

Induction of defense responses in tomato against *Pseudomonas syringae* pv. *tomato* by regulating the stress ethylene level with *Methylobacterium oryzae* CBMB20 containing 1-aminocyclopropane-1-carboxylate deaminase

Pandiyani Indiragandhi · Rangasamy Anandham · Kyounga Kim · Woojong Yim · Munusamy Madhaiyan · Tongmin Sa

Received: 28 June 2007 / Accepted: 21 September 2007 / Published online: 11 October 2007
© Springer Science+Business Media B.V. 2007

Abstract This study aimed to examine the induction of defense responses in tomato elicited by *Methylobacterium oryzae* CBMB20 as a consequence of reduced stress ethylene level possibly through its ACC deaminase activity. Significantly increased activities of pathogenesis-related (PR) proteins and defense enzymes such as β -1,3-glucanase, phenylalanine ammonia-lyase, peroxidase and polyphenol oxidase were noted in *M. oryzae* CBMB20 pretreated and challenged with *Pseudomonas syringae* pv. *tomato* (Pst) compared to either control or *M. oryzae*-treated tomato plants in both growth chamber and greenhouse conditions. Increased PR proteins and defense enzyme activities were correlated with the reduction of stress ethylene level. *M. oryzae* CBMB20 reduced the stress ethylene level about 27% and 55% when challenged with Pst, in growth chamber and greenhouse on day 7 respectively and the effect was comparable to that of the chemical ethylene biosynthesis inhibitor AVG, L- α -(2-aminoethoxyvinyl)-glycine hydrochloride. As a consequence of reduced stress ethylene level and its effect on defense response in crop plants, the disease severity was reduced 26% in *M. oryzae* CBMB20-treated plants challenged with pathogen. Therefore, inoculation of *M. oryzae* CBMB20 would induce the defense enzymes and contribute to the enhanced resistance of tomato plants against the pathogen Pst.

Keywords *Methylobacterium oryzae* · Stress ethylene · Defense enzymes · *Pseudomonas syringae* pv. *tomato*

Introduction

Tomato (*Lycopersicon esculentum* Mill L.) is one of the most important vegetable crops of South Korea, cultivated over a 5,624 ha area under greenhouse conditions. Ninety nine percent of the tomatoes produced in Korea are exported to Japan (Ministry of Agriculture, Nature and Food quality, Republic of Korea 2006). Bacterial speck disease caused by *Pseudomonas syringae* pv. *tomato*, decreases production under greenhouse conditions despite its moderate economic importance to tomato production (Bashan and de-Bashan 2002). Earlier studies have demonstrated the efficacy of chemical sprays against bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato*. However, use of agrochemicals to combat plant pathogens is environmentally and economically not benign, in addition to causing resistance development in pathogens and reduction in the beneficial organism population (Glick and Bashan 1997). Therefore, biological approaches as an alternative strategy for the use of synthetic chemicals to manage diseases of crop plants are receiving more attention.

Bio-control agents, mainly bacterial inoculants are believed to induce systemic defense responses in the plants besides other mechanisms including direct antagonism, antibiosis and siderophore production. Induction of defense responses by plant-growth-promoting bacteria (PGPB) is largely associated with the production of pathogenesis-related (PR) proteins like β -1,3-glucanase and the defense enzyme phenylalanine ammonia-lyase (PAL 4.3.1.5) and oxidative enzymes like peroxidase (PO) and polyphenol

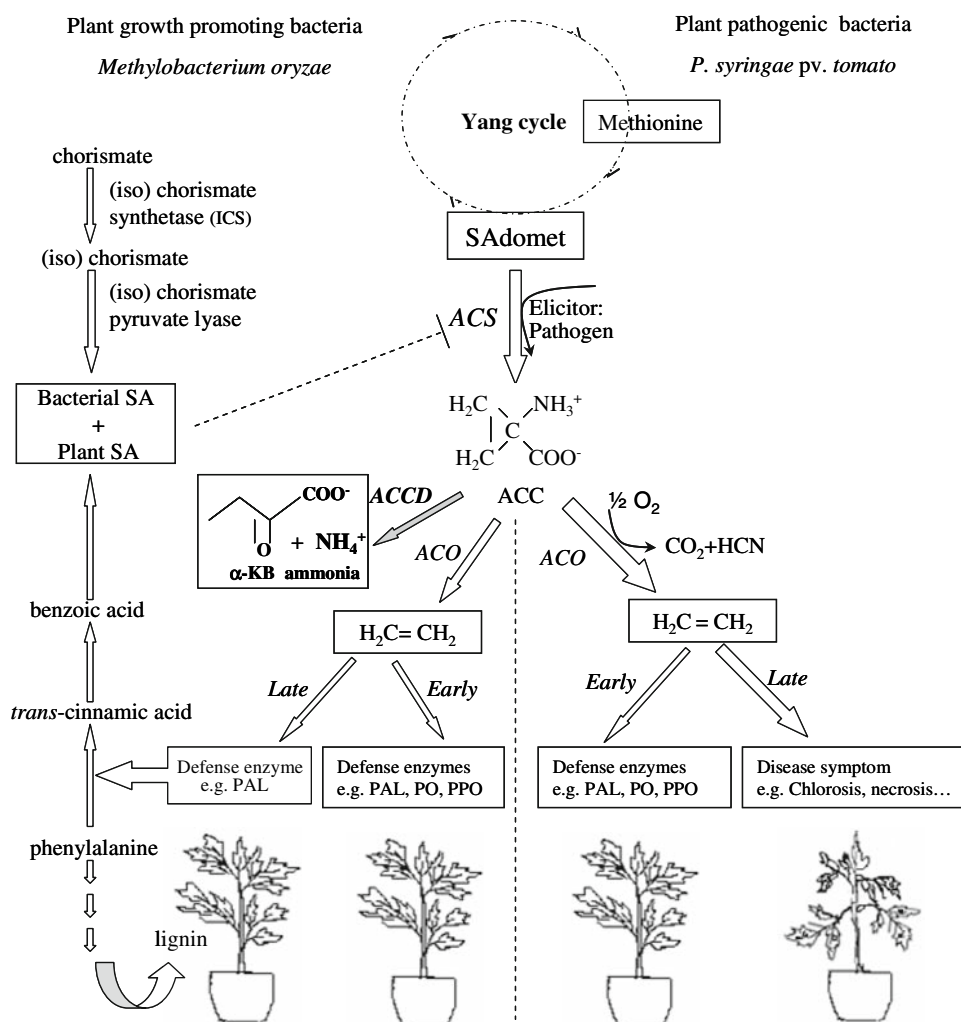
P. Indiragandhi · R. Anandham · K. Kim · W. Yim · M. Madhaiyan · T. Sa (✉)
Department of Agricultural Chemistry,
Chungbuk National University, Cheongju 361763,
Republic of Korea
e-mail: tomsa@chungbuk.ac.kr

oxidase (PPO) (Compant et al. 2005). However, increased activities and accumulation of these enzymes depends mainly on the plant hormone ethylene (Boller 1991; Chen et al. 2003). Ethylene emission is an early response of plants to pathogen attack and it has been suggested that it acts as a signal for defense response during plant-microbe interactions (Fig. 1). On the other hand, timing of increased ethylene emission is paralleled with the development of disease symptoms in crop plants (Chen et al. 2003). The chemical ethylene biosynthesis inhibitor AVG is used to reduce the ethylene levels and eventually the disease symptoms. It is established that PGPB possessing 1-aminocyclopropane-1-carboxylate deaminase activity (EC 3.5.99.7) could reduce the stress ethylene level through the hydrolytic cleavage of the ethylene biosynthesis precursor 1-aminocyclopropane-1-carboxylate (Glick et al. 1998; Madhaiyan et al. 2006a) and yet be able to maintain the ethylene level sufficient for biological processes till the later stage of crop growth, thus reducing the disease

symptoms in crop plants as proposed in Fig. 1 (Robison 2001; Wang et al. 2000).

The genus *Methylobacterium* of class *alphaproteobacteria*, sometimes referred as pink pigmented facultative methylotrophs (PPFMs) are a physiologically interesting group of bacteria, able to grow on methanol, methylamine as well as on a variety of C₂, C₃ and C₄ compounds (Lidstrom 2001) is receiving more attention as PGPB. Several beneficial aspects such as stimulation of seed germination, plant growth promotion, production of phytohormones and induction of defense responses in rice and peanut against *Rhizoctonia solani* and *Aspergillus niger/Sclerotium rolfsii* have been reported for *Methylobacterium* (Omer et al. 2004; Madhaiyan et al. 2004; 2006b). More recently, Ryu et al. (2006) and Anandham et al. (2007) documented the production of IAA, cytokinin and oxidation of sulfur by *Methylobacterium oryzae* CBMB20. Also, the lessening of plant stress ethylene level through the action of the bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC)

Fig. 1 A proposed model for the lowering of plant ethylene level by plant growth promoting *Methylobacterium oryzae* leading to eventual increase in defense enzymes activity as a consequence of induction systemic resistance against invading pathogen (based on Glick and Penrose 1998; Boller 1991; Huang et al. 1993). SAdomet is S-adenosylmethionine, SA is salicylic acid, ACS is 1-aminocyclopropane-1-carboxylate synthase, ACC is 1-aminocyclopropane-1-carboxylate, ACO is 1-aminocyclopropane-1-carboxylic acid oxidase, ACCD is 1-aminocyclopropane-1-carboxylate deaminase, α -KB is α -ketobutyrate. \perp Symbol shows the inhibition of ACS by salicylic acid (SA)



deaminase was reported for *M. oryzae* strains CBMB20 and CBMB110 (Glick et al. 1998; Madhaiyan et al. 2006a). This work aimed to test the induction of the defense response in tomato provided by *M. oryzae* CBMB20 against the foliar pathogen-*Pseudomonas syringae* pv. *tomato* as a consequence of reducing the stress ethylene level through its ACC deaminase activity.

Materials and methods

Bacterial strains, culture conditions and plant material

Methylobacterium oryzae strains CBMB20 (AY683045) and CBMB110 (AY683046) were grown in ammonium mineral salts (AMS) medium (Whittenbury et al. 1970) supplemented with 0.5% methanol (v/v) and cycloheximide (20 µg/ml). The bacterial pathogen *P. syringae* pv. *tomato* (Pst) maintained in King's B (KB) liquid medium was obtained from the Department of Plant Medicine, Chungbuk National University, Republic of Korea. Tomato seeds (*Lycopersicon esculentum* cv. rokusanmaru) and seedlings susceptible to bacterial speck were obtained from commercial sources for growth chamber and greenhouse experiments respectively.

Siderophore production and antagonistic activity of *M. oryzae*

Siderophore production by *M. oryzae* strains CBMB20 and CBMB110 was tested on universal chrome azural S (CAS) agar plates (Schwyn and Neilands 1987). The antagonistic activity was tested by a dual culture method. Filter paper discs (6 mm) saturated with *Methylobacterium* suspension were placed over a bacterial lawn of Pst (10^8 c.f.u./ml) in nutrient agar medium. The plates were incubated for 3–5 days at 30°C and observed for clear inhibition zones around the discs.

In vitro salicylic acid (SA) production by *M. oryzae*

M. oryzae strains CBMB20 and CBMB110 were grown in 25 ml of liquid medium (Davis and Mingioli 1950) supplemented with 0.5% methanol at 30°C for 72 h in shaking incubator (150 rev/min). Bacterial cultures were harvested by centrifugation at 2,800g for 10 min at 4°C. The resulting supernatant was acidified with 1 M HCl to pH 1.5–2.0 and extracted with an equal volume of ethyl acetate. After evaporation of the organic phase, the dry residue was suspended in 3 ml of 97% methanol. SA concentration was determined by adding 5 µl of 2 mol FeCl₃ and 3 ml of

water to 1 ml of concentrated extract. The absorbance of the purple iron-SA complex, which developed in the aqueous phase, was measured at 527 nm (UV-spectrophotometer, UV-1601, Shimadzu, Japan) and compared with a standard curve of SA dissolved in ethyl acetate (Meyer and Abdallah 1992).

Antimicrobial effect of Salicylic acid (SA)

The antibacterial activity of SA on Pst was tested according to Cameron and Zaton (2004). Briefly, a single colony from a KB agar plate on which it had been maintained for 24 h, was grown in KB liquid medium containing SA (0, 0.01, 0.01, 1.0 and 5 mM) at 120 rev/min for 28 h at 30°C. Bacterial culture growth was measured spectrophotometrically (O.D._{600 nm}) at 8, 20, 22, 24 and 28 h.

Growth chamber bioassay

Bacterial cells of 72-h-old cultures of *M. oryzae* strains CBMB20 and CBMB110 were harvested by centrifugation at 10,000g for 10 min at 4°C, washed twice and suspended in 0.03 M MgSO₄. Homogenous bacterial suspensions were adjusted to O.D._{600 nm} = 0.15 (10^8 c.f.u./ml). The surface disinfected (70% ethanol-10 min; 1% sodium hypochlorite-5 min) tomato seeds were subjected to the following treatment for 4 h: (i) *M. oryzae* CBMB20, (ii) CBMB110, (iii) 1×10^{-4} M AVG (L-α-(2-aminoethoxyvinyl)-glycine hydrochloride) (Sigma-Aldrich Co. St Louis, Mo, USA) a chemical ethylene biosynthesis inhibitor (as positive control), and (iv) 0.03 M MgSO₄ (as negative control).

Treated tomato seeds were axenically dried and placed in a Petri dish with moist sterile filter paper and incubated for 7 days at 24°C under dark. On day 7 germinated tomato seeds were transplanted into 150-ml plastic pots containing oven-sterilized commercial pot mixture (Sun Gro Horticulture Distribution, Inc., Seba Beach, Canada). Overnight-grown bacterial pathogen Pst cells were harvested by centrifugation at 10,000g for 10 min at 4°C and washed twice and suspended in saline (0.85% NaCl). Bacterial suspension was prepared by mixing 1 ml of saline-suspended bacterial cells with 20 ml of 0.025% Triton X-100 to a final concentration of 10^8 c.f.u./ml (O.D._{600 nm} = 0.4) and used as foliar spray on 4-week-old tomato plants until run-off by using a hand-held pneumatic sprayer. Pots were placed in growth chambers at $20 \pm 1^\circ\text{C}$, photoperiod starting with 12 h dark followed by 12 h lights ($18 \mu\text{mol}/\text{m}^2/\text{S}$) in a completely randomized design with three replications. Plants were watered regularly with sterile distilled water.

Greenhouse bioassay

Twenty one day old tomato seedlings were transplanted into 300-ml pots containing an oven-sterilized commercial pot mixture. After establishment the plants were subjected to the following treatments: (i) Control (0.03 M MgSO_4), (ii) CBMB20 (O.D._{600 nm} = 0.15 = 10^8 c.f.u./ml), (iii) CBMB20 (10^8 c.f.u./ml) + Pst (O.D._{600 nm} = 0.4 = 1.5×10^7 c.f.u./ml) and (iv) Pst alone (1.5×10^7 c.f.u./ml). *Methylobacterium* and pathogen inocula were prepared as described for growth chamber bioassay and inoculation was performed with a hand-held pneumatic sprayer until run-off on 28- and 30-day-old tomato plants respectively. Plants were maintained in the greenhouse with a temperature regime of 28/22°C (day/night) and natural illumination. Twelve pots per treatment, each with a single plant were arranged in a completely randomized design with three replications.

Disease scoring was started after the initial development of the bacterial speck on 5 and 3 days after pathogen inoculation in growth chamber and greenhouse respectively. Disease severity was evaluated visually and scored using a disease index with a range of 0–3 (0-signifies a leaf showing no symptom or speck; 1-signifies 2–5 lesions/leaf; 2-signifies 6–10 lesions; and 3-signifies more than 10 lesions together or spread over each leaf) as described previously in Bashan and de-Bashan (2002). Leaf samples were collected on days 5, 10 and 1, 3, 5, 7, 10, 15 days respectively from growth chamber and greenhouse after challenge inoculation of the pathogen for ethylene, PR-protein and defense enzymes analysis.

Ethylene analysis

Tomato leaves (1 g) collected from growth chamber and greenhouse experiments were placed in 120 ml vials for 30 min to allow for the escape of wound ethylene and sealed with a rubber septum for 4 h. One ml sample of the head-space was injected into a Gas Chromatograph (dsCHROM 6200, Donam Instruments Inc., Republic of Korea) packed with Poropak-Q column at 70°C and equipped with a flame ionization detector. The amount of ethylene emission was expressed as nmol or pmol of ethylene/h/g fresh weight by comparing the standard curve generated with pure ethylene (Praxair, Praxair Korea Co., Ltd).

Enzyme extraction, PR-protein and defense enzyme assay

Leaf samples (1 g) collected from tomato plants were snap frozen and homogenized immediately with liquid nitrogen.

The resulting powder was macerated for 30 s with 100 mM potassium phosphate buffer, pH 7.0 (1:1.25 w/v). Then the crude homogenates were centrifuged at 20,000g for 20 min at 4°C. The supernatants were kept in an ice bath and used for the determination of PR proteins and defense enzyme and dosing for Lowry protein estimation using bovine serum albumin (BSA) as a standard.

β -1,3-Glucanase activity was assayed using laminarin (from *Laminaria digitata*) (Sigma-Aldrich Co. St. Louis, Missouri, USA) as substrate (Liang et al. 1995). The assay mixture 0.1 ml consisted of 50 μ l enzyme extract, 50 μ l laminarin (10 mg/ml) in 50 mM sodium acetate buffer (pH 5.0) and was incubated at 37°C for 1 h and the amount of reducing sugar released from laminarin was determined by the termination of reaction with 1.5 ml dinitrosalicylic acid (DNS) reagent and boiling the mixture in a water bath for 5 min. Reducing sugar equivalents were measured at 530 nm. Enzyme activity was expressed as μ g glucose/min/mg protein. Phenylalanine ammonia-lyase activity was measured according to the procedure described by El-Shora (2002). The assay mixture consisted of 1.9 ml of 100 mM Tris-HCl buffer (pH 8.5), 1 ml of 15 mM L-phenylalanine and 100 μ l enzyme extract was incubated at 30°C for 15 min. The reaction was terminated by adding 200 μ l of 6 M HCl and the absorbance of the solution was measured at 290 nm. One unit represents the conversion of 1 μ mol L-phenylalanine to *trans*-cinnamic acid per min. The amount of *trans*-cinnamic acid synthesized was calculated using its molar absorptivity of $9630 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol *trans*-cinnamic acid/min/mg protein.

Peroxidase activity was determined by mixing the 50 μ l of enzyme extract with 2.85 ml of 100 mM phosphate buffer (pH 7.0) and 50 μ l of 20 mM guaiacol (Fu and Huang 2001). The reaction was started by adding 20 μ l of 40 mM H_2O_2 to the mixture and the initial rate of increase in the absorbance was measured at 470 nm over 1 min. One unit of activity was defined as a change in absorbance of 0.001/min. Polyphenol oxidase activity was determined according to Mayer et al. (1965). The reaction mixture consisted of 200 μ l enzyme extract and 1.5 ml 0.1 M sodium phosphate buffer (pH 6.5). The reaction was started with the addition of 200 μ l 0.01 M catechol and the activity was measured by observing the changes in absorbance at 420 nm. The activity of PO and PPO was expressed as changes in absorbance/min/mg protein.

For estimation of the total phenolics, fresh tomato leaves (1 g) were extracted with 10 ml of 80% methanol and agitated for 15 min at 70°C (Zieslin and Ben-Zaken 1993). One ml of the methanolic extract was added to 5 ml of distilled water and 250 μ l of Folin-Ciocalteu reagent (1 M) and kept for incubation at 25°C for 30 min. The absorbance of the developed blue color was measured at 725 nm

and expressed as μg of catechol/mg protein by using catechol as standard.

Statistical analysis

The data were statistically analysed by analysis of variance (ANOVA) using the general linear model version 9.1 developed by SAS institute Inc., Cary, NC, USA (SAS 2004). Means were compared using the least significant difference (LSD) $P \leq 0.05$.

Results

Siderophore-negative *M. oryzae* strains CBMB20 and CBMB110 were found to produce 10.5 ± 0.40 and $8.0 \pm 0.57 \mu\text{g}$ salicylic acid/ml of culture media. Though the strains were not able to show antagonism toward Pst in dual culture technique, SA produced by the *M. oryzae* CBMB20 inhibited the growth of Pst about 2-fold that of control over 20 h of incubation (Fig. 2).

In the growth chamber experiment, *M. oryzae* CBMB20 significantly ($P \leq 0.05$) increased the PR protein and defense enzymes activity in tomato plants inoculated with pathogen. The level of ethylene recorded in plants pretreated with *M. oryzae* CBMB20 and challenge-inoculated with pathogen (3.52 ± 0.18) was on par with the ethylene level recorded in AVG and challenge-inoculated with pathogen (3.40 ± 0.16) and significantly less than that of the ethylene level in plants treated with pathogen alone (4.86 ± 0.20). The increased activity of β -1,3-glucanases,

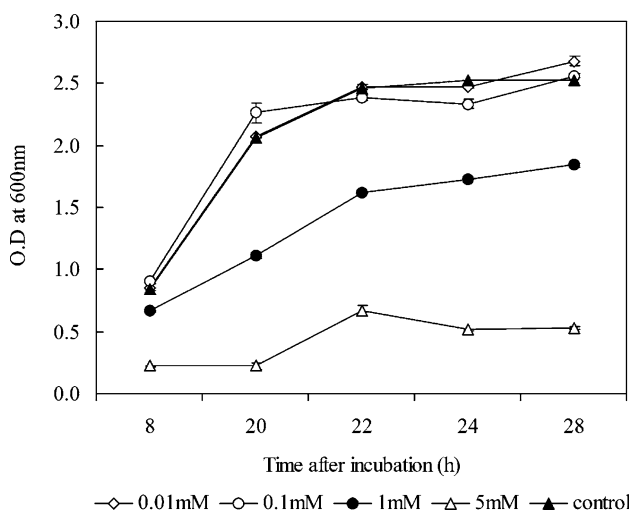


Fig. 2 Antimicrobial effect of different concentration of salicylic acid on growth of *Pseudomonas syringae* pv. *tomato* in King's B liquid medium at 30°C. Each value represents the mean of three replications per treatment. Error bars indicate \pm standard error (SE)

PAL, PO, PPO was noted in plants pretreated with *M. oryzae* CBMB20 compared with that of *M. oryzae* CBMB110 and comparable to that of AVG (Table 1). Based on the significant performance in the growth chamber experiment, *M. oryzae* CBMB20 was chosen for the greenhouse experiment.

Under greenhouse conditions, ethylene production in uninoculated plants remained low compared to pathogen-inoculated plants and the latter showed a significant increase in ethylene level on a day after inoculation and reached maximum on day 7 (Fig. 3a). During this time period, pathogen-inoculated plants showed small, water-soaked lesions. Ethylene production then began to decrease and yellow haloes appeared around the water-soaked lesions on day 9 after pathogen inoculation (Fig. 4a and b). On day 12 the lesions coalesced and almost all of the leaf tissue was damaged, coinciding with a second peak in ethylene production (Figs. 3a and 4c). Plants pretreated with *M. oryzae* CBMB20 and challenge-inoculated with pathogen showed reduced ethylene level (Fig. 3a) and a concurrent increase in the activity of β -1,3-glucanases (204.2–288.8 ng glucose/min/mg protein) was also recorded (Fig. 3b).

The activity of PAL started to increase from 30.25 to 44.79 nmol of *trans*-cinnamic acid/min/mg protein on day 1 through day 5 after pathogen inoculation in plants pretreated with *M. oryzae* CBMB20. The increase in PAL activity was stable till day 12 (Fig. 3c). PO and PPO activities were extremely high in treatment with *M. oryzae* CBMB20 and challenged with pathogen, compared to treatment with either pathogen or *Methylobacterium* alone (Fig 3d and e). Treatment with *M. oryzae* CBMB20 and challenge-inoculated with Pst increased the total phenols of the plants, ranging between 6.32 and 6.76 μg catechol/mg of protein and the increase was noticed throughout the experimental period. Plants treated with Pst alone showed a gradual decline in phenolic content (Fig. 3f).

In growth chamber experiments, the percent disease index (PDI) value observed in seedlings of CBMB20-treated seeds challenged with Pst was 10.5% and was comparable to that of plants treated with AVG. whereas, PDIs in the pathogen-treated plants was as high as 22% under growth chamber and 28% for greenhouse conditions (Fig. 5).

Discussion

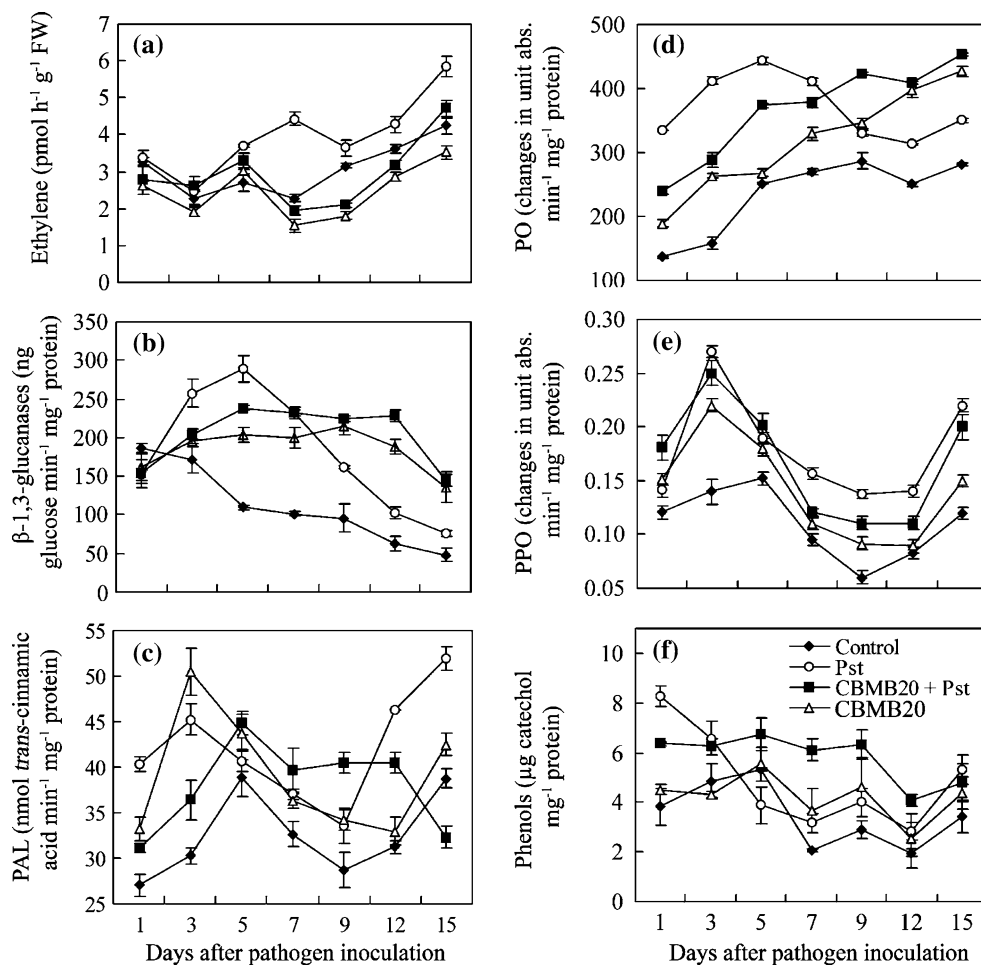
It is established that the antagonistic activity of the PGPB was characterized by the production of siderophore (Compant et al. 2005). Therefore, the absence of antagonism against the Pst by the siderophore-negative *M. oryzae* strains CBMB20 and CBMB110 is not surprising. It was

Table 1 Induction of defense enzymes in tomato against bacterial pathogen *P. syringae* pv. *tomato* elicited by *M. oryzae* under growth chamber condition

Treatment	Ethylene (nmol/h/g fresh weight)	β -1,3-glucanase (ng glucose/min/mg protein)	Phenylalanine ammonia-lyase (nmol <i>trans</i> -cinnamic acid/min/mg protein)	Peroxidase (changes in unit abs./min/mg protein)	Polyphenol oxidase (changes in unit abs./min/mg protein)	Phenols (μ g catechol/min/mg protein)
Control	1.11 \pm 0.06e	29.7 \pm 1.16e	3.36 \pm 0.07f	37.7 \pm 1.21g	0.06 \pm 0.01f	37.2 \pm 0.72e
Pst	4.86 \pm 0.20a	45.3 \pm 1.74d	7.45 \pm 0.14b	86.1 \pm 0.37f	0.15 \pm 0.02c	33.8 \pm 1.94f
CBMB20	1.47 \pm 0.09d	41.0 \pm 0.77c	6.62 \pm 0.12c	108.3 \pm 1.23e	0.07 \pm 0.01ef	40.3 \pm 0.79d
CBMB110	3.04 \pm 0.05c	39.5 \pm 0.47f	6.45 \pm 0.06dc	108.1 \pm 1.00d	0.05 \pm 0.01f	30.2 \pm 0.47h
AVG	1.47 \pm 0.02d	45.3 \pm 1.10f	4.32 \pm 0.06e	150.3 \pm 1.05e	0.09 \pm 0.01e	32.6 \pm 1.38g
CBMB20 + Pst	3.52 \pm 0.18b	101.0 \pm 0.41f	8.07 \pm 0.05a	218.8 \pm 0.75a	0.23 \pm 0.05a	54.3 \pm 1.22a
CBMB110 + Pst	4.66 \pm 0.24a	68.3 \pm 2.56b	6.00 \pm 0.58d	175.5 \pm 0.63b	0.13 \pm 0.01d	50.1 \pm 0.52b
AVG + Pst	3.40 \pm 0.16b	80.0 \pm 6.21a	8.51 \pm 0.28a	159.5 \pm 0.58c	0.20 \pm 0.04 b	47.4 \pm 1.27c
LSD ($P \geq 0.05$)	0.24	5.83	0.54	1.00	0.05	1.52

Treatment details: Control, 0.03 mol MgSO₄; Pst, *Pseudomonas syringae* pv. *tomato*; CBMB20 and CBMB110 are *M. oryzae* strains; AVG, 1 \times 10⁻⁴ M L- α -(2-aminoethoxyvinyl) glycine hydrochloride; Values are mean \pm standard error (SE) of three replications per treatment observed on day 5 and 10. Means followed by the same letter are not significantly different from each other at 5% (LSD)

Fig. 3 Induction of defense response in tomato by *Methylobacterium oryzae* CBMB20 against *Pseudomonas syringae* pv. *tomato* under growth chamber condition resulting from reduced (a) ethylene level and increased activity of (b) β -1,3-glucanase, (c) Phenylalanine ammonia-lyase, (d) Peroxidase, (e) Polyphenol oxidase and (f) Phenolics. Each value represents the mean of three replications per treatment. Error bars indicate \pm standard error (SE)



evident from the growth chamber experiment, where the spatial separation of *Methylobacterium* in rhizosphere and the bacterial pathogen in the phyllosphere excludes the

possibility of direct antagonism leads to the conclusion that the defense response was induced. Induction of defense response through the application of PGPB often induces

Fig. 4 Efficacy of *Methylobacterium oryzae* on the severity of bacterial speck caused by *P. syringae* pv. *tomato* in tomato leaves on day (a) 3 after pathogen inoculation, (b) 9 after pathogen inoculation and (c) 12 after pathogen inoculation. (Top: *P. syringae* pv. *tomato* alone, Bottom: *M. oryzae* CBMB20 + Pst)

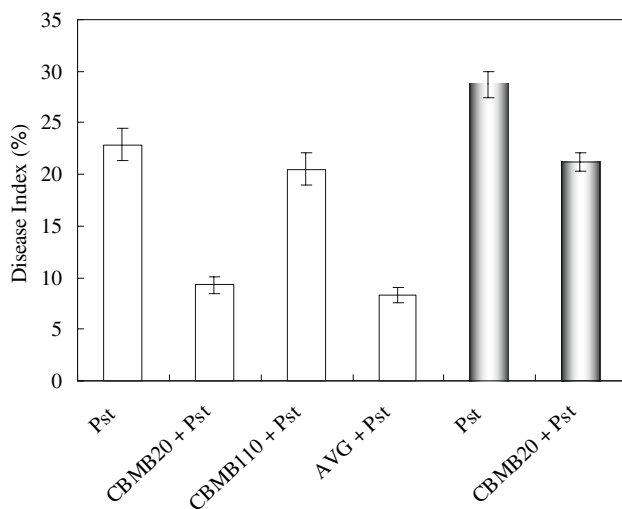
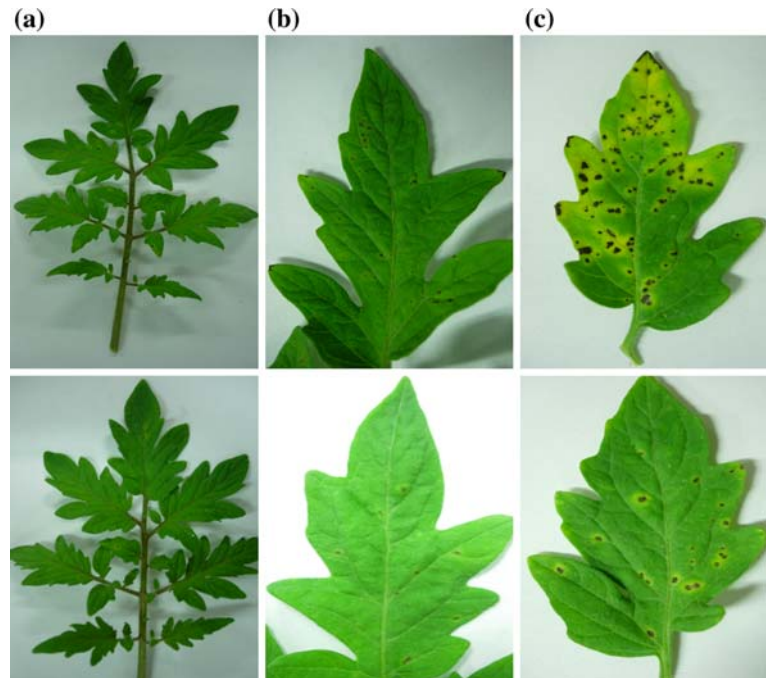


Fig. 5 Percent disease index of tomato plants treated with *Methylobacterium oryzae* strains CBMB20 and CBMB110 and challenged with *P. syringae* pv. *tomato*. A hollow and solid bar indicates under growth chamber and greenhouse conditions respectively. Each value represents means of three replicates per treatment. Error bars indicate \pm standard error (SE)

changes in plant chemistry that are correlated with an increase in disease resistance, which is more advantageous than antagonistic activity. *Methylobacterium*-elicited defense responses have been demonstrated in rice and peanut (Madhaiyan et al. 2004; 2006b). *M. oryzae* CBMB20 probably reduced the level of stress ethylene induced by pathogen attack and thus confers resistance to stress posed by phytopathogens, which is congruent with

earlier findings (Robison et al. 2001; Wang et al. 2000; Glick et al. 1998).

The possible mechanism behind this protection might be due to the hydrolytic cleavage of the ethylene biosynthesis precursor ACC into α -ketobutyrate (α -KB) and ammonia by the bacterially produced ACC deaminase (Glick et al. 1998; Madhaiyan et al. 2006a), since the reduced level of ethylene is enough to induce PR proteins and defense enzymes; but not sufficient for the proliferation of the pathogen and eventually disease symptom development (Chen et al. 2003). It is evident from our study that treatment with *M. oryzae* CBMB20 showed reduced ethylene level (1.47 nmol/h/g fresh weight), which is within the level of 0.05 μ l/l, enough to activate the biological processes in crop plants (Abeles 1992) and increased activity of PR proteins. The increased activity of defense enzymes and reduced level of ethylene in *M. oryzae* CBMB20 compared with *M. oryzae* CBMB110 could be explained by the magnitude of ACC deaminase activity of 94.48 and 24.74 nmol α -KB/h/mg protein respectively (Madhaiyan et al. 2006a). In a previous study, transgenic tomato expressing a bacterial ACC deaminase showed reduced symptoms of *Verticillium* wilt (Robison et al. 2001). Also, the inoculation of *Methylobacterium* to rice and peanut plants increased the activity of PR proteins (Madhaiyan et al. 2004; 2006b).

Ethylene production as a defense response immediately after pathogen attack might be originate from the portion of plant's existing pool of ACC by ACC oxidase (Fig. 1). But the subsequent increase in ethylene level is due to the transcription of ACS by pathogen leading to disease

symptom development (Hase et al. 2003). In this study, the reduced level of ethylene in plants treated with either CBMB20 alone or challenged with pathogen might be due to the action of ACC deaminase present in the *Methylobacterium*. Though we did not determine the SA level in plant after *Methylobacterium* treatment, it can be explained that the reduced stress ethylene level may also be due to the inhibition of ACS by SA (Huang et al. 1993) (Fig. 1) besides its antimicrobial activity (Cameron and Zaton 2004). It has been established that the plant growth-promoting bacteria able to produce SA are well known for their role in the induction of PR proteins and defense enzymes (Compant et al. 2005).

In tomato, seed treatment with *Methylobacterium* induced the plants to synthesize β -1,3-glucanase, PAL, PO and PPO. An additional increase in β -1,3-glucanase synthesis was observed in *M. oryzae* CBMB20-pretreated plants challenged with Pst in both growth chamber and greenhouse experiments. The increased activity of β -1,3-glucanase parallel with the level of ethylene reflects the role of ACC deaminase-containing *Methylobacterium* in induction of the defense response. Increased activity of β -1,3-glucanase was reported for rice and peanut when the seedlings raised from *Methylobacterium*-bacterized seeds and challenged with *Rhizoctonia solani* and *Aspergillus niger/Sclerotium rolfii* respectively (Madhaiyan et al. 2004; 2006b).

Cinnamic acid, the product of PAL, is directly linked to cell lignification processes and the highest levels of PAL activity usually occur about one day after initial infection of pathogen (Podile and Laxmi 1998). In this study also the highest PAL activity occurred about one day after initial infection of the pathogens and the activity was increased constantly in *Methylobacterium*-treated plants up to day 12, which is congruent with the results reported by earlier researchers (Podile and Laxmi 1998; Madhaiyan et al. 2006b). It is possible that the product of PAL, cinnamic acid directs the synthesis of the antimicrobial agent SA (Fig. 1). The PO and PPO activities are linked to lignification and generation of hydrogen peroxides at later stages of infection, which inhibit pathogens directly, or generation of other free radicals with antimicrobial activity, that restrict the development of pathogenic bacteria (Silva et al. 2004). Similar results were obtained in rice and peanut with a high accumulation of phenolic compounds when treated with *Methylobacterium* and *R. solani* and *A. niger/S. rolfii* (Madhaiyan et al. 2004; 2006b).

The probable reason for the reduced bacterial speck disease symptoms in *M. oryzae* CBMB20-pretreated plants challenged with Pst might be due to the reduced ethylene levels resulting from the deaminase activity of *M. oryzae* CBMB20. The enhanced accumulation of PR proteins and defense enzymes as a result of reduced ethylene level

substantiates the potential role of *M. oryzae* CBMB20 in disease resistant development in crop plants besides its potential for the direct plant growth-promoting activity through the production of phytohormone (IAA, cytokinin) and sulfur oxidation. The tested strain had been originally isolated from the surface-sterilized stem of rice plants, thus it may have the potential to grow endophytically within the plant system and could arrest the proliferation of the bacterial pathogens for instance, Pst causing systemic disease in crop plants. The findings of the present study support the use of *M. oryzae* CBMB20 as a potential biocontrol agent against bacterial speck. However, further studies under field conditions need to be conducted to test the efficacy of this biocontrol agent against bacterial speck and other foliar diseases of tomato.

Acknowledgements The financial support provided by Brain Korea (BK21) through Ph.D. scholarship to P.I is greatly acknowledged. R.A and M.M. are supported by Korea Research Foundation. We acknowledge the partial financial support provided by Rural Development Administration. Critical reading of this paper by the learned referees is thankfully acknowledged.

References

- Anandham R, Indiragandhi P, Kim K, Yim W, Madhaiyan M, Saravanan VS, Chung J, Sa TM (2007) Thiosulfate oxidation and mixotrophic growth of *Methylobacterium oryzae*. Can J Microbiol 53:869–876
- Abeles FB, Morgan PW, Saltveit ME Jr. (1992) Ethylene in plant biology, 2nd edn. Academic Press, New York
- Bashan Y, de-Bashan LE (2002) Protection of tomato seedlings against infection by *Pseudomonas syringae* pv. *tomato* by using the plant growth promoting bacterium *Azospirillum brasilense*. Appl Environ Microbiol 68:2637–2643
- Boller T (1991) Ethylene in pathogenesis and disease resistance. In: Mattoo AK, Suttle JC (eds) The plant hormone ethylene. CRC Press, Boca Raton, FL, pp 293–314
- Cameron RK, Zaton K (2004) Intercellular salicylic acid accumulation is important for age-related resistance in *Arabidopsis* to *Pseudomonas syringae*. Physiol Mol Plant Pathol 65:197–209
- Chen N, Goodwin PH, Hsiang T (2003) The role of ethylene during the infection of *Nicotiana tabacum* by *Colletotrichum destructivum*. J Exp Bot 54:2449–2456
- Compant S, Duffy B, Nowak J, Clément C, Barka EA (2005) Use of plant growth promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71:4951–4959
- Davis BD, Mingioli ES (1950) Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. J Bacteriol 60:17–28
- El-Shora HM (2002) Properties of phenylalanine ammonia-lyase from marrow cotyledons. Plant Sci 162:1–7
- Fu JM, Huang BR (2001) Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ Exp Bot 45:105–114
- Glick BR, Bashan Y (1997) Genetic manipulation of plant growth promoting bacteria to enhance biocontrol of phytopathogens. Biotechnol Adv 15:353–378
- Glick BR, Penrose DM, Li J (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J Theor Biol 190:63–68

- Hase S, van Pelt JA, van Loon LC, Pieterse CMJ (2003) Colonization of *Arabidopsis* roots by *Pseudomonas fluorescens* primes the plant to produce higher levels of ethylene upon pathogen infection. *Physiol Mol Plant Pathol* 62:219–226
- Huang YF, Chen CT, Kao CH (1993) Salicylic acid inhibits the biosynthesis of ethylene in detached rice leaves. *Plant Growth Regul* 12:79–82
- Liang ZC, Hseu RS, Wang HH (1995) Partial purification and characterization of a 1,3- β -D-glucanase from *Ganoderma tsugae*. *J Ind Microbiol* 14:5–9
- Lidstrom ME (2001) The aerobic methylotrophic bacteria. In: Dworkin M (ed) *The prokaryotes*. Berlin, Springer-Verlag, pp 223–244
- Madhaiyan M, Poonguzhali S, Senthikumar M, Seshadri S, Chung H, Yang J, Sundaram S, Sa TM (2004) Growth promotion and induction of systemic resistance in rice cultivar Co-47 (*Oryza sativa* L.) by *Methylobacterium* spp. *Bot Bull Acad Sin* 45: 315–324
- Madhaiyan M, Poonguzhali S, Ryu JH, Sa TM (2006a) Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase containing *Methylobacterium fujisawaense*. *Planta* 224:268–278
- Madhaiyan M, Suresh Reddy BV, Anandham R, Senthikumar M, Poonguzhali S, Sundaram SP, Sa TM (2006b) Plant growth-promoting *Methylobacterium* induces defense responses in groundnut (*Arachis hypogaea* L.) compared with rot Pathogens. *Curr Microbiol* 53:270–276
- Mayer AM, Harel E, Shaul RB (1965) Assay of catechol oxidase a critical comparison of methods. *Phytochemistry* 5:783–789
- Meyer JM, Abdallah MA (1992) The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *J Gen Microbiol* 107:319–328
- Omer ZS, Tombolini R, Broberg A, Gerhardson B (2004) Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria. *Plant Growth Regul* 43:93–96
- Podile AR, Laxmi VDV (1998) Seed bacterization with *Bacillus subtilis* AF1 increases phenylalanine ammonia-lyase and reduces the incidence of fusarial wilt in pigeon pea. *J Phytopathol* 146:255–259
- Robison MM, Shah S, Tamot B, Pauls KP, Moffatt BA, Glick BR (2001) Reduced symptoms of *Verticillium* wilt in transgenic tomato expressing a bacterial ACC deaminase. *Mol Plant Pathol* 2:135–145
- Ryu JH, Madhaiyan M, Poonguzhali S, Yim WJ, Indira Gandhi P, Kim KA, Anandham R, Young JC, Kim KH, Sa TM (2006) Plant growth substances produced by *Methylobacterium* spp. and their effect on tomato (*Lycopersicon esculentum* L.) and red pepper (*Capsicum annum* L.) growth. *J Microbiol Biotechnol* 16:1622–1628
- SAS Institute Inc. SAS 9.1[®] (2004) Companion for windows SAS. SAS Institute Inc., Cary, NC
- Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160:47–56
- Silva HS, Aromeiro RS, Macagnan D, Halfeld-Vierira BA, Pereira MCB, Mouteer A (2004) Rhizobacterial induction of systemic resistance in tomato plants: non-specific protection and increase in enzyme activities. *Biol Control* 29:288–295
- Wang C, Knill E, Glick BR, Défago G (2000) Effect of transferring 1-aminocyclopropane-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its *gacA* derivative CHA96 on their growth-promoting and disease-suppressive capacities. *Can J Microbiol* 46:898–907
- Whittenbury R, Davies SL, Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* 61:205–218
- Zieslin N, Ben-Zaken R (1993) Peroxidase activity and presence of phenolic substances in peduncles of rose flowers. *Plant Physiol Biochem* 31:333–339