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Different Mechanisms of Induced Systemic Resistance and Systemic Acquired Resistance Against *Colletotrichum orbiculare* on the Leaves of Cucumber Plants

Yong-Chull Jeun, Kyungseok Park* and Choong-Hoe Kim

Plant Pathology Division, National Institute of Agricultural Science and Technology, Suwon, 441-707, Korea

Defense mechanisms against anthracnose disease caused by *Colletotrichum orbiculare* on the leaf surface of cucumber plants after pre-treatment with plant growth promoting rhizobacteria (PGPR), amino salicylic acid (ASA) or *C. orbiculare* were compared using a fluorescence microscope. Induced systemic resistance was mediated by the pre-inoculation in the root system with PGPR strain *Bacillus amylolquefaciens* EXTN-1 that showed direct antifungal activity to *C. gloeosporioides* and *C. orbiculare*. Also, systemic acquired resistance was triggered by the pre-treatments on the bottom leaves with amino salicylic acid or conidial suspension of *C. orbiculare*. The protection values on the leaves expressing SAR were higher compared to those expressing ISR. After pre-inoculation with PGPR strains no change of the plants was found in phenotype, while necrosis or hypersensitive reaction (HR) was observed on the leaves of plants pre-treated with ASA or the pathogen. After challenge inoculation, inhibition of fungal growth was observed on the leaves expressing both ISR and SAR. HR was frequently observed at the penetration sites of both resistance-expressing leaves. Appressorium formation was dramatically reduced on the leaves of plants pre-treated with ASA, whereas EXTN-1 did not suppress the appressorium formation. ASA also more strongly inhibited the conidial germination than EXTN-1. Conversely, EXTN-1 significantly increased the frequency of callose formation at the penetration sites, but ASA did not. The defense mechanisms induced by *C. orbiculare* were similar to those by ASA. Based on these results it is suggested that resistance mechanisms on the leaf surface was different between on the cucumber leaves expressing ISR and SAR, resulting in the different protection values.

KEYWORDS: **Amino salicylic acid (ASA), *Colletotrichum orbiculare*, Cucumber plants, Induced systemic resistance (ISR), Plant growth-promoting rhizobacteria (PGPR), Systemic acquired resistance (SAR)**

Defense reactions of plants are enhanced when the plants are exposed to exogenous stimuli, such as plant pathogens, non-pathogens or chemicals (Ryals *et al.*, 1992). This resistance by the enhanced defense reactions is expressed after inoculation with virulent pathogen not only locally but also systemically (Sticher *et al.*, 1997). The systemically induced resistance, which defined as systemic acquired resistance (SAR), has been focused by plant pathologists, physiologist and biochemists in the last decade because it could be useful to protect crops against plant pathogens. As a result of intensive studies concerning with SAR, a number of plant-pathogen interactions expressing SAR have been reported (Sticher *et al.*, 1997).

Although the mechanisms of SAR are not yet clearly explained, trials to illustrate the mechanisms of SAR including signal pathway have been continued. Based on the previous studies of SAR, it is known that a certain chemical compound such as salicylic acid (SA) is endogenously produced on the stimulus-exposed part of the plants and plays a role for signaling to the distant part of the plants (Ryals *et al.*, 1992; Sticher *et al.*, 1997). The significance of SA in expression of SAR could be clearly explained by using transgenic tobacco plants expressing the *nahG*-gene from *Pseudomonas putida*, which encodes a salicylate hydroxylase. This enzyme catalyzes the degradation of SA to catechol. In the NahG tobacco plants SAR was not triggered after pre-inoculation with *tobacco mosaic virus* (TMV) whereas SAR was expressed in the Xanthi-nc tobacco plants (Gaffney *et al.*, 1993).

However, it is not likely that SA acts as a signal compound in some cases of SAR expression. Treatment with 2,6-dichloroisonicotinic acid (INA) or benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) triggers SAR in plants without accumulation of SA (Lawton *et al.*, 1996). Furthermore, in the NahG tobacco plants SAR can be induced by the treatment with INA or BTH, indicating that SA may be not necessary for expression of SAR in these systems (Friedrich *et al.*, 1996). Based on these findings it is speculated that SA may only play a role for synthesis of an unknown signal compound.

Recently, it is found that resistance can be also induced on the aerial parts of plants by pre-inoculation with plant growth-promoting rhizobacteria (PGPR) in the root system (van Loon *et al.*, 1998a). This type of resistance is defined as induced systemic resistance (ISR) whose signal pathway is different from that found in the classic induced resistance, SAR (Knoester *et al.*, 1999; Pieterse *et al.*, 1998; 1999). In the transgenic Arabidopsis or tobacco NahG plants, ISR was triggered by pre-inoculation with PGPR strains indicating independence on SA for ISR expression (Pieterse *et al.*, 1998). However, in the jasmonic acid or ethylene insensitive mutant of Arabidopsis

*Corresponding author <E-mail: kspark@rda.go.kr>

plants containing *jah1*- and *etr1*-gene, respectively, resistance could not be induced by pre-inoculation with PGPR strains (Pieterse *et al.*, 1998). Furthermore, necrosis or hypersensitive reaction (HR) on the inducer-treated leaves is necessary for expression of SAR whereas ISR is expressed without any damage of plants but with promoting of the plant growth (Sticher *et al.*, 1997). These findings indicate that ISR may be triggered by different signal pathways to those of SAR.

The reason for differentiation of ISR from SAR lies in also some different defense mechanisms. It is reported that PGPR can directly inhibit the growth of plant pathogen by producing siderophores, whereas the inducers triggering SAR have no potential of antifungal activity (Van Loon *et al.*, 1998a). Moreover, pathogenesis-related proteins (PR-protein) are normally synthesized in the leaves expressing SAR (Sticher *et al.*, 1997). These proteins are known to play a role directly or indirectly in the expression of SAR (Van Loon, 1999). In contrast to SAR, Arabidopsis plants expressing ISR showed no PR-1 gene expression (Pieterse *et al.*, 1998). Based on these observations, it is assumed that there are various defense mechanisms that are differently expressed depending on the treatment with the SAR-inducing factors or by the pre-inoculation with PGPR strains.

Surprisingly, only a few studies on the expression of the systemically induced resistance have been carried out to compare between the plants expressing SAR and ISR. Comparison of biochemical and cytological characteristics of the plants expressing SAR and ISR may be required to illustrate the expression mechanisms of the systemically induced resistance. In the present study, protection effects against cucumber anthracnose caused by *C. orbiculare* were observed when the plant expressed SAR by pre-treatment with ASA, *C. orbiculare* or PGPR strains. Furthermore, infection structures of the pathogen and defense responses of the cucumber plants were also cytologically examined on the leaf surfaces of the plants treated with the different resistance inducers.

Materials and Methods

Plant and pathogen. Cucumber seeds (*Cucumis sativus* L. cv. Eun Sung) were sown in plastic pots (10-cm in diameter) filled with a commercial potting mixture (TKS 2, Flora gard®, Germany) containing 10% of perlite (Parat). Cucumber seedlings were grown in the greenhouse maintaining 28°C at daytime and 25°C at night. Plants at 2-leaf stage were used in this study.

Anthracnose pathogen, *C. orbiculare* was grown in green beans agar for 5 days. The fungal conidia were harvested with H_2O and the conidial concentration were adjusted to 5×10^5 conidia/m*l*. This conidial suspension with 0.01% (v/v) of Silwet L-77, which enhance the adhesion of conidia on leaf surface, was used as inoculum for challenge inoculation on cucumber leaves.

Triggering of ISR and SAR. PGPR strain *Bacillus amylolquefaciens* EXTN-1 was isolated from rhizosphere of the plants sampled in Suwon, Korea. *Bacillus pumilus* INR-7, whose treatment showed protection effect against *C. orbiculare* in cucumber (Raupach and Kloepper, 1998), was provided by J. W. Kloepper, Department of Plant Pathology, Auburn University, U.S.A. Both strains were grown in tryptic soy agar at 28°C for 24 h. The concentration of each PGPR strain was adjusted to 10^8 colony forming unit (cfu)/m*l* according to the methods described by Park and Kloepper (2000). Thirty m*l* of the bacterial suspension was soil-drenched per cucumber plant before challenge inoculation with *C. orbiculare*. For the control, H_2O was applied on the cucumber plants instead of the bacterial suspension.

For triggering SAR, suspension of 50 mM amino salicylic acid (ASA) was sprayed on the first leaves of cucumber plant before challenge inoculation with *C. orbiculare*. As biotic agent for triggering SAR, conidial suspension of *C. orbiculare* (5×10^5 conidia/m*l*) was inoculated on the first leaves. For negative control, non-treated healthy plants at the same growth stadium were used.

Challenge inoculation and disease assessment. The conidial suspension of *C. orbiculare* (5×10^5 conidia/m*l*) was sprayed on the aerial cucumber leaves 5 days after inoculation with PGPR strains and 7 days after application with ASA and the same pathogen, respectively. The plants challenge-inoculated were kept in a humid chamber maintaining 100% RH for 24 h and then transferred to the greenhouse.

Number of anthracnose lesions on the inoculated leaves was counted and assessed 7 days after challenge-inoculation. Protection rate was calculated as described by Cohen (1994), that is the rate (%) = 100 (1 - x/y) in which x and y are number of lesions on the leaves of treated and non-treated plants, respectively.

Antifungal effect of strain EXTN-1. Two species of anthracnose pathogens *C. gloeosporioides* and *C. orbiculare* and late blight pathogen *Phytophthora capsici* were grown on potato dextrose agar for 7 days. Using a cork borer (5 mm ∅) a piece of tryptic soy agar on which strain EXTN-1 grown was taken up and transferred on the middle of half side of potato dextrose agar. On the other side, a piece of agar block grown *C. gloeosporioides*, *C. orbiculare* or *P. capsici* was transferred and incubated at 28°C. Seven and 3 days after incubation of anthracnose and late blight pathogens, respectively, antifungal effect of EXTN-1 was measured by visible growth inhibition of hyphae of fungi.

Light microscopy of infection structures. Second leaves of the inoculated cucumber plants were detached 3 days after challenge-inoculation. The leaves were cut with a cork borer (5-mm ϕ) and fixed with 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 h. The leaf tissues were stained using the method described by Jeun *et al.* (2000). The leaves were cut with a cork borer (5 mm ϕ) and fixed with 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 h. After washing in the phosphate buffer three times, for 10 min each, the leaf disks were stained with 0.02% Uvitex 2B (w/v) (Diethanol) and 0.005% (w/v) aniline blue for 20 min each, in order to observe the fungal structures and plant leaves response forming callose, respectively. After washing in the phosphate buffer the leaf disks were mounted on glass slides in 50% glycerin. The infection structures of the anthracnose fungus and callose deposition at the penetration sites were observed using fluorescent microscopy (Zeiss) equipped with filter set 05 (BP 400-440, FT 460, LP 470) for fungal structures and 09 (BP 450-490, FT 510, LP 520) for callose deposition. Numbers of total conidia, germinated conidia, appressoria and callose depositions were counted on the leaf surfaces of the plants non-treated, sprayed with chemicals or fungus or drenched with PGPR strains.

Data analysis. All data of numbers of lesions and microscopical observations were statistically analyzed in the inducer-treated and the non-treated plants using a paired *t*-test. Significance levels at $P = 0.05$ or 0.001 were used for all statistical tests.

Results

Antifungal effect by strain EXTN-1 *in vitro* test. A direct antifungal effect of EXTN-1 was revealed by the cultivation with anthracnose pathogens *C. gloeosporioides* or *C. orbiculare*. Hyphal growth of both fungi adjacent to EXTN-1 was strongly inhibited whereas hyphae of other part of both colonies were vegetatively grown without any growth inhibition (Fig. 1A left and middle). The colony of *C. orbiculare* formed a band near EXTN-1 indicating the unfavorable condition for fungal growth (Fig. 1A, middle, see arrow).

In contrast to the case of anthracnose, fungus EXTN-1 could not inhibit the hyphal growth of a Oomycete fungus *Phytophthora capsici*, which has a less chitin compound in its cell walls (Fig. 1A right). In 5 days after incubation the fungal mycelium grew over the EXTN-1 colony, indicating no affect of EXTN-1 to hyphal growth of *P. capsici* (data not shown).

Growth promoting by PGPR strains and necrosis by SAR inducers. PGPR strains have been known to promote plant growth as well as to induce resistance against plant pathogens. To test weather the baterial strain EXTN-1 shows the growth-promoting effect, the cucumber plants were observed after treatment with EXTN-1. Two weeks after drenching with both PGPR strains EXTN-1 and INR-7 to the root system of cucumber plants resulted in growth promotion of the plants (data not shown). There was no visible difference in phenotype between the plants treated with both PGPR strains and the untreated control plants.

In the classic induced resistance, termed SAR, the forming of necrosis on the treated part of plants plays a great role for triggering an effective defense mechanism against an attack of pathogens. The abiotic activator ASA caused the forming of necrotic spots on the sprayed leaves at 3 days after treatment. The lesion spots by the biotic inducer anthracnose pathogen were visible at least 5 days after inoculation.

Decrease of anthracnose severity by ISR and SAR. Five days after inoculation with *C. orbiculare*, visible lesion spots were formed on the leaves of the control as well as the PGPR-treated plants. Number of lesions, however, was reduced in the leaves of the PGPR-treated plants compared with the control plants (Table 1). Furthermore, the size of lesions on the leaves of untreated plants increased rapidly, indicating an enhancement of development of the fungal growth. The development of anthracnose fungus was restricted on the leaves of plants treated with PGPR strain EXTN-1 at approximately 33.4% whereas the treatment with INR-7 could not effectively depress the disease development (Table 1).

By the treatment with ASA, resistance against cucumber anthracnose was effectively mediated in the upper parts of the plants (Table 1). The foliage treatment with ASA protected the plants against anthracnose disease much more effectively than that by treatment with EXTN-1 (Table 1). Similarly, the protection value by *C. orbiculare* was comparable with that of ASA (Table 1).

Microscopical observation on the leaf surface. Using a fluorescence microscope, infection structures were examined after challenge inoculation with *C. orbiculare* on the leaf surface and in the epidermal cell layer of cucumber plants mediated ISR or SAR. The conidia of *C. orbiculare* geminated and formed appressoria on the leaf surfaces of untreated plants 24 h after inoculation. Some germinated conidia failed to form appressoria. Most of appressoria formed melanin, which was identified as black under the microscope. A few appressoria did not form melanin. No visible responses were found in the leaf tissues a day after inoculation. Three days after inoculation, some penetration sites became fluorescent, indicating callose formation in the plant cell walls stained by aniline

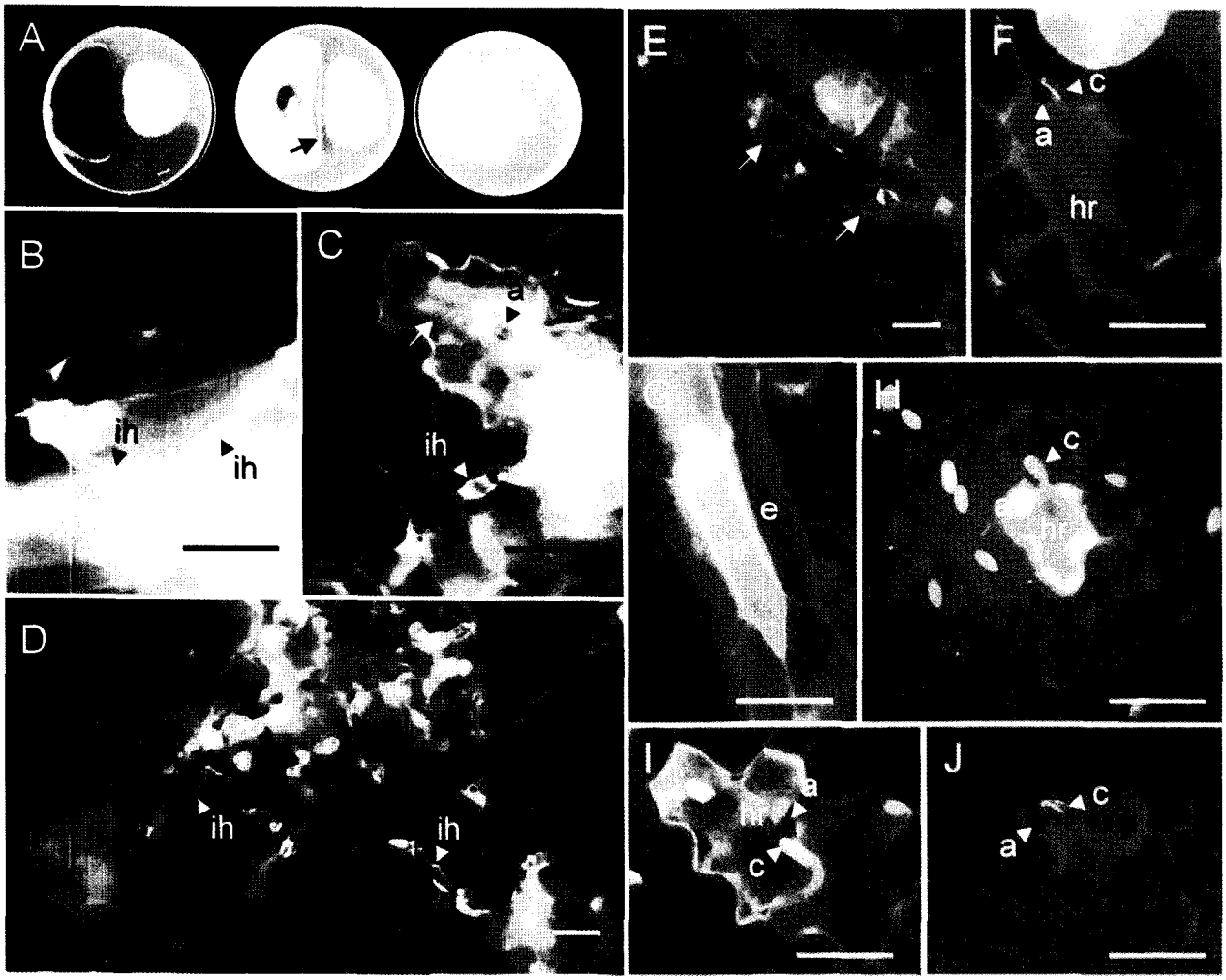

Fig. 1. Effect of the PGPR strain EXTN-1 to hyphal growth of fungal pathogens *Colletotrichum gloeosporioides* (left), *C. orbiculare* (middle) or *Phytophthora capsici* (right) growing on potato dextrose agar *in vitro* (A). Fluorescence microscopical observations of infection structures on the leaves of the cucumber plants 3 (B, C, E-J) and 5 (D) days after inoculation with conidial suspension of *C. orbiculare* (5×10^5 conidia/m*l*). Infection structures at penetration site on the leaves of untreated control (B-D) and on the leaves of PGPR strains EXTN-1 (E, F) and INR-7 (G) pre-inoculated plants. Infection structures on second leaves of plants pre-treated with amino salicylic acid (ASA) (H and I) as well as pre-inoculation with *C. orbiculare* (J) on the first leaves. Drench with the suspension of PGPR strains (10^8 cfu/m*l*), treatment with 50 mM ASA and pre-inoculation with *C. orbiculare* (5×10^5 conidia/m*l*) on the first leaves were carried out 5 and 7 days before the challenge inoculation, respectively. The bars = 20 μm. Abbreviations: a, appressorium; c, conidium; e, epidermal cell; hr, hypersensitive reaction; ih, intercellular hypha.

blue. Some epidermal cells reacted with hypersensitivity against the fungal pathogen attack. However, frequency of appressorium formation did not increase, as compared to that found on the leaves a day after inoculation (data not shown). Intercellular hyphae were detected at some penetration sites of the inoculated leaves 3 days later (Fig. 1B and C). Most of the penetration sites were not intensively fluorescent with aniline blue, indicating no more defense reaction of the host cells (Fig. 1C, arrow). The intercellular hyphae spread broadly into the plant tissues 5 days after inoculation (Fig. 1D).

Frequency of conidial germination on the leaves of the plants treated with PGPR strain EXTN-1 was significantly decreased compared to those on the untreated control plants (Fig. 2A). Most of appressoria formed melanin at the level similar to that of the untreated plants. There was no difference in the frequency of appressoria formation between the EXTN-1 treated and untreated control plants (Fig. 2B). However, callose as a β-1,3-glucan polymer, was frequently deposited at the penetration sites on the leaves of plants treated with EXTN-1 (Fig. 1E, arrows and Fig. 2C), suggesting a defense reaction of plants against fungal pathogen attack. Furthermore, epidermal cells of EXTN-1 treated plants showed frequently HR against the invasion of pathogen, which was also found in the INR-7 treated plants (Fig. 1F and G). HR was not fre-

Table 1. Reduction of lesion number and protection rate on the second leaves of cucumber plants expressing induced systemic resistance by drench with PGPRs EXTN-1 or INR-7 and acquired systemic resistance by treatment with amino salicylic acid (ASA) or *Colletotrichum orbiculare* 7 days after challenge inoculation with the anthracnose pathogen

Treatment[a]	Number of lesions	Protection (%)[c]
EXTN-1	71.2±47.3[b]	33.4
INR7	86.9±39.3	18.7
Control	106.9±42.1	-
ASA	9.0±9.7	48.0
C. orbiculare	7.8±8.5	54.9
Control	17.3±13.0	-

[a]Inoculation with 10^8 cfu/m*l* of PGPR strains *Bacillus amyloliquefaciens* EXTN-1, and *B. pumilus* INR7 were carried out 5 days before the challenge-inoculation. Inoculation with *C. orbiculare* (5×10^5 conidia/m*l*) and treatment with 1 mM amino salicylic acid (ASA) on the first leaves were carried out 7 days before the challenge inoculation.
[b]Values represent means of values±standard deviation of two separated experiments each containing 12 plants per treatment.
[c]Percentage was calculated by the formula, protection (%) = 100 (1 - *x*/*y*) in which *x* and *y* are number of lesions on the leaves of treated and non-treated plants, respectively.

quently observed on the control plants. Treatment with strain INR-7 showed no significant difference in the percent of conidial germination, appressorium formation and callose formation (Fig. 2A-C).

In contrast to PGPR, application of ASA strongly decreased germination of fungal conidia on the second leaves (Fig. 1H, I and 3A). Furthermore, appressorium formation was dramatically reduced on the upper leaves of plant sprayed ASA (Fig. 3B). However, frequency of callose deposition did not increase on the leaves of plant treated with ASA at the penetration sites (Fig. 3C). HR of the epidermal cells of ASA treated plants was observed as frequently as that detected on the leaves of PGPR treated plants (Fig 1H and I).

On the upper leaves of cucumber plants pre-inoculated with *C. orbiculare* on the bottom leaf, germination rate of conidia was decreased after challenge inoculation, although the decrease was not dramatically as much as that observed on the leaves of plants treated with ASA (Fig. 3A). Appressorium formation was also slightly decreased on the leaves expressing resistance by *C. orbiculare* compared with untreated corresponding leaves (Fig. 3B). Similar to the case of ASA, *C. orbiculare* could not be caused an enhancement of callose formation at the penetration sites (Fig. 1J and Fig. 3C).

Discussion

Although signal pathway for expression of induced resistance is not clearly explained, some possible mechanisms

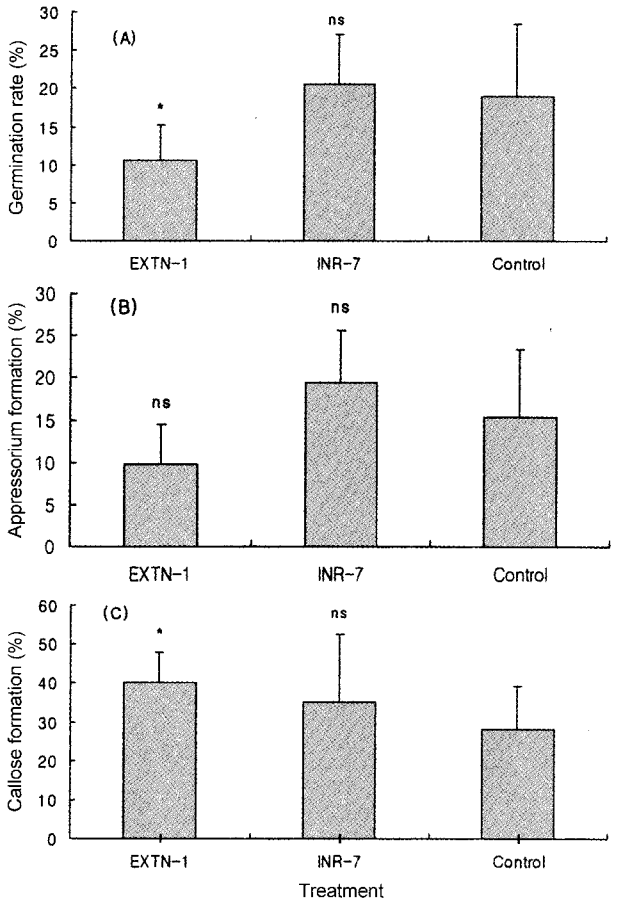

Fig. 2. Frequency of conidial germination, appressorium formation and collose formation at the penetration site on the leaves of cucumber plants applied with PGPR strains EXTN-1 and INR-7 as well as untreated control plants 3 days after inoculation with *Colletotrichum orbiculare* (5×10^5 conidia/m*l*). The inoculation with PGPR strains (10^8 cfu/m*l*) was carried out 7 days before the challenge inoculation. The vertical bars indicate the standard deviation of the three separated experiments each containing four leaf discs from six plants per treatment. ns = non-significant; * = significant at the 5% probability level.

are proposed that an accumulation of SA is necessary for expression of SAR (Malamy *et al.*, 1990; Metraux *et al.*, 1990), whereas SA does not play an important role for ISR mediated by PGPR strains. Differences of defense mechanisms were also observed in Arabidopsis plants between expressing SAR and ISR (Pieterse *et al.*, 1998). It seems that defense mechanisms expressing by systemic induced resistance are various to the plant speices (Sticher *et al.*, 1997; Van Loon *et al.*, 1998a). In this study the defense mechanisms were compared in the leaves of cucumber plants in the aspect of SAR and ISR.

In contrast to the case of SAR, some PGPR strains mediating systemic resistance have direct antifungal activity. In the ISR expressing plants, growth of fungal pathogen may be limited by the competition of ion (Fe) which

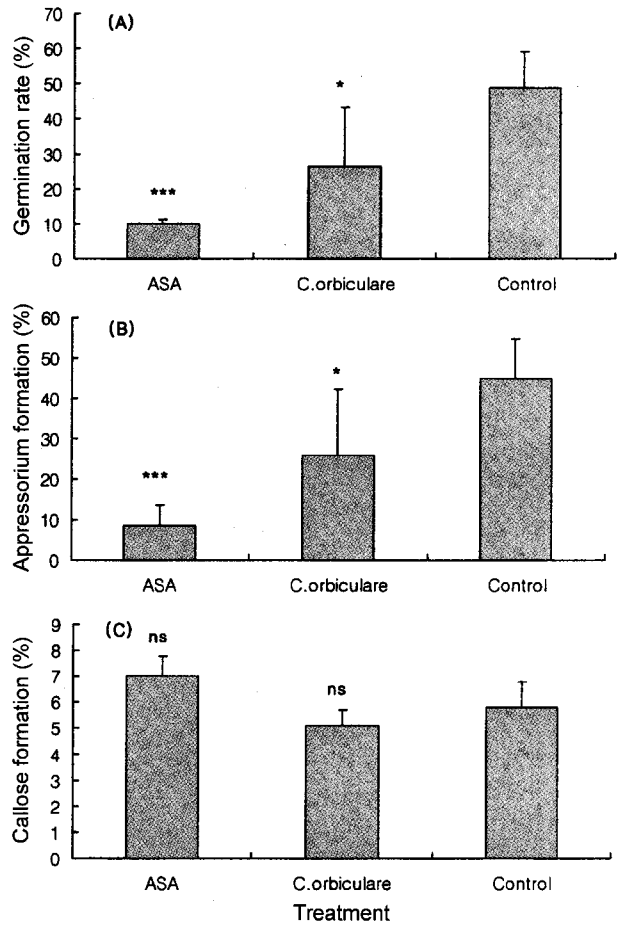

Fig. 3. Frequency of conidial germination, appressorium formation and collose formation at the penetration site on the leaves of cucumber plants applied with amino salicylic acid (ASA), pre-inoculated with *Colletotrichum orbiculare* as well as untreated control plants 3 days after inoculation with *C. orbiculare* (5×10^5 conidia/m*l*). Application with ASA (50 mM) and pre-inoculation with *C. orbiculare* (5×10^5 conidia/m*l*) were carried out 7 days before the challenge inoculation. The vertical bars indicate the standard deviation of the three separated experiments each containing four leaf discs from six plants per treatment. ns = non-significant; * = significant at the 5% probability level; *** = significant at the 0.1% probability level.

is easily captured by siderophores produced by PGPR (Maurhofer *et al.*, 1994; Van Loon *et al.*, 1997; 1998b). The present results showed that the hyphal growths of both anthracnose pathogens growing on the agar medium were inhibited by EXTN-1 (Fig. 1A), indicating a direct antifungal effect of EXTN-1 to anthracnose pathogens. However, EXTN-1 did not suppress the hyphal growth of the Oomycete fungus *P. capsici* (Fig. 1A). Similar results were also observed *in vitro* test in which PR-proteins β-1,3-glucanase and chitinase could inhibit the growth of various fungal pathogens except Oomycetes (Mauch *et al.*, 1988). These findings lead to the suggestion that the antifungal effect of PGPR may be similar to that of PR-proteins.

In contrast to PGPR strains, ASA shows no growth inhibition of fungal pathogens. *In vitro* test with filter paper disks soaked in the high concentration of ASA solutions (1%, w/v) showed no antifungal effect in the hyphal growth of *C. orbiculare* on the potato dextrose agar (data not shown). Nevertheless, the treatment with ASA caused the expression of SAR in cucumber plants against *C. orbiculare* (Table 1). Based on these results it is strongly speculated that there are various defense mechanisms of induced resistance, which are differently expressed by treated inducers.

The possibility of different triggering between SAR and ISR may be supported by the phenotype of the plants after inducer treatments. For the effective expression of SAR necrosis or HR is necessary on the treated part of plants (Sticher *et al.*, 1997; Siegrist *et al.*, 2000). In this study necrotic and lesion spots were also observed on ASA treated and *C. orbiculare* inoculated leaves, respectively. However, the inoculation with EXTN-1 or INR-7 caused the growth promotion of cucumber plants without any necrosis nor HR. Though the different phenotypes of cucumber plant, the systemically induced resistance was expressed on the leaves of all pre-treated plants after challenge inoculation with *C. orbiculare* (Table 1).

The differences between both induction systems were detected not only in signal pathways but also in expression of resistance. The protection values by SAR were higher compared to those by ISR (Table 1). Similarly, Arabidopsis plants were more effectively protected by the pressure infiltration with an avirulent *Pseudomonas syringae* pv *tomato* compared to the protection by treatment with a PGPR strain *P. fluorescens* (Knoster *et al.*, 1999). The different protection values support the suggestion of various defense mechanisms that are differently expressed in both induction systems. In fact, in the Arabidopsis plants expressing ISR PR-proteins were not accumulated whereas PR-1a mRNA was found in the SAR expressing Arabidopsis plants (Pieterse *et al.*, 1998).

In the present study, the further differences between SAR and ISR expression were observed by cytological methods using a fluorescence microscope. To penetrate the host cell wall, anthracnose pathogen *C. orbiculare* forms a special infection structure called appressorium that contains a melanin layer inner its cell wall. The melanin layer plays an important role for penetration into host cells forming a high-pressure inner the appressorium (Howard *et al.*, 1989; 1991). Therefore, the defense mechanism can be expressed by the suppression of melanin biosyntheses. Unfortunately, using microscope it was impossible to evaluate the melanin formation of appressorium on the leaf surfaces.

It was detected that the conidial germination was signif-

icantly suppressed on the leaves of plants pre-inoculated with EXTN-1 (Fig. 2A). Moreover, on the leaves expressing SAR by ASA the germination of conidia was dramatically reduced (Fig. 3A). Reduction of spore germination had been demonstrated in several incompatible in- teractions (Doke *et al.*, 1987; Kovats *et al.*, 1991b). Probably, the quantitative decrease of the germination rates in the SAR expressing leaves may cause the more effective protection than ISR expressing leaves (Table 1).

The apparent difference of infection structures between leaves expressing ISR and SAR lay in the frequency of appressorium formation at the penetration sites. EXTN-1 pre-inoculation lead no suppression of appressorium formation compared to untreated control plants (Fig. 2B), whereas on the leaves expressing SAR the rate of appressoria formation was strongly decreased (Fig. 3B). Because appressorium is formed only at the penetration site, the low frequency of appressorium formation means decrease of fungal penetration. Suppression of appressorium formation had also been demonstrated in some plants expressing SAR against plant pathogens (Kovats *et al.*, 1991a). Although the appressioum is not always involved in the resistance expression (Jeun *et al.*, 2000; Kovats *et al.*, 1991b), the lower formation rate of appressorium in the SAR-expressing leaves may cause the more effective protection compared to the leaves expressing ISR (Table 1).

One of the active defense mechanisms of plants against fungal attack is papillae formation at the penetration sites (Deising *et al.*, 1996). It is known that papillae is composed with callose, a polymer of β-1,3-glucans (Kauss, 1992). Using fluorescence microscopy the deposition of callose formation could be detected in the leaves stained with aniline blue (Fig. 1F-I). Much more number of callose depositions was found on the leaves of EXTN-1 pre-inoculated plants compared to that of control plants (Fig. 2C). The enhancement of callose formation was also observed in the leaves of tomato plants that were pre-inoculated with tobacco necrosis virus (TNV) (Jeun *et al.*, 2000). However, ASA could not enhance callose formation (Fig. 3C). Similarly, the leaves expressing classic SAR mediated by *C. orbiculare* the callose deposition was not increased compared to that in control leaves (Fig. 3C). From the results it is speculated that the promotion of callose formation may not always necessary to enhance defense mechanisms in the host pathogen interaction.

Interestingly, the degree of reduction of germination rate as well as appressorium formation on the leaves expressing classic SAR by anthracnose pathogen were less than those found on the SAR leaves mediated by ASA (Fig. 3A and B). However, compared to the control plants the fungal germination and appressorium were significantly reduced on the leaves expressing classic SAR by the pathogen (Fig. 3A and B). Furthermore, tendency of the microscopical results was similar between both leaves expressing SAR by pathogen and by ASA (Fig. 3A-C). Based on these observations it is suggested that the defense mechanisms by the pathogen may similar with those by chemical. The similarity of the defense mechanisms of SAR mediated by biotic or abiotic factors was also reported in the other host pathogen interactions (Jeun *et al.*, 2000; Siegrist *et al.*, 2000).

The PGPR strain INR-7, which successfully mediated resistance in cucumber plants against *C. orbiculare* (Raupach and Kloepper, 1998), could not effectively trigger ISR in this experiment (Table 1). Although the pre-inoculation with INR-7 caused the growth promotion of plants and HR (Fig. 1G), there were no differences in the germination rate, appressorium formation and callose formation between on the leaves of plants pre-inoculated with INR-7 and untreated control plants (Fig. 3A-C). Perhaps, in this study the resistance induction by INR-7 may be broken down through the environmental factors such as soil composition.

In conclusion, the resistance mechanisms on the leaf surfaces were differently expressed by the pre-inoculation with PGPR strain EXTN-1 mediating ISR and by the pre-treatment with ASA or *C. orbiculare* inducing SAR. These different defense mechanisms including the other factors such as PR-proteins (Jeun, 2000; Hwang *et al.*, 1997) may cause the different protection values.

Acknowledgments

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References

Cohen, Y. 1994. Local and systemic control of *Phytophthora infestans* in tomato plants by DL-3-amino-n-butanoic acids. *Phytopathology* **84**: 55-59.

Deising, H., Heiler, S., Rauscher, M., Xu, H. and Mendgen K. 1996. Cellular aspects of rust infection structure differentiation. Spore adhesion and fungal morphogenesis. In: Histology, Ultrastructure and Molecular Cytology of Plant-Microorganism Interactions, M. Nicole and V. Gianinazzi-Pearson (eds.) pp. 135-156, Kluwer Academic Publishers.

Doke, N., Ramirez, A. V. and Tomiyama, K. 1987. Systemic induction of resistance in potato plants against *Phytophthora infestans* by local treatment with hyphal wall components of the fungus. *J. Phytopathol.* **119**: 232-239.

Friedrich, L., Lowton, K., Ruess, W., Masner, P., Specker, N., Rella, M. G., Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, J.-P., Kessmann, H. and Ryals, J. 1996. A benzothiazole derivative induces systemic acquired resistance in tobacco.

Plant J. **10**: 61-70.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* **261**: 754-756.

Howard, R. J. and Ferrari, M. A. 1989. Role of melanin in appressorium function. *Exp. Mycol.* **13**: 403-418.

Howard, R. J., Ferrari, M. A., Roach, D. H. and Money, N. P. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures. Proc. *Natl. Acad. Sci. USA* **88**: 11281-11284.

Hwang, B. K., Sunwoo, J. Y., Kim, Y. J. and Kim, B. S. 1997. Accumulation of β-1,3-glucanase and chitinase isoforms, and salicylic acid in the DL–β-amino-n-butyric acid-induced resistance response of pepper stems to *Phytophthora capsici*. *Physiol. Mol. Plant Pathol.* **51**: 305-322.

Jeun, Y. C. 2000. Immunolocalization of PR-protein P14 in leaves of tomato plants exhibiting systemic acquired resistance against *Phytophthora infestans* induced by pretreatment with 3-aminobutyric acid and preinoculation with *Tobacco necrosis virus*. *J. Plant Disease and Protection* **107**: 352-367.

Jeun, Y. C., Siegrist, J. and Buchenauer, H. 2000. Biochemical and cytological studies on mechanisms of systemic induced resistance in tomato plant against *Phytophthora infestans*. *J. Phytopathol.* **148**: 129-140.

Kauss, H. 1992. Callose and callose synthase. In: Gurr S. J., McPherson M. J. and Bowles D. J. eds. Molecular Plant Pathology: A practical Approach IRL Press, Vol. II, Pp 1-8.

Knoester, M., Pieterse, C. M. J., Bol, J. F. and Van Loon, L. C. 1999. Systemic resistance in Arabidopsis induced by rhizobacteria requires ethylene-dependent signaling at the site of application. *Mol. Plant Microb. Inter.* **12**: 720-727.

Kovats, K., Binder, A. and Hohl, H. R. 1991a. Cytology of induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Planta* **183**: 484-490.

Kovats, K., Binder, A. and Hohl, H. R. 1991b. Cytology of induced systemic resistance of tomato to *Phytophthora infestans*. *Planta* **183**: 491-496.

Lawton, K., Friedrich, L., Hunt, M., Weymann, K. and Delaney, T. 1996. Bezothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant Journal.* **10**: 71-82.

Malamy, J., Carr, J., Klessig, D. and Raskin, I. 1990. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250**: 1002-1004.

Maurhofer, M., Hase, C., Meuwly, P., Mraux, J.-P. and Defago, G. 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHAO: influence of the *gacA*-gene and of pyoverdine production. *Phytopathology* **84**: 139-146.

Mauch, F., Mauch-Mani, B. and Boller, T. 1988. Antifungal hydrolases in pea tissue II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. *Plant Physiology* **88**: 936-942.

Métraux, J. P., Signer, H., Ryals, J., Ward, E. and Wyss-Benz, M. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* **250**: 1004-1006.

Park, K. S. and Kloepper, J. W. 2000. Activation of PR-1a promoter by rhizobacteria that induce systemic resistance in tobacco against *Pseudomonas syringae* pv. *tabaci*. *Biol. Control* **18**: 2-9.

Pieterse, C. M. J. and Van Loon, L. C. 1999. Salicylic acid-independent plant defence pathways. *Trends in plant science* **4**: 52-58.

Pieterse, C. M. J., Van Wees, S. C. M., Van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P. J. and Van Loon, L. C. 1998. A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell* **10**: 1571-1580.

Raupach, G. S. and Kloepper, J. W. 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* **88**: 1158-1164.

Ryals, J., Ward, E., Ahl-coy, P. and Metraux, J. P. 1992. Systemic acquired resistance: an inducible defence mechanism in plants. Society for Experimental Biology Seminar Series 49: In Inducible Plant Proteins, ed. J.L. Wray. Cambridge University Press, Pp. 205-229.

Siegrist, J., Orober, M. and Buchenauer, H. 2000. β-Aminobutyric acid-mediated enhancement of resistance in tobacco to tobacco mosaic virus depends on the accumulation of salicylic acid. *Physiol. Mol. Pl. Pathol.* **56**: 95-106.

Sticher, L., Mauch-Mani, B. and Métraux, J. P. 1997. Systemic acquired resistance. *Annu. Rev. Phytopathol.* **35**: 235-270.

Van Loon, L. C., Bakker, P. A. H. M. and Pieterse, C. M. J. 1998a. Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* **36**: 453-483.

Van Loon, L. C., Bakker, P. A. H. M. and Pieterse, C. M. J. 1998b. Induction and expression of PGPR-mediated induced resistance against pathogens. In: Duffy, B., Rosenberger, U. and Defago, G. eds. Molecular Approaches in Biological Control. Delemont: IOBC/OILB, Pp 103-110.

Van Loon, L. C., Bakker, P. A. H. M. and Pieterse, C. M. J. 1997. Mechanisms of PGPR-induced resistance against pathogens. In: Ogoshi, A., Kobayashi, Y., Homma, Y., Kodama, F., Kondo, N. and Akino, S. eds. Plant Growth-Promoting Rhizobacteria-Present status and future prospects. Sapporo: OECD, Pp 50-57.

Van Loon, L. C. 1999. Occurrence and properties of plant pathogenesis-related proteins. In: Swapan, K. D. and Subbaratnam M. eds. Pathogenesis-related proteins in plants. Pp 1-20.