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Subcellular Responses in Nonhost Plant Infected with Pathogenic and Non-pathogenic Strains of *Xanthomonas axonopodis* pv. *glycines*

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***Xanthomonas axonopodis* pv. *glycines*, the causal agent of bacterial pustule of soybean, induces hypersensitive response (HR) in a non-host plant, hot pepper (*Capsicum annuum*). A wild-type strain (8ra) and its non-pathogenic mutant (8-13) of *X. axonopodis* pv. *glycines* were inoculated into the pepper leaf tissues and their subcellular responses to the bacterial infections were examined by electron microscopy. Ultrastructural changes related to HR were found in the leaf tissues infected with 8ra from 8 h after inoculation, characterized by separation of plasmalemma from the cell wall, formation of small vacuoles and vesicles, formation of cell wall apposition, and cellular necrosis. No such responses were observed in the tissues infected with the mutant. In 8ra, the bacterial cells were attached to the cell walls, with the cell wall material dissolved into and appearing to encapsulate the bacterial cells. The bacterial cells later became entirely embedded in the cell wall material. On the other hand, in 8-13, the bacterial cells were usually not attached tightly to the plant cell wall, and no or poor encapsulation of the bacteria by the wall material occurred, although these were encircled by rather loose wall materials at the later stages.**

Keywords : cell death, electron microscopy, hot pepper, hypersensitive response, *Xanthomonas axonopodis* pv. *glycines*.

Xanthomonas axonopodis pv. *glycines* (formerly *X. campestris* pv. *glycines*) is the causal agent of bacterial pustule of soybean, and has been reported in most countries where soybean is grown. It occurs severely in soybean fields, when warm temperatures and frequent rains prevail during the growing season, and causes premature leaf defoliation resulting in reduction of seed production (Hwang and Lim, 1992). In susceptible plants, spots of minute specks to large, irregular, mottled brown areas are formed on leaves with or without pustules, and the leaves become ragged and defoliate later.

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Various factors have been identified in relation to pathogenicity of the bacterium. However, single hormonal or enzymatic factors do not totally govern the pathogenicity (Fett and Dunn, 1987; Hwang et al., 1992). Lindgren et al. (1986) found that genes responsible for pathogenicity and hypersensitive response (HR) in non-host plants in a Gram-negative bacterium *Pseudomonas syringae* pv. *phaseolicola* are known as *hrp* (hypersensitive response and pathogenicity) genes. Major phenotypes of *hrp* mutants that have lost pathogenicity also involve the inability of causing HR in non-host plants. The loss of pathogenicity of *hrp* mutants is associated with reduced multiplication of *X. axonopodis* pv. *glycines* in host tissues (Hwang et al., 1992).

For *X. axonopodis* pv. *glycines*, HR was lost or reduced in non-host plants inoculated with transposon mutants of *X. axonopodis* pv. *glycines* 8ra (a wild-type strain) (Park and Hwang, 1999). Identified *hrp* genes from this bacterium were different from other Gram-negative bacteria. All other known *hrp* mutants of other plant-pathogenic bacteria are non-pathogenic and unable to cause HR (Hueck, 1998). However, some *hrp* mutants of *X. axonopodis* pv. *glycines* lose the ability to cause HR (Han et al., 2001). The above aspects suggest that in *X. axonopodis* pv. *glycines*, genes for governing pathogenicity in host plant may be different from those for HR in non-host plant.

The pathogenicity loss of *X. axonopodis* pv. *glycines* by transposon mutation of *hrp* genes was related to the inability of multiplication *in vivo* (Park and Hwang, 1999). However, it is unlikely that the loss of HR in non-host plants was due to bacterial multiplication because both pathogenic and non-pathogenic bacterial strains do not induce bacterial diseases in non-host plants. There may be differences in the host-parasite relations that can discriminate between HR and non-HR in non-host plants. Therefore, this study aimed to examine differential subcellular responses in pepper, a non-host plant, infected with pathogenic and non-pathogenic strains of *X. axonopodis* pv. *glycines*.

Materials and Methods

Bacterial strains and plant. The bacterial strains used in this

study were *X. axonopodis* pv. *glycines* wild-type strain 8ra and its transposon mutant 8-13, which is non-pathogenic (Park and Hwang, 1999). The bacterial strains were grown on yeast extract calcium carbonate (YDC) agar (Schaad, 1988) at 28°C for 3 days before use.

Inoculation. Pepper (*Capsicum annuum* L. cv. Dabokkun) plants were grown at 25°C in a greenhouse. Bacterial cells on the agar medium were suspended in sterilized distilled water and adjusted to 2-5×10^8 cells/ml, and injected into fully expanded leaves of whole plants. The inoculated plants were placed at 25°C in the greenhouse.

Electron microscopy. Leaf tissues injected with the bacterial cells were excised and fixed in Karnovsky's fixative in 0.05 M cacodylate buffer (pH 7.2) for 2 h at 4, 8, 12, and 18 h after inoculation. The fixed leaf tissues were rinsed in the same buffer solution three times each for 20 minutes, and post-fixed in 1% osmium tetroxide for 2 h. The fixed samples were washed briefly in distilled water and stained *en bloc* with 0.5% uranyl acetate overnight. These were then dehydrated in an ethanol series (30%, 50%, 80%, 95%, and 100%), and embedded in Spurr's epoxy resin (Spurr, 1969). Ultrathin sections of 80-90 nm in thickness were made with a diamond knife. The sections were stained with uranyl acetate and lead citrate, and observed under a JEM-1010 electron microscope (JEOL, Japan) at 80 kV.

Results

Pepper leaves injected with the bacterial suspension of *X. axonopodis* pv. *glycines* 8ra had no symptom of hypersensitive response at less than 12 h after inoculation. However, water-soaking area was formed around the inoculation site after 12 h, and HR was noted 16-20 h after inoculation, which was characterized by shiny appearance on the water-soaked area. No such HR was formed by the mutant non-pathogenic bacterial strain 8-13. In the leaf tissue areas inoculated with both bacterial strains, no significant bacterial population changes occurred at 30 h after inoculation, regardless of pathogenic and non-pathogenic strains (unpublished data).

At 4 h after inoculation, no structural changes related to programmed cell death were noted both in 8ra (Fig. 1) and 8-13 strains (Fig. 2). The cells adjacent to infecting bacteria were intact, containing healthy chloroplasts and mitochondria. Cytoplasm was also intact. In case of *X. axonopodis* pv. *glycines* 8ra, the bacterial cells were rather tightly attached to the cell walls, from which the cell wall material was dissolved and encapsulate the bacterial cells (Fig. 1A, B). Sometimes bacterial cells were entirely embedded in the cell wall material especially in the diverging area of intercellular space (Fig. 1C). The bacterial cells of mutant strain 8-13, on the other hand, were usually not attached tightly to the plant cell wall or dispersed in the intercellular space (Fig. 2). As in 8ra, the material was occasionally dissolved from the cell walls (Fig. 2A, B) or

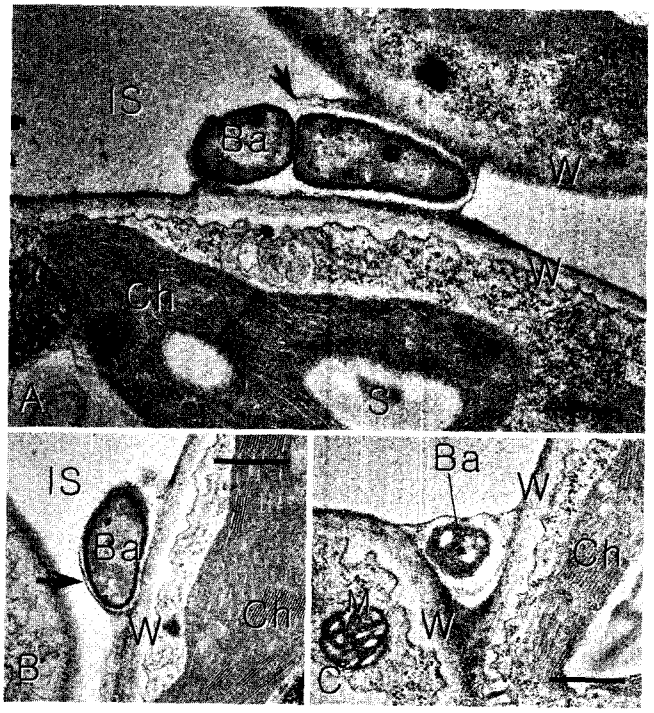

Fig. 1. Pepper leaf tissue infected with *Xanthomonas axoropodis* pv. *glycines* 8ra 4 h after inoculation, showing bacterial cells (Ba) attached to plant cell walls (W). Note a material (arrow) probably formed by hydrolysis or enzymolysis encircling the bacterial cells. In (C), the bacterial cell is embedded in the wall material. Cytoplasm adjacent to the bacterial cells is intact, containing intact chloroplast (Ch) and mitochondria (M). IS: intercellular space, S: starch. Bars = 0.5 μm.

no material was formed from the cell wall (Fig. 2C, D). However, it did not encircle the bacterial cells. Electron-dense materials were deposited around the bacterial cells of 8-13 (Fig. 2A, C, D).

At 8 h after inoculation, the bacterial cells of *X. axonopodis* pv. *glycines* 8ra were embedded singly or in group in the encapsulation material, which were firmly attached to the cell wall (Fig. 3). Plant cells affected by the bacterial infection showed a kind of hypersensitive response (HR), characterized by formation of various vesicles (vacuolation) and separation of plasmalemma from the cell wall (Fig. 3A-C). Cytoplasm was granulated and contained numerous vesicles (Fig. 3C). Sometimes vesicles were opened to the space between the cell wall and plasmalemma, and empty or filled vesicles were numerous in the space (Fig. 2C). On the other hand, no such hypersensitive responses were found in leaf cells infected with *X. axonopodis* pv. *glycines* 8-13 at this time of infection (not photographed).

At 12-18 h after inoculation with *X. axonopodis* pv. *glycines* 8ra, the bacterial cells were completely embedded in the cell wall material and prominent cell wall apposition

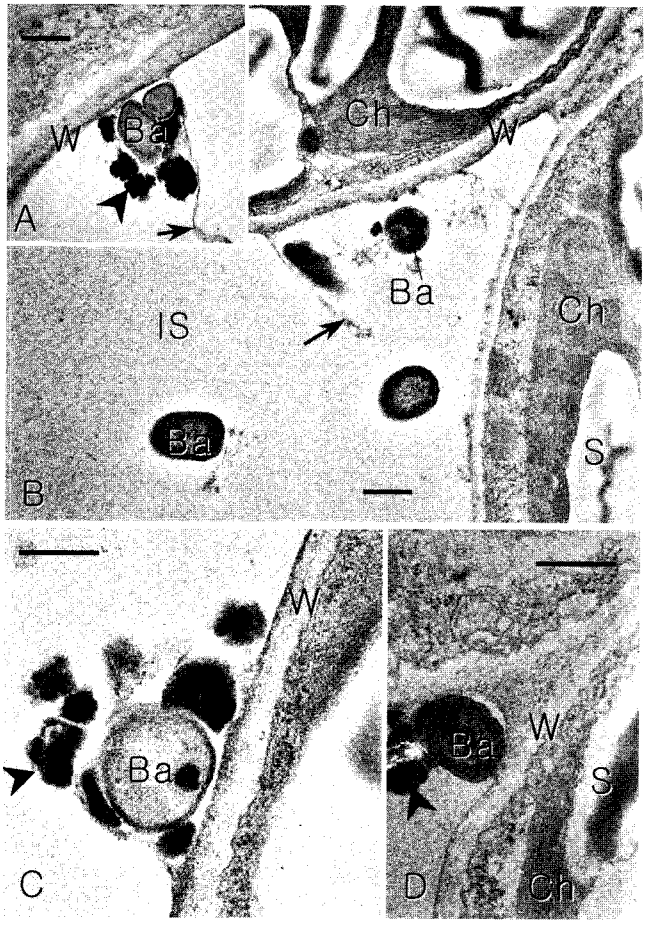

Fig. 2. Pepper leaf tissue infected with *Xanthomonas axonopodis* pv. *glycines* 8-13 4 h after inoculation, showing bacterial cells (Ba) dispersed in the intercellular space (IS) or attached to the cell wall (W). Note a material (arrow) probably formed by hydrolysis of enzymolysis of cell wall, but does not encircle the bacterial cells. Electron dense materials (arrowhead) accumulated around the bacterial cells (A, C, D). Cytoplasm adjacent to the bacterial cells is intact. Ch: chloroplast, S: starch. Bars = 0.5 μm.

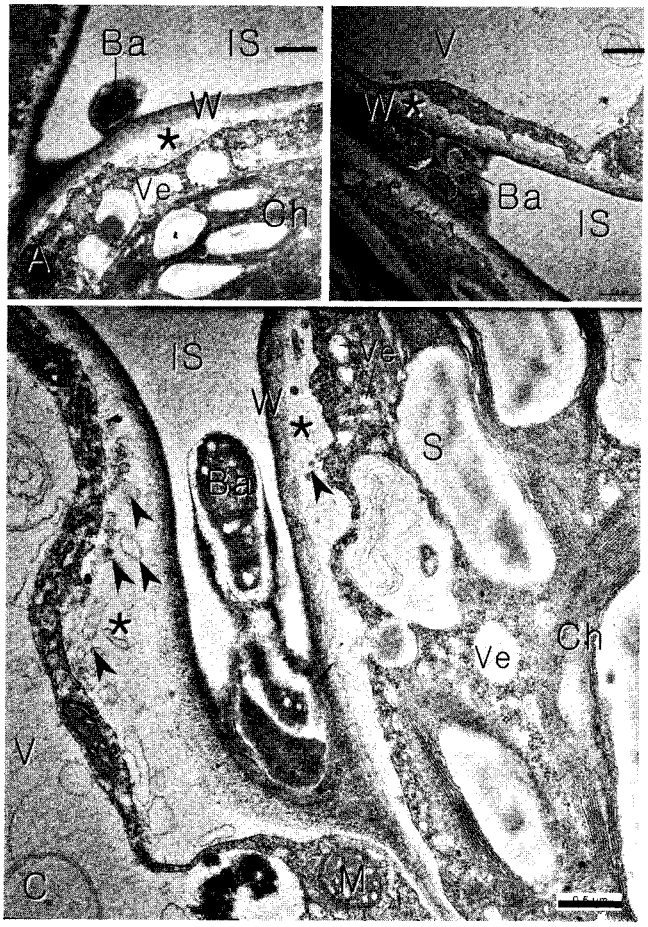

Fig. 3. Pepper leaf tissue infected with *Xanthomonas axonopodis* pv. *glycines* 8ra 8 h after inoculation, showing bacterial cells (Ba) cemented to plant cell walls (W). The plant cells show plasmalemma separation (asterisk) from cell wall and formation of numerous vesicles (Ve) in the cytoplasm (vacuolation). Numerous empty or filled vesicles in the space between the cell wall and plasmalemma indicate apposition of wall materials to the primary cell wall. Note that the bacterial cells are embedded in the wall material. Ch: chloroplast, M: mitochondria, V: vacuole, S: starch. Bars = 0.5 μm.

was formed on the inside cell wall (Fig. 4A, C). Cytoplasm was granulated with small vesicles (Fig. 4B) or necrotized (Fig. 4C). However, leaf tissues inoculated with the nonpathogenic strain 8-13 had no indication of cell wall apposition and cellular necrosis (Fig. 5). The bacterial cells of 8-13 were attached to the cell walls, which were encapsulated loosely by the cell wall material (Fig. 5B). Generally cytoplasm was intact (Fig. 5A), although at a later stage, some HR indications such as cell membrane separation and formation of vesicles were noticed (Fig. 5C, D).

Discussion

Interactions between plants and plant-pathogenic bacteria are largely divided into susceptible, resistant, and null responses. The *hrp* gene clusters of plant-pathogenic bacteria are associated with pathogenicity in host plants and resistance in resistant plants in which hypersensitive reaction is elicited. The localized HR in the infection sites of resistant plants are genetically governed by Flor's gene-for-gene hypothesis (Flor, 1946; Keen, 1990), which determines resistance by the combination of plant resistance (*R*) and pathogen avirulence (*avr*) genes. Null responses are only phenotypic views, which often occur when *hrp* gene(s) are mutated to lose pathogenicity. Strain 8-13 has a mutation in a *hrp* gene homologous to *hrpF* of *X. campestris* pv. *vesicatoria* (Park and Hwang, 1999). An *hrcU*-homologous gene mutant of *X. axonopodis* pv.

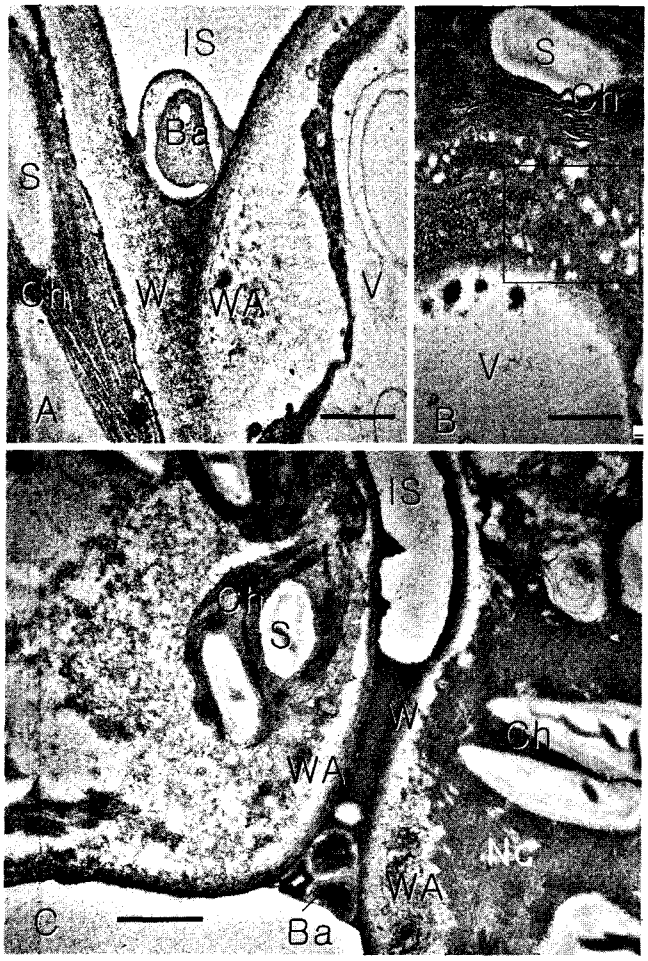

Fig. 4. Pepper leaf tissue infected with *Xanthomonas axonopodis* pv. *glycines* 8ra 12 h (A, B) and 18 h (C) after inoculation, showing bacterial cells (Ba) embedded in the material from plant cell wall (W). Note prominent cell wall appositions (WA) and necrotic cytoplasm (Nc). The rectangle in (B) shows the formation of various vesicles in granulated cytoplasm. Ch: chloroplast, M: mitochondria, V: vacuole, S: starch. Bars = 0.5 μm.

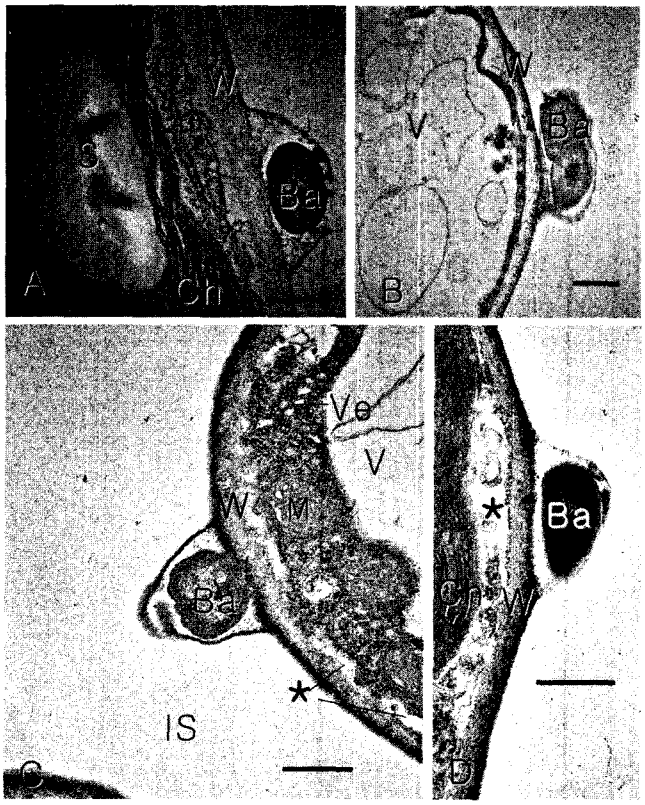

Fig. 5. Pepper leaf tissue infected with *Xanthomonas axonopodis* pv. *glycines* 8-13 12 h (A, B) and 18 h (C) after inoculation, showing bacterial cells (Ba) embedded in the loose material from plant cell wall (W). Note that intact cytoplasm in (A), (C) and (D) cellular modifications similar to initial hypersensitive responses, cell membrane separation (asterisk) and formation of vesicles are noted. Ch: chloroplast, M: mitochondria, V: vacuole, S: starch. Bars = 0.5 μm.

glycines which is non-pathogenic can elicit HR on non-host plants (Oh et al., 1999). Mutations in a *hrp* gene result in delayed plant responses both in susceptible and resistant host plants (Noel et al., 2002).

In this study, HR-type symptoms were observed in the non-host plant (pepper) by the pathogenic strain of *X. axonopodis* pv. *glycines* after 12 h of inoculation, but not by the non-pathogenic mutant. Likewise, HR responses in cellular level were only prominent in pepper leaf tissues inoculated with the pathogenic strain. Cellular responses indicating HR in the prenecrotic tissue were noticed 8 h after inoculation, which were earlier than visual symptom expression. The non-pathogenic strain 8-13 did not cause any significant cellular changes related to HR even at the later stages of infection.

Initial cellular reactions associated with HR involve various processes such as Ca^{++} influx, K^+ efflux/H^+ influx, active oxygen generation, synthesis of phytoalexins and degradative enzymes, deposition of cell wall materials, hallmarks of programmed cell death (PCD), etc. (Dangl and Jones, 2001). H_2O_2 accumulates in the cell wall adjacent to infecting bacteria during the oxidative burst in tobacco challenged with *Pseudomonas syringae* pv. *phaseolicola* (Mur et al., 2000), in which the transient rise of salicylic acid (SA) has potentiated oxidative burst, resulting in rapid cell death. Ion leakage and membrane disruption are two measures for cell death.

The initial noticeable structural changes related to the HR in this study were similar to those in soybean cultivars infected with avirulent strains of separation of plasma membrane from the cell wall (plasmolysis) and cytoplasmic vacuolation (Jones and Fett, 1985). In some cells adjacent to the bacteria, dilated endoplasmic reticulum (ER) was prominently formed (not photographed). In the study of

Mittler et al. (1997), condensation and vacuolization of the cytoplasm and cleavage of nuclear DNA were observed in programmed cell death (PCD). This phenomenon was found in a resistant soybean infected with soybean cyst nematode, showing plasmalemma separation from cell wall in syncytial cells due to nuclear degeneration (Kim et al., 1987). In tobacco, the membrane separation was also found in *Tobacco mosaic virus* (TMV)-infected cells with PCD (Shin et al., 2001). Plasmolysis is the indication of ion leakage from the cells, which indicates the relative amount of cell death in plant tissues (del Pozo and Lam, 1998). Therefore, these structural features may be characteristic features of initial PCD in pepper leaf tissues infected with *X. axonopodis* pv. *glycines.*

Cell wall apposition was formed and cytoplasm was necrotized at later stages, which corresponded to the time of the visual HR symptom expression. Cell wall apposition together with necrosis is a common phenomenon in incompatible host parasite interactions (Kim et al., 1987; Riggs et al., 1973; Tu and Hiruki, 1971). Together with membrane separation, wall appositions may wall off the plasmodesmata and prevent cell-to-cell movement of materials, which would lead to a deficit in food supply and build-up of toxic by-products within the cells. Formation of numerous vesicles in the cytoplasm and in the space between the cell wall and plasmalemma at the stage prior to the formation of cell wall apposition may indicate the deposition of cell wall materials to the primary cell wall.

Plant cell walls are a source of signaling molecules that elicit various cell behaviors, such as defense responses to fungal and bacterial pathogen parenchyma cells. In the non-host plant tissues in this study, some materials from the cell wall encircled the infecting bacteria, which is called immobilization (Jones and Fett, 1985). These cytological responses were only prominent in 8ra. In 8-13, the structures were formed later and were looser than in 8ra. It is known that parenchyma cells produce a fibrillar material that surrounds the bacteria, preventing further multiplication as an induced resistance. The material appeared to result from hydrolysis or enzymolysis of primary cell wall in this study. Jones and Fett (1985) suggested that fibrillar or electron-dense amorphous materials on cell surface are derived from the cell wall. Darvill et al. (1995) also suggested that fragments of polysaccharides from primary cell wall by hydrolysis evoke defense responses in plants and activate mechanisms for resisting potential pathogens. The cell wall modifications may be related to recognition of the pathogen by the plant. Considering that earlier recognition results in faster mobilization of plant defense, plant cells may have recognized the attack of 8ra earlier than 8-13, which may be related to the difference in the amount of elicitor(s) between the wild-type and mutant strains (unpublished data).

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