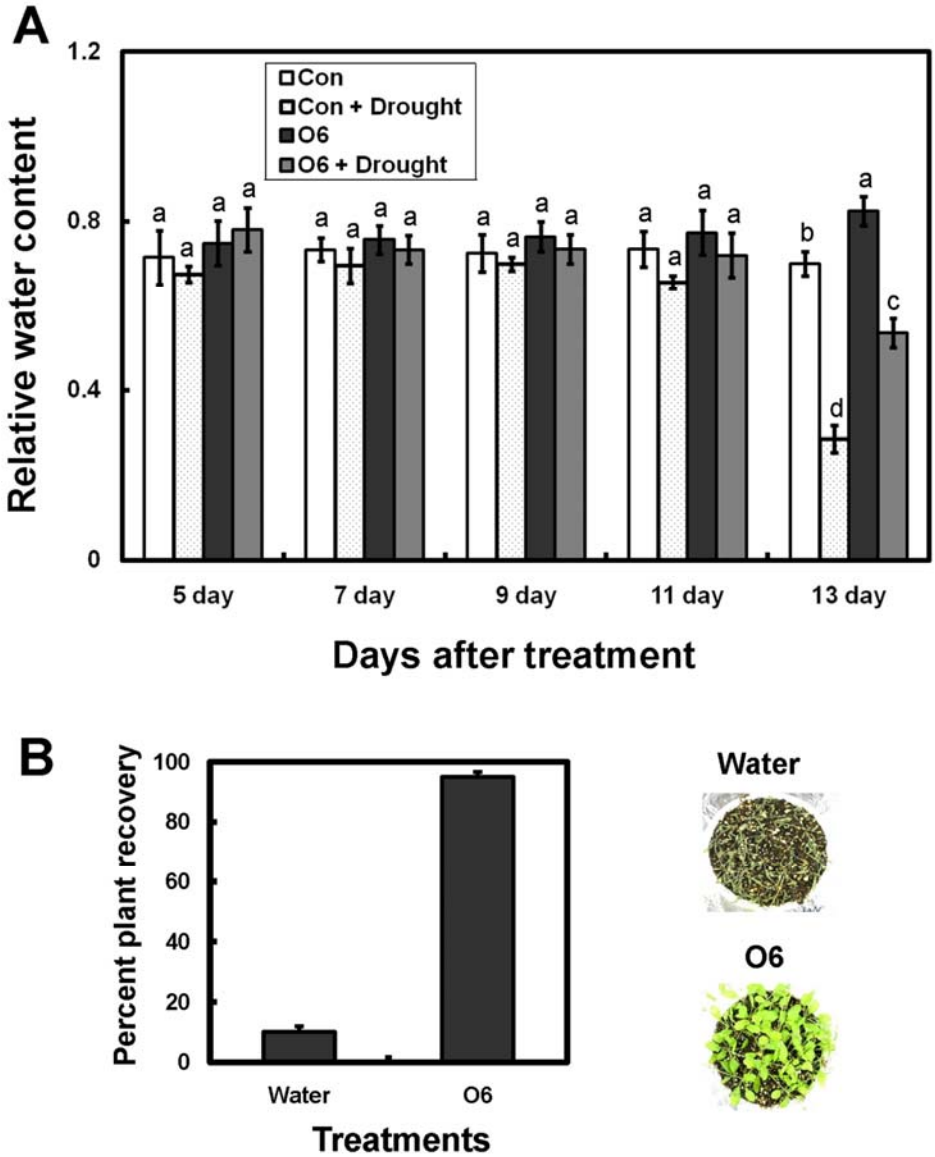

Fig. 1. A, Relative water content and **B,** induced drought resistance of *Arabidopsis* seedlings grown with roots colonized by *Pseudomonas chlororaphis* O6 (O6). **A,** Plants were grown in pots containing soil-less medium. Water was withheld from one set of plants, beginning at 7 days after root inoculation with *P. chlororaphis* O6 for the colonized and noncolonized plants (O6 + drought or Con + drought). Nondrought control plants had a regular watering schedule (Con or O6). After 5, 7, 9, 11, and 13 days, relative water content was assessed. Three independent experiments were performed from at least five leaflets randomly cut from 30 plants/treatment. Each data point represents the mean ± standard deviation from three replicate experiments. At day 13, data represented by different letters are statistically different at $P = 0.05$. **B,** Plants grown in soil-less medium were used for this study with or without colonization by *P. chlororaphis* O6. Water was withheld for 16 days before reapplication to simulate drought conditions. Percent plant recovery was assessed after 2 days of reapplication. The data are the means of three independent studies, with at least 100 plants/treatment. Representative photographic images of plants following 16 days of induced drought and 2 days of rehydration are shown.

(Fig. 2C). *P. chlororaphis* O6-elicited stomatal closure also was apparent in the plants from which water was withheld; the proportion of closed stomates increased to 70 to 80% in the strain O6-colonized plants (Fig. 2C).

Induced stomatal closure is correlated with resistance to drought.

We determined whether stomatal closure induced by other treatments also correlated with induced drought tolerance. Treatments with the MAMPs (flg22 or LPS), ABA, and the priming agent, BABA, caused stomatal closure in the epidermal peels (Fig. 3A). Assessment, upon water withholding, of the survival of seedlings exposed to the same treatments showed increased survival for all plants (Fig. 3B). Between 60 and 80% plants treated with either the MAMPs or *P. chlororaphis* O6 survived under water withholding; survival for the plants without treatment or treated with mock solutions was lower (10 to 20%) (Fig. 3B). These results implicate a correlation between induced drought tolerance and induced stomatal closure.

Bacterial production of 2R,3R-butanediol is a major factor in induced drought tolerance.

We examined whether 2R,3R-butanediol was involved in induction of drought tolerance. Direct application of authentic

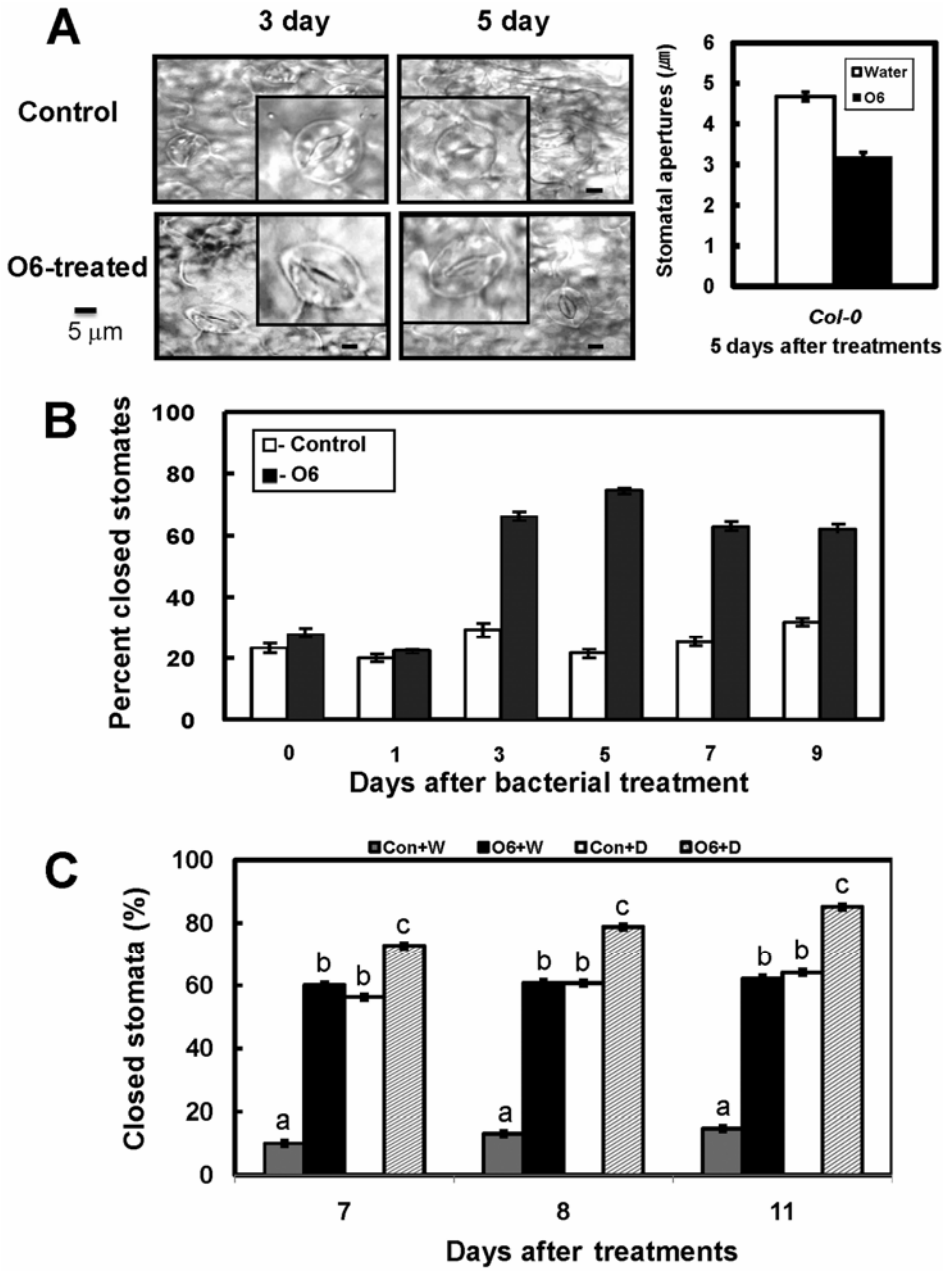

Fig. 2. Effect of root colonization of *Pseudomonas chlororaphis* O6 on stomatal closure of *Arabidopsis thaliana* Col-0. **A,** Colonization of *Arabidopsis thaliana* Col-0 by *P. chlororaphis* O6 (O6) decreases stomatal apertures and **B,** increases the proportion of closed stomates under normal growth conditions with adequate water in plants grown with and without colonization by *P. chlororaphis* O6 in soil-less medium. The apertures of at least 100 stomata/plant were counted and five plants per treatment were used. Each data point represents the mean ± standard error from three experiments. **C,** Stomatal closure in wild-type *Arabidopsis* in response to drought in plants with (O6) or without (Con) root colonization by *P. chlororaphis* O6. The plants were grown in pots containing soil-less medium for 2 weeks. Water was withheld from one set of plants (D) for 11 days (beginning 7 days after root treatments). Stomatal closure was measured by microscopy at days 7, 8, and 11. At least 100 stomata/plant were counted from five plants per treatment. Differences in letters indicate a significant difference among treatments based on Duncan's multiple range test at $P = 0.05$. Data are the means ± standard deviations from each treatment.

2R,3R-butanediol to epidermal peels caused stomatal closure, to less than 3 μm, and its application to plants increased the proportion of closed stomates approximately twofold (Fig. 4). Additionally, 2R,3R-butanediol treatment of *Arabidopsis* plants increased survival rate from 20% to over 60% after water withholding (Fig. 4). Thus, 2R,3R-butanediol caused the same effects as root colonization with *P. chlororaphis* O6.

To provide more evidence that 2R,3R-butanediol production during root colonization was a factor mediating *P. chlororaphis* O6-induced drought resistance, we examined the drought tolerance response in plants colonized with bacterial strains deficient in butanediol production. At present, we do not have mutants in *P. chlororaphis* O6 with deletions in the biosynthetic genes for 2R,3R-butanediol production. However, 2R,3R-butanediol production in *P. chlororaphis* O6 was deficient in a *gacS* mutant (Han et al. 2006). Additionally, we utilized *Bacillus subtilis* 168 mutants BSIP1173 and BSIP1174, with deficiencies in the 2R,3R-butanediol biosynthetic genes (Ramos et al. 2000), as well as mutant BSIP1171, that overproduced 2R,3R-butanediol. The wild-type *B. subtilis* 168 strain and its mutants were equally rhizosphere competent (Ryu et al. 2004). Only strains that produced 2R,3R-butanediol (wild-type and the complemented *gacS* strains of *P. chlororaphis* O6, and the wild-type and overproducing strains of *B. subtilis* 168) induced drought tolerance above noninoculated control plants (Fig. 5).

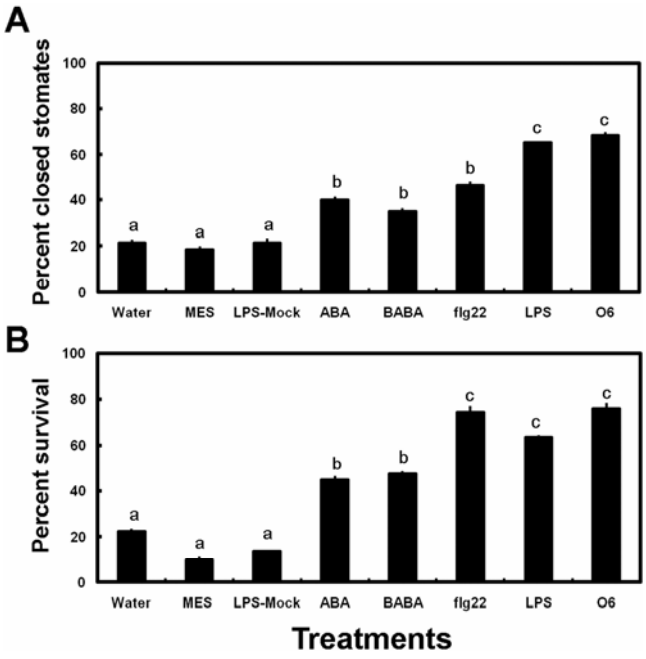

Fig. 3. A, Stomatal closure in epidermal peels of wild-type *Col*-0 plants and **B,** induced drought resistance in seedlings. **A,** Treatments were applied in 1-ml aliquots to epidermal peels prepared from plants grown for 2 weeks in well plates. The treatments were water or 2-N-morpholinoethanesulfonic acid (MES) buffer (as controls), 10 μM abscisic acid (ABA), 300 μM β-aminobutyric acid (BABA), 5 μM flg22, 100 ng/μl LPS, or *Pseudomonas chlororaphis* O6 cells (O6, 1×10^8 CFU/ml). After 2 h, stomata were observed microscopically to determine the percentage of stomates that were closed. At least 100 stomata each for five plants per treatment were examined. Each data point represents the mean ± standard error for three experiments. Data showing the same letter are not significantly different at $P = 0.05$. **B,** Induced drought tolerance was assessed in seedling grown on Whatman filter paper overlaid onto one-half-strength Murashige and Skoog solid medium. Two-week-old seedlings were treated as described in A and, after 3 days, the plants were exposed to drought stress by transferring them to open petri dishes. The survival of the seedlings was assessed after 2 h. Data showing the same letter are not significantly different at $P = 0.05$.

Involvement of ABA-, SA-, JA-, and ethylene-signaling pathways in induced drought tolerance.

We examined induced resistance after *P. chlororaphis* O6 colonization in *Arabidopsis* cell lines deficient in one component of the SA-, JA-, or ethylene-regulated pathways (Fig. 6A). Response variation between these lines was not due to significant differences in colonization levels of *P. chlororaphis* O6 on the roots; a colonization level of approximately 10^7 CFU/g fresh root weight was observed for all plants (data not shown).

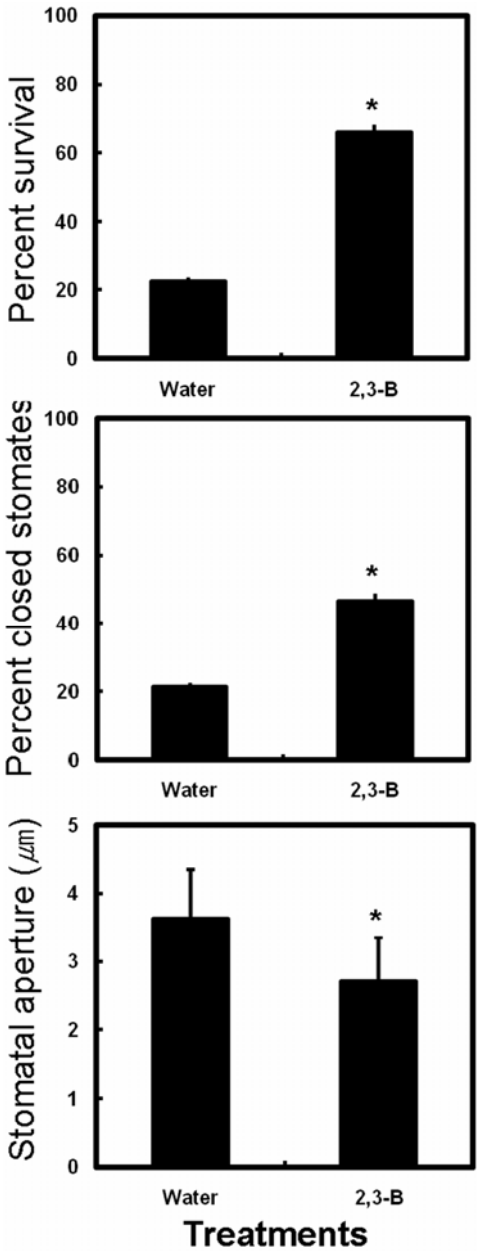

Fig. 4. Treatment with 2R,3R-butanediol (2,3-B) increased survival upon water withholding, increased the percentage of closed stomata, and altered stomatal apertures in *Arabidopsis* Col-0. *Arabidopsis* plants were grown on sterile Whatman filter paper overlaid onto one-half-strength Murashige and Skoog solid medium for 2 weeks. Three days after 2R,3R-butanediol treatment of their roots, one set of plants was used to assess tolerance to drought by transferring plants to open petri dishes and observing the extent of wilting after 2 h. For stomatal studies, epidermal peels were treated with 100 μM 2R,3R-butandiol or water as a control. After 2 h, at least 100 stomata/treatment were observed to assess width of the stomatal aperture and the percent of closed stomata. Each data point is the mean of two experiments, and the asterisk indicates a significant difference between conditions according to the Student's *t* test at $P = 0.05$.

P. chlororaphis O6 root colonization did not induce systemic drought tolerance in the JA-insensitive mutant (*jar1-1*) (Fig. 6A). In addition, no induction was observed in the SA pathway mutants: *nahG*, deficient in SA accumulation (Ryals et al. 1996) (Fig. 6A) or *eds16-1*, altered in SA-signaling (Wildermuth et al. 2001) (data not shown). Similarly, root colonization by *P. chlororaphis* O6 did not increase survival in two ethylene-insensitive lines, *etr1-1* and *ein2-1* (Alonso et al. 1999; Guo and Ecker 2004). However, the mutant line *eir1-1,* deficient in an auxin-efflux carrier in the plant roots (Sieberer et al. 2000), displayed increased tolerance to drought stress when *P. chlororaphis* O6 colonized the roots of the mutant line. Drought tolerance also was observed when *P. chlororaphis* colonized the roots of the *ctr1-1* line exhibiting a constitutive ethylene response (Gao et al. 2003). Treatments of plant lines defective in ethylene, SA, and JA signaling with 2R,3R-butanediol did not induce drought tolerance (Fig. 6C). These results demonstrate that *P. chlororaphis* O6-mediated drought tolerance involved the SA, JA, and ethylene pathways.

To investigate the role of ABA in induced drought tolerance, we utilized two *Arabidopsis* mutants lines with reduced responses to ABA for stomatal closure: *aba1*, impaired in ABA synthesis (Barrero et al. 2005; Merlot et al. 2002), and *ost1-1,* deficient in an ABA-response kinase (Merlot et al. 2002; Mustilli et al. 2002). Without bacterial root colonization, the *aba-1* mutant showed reduced drought tolerance compared with its parental line and *P. chlororaphis* O6-colonization did not increase drought tolerance (Fig. 6B). Similarly, the *ost1-1* line showed no increased tolerance (Fig. 6B). However, *P. chlororaphis* O6 root colonization induced drought tolerance in ABA-insensitive mutant lines *abi3* and *abi4*, similar to the wild-type *Arabidopsis* ecotypes (Fig. 6B).

Drought tolerance induced with *P. chlororaphis* O6 and 2R,3R-butanediol is correlated with increased accumulation of SA but not ABA.

As anticipated, ABA levels in noncolonized plants were increased in response to drought stress. The ABA content was 2 ng/g fresh weight in plants grown under normal conditions with regular watering, whereas the ABA content was increased to 18 and 28 ng/g fresh weight after 13 and 15 days of water withholding, respectively (Fig. 7). Plants with roots colonized by *P. chlororaphis* O6 even after 13 days of water withholding displayed ABA levels similar to levels of the colonized plants without drought stress. At 15 days of water withholding, ABA levels increased to 16 ng/g fresh weight in the colonized plants but the levels were significantly less than those in the noncolonized plants (Fig. 7).

Root colonization by *P. chlororaphis* O6 increased free SA approximately fourfold when the plants were grown with adequate water (Table 1). Upon water withholding for 13 and 15 days, the SA level was increased approximately twofold in the control plants (Table 1); this level was still lower than in the *P. chlororaphis* O6-colonized plants. The SA levels in the O6-colonized plant did not change significantly upon prolonged water withholding (Table 1). 2R,3R-butanediol also affected SA levels; in plants treated with 100 μM 2R,3R-butanediol followed by water-withholding, SA levels were 1,665 and 2,014 ng/g fresh weight (at day 3 and 7 water withholding, respectively) compared with values of less than 100 ng/g fresh weight in plants without 2R,3R-butanediol treatment.

DISCUSSION

Root colonization with *P. chlororaphis* O6 induced tolerance to drought stress that correlated with reduced water loss by transpiration, as mediated by a decrease in stomatal aperture size and increased proportions of closed stomates on the leaf. Decreased stomatal opening was detectable as early as 3 days after root colonization with *P. chlororaphis* O6. Our findings paralleled the anticipated effects of treatment with ABA, we also demonstrated that treatment with bacterial surface MAMPs flg22 and LPS (Melotto et al. 2006; Underwood et al. 2007) induced drought tolerance.

Drought tolerance induced by *P. chlororaphis* O6 was systemic because colonization of aerial tissues has not been detected with this bacterium. However, we demonstrated a significant role in induced drought tolerance for the production of 2R,3R-butanediol by the root-associated *P. chlororaphis* O6 cells. Because 2R,3R-butanediol was a metabolite emitted from *P. chlororaphis* O6 when it colonized roots (Han et al. 2006), we propose that 2R,3R-butanediol represents another class of effectors of plant innate immunity. Whether additional factors from *P. chlororaphis* O6 also influenced induced systemic drought tolerance is under investigation.

Stomatal closure is regulated by a complex hormonal net work, with a requirement for ABA and the involvement of SA, ethylene, and methyl jasmonate (Desikan et al. 2006; Evans 2003; Leung and Giraudat 1998; Mori et al. 2001; Pandey et al. 2007; Suhita et al. 2004). We found that induction of drought tolerance by root colonization of *P. chlororaphis* O6 or applications of 2R,3R-butanediol required functional SA-, JA-, and ethylene-mediated responses. Thus, our results also support the findings that MAMP-induced stomatal closure required a functional SA pathway (Melotto et al. 2006; Tsuda et al. 2008). We observed levels of SA but not ABA to be modulated in *Arabidopsis* by root colonization with *P. chloro raphis* O6. An SA-dependent pathway has been implicated in both stomatal closure and tolerance to drought in other plants (Bezrukova et al. 2001; Manthe et al. 1992; Mori et al. 2001; Rait et al. 1986).

Drought tolerance induced by root colonization of *P. chlororaphis* O6 required the plant to respond to ABA with stomatal closure; both *aba-1* and the *ost1-1* mutant lines showed no enhanced tolerance to drought upon *P. chlororaphis* O6-root colonization. Melloto and associates (2006) also found that

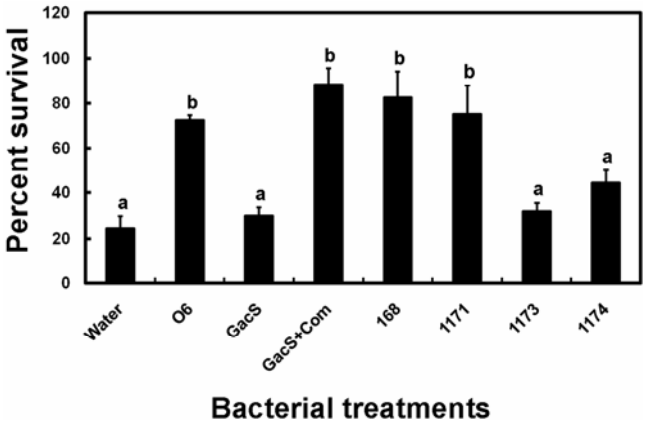

Fig. 5. 2R,3R-butanediol production from rhizobacteria is essential for induced drought resistance in plants. *Arabidopsis* plants were grown on sterile Whatman filter paper overlaid onto one-half-strength Murashige and Skoog solid medium for 2 weeks. Roots of the seedlings were treated with water as a control or cell suspensions of wild-type *P. chlororaphis* O6, the complemented *gacS* mutant (GasS+Com), or the *gacS* mutant (GacS), as well as wild-type 2R,3R-butandiol-producing *Bacillus* sp. 168 (168), the 2R,3R-butanediol-overproducing mutant BSIP1171 (1171), and the nonproducing mutants BSIP1173 (1173) and BSIP1174 (1174). Three days after root treatments, the plants were exposed to drought stress by transferring the plants to open petri dishes. The extent of wilting was assessed after 2 h. The data are the means from three independent studies, with at least 30 plants/treatment. Data showing the same letter are not significantly different at $P = 0.05$.

stomatal closure by application of flg22 and LPS required the Ost-1 kinase. The induction of drought tolerance in the ABA-insensitive lines *abi-3* and *abi-4* was consistent with the concept that the ABA-related transcription factors deficient in these lines are not involved in guard cell functions (Soderman et al. 2000). Because the OST1 kinase is upstream of the production of reactive oxygen species (ROS) (Mustilli et al. 2002), we are investigating the roles of ROS in induced drought tolerance by root colonization of *P. chlororaphis* O6 or application of 2R,3R-butanediol. Interestingly, we find that 2R,3R-butanediol induced the production of hydrogen peroxide and nitric oxide in the guard cells (*unpublished data*).

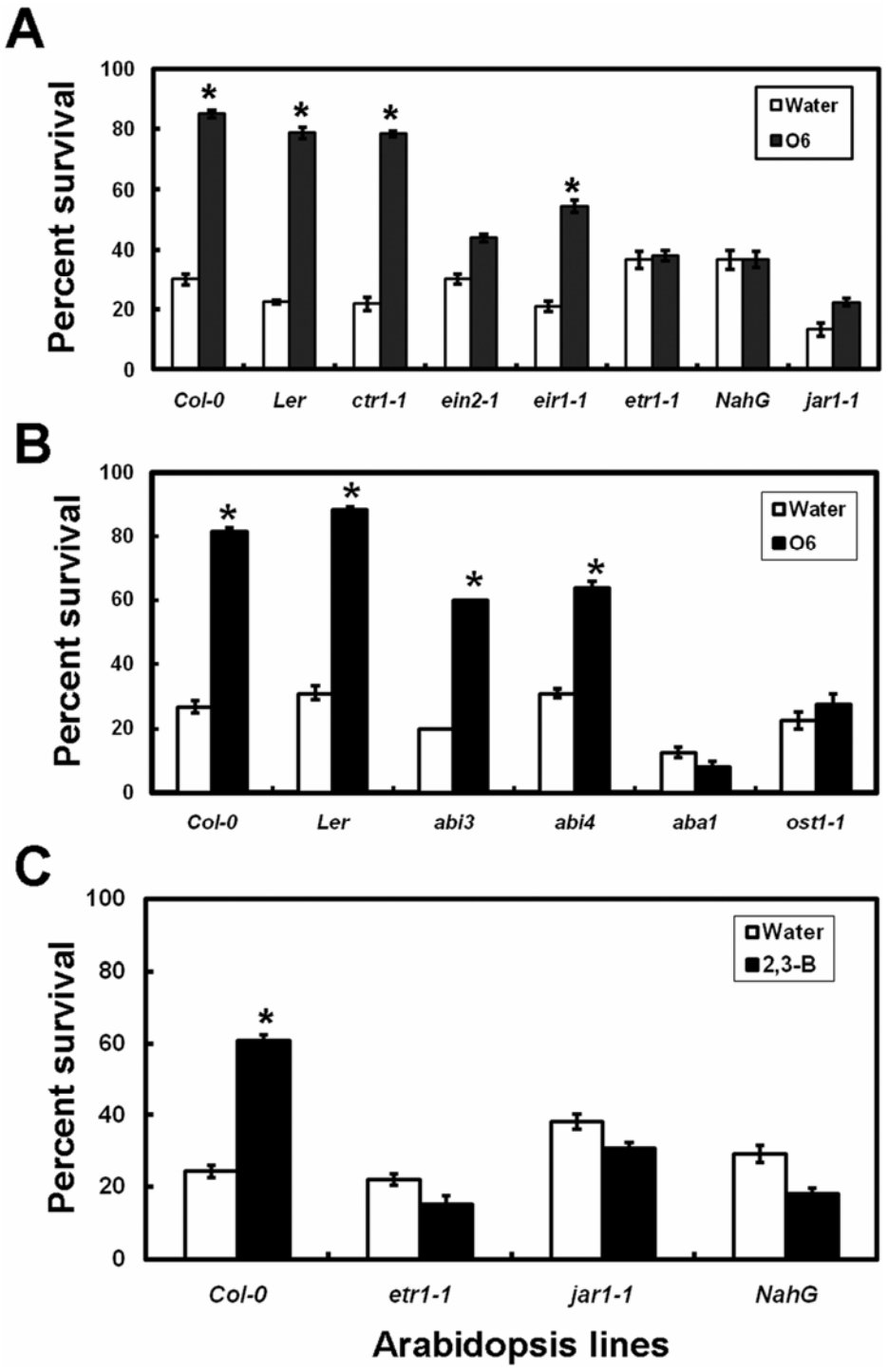

Fig. 6. Induced drought resistance in *Arabidopsis* wild-type and mutants by root colonization with *Pseudomonas chlororaphis* O6 or treatment with 2R,3R-butanediol. **A,** *Arabidopsis* lines altered in the salicylic acid (SA), ethylene and jasmonate (JA) pathways. **B,** *Arabidopsis* lines altered in abscisic acid (ABA)-related functions. **C,** Treatment with 2R,3R-butanediol. *Arabidopsis* plants were grown on sterile Whatman filter paper overlaid onto one-half-strength Murashige and Skoog solid medium for 2 weeks. The roots were inoculated with sterile water as a control (water) or *P. chlororaphis* O6 (O6) for studies A and B. After 1 week, the plants were drought stressed by transferring them to open petri dishes. In study C, plants were treated with 100 μM 2R,3R-butanediol (2,3-B) or water as a control and transferred to open petri dishes after 3 days. Plants that wilted within 2 h were considered drought sensitive. Three independent experiments were performed, with at least 25 plants/treatment. Asterisks indicate survival rates that were significantly different from the control-inoculated plants according to Duncan's multiple range test at $P = 0.05$.

In conclusion, this study provided evidence implicating a microbial volatile, 2R,3R-butanediol, in induced drought resistance. The production and great effect on plants of 2R,3R-butanediol during root colonization by *P. chlororaphis* O6 might indicate that plant performance is influenced significantly by saprophytic microbes and the compounds they produce.

MATERIALS AND METHODS

Chemicals.

Authentic chemicals were used at the following concentrations: 5 μM flg22 peptide (Alpha Dignostics, Inc., San Antonio, TX, U.S.A.), 100 μM butanediol (Sigma-Aldrich, St. Louis), 10 μM ABA (Sigma-Aldrich), and 300 μM BABA (Sigma-Aldrich), dissolved in 25 mM 2-N-morpholino-ethanesulfonic acid (MES)-KOH buffer (pH 6.15) with 10 mM KCl. LPS (100 ng/μl) from *P. aeruginosa* (Sigma-Aldrich) was used in MES-KOH buffer with 0.25 mM $MgCl_2$ and 0.1 mM $CaCl_2$.

Plant materials and growth conditions.

Parental *Arabidopsis thaliana* ecotypes Columbia (Col-0) and Landsberg erecta (Ler), were obtained from the Ohio State University Stock Center (Ohio State University, Columbus). Mutant lines used were as follows: *eir1* (auxin transport defi cient and ethylene insensitive) (Luschnig et al. 1998), *ein2* (ethylene insensitive) (Alonso et al. 1999)], *etr1* (ethylene insensitive) (Chang et al. 1993) (Ohio State University Stock Center), *abi3-1* (ABA insensitive, acidic domain transcription factor), *abi4-1* (ABA insensitive, APETALA2 domain tran scription factor) (Salk Institute, La Jolla, CA, U.S.A.), *nahG* (transgenic line that degrades salicylic acid, provided by J. W. Ryals, Paradigm Genetics, Research Triangle Park, NC, U.S.A.), *jar1-1* (insensitive to jasmonic acid and methyl jas monic acid, provided by X. Dong, Duke University; Durham, NC, U.S.A.), *ost1-1* (deficient in the protein kinase mediating stomatal regulation in response to drought, provided by S. Merlot, ISV-CNRS, France), *eds16-1* (SA-deficient mutant, provided by M. C. Wildermuth, University of California, Berkeley, U.S.A.), and *aba1* (mutant with reduced ABA syn thesis) (Kang et al. 2002). The *abi2*, *abi4*, *ctr1-1*, *eds16-1*, *etr1*, *eir1-1*, *ost1-1*, *nahG*, and *jar1-1* mutants had a *Col*-0 background, while *aba1*, *abi3*, and *ein2-1* were in the *Ler* eco type.

Plants were grown on agar or in a soil-less mixture. All seed were surface sterilized by soaking in 70% ethanol for 2 min and 1% sodium hypochlorite for 20 min. After an extensive wash with sterile distilled water, the seed were incubated in sterile water for 3 days in the dark at 4°C to promote germination.

For growth in microtiter plates (12-well plates; SPL Lifescience Co., Pecheon, Korea), single germinated seed were placed onto medium containing half-strength Murashige and Skoog salt (MS) medium (Murashige and Skoog 1962), consisting of 0.3% Phytagel and 3% sucrose adjusted to pH 5.7. Where indicated, seed were transferred to the surface of sterile Whatman no. 1 filter paper placed over the agar. Plates were sealed with Parafilm to prevent drying, and no additional water was supplied during the experiment.

Other seed were planted into sterile soil-less medium (peat moss:vermiculite/perlite, 7:3:3, vol/vol) using 500 cm^3 of medium for each 10.5-by-10.5-by-9-cm pot, with 15 to 30 seeds/pot. These pots were watered with 20 ml of sterile water every 2 days. Seedlings were grown with a cycle of 16 h of light and 8 h of darkness under 40-W fluorescent lights (2,000 lux, 80 μmol photons m^{-2} s^{-1}). The temperature was maintained at 22 ± 1°C with a relative humidity of 50 to 60%.

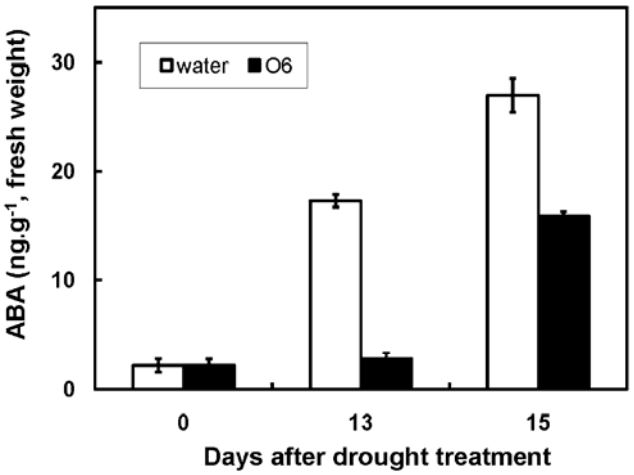

Fig. 7. Abscisic acid (ABA) concentration in *Arabidopsis* leaves from plants grown with and without root colonization by *Pseudomonas chlororaphis* O6. *Arabidopsis* plants were grown in sterile soil-less medium for 2 weeks before inoculation with *P. chlororaphis* O6 or water as a control. After 7 days, water was withheld from all plants for 13 and 15 days. ABA was extracted from leaves and ABA levels were measured. Data are the mean ± standard deviations of two replicates, with 30 plants/treatment.

Bacterial strains and culture conditions.

The strains used in this study were *P. chlororaphis* O6 and its *gacS* mutant (Spencer et al. 2003). Other bacteria included *B. subtilis* 168 wild type that produced 2R,3R-butanediol; an overproducing mutant, BSIP 1171; and two 2R,3R-butanediol-deficient mutants, BSIP 1173 and BSIP 1174, provided by D. Jahn (Braunschweig University, Braunschweig, Germany) (Ramos et al. 2000). For storage, bacteria were maintained at –80°C in Luria-Bertani broth containing 20% glycerol. For experimental use, all bacteria were streaked onto King's medium B (KB) agar plates with 2% agar and incubated at 28°C for 2 days. To prepare inocula, bacteria were grown overnight in KB broth, pelleted by centrifugation at 10,000 × *g* for 10 min, washed once with sterile water, and suspended to 1×10^8 CFU/ml. The *Bacillus* isolates were grown on tryptic soy agar plates (Difco Laboratories, Detroit) containing 2% agar.

Measurement of transpiration rate and water status.

To measure water status, rosette leaves were cut from pot-grown plants at 3 to 13 days after root treatments (inoculation

Table 1. Free salicylic acid (SA) levels in whole *Arabidopsis* plants, grown with and without root colonization by *Pseudomonas chlororaphis* O6, and with or without water withholding[a]

	SA concentrations (ng/g fresh weight) at days after water withholding[a]					
	0 days		13 days		15 days	
Levels of	Water	O6	Water	O6	Water	O6
Free SA	40 ± 5 a	171 ± 18 c	97 ± 36 b	191 ± 17 c	80 ± 1 ab	171 ± 13 c

[a] Roots of plants grown in soil-less medium were inoculated with *P. chlororaphis* O6 (O6) or sterile water (Water; no colonization). After 7 days, water was withheld from test plants for 13 and 15 days before extraction to determine free SA levels. The data are means ± standard deviations of two replicates each, with 30 plants/replicate. Numbers followed by different letters indicate significant differences between colonized and noncolonized growth conditions based on Duncan's multiple range test at $P = 0.05$.

with *P. chlororaphis* O6 or water, the control). Fresh weight (FW) was recorded, and dry weight (DW) was determined after drying the tissues for 2 h in an 80°C oven. For each leaf, the fully turgid weight (FTW), defined as the weight of the leaf following incubation at 100% humidity at room temperature and overnight, was recorded. The relative water content (RWC) was calculated according to the equation RWC = (FW – DW)/(FTW – DW) (Mayak et al. 2004). Three independent experiments were performed, each involving at least five leaflets cut randomly from each of 30 plants per treatment.

Measurement of induced systemic drought tolerance.

The roots of 2-week-old pot-grown *Arabidopsis* seedlings were inoculated with 35 ml of *P. chlororaphis* O6 suspended to 1×10^8 CFU/ml. As a control, sterile water was applied to the roots. Seven days after root treatment, pot-grown plants were exposed to drought stress by withholding water for 16 days, followed by rehydration with sterile water for 2 days to determine seedling survival. Three independent experiments were performed, with at least 100 plants per treatment.

A second method to study induced drought tolerance involved plants grown on the filter papers in well plates. One week after root treatments, the plants 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 study was performed three times with 12 plants per treatment.

Observation of stomatal closure.

A. thaliana plants were grown in pot cultures and colonized as described above for 1 week. The plants were divided into two groups; one group was exposed to drought stress by withholding water and the other group was watered with 20 ml of sterile water every 2 days. To maximize stomatal opening, plants were maintained under light (100 $\mu E/m^2/s$) for at least 3 h before microscopic analysis. The leaves were stained with 0.5% safranin O (Sigma-Aldrich) for 0.5 to 1 min and washed three times with sterile distilled water. The samples were observed under light microscopy (Zeiss Model DE/AXIOLAB-POI, Gena, Germany) and imaged (Image-Pro plus 4.5.1; Media Cybernetics Inc., Silver Spring, MD, U.S.A.). Stomatal aperture size was measured using the software Image-Pro (version 2.32 for Windows; Cybernetics, Inc.). At least 100 stomata/plants were counted, with each experimental replication consisting of five plants per treatment.

We also observed the stomatal aperture in excised epidermal peels from fully expanded leaves. The peels were collected and transferred onto glass slides, with the cuticle side in contact with water or MES buffer (as controls), solutions of the experimental chemicals, or aqueous suspensions of bacterial cells, when indicated. Images were taken across the peels as described above, and the width of the stomatal aperture was measured using the software Image-Pro (version 2.32 for Windows). At least 100 stomata/plants were counted, with five plants per treatment used in each experimental replicate.

Measurements of ABA and SA contents.

ABA and SA contents were determined from plants grown with and without root colonization by *P. chlororaphis* O6 and with and without water withholding. ABA extraction and measurement were performed as previously described (Arenas-Huertero et al. 2000). Briefly, leaves were homogenized in 1 ml of buffer (10 mM HCl and 1% [wt/vol] polyvinyl polypyrrolidone in methanol) and extracted for 14 h at 4°C. The supernatant was collected after centrifugation, and 15 µl of 1 M NaOH/ml was added to neutralize the pH. ABA content was quantified using the Phytodeteka-ABA protocol (Agdia Inc., Elkhart, IN, U.S.A.) but with the addition of Tris-buffered saline and 0.1% gelatin to all samples. ABA levels were standardized according to the mass of the leaves from which they were measured.

SA was determined at various time points in plants grown in soil-less medium. Plants were inoculated with *P. chlororaphis* O6, or treated with water as a control, 1 week before half the plants were subjected to water withholding. Other plants were treated with 100 µM 2R,3R-butanediol before water withholding. Free SA was measured in whole-plant extracts by high-performance liquid chromatography (HPLC), using a modification of methods described previously (Bowling et al. 1994). The seedlings (0.5 g of fresh weight) were frozen in liquid nitrogen, ground to a fine powder, and sequentially extracted in 3 ml of 90 and 100% methanol. The combined methanol extracts were vacuum dried, resuspended in 250 µl of 20% methanol, vortexed, sonicated for 5 min, and filtered through a 0.22-µm nylon filter. SA levels were determined by fluorescence (excitation 301 nm, emission 412 nm) after separation on a C18 reverse-phase HPLC column (Waters Inc., Milford, MA, U.S.A.), using authentic SA as a standard. Briefly, the column was maintained at 40°C and equilibrated in 0.5% glacial acetic acid/methanol (75:25, vol/vol), with a flow rate of 1.5 ml/min. Three minutes after injection, a methanol gradient (25 to 60%) was applied over 7 min, after which the methanol concentration was restored to 25%. All data were standardized for SA recovery efficiency, as determined by samples spiked with a known concentration of SA.

Statistical analysis.

Data were analyzed by analysis of variance using JMP 4.0 software (SAS Institute; Cary, NC, U.S.A.). The significance of the observed effects was determined by Duncan's multiple range test ($P = 0.05$).

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