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Ultrastructures of the Leaves of Cucumber Plants Treated with DL-3-Aminobutyric Acid at the Vascular Bundle and the Penetration Sites after Inoculation with *Colletotrichum orbiculare*

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Pre-treatment with DL-3-aminobutyric acid (BABA) in the cucumber plants caused the decrease of disease severity after inoculation with anthracnose pathogen *Colletotrichum orbiculare*. In this study, ultrastructures of the vascular bundle and the infection structures in the leaves of BABA-treated as well as untreated cucumber plants were observed after inoculation with the anthracnose pathogen by electron microscopy. The ultrastructures of vascular bundle in the leaves of BABA-treated plants were similar to those of the untreated plants except plasmodesmata. In the BABA-treated plants, the plasmodesmata were more numerous than in the untreated plants, suggesting that the BABA treatment may cause the active transfer of metabolites through the vascular bundle. In the leaves of untreated plants, the fungal hyphae were spread widely in the plant tissues at 5 days after pathogen inoculation. Most cellular organelles in the hyphae were intact, indicating a compatible interaction between the plant and the parasite. In contrast, in the leaves of BABA pre-treated plants the growth of most hyphae was restricted to the epidermal cell layer at 5 days after inoculation. Most hyphal cytoplasm and nucleoplasm was electron dense or the intracellular organelles were degenerated. The cell walls of some plant cells became thick at the site adjacent to the intercellular hyphae, indicating a mechanical defense reaction of the plant cells against the fungal attack. Furthermore, hypersensitive reaction (HR) of the epidermal cells was often observed, in which the intracellular hyphae were degenerated. Based on these results it is suggested that BABA causes the enhancement of defense mechanisms in the cucumber plants such as cell wall apposition or HR against the invasion of *C. orbiculare*.

Keywords : DL-3-aminobutyric acid (BABA), *Colletotrichum orbiculare*, cucumber, systemic acquired resistance (SAR), ultrastructure

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The systemic acquired resistance (SAR) can be triggered in plants by inoculation with necrosis forming pathogens or with nature originated chemicals (Sticher et al., 1997). SAR is effective simultaneously against a broad spectrum of plant diseases caused by fungi, bacteria, and viruses (Sticher et al., 1997). Because of this benefit, application of SAR in crop cultivation has been regarded as one of the new strategies for plant protection (Kuc, 1995). Especially, using SAR is important to protect the diseases caused by viruses because there is no chemical effective directly to viruses, yet. Actually, effective SAR has been reported in many cases of host-parasite interactions until now (Sticher et al., 1997). Moreover, some of activators inducing SAR such as benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH; Bion®) have been commercially developed and applied to the field in Europe (Görlach et al., 1996).

It has been tried to illustrate the signal pathway of SAR during the recent decade and some part of the pathway were explained through the experiments using various activators inducing SAR (Malamy et al., 1990; Vernooij et al., 1994; Friedrich et al., 1996). Accumulation of endogenous salicylic acid (SA) is necessary to express SAR by pre-inoculation with a biotic inducer such as plant pathogen (Malamy et al., 1990). It has been well revealed by the experiment using the NahG plant (Gaffney et al., 1993). This is a transgenic plant in which SA is converted to catechol resulting in no accumulation of SA. In the NahG plants SAR was not triggered even by the pre-inoculation of plant pathogens forming necrosis. However, SAR can be also triggered without accumulation of SA in the plants pre-treated with the chemical activators such as 2,6-dichloro-isonicotinic acid (INA) or BTH (Friedrich et al., 1996; Lawton et al., 1996). This means that INA or BTH play a role in the downstream of SA in the signal pathway of SAR (Sticher et al., 1997).

DL-3-aminobutyric acid (BABA) is well known as one of the chemical activators inducing resistance against several plant pathogens (Cohen, 2002). It has previously been shown that pre-treatment with BABA in the cucumber

plants caused the decrease of disease severity after inoculation with anthracnose pathogen *Colletotrichum orbiculare* (Jeun et al., 2001). Also, 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). However, the signal pathway of resistance mediated by BABA is not yet clearly illustrated.

Using the fluorescent microscope it was shown that either germination rate or appressorium formation of the *C. orbiculare* was dramatically reduced on the leaf surface of the plants pre-treated with BABA compared with those of untreated plants after the fungal inoculation (Jeun et al., 2001). The suppression of fungal growth on the surface of the leaves may result in the resistance expression of the BABA-treated plants (Jeun et al., 2001). However, to express resistance in the plants more resistance mechanism may be involved in symplast such as accumulation of phenolic compounds, phytoalexin, pathogenesis-related proteins and lignification of cell walls (Park and Kloepper, 2000; Siegrist et al., 1994; Hwang et al., 1997).

In the present studies, the ultrastructures of the vascular bundle in the leaves of untreated as well as BABA-treated cucumber plants before fungal infection were examined by electron microscopy. In order to explain the resistance mechanisms in symplast, the ultra-structures of the infection structures at the penetration sites were observed in the cucumber leaves expressing systemic acquired resistance (SAR) by BABA treatment as well as in the leaves of untreated plants after inoculation with the anthracnose pathogen.

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 commercial soil (TKS-2®, Floragard, Germany) containing 10% of perlite (Parat). Cucumber seedlings were grown in a greenhouse at 28°C at daytime and 25°C at night. Plants were watered once daily with about 30 ml per plant and fertilized once a week with 1% Wuxal super® (N:P:K, 12:4:6; Aglukon, Duesseldorf, Germany).

Anthracnose pathogen, *Colletotrichum orbiculare*, was incubated on green beans agar medium at 28°C for 5 days. Ten ml of distilled water was poured in the fungal culture and then fungal conidia were harvested by using a brush. The conidial concentration was adjusted to 2.5×10^5 conidia/ml. This conidial suspension with 100 µl/L Silwet L-77, which enhances the adhesion of conidia on leaf surface, was used as inoculum for challenge inoculation on cucumber leaves.

Treatment with BABA, challenge inoculation and disease assessment. Thirty ml of DL-3-aminobutyric acid (BABA, 10 mM) solution was soil-drenched per plant 3 days before challenge inoculation with *C. orbiculare*. Water treated plants were used as control.

The conidial suspension of *C. orbiculare* (2.5×10^5 conidia/ml) was sprayed on the aerial cucumber leaves 3 days after the treatment with BABA solution. The plants inoculated with the conidial suspension of the fungus were kept in a humid chamber of 100% RH for 24 h and transferred to the greenhouse at 28°C during the day and 25°C at night and with 60% humidity. The number of anthracnose lesions on the inoculated leaves was counted 7 days after challenge inoculation by visual observation.

Tissue processing for electron microscopy. First leaves of plants treated with BABA as well as the corresponding leaves of control plants were detached 5 days after the challenge inoculation with *C. orbiculare*, respectively. Infected leaf areas with necrotic lesions were cut (1×3 mm^2) using a razor blade. Fixation, dehydration and embedding of the leaf materials were performed according to Hayat (1989). The leaf sections were fixed in 2%(v/v) glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, for 2 h. Post fixation was performed in 2%(w/v) osmium tetroxide in the phosphate buffer for 2 h at room temperature and dehydrated through an alcohol series (30, 50, 70, 90 and 100% every two times for 30 min., respectively). Then the samples were embedded in an epoxy resin (vinylcyclohexene dioxide, ERL 4206) (Spurr, 1969) and polymerized at 70°C for 8 h.

The embedded block was further ultra-thin sectioned (60 nm) using an ultramicrotome (MTX, RMC). The sections were mounted on copper grids. For enhancement of contrast, the sections were stained with 2%(w/v) uranyl acetate in H_2O and lead citrate according to Reynolds (1963). The ultrastructures of the plant tissues were examined using a transmission electron microscope (TEM; JEM1010, JEOL) at 80 kV.

Image processing. The TEM images were read by 25 µm-pixel-size digital imaging plates (IP) and delivered by an IP reader (Fujifilm FDL 5000). The digital image data were transferred to a computer and printed out by a photographic-grade printer (Pictrography 3000).

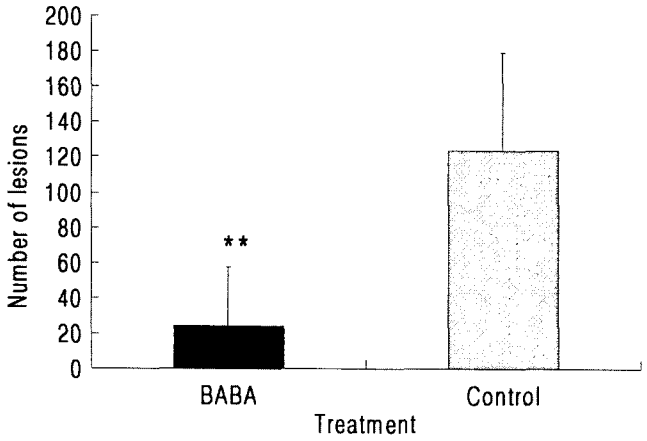

Fig. 1. Reduction of lesion numbers on the leaves of cucumber plants pre-treated with DL-3-aminobutyric acid 7 days after inoculation with *C. orbiculare*. The vertical bars indicate the standard deviations of the three separated experiments each containing 6 plants per treatment.
**significant at the 0.1% probability level by T-test

Results

The pre-treatment with BABA in the cucumber plants caused the decrease of disease severity after inoculation with anthracnose pathogen *Colletotrichum orbiculare* (Fig. 1). The number of lesions on a leaf was only about 20 in BABA pre-treated plants, whereas approximately 120 in untreated plants. The result indicated a significant reduction of the disease severity by the treatment of BABA.

The vascular bundle of the leaves of untreated plants consisted of the tracheary elements of xylem, sieve elements and companion cells (Fig. 2A). The companion cell contained the numerous large mitochondria and the abundant small vacuoles (Fig. 2A). Only a few plasmo-

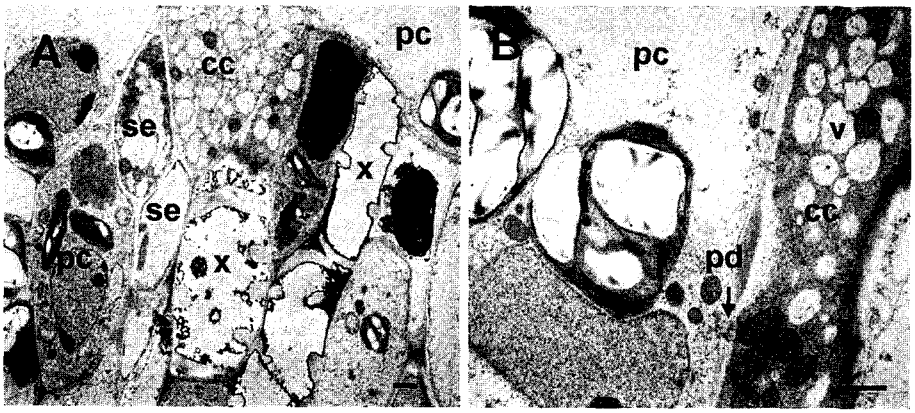

Fig. 2. Electron microscopical observations of the leaf vascular bundle in untreated cucumber plant. **A**: The vascular bundle consists of parenchyma cell, companion cell, sieve element, and xylem. **B**: Only a few plasmodesmata were found. All bars = 1 μm (cc, companion cell; m, mitochondria; pc, parenchyma cell; pd, plasmodesmata; se, sieve element; v, vacuole; x, xylem).

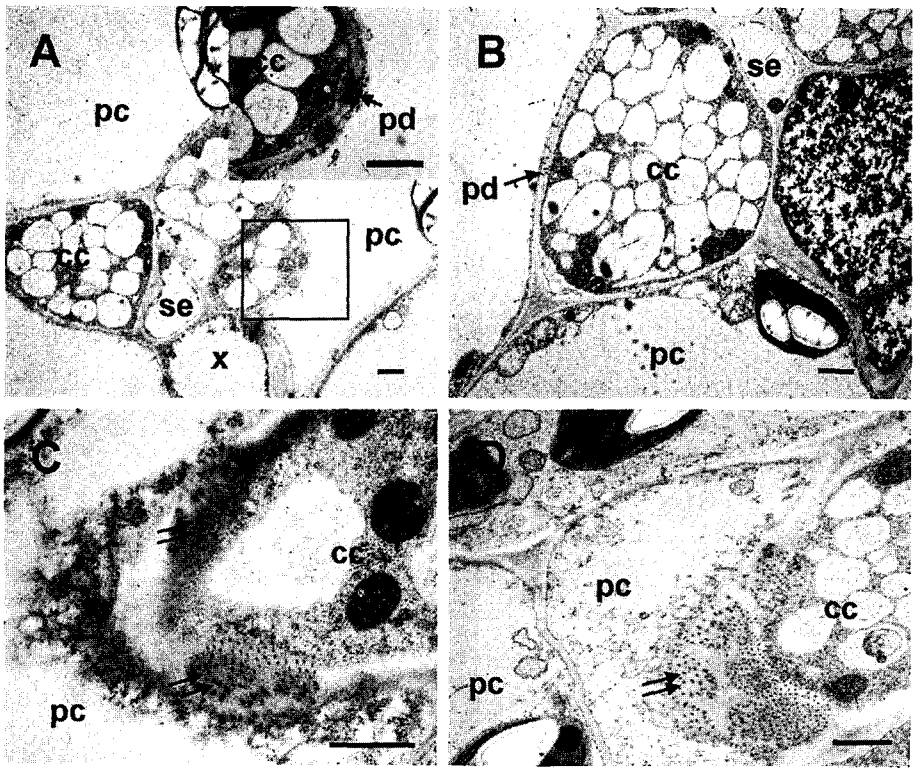

Fig. 3. Electron microscopical observations of the leaf vascular bundle in BABA pre-treated cucumber plant. **A** and **B**: Between the companion and parenchyma cell the plamodesmata were well developed. The square box of upper right is higher magnification of the squared region. **C** and **D**: Abundant particles were found in the companion cell (double arrows). All bars = 1 μm (cc, companion cell; pc, parenchyma cell; pd, plasmodesmata; se, sieve element; x, xylem).

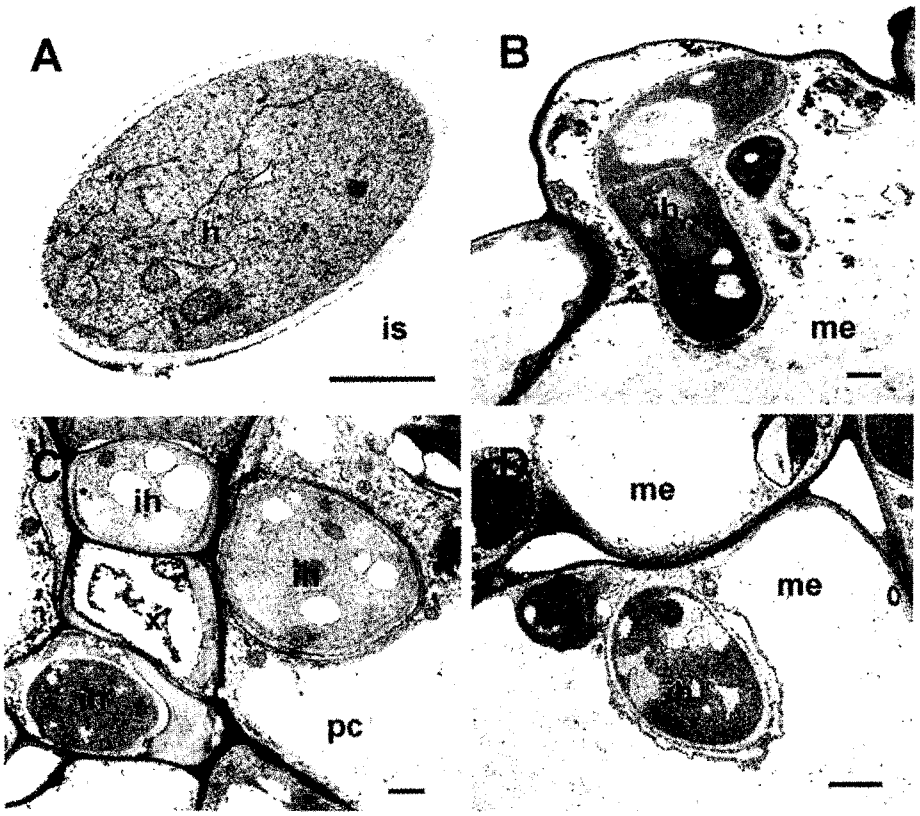

Fig. 4. Electron microscopical observations of the infected leaves in untreated cucumber plants at 5 days after inoculation with *Colletotrichum orbiculare*. **A**: In the intercellular hypha, the cell organelles can be well recognized. **B**: The intracellular hypha was not degenerated. **C**: The fungal hyphae were found in the parenchyma cell and xylem vessel. **D**: Some mesophyll cells were not degenerated after the invasion of the fungal hyphae. All bars = 1 μm (h, intercelluar hypha; ih, intracellular hypha; is, intercellular space; m, mesophyll cell; pc, parenchyma cell; x, xylem).

desmata, through which the photosynthate is transferred, were observed (Fig. 2B). The ultrastructures of vascular bundle in the leaves of BABA-treated plants were similar to those of the untreated plants except the plasmodesmata. In the BABA-treated plants the plasmodesmata were more frequently formed compared to those of untreated plants (Fig. 3A and 3B). Also, numerous particles were observed in the companion cells (Fig. 3C and 3D, arrows), which were not found in the untreated plants.

In the leaves of untreated plants the fungal hyphae were found through out leaf tissues; e.g. in the epidermal, palisade and spongy parenchyma, bundle sheath cells and intercellular spaces at 5 days after inoculation (Fig. 4A, B, C, D). Most organelles in the hyphae as well as the plant cells were apparent and not degenerated (Fig. 4B, C, D), indicating a compatible interaction between the plant and the parasite.

In contrast to the untreated plants, in the leaves of BABA pre-treated plants most hyphal growth was restricted to the epidermal cell layer at 5 days after the fungal inoculation (Fig. 5C, D, E). The fungal hyphae were mostly electron dense (Fig. 5A, B, C, D) and cellular organelles of some intracellular hyphae were degenerated (Fig. 5E). Some plant cell adjacent to the intercellular hyphae became thickened (Fig. 5A and B), indicating an active defense reaction of the plant cells against the fungal attack. The organelles of some infected plant cells were rapidly destroyed (Fig. 5C and F) and sometimes a number of vesicles were formed at the penetration site (Fig. 5D). Furthermore, hypersensitive reaction (HR) of the epidermal cells was often observed in which the intracellular hypha was also degenerated (Fig. 5E), indicating the plant cell react actively against the attack of the fungal hyphae.

Discussion

The systemic acquired resistance (SAR) by BABA has been well known in many host-parasite interactions including cucumber-anthracnose (Cohen, 2002). However, the SAR mechanism by BABA is not clearly demonstrated yet. To illustrate the resistance mechanism mediated by BABA in cucumber plants, the cytological study was carried out

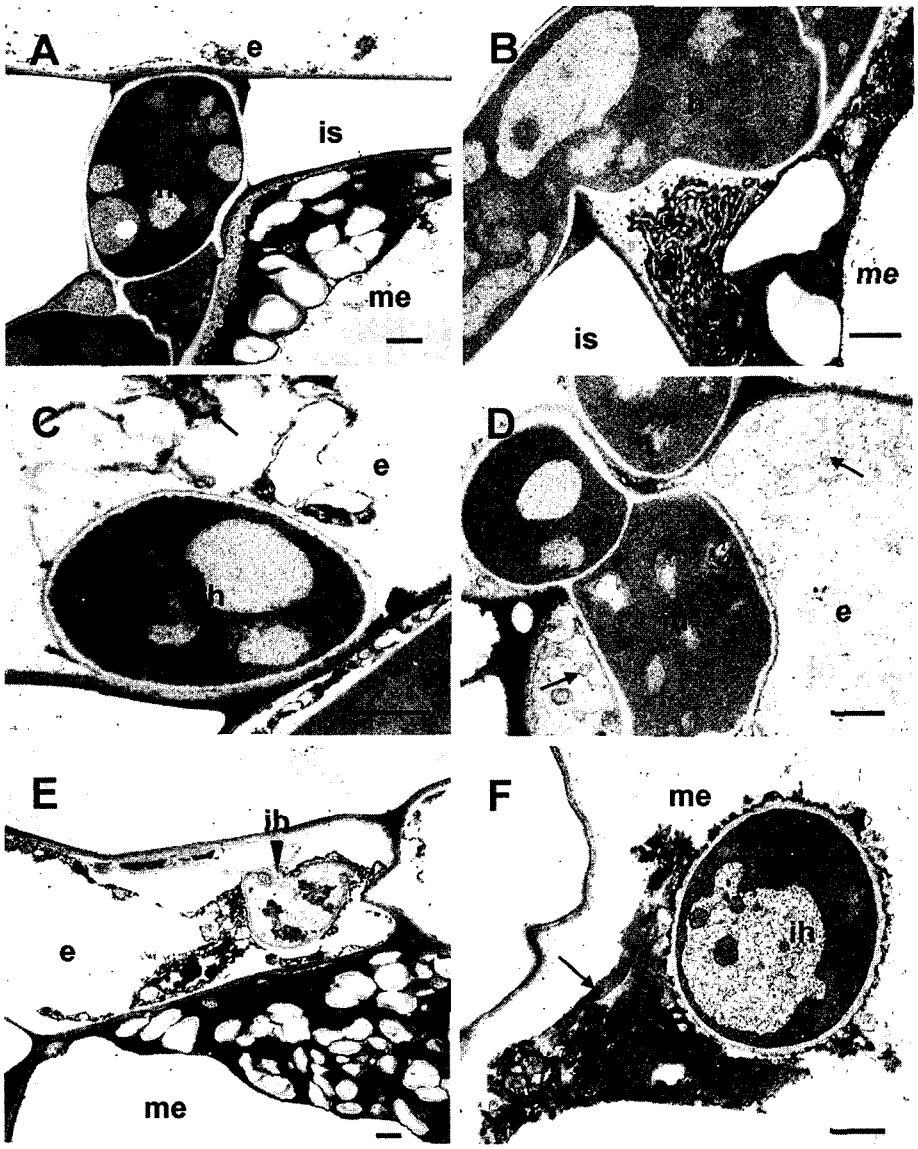

Fig. 5. Electron microscopical observations of the infected leaves in the cucumber plants pre-treated with BABA at 5 days after inoculation with *C. orbiculare*. **A**: The cell wall of the mesophyll cell was thickened against the attack of the fungal hypha (arrow). **B**: An aggregate was formed from the mesophyll cell adjacent to the fungal hypha (arrow). **C**: The intracellular hypha was electron dense and the organelles of the host cell were degenerated (arrow). **D**: Abundant vesicles were found at the penetration site (arrows). **E**: The epidermal cell died rapidly in which intracellular hypha was degenerated. **F**: The cytoplasm of host cell was degenerated after invasion of the fungal hypha (arrow). All bars = 1 μm (e, epidermal cell; h, intercelluar hypha; ih, intracellular hypha; is, intercellular space; m, mesophyll cell).

using an electron microscopy.

Unlike to other chemical inducers INA or BTH, BABA caused the accumulation of SA in tobacco plants before challenge inoculation with anthracnose pathogen (Sigriest et al., 2000). It is well known that SA accumulation is necessary for expression of SAR by biotic inducers such as plant pathogens forming necrosis (Sticher, et al., 1997). This indicates that BABA induces resistance in the plants through the signal pathway, which is different from that by other chemicals (Cohen, 2002). From the phenomenon of the SA accumulation by BABA treatment it is suggested that the active physiological changes occurred in the cucumber plants from which abundant metabolites including SA excluded out (Cohen, 2002). Perhaps, these metabolites such as SA might be concerned with the expression of SAR against the anthracnose pathogen.

This study revealed that the treatment with BABA caused the development of plasmodesmata in the vascular system (Fig. 3A and B). Moreover, the abundant particles between the companion cells and the parenchyma cells were observed

(Fig. 3C and D). These results lead to the suggestion that the physiological changes by BABA might be involved with development of vascular bundle so that abundant metabolite including SA can be actively transferred.

The previous study using a fluorescence microscope revealed that on the leaf surface of cucumber plants pre-treated with BABA either germination rate or appressorium formation of the anthracnose pathogen was dramatically reduced after challenge inoculation (Jeun et al., 2001). Similar results were observed on the leaf surfaces of cucumber plants pre-treated with amino salicylic acid (Jeun et al., 2001). These observations indicated that the SAR might be expressed on the leaf surface before the fungal attack into the leaf tissues.

On the other hand, the SAR seems to be also expressed in the leaf tissues by the treatment with BABA (Fig. 5). It was revealed that in the leaves of BABA pre-treated cucumber plants the fungal growth were restricted to epidermal cell layer (Fig. 5C, D, E) whereas the fungal hyphae grew extensively in the leaves of the untreated plants (Fig. 4B, C, D). In the mesophyll cells of BABA pre-treated plants rapid cytoplasmic degeneration (Fig. 5F) and the formation of the numerous vesicles were found in the epidermal cell (Fig. 5D). The hypersensitive reaction of the plant cell or the accumulation of vesicles at the penetration sites is well known as an incompatible reaction (Hohl and Suter, 1976). Moreover, cell wall apposition of the plant at the penetration sites were often observed in the BABA pre-treated plants (Fig. 5A and B) in which the attack of fungal hyphae into the plant cells might be mechanically inhibited.

Besides, most of fungal hyphae detected in the BABA treated plants were electron dense (Fig. 5A-F), indicating the environment of the BABA treated plant may not compatible for the survival of the anthracnose pathogen. The hyphae showing electron dense were rarely observed in the untreated plant cells (Fig. 4A-D). Based on these observations it is suggested that BABA caused the enhancement of defense mechanisms in the cucumber plants such as cell wall apposition or hypersensitive reaction (HR) against the invasion of *C. orbiculare*.

In summary, this study revealed that the treatment of BABA to the cucumber plants may cause the ultrastructural change of the vascular bundle and that the SAR mediated by BABA in the cucumber plants may be expressed not only by the biochemical resistance mechanisms such as an accumulation of SA (Zimmerli et al., 2000), pathogenesis related proteins (PR-proteins) (Hwang et al., 1997; Jeun and Buchenauer, 2001), phytoalexin (Siegrist and Muhlenbeck, 2002), reactive oxygen species (Park et al., 1998), etc., but also by the structural defense responses such as cell well apposition at the penetration sites and HR.

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Referances

Cohen, Y. R. 2002. β-aminobutyric acid-induced resistance against plant pathogens. *Plant Dis.* 86:448-457.

Friedrich, L., Lawton, 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.

Görlach, J., Volrath, S., Knaufbeiter, G., Hengy, G. and Beckhove, U. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* 8:629-643.

Hayat, M. A. 1989. Principles and techniques of electron microscopy. Biological applications, Third edition. The MacMillan Press, LTD.

Hohl, H. R. and Suter, E. 1976. Host-parasite interfaces in a resistant and a susceptible cultivar of *Solanum tuberosum* inoculated with *Phytophthora infestans*: leaf tissue. *Can. J. Bot.* 54: 1956-1970.

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

Jeun, Y. C. and Buchenauer, H. 2001. Infection structures and localization of the pathogenesis-related protein AP24 in leaves of tomato plants exhibiting systemic acquired resistance against *Phytophthora infestans* after pre-treatment with 3-aminobutyric acid or *Tobacco necrosis virus*. *J. Phytopathol.* 149: 141-154.

Jeun, Y. C., Park, K. S. and Kim, C. H. 2001. Different mechanisms of induced systemic resistance (ISR) and systemic acquired resistance (SAR) against *Colletotrichum orbiculare* on the leaves of cucumber plants. *Mycobiology* 29:19-26.

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

Kuc, J. 1995. Induced systemic resistance- An overview. in: Induced Resistance to Disease in Plants. R. Hammerschmidt and J. Kuc, eds. Kluwer Academic Publishers. pp 169-175.

Lawton, K., Friedrich, L., Hunt, M., Weymann, K. and Delaney, T. 1996. Benzothiadiszole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* 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.

Park, H. J., Doke, N., Miura, Y., Kawakita, K., Noritake, T. and Komatsubara, H. 1998. Induction of a sub-systemic oxidative burst by elicitor-stimulated local oxidative burst in potato plant tissues: a possible systemic signaling in systemic acquired resistance. *Plant Sci.* 138:197-208.

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.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in eletron microscopy. *J. Cell Biol.* 17: 208.

Siegrist, J., Jeblick, W. and Kauss, H. 1994. Defense responses in infected and elicited cucumber (*Cucumis sativus* L.) hypocotyl segments exhibiting acquired resistance. *Plant Physiol.* 105: 1365-1374.

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

Siegrist, J. and Muhlenbeck, S. 2002. Rapid testing of resistance-inducing agents with cultured parsley cells. Modern Fungicides and antifungal compounds II. Intercept Ltd., Andover, UK. 381 p.

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31-43.

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

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals J. 1994. salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* 6:959-965.

Zimmerli, L., Jakab, G., Métraux, J. P. and Mauch-Mani, B. 2000. Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by β-aminobutyric acid. *Proc. Natl. Acad. Sci. USA* 97:12920-12925.