Infection Structures on the Infected Leaves of Potato Pre-inoculated with Bacterial Strains and DL-3-amino Butyric Acid after Challenge Inoculation with Phytophthora infestans

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Infection structures were observed using a fluorescence microscope at the penetration sites on the leaves of potato plants pre-inoculated with the bacterial strains Pseudomonas putida TRL2-3, Micrococcus luteus TRK2-2, and Flexibacteraceae bacterium MRL412, which mediated an induced systemic resistance on potato plants against late blight disease caused by Phytophthora infestans. In order to compare the infection structures on the leaves expressing systemic acquired resistance, the leaves of potato plants pre-treated with DL-3-amino butyric acid (BABA) were also observed after challenge inoculation with the same pathogen. The infection structures were investigated. The total number of germination and appressorium formation of P. infestans were counted. Furthermore, the frequencies of fluorescent epidermal cells at the penetration sites, which indicate a defense response of plant cell, were estimated. There were no differences on the germination rates of the fungal cysts among the untreated control, BABA pre-treated, and bacterial strains pre-inoculated plants. However, appressorium formation was slightly decreased on the leaves of BABA pre-treated plants compared to those of untreated as well as bacterial strains pre-inoculated plants. Furthermore, the frequencies of fluorescent cells of BABA pre-treated and bacterial strains pre-inoculated were higher than that of untreated plants, indicating an active defense reaction of the host cells against the fungal attack. On the other hand, the pre-treatment with BABA caused a stronger fluorescent of epidermal cells at the penetration sites compared to the pre-inoculation with the bacterial strains. Interestingly, the frequency of fluorescent cells by BABA, however, was lower than that by the bacterial strains. Based on the results it is suggested that the infection structures showing resistance reaction on the leaves of potato plants were different between by pre-inoculation with bacterial strains and by pre-treatment with BABA against the late blight pathogen.

Keywords: DL-3-amino butyric acid, Induced systemic resistance, Infection structures, Phytophthora infestans, Plant growth-promoting rhizobacteria, Potato, Systemic acquired resistance

Late blight disease caused by Phytophthora infestans is one of the diseases, which are difficult to control in the potato field. Some resistance varieties, which are effective against only one or a few races of the pathogen, can easily become susceptible after attacking by a new virulent race of the pathogen. Beside to the vertical resistance, some varieties possess horizontal resistance of varying degree, resulting in effective against various races of P. infestans. However, cultivating resistant varieties is not sufficient to control the late blight disease, since under favorable conditions even the resistant varieties can be severely infected by P. infestans.

One of the strategies for plant protection against late blight disease may be using crops expressing a induced systemic resistance (ISR), which can be triggered in the plant by pre-inoculation with plant growth-promoting rhizobacteria (PGPR) (van Loon et al., 1998a). Free-living root colonizing bacteria (rhizobacteria) have been studied for the past century as possible inoculants for increasing plant productivity and controlling microbial pathogens (Kloepper, 1992). Soil or seed applications with PGPR have been used to enhance the growth of several crops (Glick, 1995) as well as to suppress the growth of plant pathogens (Kloepper et al., 2004). PGPR that colonize root systems through seed applications and protect plants from foliar diseases include Pseudomonas spp., Bacillus spp., Paebacillus spp., and Serratia sp. (Kloepper et al., 2004). PGPR are known to survive both in the rhizosphere and phyllosphere (Krishnamurthy and Gnanamaniickam, 1998). The PGPR-mediated resistance has been defined as induced systemic resistance (van Loon et al., 1998a), in which the plant’s defense mechanisms are stimulated and primed to the infection by pathogens (Conrath et al., 2002). ISR is distinguished from systemic acquired resistance

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(SAR) that triggers systemically plant defense response following hypersensitive response after inoculation of plant pathogens (Durrant and Dong, 2004; van Loon et al., 1998a). SAR by chemicals such as DL-3 amino butyric acid (BABA), 2,6-dichloro-isonicotinic acid (INA) or benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) has been also documented (Cohen, 2002; Friedrich et al., 1996; Lawton et al., 1996).

In recent years, the use of PGPR as an inducing agent of systemic resistance in crop plants against different pathogens has been demonstrated under field conditions (Bharathiraj et al., 2004). ISR is considered natural, eco-friendly and safe, and provides resistance against a broad spectrum of pathogens (Radijacommare et al., 2002). In the previous study bacterial strains that were isolated from the rhizosphere of some plants and the anti-fungal activities of the isolates against several plant pathogens were tested (Lee et al. 2003). The bacterial isolates Pseudomonas putida (TRL2-3), Micrococcus luteus (TRK2-2) and Flexibacteraceae bacterium (MRLA42) were selected as ISR inducing agent in potato plants against late blight disease (Kim and Jeun, 2006). In this study, infection process of the pathogen and defense responses of potato plant by the three bacterial strains as well as pre-treated with BABA were microscopically observed on the leaf surfaces after fungal challenge inoculation.

Materials and Methods

Plants. Seed tuber of potatoes (Solanum tuberosum L. cv. Deajima) were grown in a styrofoam bed (volume 0.032 m³, W × L × D = 31 × 51 × 20 cm in size) filled with perlite (Parat®, Sam son, Korea) and peat-moss (Tuknami®, Nong-woogreentec, Korea) mixture (1 : 2, v/v). Each bed was fertilized with 80 g of the essentiality microelement (Osmocote® Scotts Koream®) and covered commercial soil (Choroc Nala®, Bokyang Nongsang, Korea). Eight potato plug seedling was planted at intervals of 12 cm in one bed. Plants were grown in a glass greenhouse at 25°C during the day and at 20°C during the night for 90 days.

Pathogen. Phytophthora infestans (Mont.) de Bary KACC 40718 was obtained from Korean Agricultural Culture Collection (KACC) and grown on oatmeal agar medium for 14 days at 15°C until formation of sporangium. For the initiation of zoospore release from the sporangia, 20 ml distilled water were added to the agar plate growing the mycelium. A spatula was used to remove air between hyphae so that sporangia were well submerged in the water. Then the plates were immediately placed in a refrigerator at 4°C until zoospores were released. The suspension containing zoospores was filtered through three times folded cheesecloth. The number of zoospores was determined using a haemocytometer under a light microscope and concentration of the suspension was adjusted to 1.0 × 10⁴ zoospores/ml. To induce encystment of the pathogen, the zoospore suspensions were intensively shaken using a vortex mixer for 2-3 min and used as an inoculum.

Treatment with three bacterial isolates and BABA in the potato plants. The bacterial strains Pseudomonas putida (TRL2-3), Micrococcus luteus (TRK2-2) and Flexibacteraceae bacterium (MRLA42) showing antifungal effect and triggering of ISR in potato plants (Kim and Jeun, 2006) were obtained from Plant Pathology Lab in Cheju University. The bacterial strains were grown in TSA medium at 28°C for 24 h. The concentration of bacterial strains was adjusted to 1.0 × 10⁷ colony forming unit (cfu)/ml. Thirty ml of the bacterial suspension was soil-drenched per each potato plants at 7 days before the challenge inoculation with P. infestans.

In order to compare the level of protection by the bacterial strains to that of other resistance inducing agent, 30 ml of DL-3-amino butyric acid (BABA; 10 mM) solution were also drenched to the root system of the plants at the same time as case of the bacterial strains. For negative control, H₂O was applied on the potato plants instead of the bacterial suspension or the BABA solution.

Challenge inoculation with pathogen. The second leaves of the bacterial strains pre-inoculated, the BABA pre-treated and the corresponding leaves of untreated control plants, respectively, were detached and inoculated with 20 μl droplets of cyst suspension of P. infestans (1.0 × 10⁴ cysts/ml) at six positions on the upper surface of the leaves. The inoculated leaves were kept in a plastic Petri dish maintaining at 100% RH in the dark for 24 h at 16°C and then at 80% RH until the sampling for the microscopic observation.

Microscopical observation of infection process of the pathogen. Observation of infection process on the infected leaves of the potato plants were performed at 24, 48 and 72 h after the challenge inoculation. Staining of leaf tissues was carried out according to the method of Jeun et al. (2000). The infected leaf tissues were cut out with a corkbore (11-mm in diameter) and fixed with 2% glutaraldehyde solution in 0.05 M phosphate buffer (pH 7.2) for 2 h. After washing in the same phosphate buffer three times, for 10 min each, the sections were stained with 0.5% (w/v) aniline blue for 20 min. Fluorescent cells at the penetration sites were detected by staining with aniline blue. After washing with phosphate buffer 3 times for 10 min, the leaf disks were stained with 0.02% Uvitex 2B (w/
v) (Diethanol) for 20 min. After washing in the phosphate buffer, the leaf disks were mounted on glass slides in 50% glycerin.

Pathogen structures were observed using a fluorescence microscope (Olympus, Japan) equipped with a ‘U’ exciter cube-filter and an ultraviolet epifluorescence filter set (BP 400-440, FT 460, LP 470). Total number of germinated cysts, appressorium formations and fluorescent cells at the penetration sites were counted on the leaf surfaces and in the epidermal cells of the potato plants pre-inoculated with the bacterial strains, pre-treated with BABA and untreated. Five leaf discs individually detached from 6 plants were observed at one experiment, and total 3 separated experiments were replicated. The number of germinate cysts and the appressorium formation as a proportion of the total cysts calculated as a percentage. And the penetration sites on the epidermal cells were counted per cent the number of appressorium formation.

Statistical analyses. The data of the germination rate, frequency of appressorium formation of the oomycetes and fluorescent epidermal cells of the leaves were statistically analyzed using Duncan’s multiple range tests (DMRT). Statistical analysis of the experimental data were conducted using the Statistical Analysis System (SAS institute, version 8.02).

Results

The infection structures *P. infestans* were observed on the leaf surfaces of the potato plants expressing systemic resistance induced by the three bacterial strains as well as by BABA. In addition, the frequencies of fluorescent epidermal cells at the penetration sites were observed after the fungal inoculation. On the leaves of untreated plants most cysts began to germinate and to form an appressorium at 24 h after the challenge inoculation (Data not shown). In most cases, germination rate and appressorium formation on the leaf surface were over 90% at 48 h after the challenge inoculation (Data not shown). Most epidermal cells did not react to the fungal attack (Fig. 1A) but only a few cells appeared fluorescent at the penetration sites at 72 h after the fungal inoculation (Fig. 4). No differences among the three trials were observed in germination rate, frequency of appressorium formation, and fluorescent epidermal cells at 72 h after the fungal inoculation (Fig. 2-4).

In the plants pre-inoculated with the bacterial strains there was no differences in the germination rate as well as in the frequency of appressorium formation compare to those of the untreated plants at 24 h, 48 h (data not shown), and 72 h after the challenge inoculation (Fig. 2 and 3). Staining with the aniline blue revealed an accumulation of yellow-fluorescing material located beneath some of the appressorium (Fig. 1). The frequencies of fluorescent cells on the bacterial strain TRL2-3 pre-inoculated plants were higher than that of untreated plants in all experiments, indicating active defense reaction of the host cells against fungal attack (Fig. 4). These defense reactions were also observed in the epidermal cells pre-inoculation with strains TRK2-2 and MRL412 especially in the experiment 3 (Fig. 4).

Similar to the case of bacterial strains, there were no differences on the rates of germination of the fungal cysts between the BABA pre-treated and untreated plants (Fig. 2). However, in contrast to the case of the bacterial strains, slight decrease of appressorium formation was observed on the leaves of BABA pre-treated in experiments 2 and 3 (Fig. 3). Although in some penetration sites the yellow fluorescent epidermal cells were very intensive (Fig. 1), the frequency of fluorescent host cell was not higher than that
Fig. 2. Germination rate of cyst of *Phytophthora infestans* on the leaves of potato plants pre-inoculated with *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacter cereacae bacterium* (MRL412) and pre-treated with DL-3-amino butyric acid (BABA) at 7 days before challenge inoculation with *P. infestans*. The concentration of all pre-inoculated bacterial strains and of pre-treated BABA were $1.0 \times 10^7$ cfu/ml and 10 mM, respectively. The leaves were attached at 72 h after challenge inoculation with *P. infestans* ($1.0 \times 10^6$ cysts/ml). The vertical bars indicate the standard deviation of the 3 separated experiments each containing six plants. The different letters on the columns present significant ($P < 0.001$) difference according to Duncan’s multiple test.

Fig. 3. Appressorium formation rate of cyst of *Phytophthora infestans* on the leaves of potato plants pre-inoculated with *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacter cereacae bacterium* (MRL412) and pre-treated with DL-3-amino butyric acid (BABA) at 7 days before challenge inoculation with *P. infestans*. The concentration of all pre-inoculated bacterial strains and of pre-treated BABA were $1.0 \times 10^7$ cfu/ml and 10 mM, respectively. The leaves were attached at 72 h after challenge inoculation with *P. infestans* ($1.0 \times 10^6$ cysts/ml). The vertical bars indicate the standard deviation of the 3 separated experiments each containing six plants. The different letters on the columns present significant ($P < 0.001$) difference according to Duncan’s multiple test.

Discussion

Using microorganisms for disease control has been considered for many years because this strategy may result in the reduction of chemical application. However, using the antagonistic microorganisms to control of plant diseases has not been successful in the field. Therefore, new strategy of the biological control such as induced systemic resistance (ISR) has been elucidated for control the plant diseases (van Loon et al., 1998b). In this study to illustrate the resistance mechanism of ISR against late blight.
bacteriaceae bacterium (MRL412) were evaluated in the potato plants against late blight disease (Kim and Jeun, 2006) as well as in cucumber plants after challenge inoculation with anthracnose pathogen Colletotrichum orbiculare (Jeun et al., 2004a). Similarly some PGPR strains such as Serratia marcescens or Pseudomonas fluorescens could effectively induce systemic resistance in cucumber plants against anthracnose disease at certain concentration (Liu et al., 1995).

The mechanisms of ISR have been compared with those of systemic acquired resistance (SAR) (Jeun et al., 2004b; Sticher et al., 1997). Nutritional competition such as ion (Fe) which is easily captured by siderophores produced in PGPR was appeared on the plants expressing ISR (Maurhofer et al., 1994; van Loon et al., 1997; 1998b), but has not been reported in the plants expressing SAR. Also, some PGPR strains that were elicited systemic resistance have direct antifungal activity. Both bacterial isolates TRL2-3 and TRK2-2 showed direct antifungal effect in vitro test (Lee et al., 2003). The other resistance mechanisms of ISR, seem to be similar with those of SAR, which is involved in the resistant gene npr1 (Pieterse and van Loon, 1999).

DL-3- amino butyric acid (BABA) is well known as an activator in many plants (Cohen, 2002; Jeun and Park, 2003; Zimmerli et al., 2000). It has been previously shown that BABA decreased disease severity in the potato and cucumber plants after inoculation with Phytophthora infestans and Colletotrichum orbiculare, respectively (Kim and Jeun, 2006; Jeun et al., 2001). The increase of salicylic acid (SA) level in the leaves of BABA-treated tomato or tobacco plants was reported (Jeun et al., 2000; Siegrist et al., 2000).

In this study, the cytological study has been carried out on the leaves of epidermal cells at the penetration sites using a fluorescence microscope. There was no different on germination rate among the bacterial strains pre-inoculated, BABA pre-treated, and untreated plants (Fig. 2). These observations indicated no suppression of germination neither by pre-inoculated with the bacterial strains nor pretreated with BABA. However BABA treatment was found a slight reduction of appressorium formation (Fig. 3). Similar results were also reported in cucumber plants pretreated with BABA after inoculation with C. orbiculare (Jeun et al., 2004b). These indicate that BABA treatment caused a suppression of appressorium formation of fungal pathogen on the leaf surface. However, the mechanism of appressorium suppression mediated by BABA is not yet clearly illustrated.

Fluorescent cell stained by aniline blue is well-known as a resistance mechanism in many host-parasite interactions (Kovats et al., 1991; Strömberg and Brishammar, 1993). In
this study the frequency of fluorescent cells on bacterial strains pre-inoculated or BABA pre-treated plants were higher than that of untreated plants (Fig. 4). The increase of fluorescent intensity indicated the thickening of cell walls as resistance expressing by the bacterial strains or by BABA. Generally, the protection rate by BABA were higher than those by bacterial strains (Jeun et al., 2004b; Kim and Jeun, 2006; Lee et al., 2005). In this study the fluorescence intensity was stronger at the penetrated site in the BABA pre-treated plants compared to those of bacterial strains pre-inoculated (Fig. 1). However, the frequency of fluorescent cells on BABA treated plants was not always higher than that of on bacterial strains pre-inoculated plants (Fig. 4). Similar results were observed in the other study, in which the frequency of fluorescent cells was increased on the leaves of cucumber plants pre-inoculated with plant growth promoting rhizobacteria (PGPR) comparing to BABA pre-treated (Jeun et al., 2004b; Lee et al., 2005). Based on these results it is suggested the level of resistance may be not dependent on how many plant cell react to the pathogen but depended on how effectively defense to the pathogen. It could be also involved in the other defense responses such as the accumulation PR-proteins (Jeun, 2000) and production of anti-fungal substance phytoalexins (Maurhofer et al., 1994; Bowles, 1990).

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