Ultrastructural Changes in the Early Stages of Interaction between Rice Leaf Parenchymatous Tissue and Infiltrated Bacteria

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Abstract

Ultrastructural changes in the early stages of interaction between rice leaf parenchymatous tissue and infiltrated bacteria such as *Xanthomonas campestris* pv. *oryzicola*, *X. c.* pv. *oryzae*, *X. c.* pv. *citri*, *X. c.* pv. *campestris* and *Pseudomonas fluorescens* were investigated. Most of the cells of all bacteria tested attached tightly on the surface of rice cells by 5 hr after infiltration. The bacteria pathogenic to rice leaves, *X. c.* pv. *oryzicola* and *X. c.* pv. *oryzae*, induced fluid sphere (FS) formation around their bodies in the intercellular spaces associating with distortion of rice plasmalemma adjacent to the bacterial cells by 10 hr after infiltration. FS was enlarged to fill up rice intercellular spaces with bacterial multiplication by 2 days after infiltration. It was conceivable that FS was composed of the liquid leaked from rice cells due to bacterial attack against rice cell wall and membrane systems. FS was also induced by the bacteria non-pathogenic to rice leaves, *X. c.* pv. *citri* and *X. c.* pv. *campestris*, but its frequency and extension were less than those in the case of the former. Rice cells adjacent to these bacteria accumulated electron dense materials (EDM-p) at periplasmic spaces and formed vesicles from membrane systems near bacterial cells. The bacteria increased their population slightly, but some of them were abnormal in shape. The cells of *P. fluorescens* did not induce FS. Some bacterial cells were surrounded by loose network fibrils on the rice cells in which EDM-p was accumulated at periplasmic space by 1-2 days after infiltration. It was suggested that FS formation was a phenomenon of compatible interaction and EDM-p accumulation was that of incompatible interaction. The rapidity and degree of FS formation and EDM-p accumulation were different depending upon compatibility between rice leaf parenchymatous cells and infiltrated bacteria.

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Key words: compatibility, rice, parenchyma, bacteria, ultrastructure.

INTRODUCTION

Ultrastructural changes in the interaction between rice leaf tissue and inoculated isolates of *Xanthomonas campestris* pv. *oryzae* different in compatibility were first investigated by Hori-no⁷⁾. He reported appearance of fibrillar material (FM) in the vessels during interaction, suggesting high correlation with incompatibility. The interactions between rice leaf parenchymatous tissue and infiltrated various bacteria different in pathogenicity from each other had been investigated also from ultrastructural viewpoints by the authors previously^{14,15)}. They have reported the phenomena which occurred between host and parasite at different stages later than 3 days of interaction and suggested that the host-parasite relationships were complicated as to be continuous from compatibility to incompatibility and hardly be defined as to be compatible or incompatible in clear-cut manner.

Since initial interactions were considered to be most important to elucidate the mechanism of compatibility, ultrastructural changes in the early stages of interactions between rice leaf

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parenchymatous tissue and infiltrated various bacteria different in pathogenicity were investigated. In this case, special attention was paid to make clear the responses of rice leaf parenchymatous cells at the site where the infiltrated bacterial cells attached.

MATERIALS AND METHODS

All of the materials and methods used in this experiment were the same as described previously^{14,15} except for the time of sampling. *X. c. pv. oryzae* Ku8101, the pathogen of bacterial leaf streak of rice, *X. c. pv. oryzae* H75373, the pathogen of bacterial leaf blight of rice, *X. c. pv. citri* Ku7502-1, the pathogen of citrus canker, *X. c. pv. campestris* I, the pathogen of black rot of crucifers, and *Pseudomonas fluorescens*, a saprophytic bacterium, which were maintained in the Laboratory of Plant Pathology, Kyushu University, were used. The bacteria cultured in PS medium¹⁴ were washed two times with sterile distilled water by centrifugation and suspended in sterile distilled water at the concentration of about 1×10^8 cells/ml. The bacterial suspension was infiltrated into intercellular spaces of rice leaf parenchymatous tissue as reported previously¹⁴. Polystyrene Latex (0.804 μ m in diameter) suspended into sterile distilled water at the concentration of about 7×10^8 particles/ml was applied as non-biotic control. The fifth leaves of rice variety "Kinmaze" at the stage of six expanded leaves were infiltrated with the suspension. At 5 hr, 10 hr, 1 day and 2 days after infiltration, the infiltrated parts of the leaves ca. 1×1 mm in size were sampled, fixed by 2.5% glutaraldehyde and 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), dehydrated in a graded series of ethanol, transferred to QY-1 (*n*-butyl glycidyl ether) and embedded in Quetol 812. Ultrathin sections obtained with glass knives on the Sorval Porter Blum MT-1 ultramicrotome were stained with 2% aqueous uranyl acetate and lead acetate, and examined with the JEM 100S electron microscope.

RESULTS

Interaction between X. campestris pv. oryzae and rice leaf parenchymatous cells

Cells of *X. c. pv. oryzae* tightly adhered to the surface of rice cells by 5 hr after infiltration (Plate I-1). Most of the rice cells attached by the bacteria did not show any remarkable changes in their cellular organs and components, but some of them showed irregularity in morphology of membrane systems adjacent to bacterial cells (Plate I-2). By 10 hr after infiltration, fluid sphere (FS) which often contained fibrils was formed around bacterial cells (Plate I-3). Remarkable changes in rice cells adjacent to the bacterial cells were characterized by retraction of plasmalemma or cytoplasm from cell wall, accumulation of small amount of amorphous electron dense material in the space between retracted plasmalemma and cell wall (Plate I-3), and extensive fragmentation of cytoplasm (Plate I-4). FS, formed around bacterial cells, increased its area in the rice intercellular spaces by 1 day after infiltration (Plate I-5). Bacterial cells in FS were normal in shape and distributed uniformly suggesting slight multiplication. In some specimens, small granules with various size were observed in periplasmic space and on the surface of rice cell wall adjacent to bacterial cell (Plate I-6). The cytoplasm and plasmalemma of these rice cells decreased in their density at the site adjacent to bacterial cells. Two days after infiltration, the bacterial cells in FS multiplied and developed markedly in the rice intercellular spaces (Plate I-7). The rice cells adjacent to bacterial cell often showed extensive distortion of plasmalemma, proliferation of abnormal vesicles near the plasmalemma, development of vacuoles, and denaturation of chloroplasts (Plate I-8).

Interaction between X. campestris pv. oryzae and rice leaf parenchymatous cells

Cells of *X. c. pv. oryzae* tightly adhered to the surface of rice cells by 5 hr after infiltration as observed in the case of *X. c. pv. oryzae* (Plate II-1A). Plasmalemma of some rice cells was uneven (Plate II-1B). By 10 hr after infiltration, FS was formed around bacterial cells

in the rice intercellular spaces (Plate II-2A). Rice cells adjacent to bacterial cell showed severe distortion of plasmalemma and formation of many abnormal vesicles (Plate II-2B). Bacteria multiplied in FS extended in the rice intercellular spaces by 1 day after infiltration (Plate II-3). Development of vacuoles, denaturation of chloroplasts and assembly of mitochondria were characteristic in rice cells adjacent to bacterial cell on 2 days after infiltration (Plate II-4A). In some specimens, rice cell wall and plasmalemma were seriously denatured at the site near bacterial cells (Plate II-4B).

Interaction between *X. campestris* pv. *citri* and rice leaf parenchymatous cells

The cells of *X. c.* pv. *citri* adhered to the rice cell wall were frequently observed at 5 hr after infiltration (Plate III-1). No remarkable changes in the rice cells occurred by this time. At 10 hr after infiltration, however, most of the bacterial cells tightly adhered to rice cell surfaces, and some rice cells showed distortion of plasmalemma, partial retraction and fragmentation of cytoplasm, appearance of abnormal vesicles and accumulation of electron dense materials (EDM-p) at the periplasmic spaces adjacent to the bacterial cell (Plate III-2, 3). FS was rarely observed around bacterial cells in the rice intercellular spaces at 10 hr after infiltration, and it became popular one day after infiltration (Plate III-4). Bacterial cells slightly multiplied in FS, but some of them showed abnormal shapes (Plate III-5). Vesicles of various size appeared abundantly in the cytoplasm of some rice cells adjacent to bacterial cell (Plate III-6). Two days after infiltration, some bacterial cells were surrounded by network fibrils (Plate III-7) or by much amount of fibrils (Plate III-8) in the rice intercellular spaces.

Interaction between *X. campestris* pv. *campestris* and rice leaf parenchymatous cells

The cells of *X. c.* pv. *campestris* tightly adhered to the surface of rice cells were observed at 5 hr, 10 hr, and 1 day after infiltration (Plate, IV-1, 2, 3, 4). By 1 day after infiltration, some of the rice cells adhered by the bacteria showed partial distortion or vesiculation of plasmalemma (Plate IV-2), accumulation of EDM-p at periplasmic spaces adjacent to bacterial cell (Plate IV-3), and formation of abnormal vesicles of various size (Plate IV-4). In some regions of the rice intercellular spaces, bacterial cells existed in FS containing much amount of fibrils and electron dense materials 2 days after infiltration (Plate IV-5). The rice cells adjacent to

Fig. 1. Possible process of FS formation in rice intercellular space induced by infiltrated compatible bacteria.

bacterial cell had Golgi vesicles in the cytoplasm near plasmalemma and accumulated EDM-p at periplasmic spaces arisen by retraction of plasmalemma (Plate IV-6). Bacterial cells surrounded by much amount of electron dense material in the intercellular spaces were often observed (Plate IV-7). Some bacterial cells located near extensively accumulated EDM-p became electron dense, surrounded by electron dense granules and disrupted (Plate IV-8).

Interaction between P. fluorescens and rice leaf parenchymatous cells

Infiltrated cells of *P. fluorescens* were commonly attached to the surface of rice cell wall without causing any remarkable changes in plant cell structures by 10 hr after infiltration (Plate V-1, 2). One day after infiltration, however, some bacterial cells loosely surrounded by network fibrils in rice intercellular spaces were observed (Plate V-3). Two days after infiltration, bacterial cells adhered to rice cell wall with (Plate V-5) or without electron dense materials (Plate V-4). In both cases, EDM-p was accumulated in the rice periplasmic spaces.

Interaction between Polystyrene Latex and rice leaf parenchymatous cells

The interaction between non-biotic particles, Polystyrene Latex, and rice leaf parenchymatous cells was observed one day after infiltration. The particles of Polystyrene Latex were observed on the surface of rice cell wall but they did not induce any responses to rice cells (Plate V-6).

DISCUSSION

The cells of all kinds of infiltrated bacteria attached tightly on the surface of rice parenchymatous cells by 5 hr after infiltration. This attachment might be caused by surface tension associated with transpiration, which attracted bacterial cells to rice cell surface. However, contrasting to this common phenomenon, the following phenomena were much different depending on the infiltrated bacteria. Fluid sphere (FS) was often observed around bacterial cells of *X. c. pv. oryzae*, *X. c. pv. citri*, and *X. c. pv. campestris* but hardly observed in the case of *P. fluorescens* and never observed in the case of Polystyrene Latex, suggesting that FS was induced only by phytopathogenic bacteria. Comparative observations on FS formation in rice leaf parenchymatous tissue in response to infiltrated bacteria were shown in Table 1. The infiltrated bacteria were divided into four groups on the basis of their modes of FS induction as follows; 1) The bacteria which induced FS frequently at an early stage after infiltration and enlarged FS to fill up rice intercellular spaces (*X. c. pv. oryzae* and *X. c. pv. citri*), 2) The bacteria which induced FS at an early stage, but its frequency and degree of enlargement were less than those of the former (*X. c. pv. citri*), 3) The bacteria which induced FS at later stage and its frequency and degree of enlargement were less (*X. c. pv. campestris*), 4) The bacteria which induced FS scarcely (*P. fluorescens*). The increase of bacterial population seems to be correlated with frequency and rapidity of FS appearance and its enlargement.

Table 1. Development of fluid sphere (FS) caused by infiltration of various bacteria into rice leaf parenchymatous tissue

Phenomena	<i>X. campestris</i> pv.				<i>P. fluorescens</i>	Polystyrene Latex
	<i>oryzae</i>	<i>oryzae</i>	<i>citri</i>	<i>campestris</i>		
Appearance of FS	+	+	+	+	—	—
Time required for FS formation after infiltration	10 h ^{b)}	10 h	10 h	2–3 d	—	—
Frequency of FS appearance	+++	+++	++	+	—	—
Development of FS	+++	+++	++	+	—	—

a) + : positive response (degree: +++ > ++ > +), — : negative response.

b) h: hours, d: days.

Simultaneously with FS formation, distortion of rice plasmalemma was often observed at the site near FS. These results suggest that FS formation might be a phenomenon associated with compatible interaction between the rice cells and infiltrated bacteria. Therefore, FS might mainly consisted of the materials or nutrients leaked from rice cells. Induction and enlargement of FS by *X. c. pv. oryzae* and *X. c. pv. oryzae* by 2 days after infiltration corresponded to the appearance of water-soaking¹⁴⁾. In other words, water-soaking is a reflection of filling up the intercellular spaces by FS which permit bacterial multiplication. Figure 1 shows the possible mechanism of FS formation by compatible bacteria. In connection with some structures or materials which wrap bacterial cells on the surface of plant cells, many workers have reported on their natures, roles, and their possible functions¹⁻¹³⁾. Sequeira *et al.*¹⁰⁾, Goodman *et al.*⁵⁾, Politis and Goodman,⁹⁾ and Cason *et al.*³⁾ reported that bacterial cells were immobilized, enveloped, or entrapped on the surface of plant cells by some plant elements such as wall cuticle or pellicle as one of plant defensive reactions. Hildebrand *et al.*⁹⁾, however, have proposed that the enveloping films for bacterial entrapment consist of plant cell wall-derived materials deposited during evaporation of water infiltrated into leaves and not directly related to a plant defense mechanism. Our results indicated that the film was originated in the materials exudated from parenchymatous cells by compatible interactions. When compatibility between rice cells and bacterial cells is not high, enlargement of FS was limited. This phenomenon would be misunderstood as entrapping of bacterial cells by some previous workers. The lack or insufficient enlargement of FS by bacteria may suggest the lack or insufficient activity of bacteria to rice membrane systems.

Rice cells adjacent to the cells of *X. c. pv. citri* and *X. c. pv. campestris* accumulated electron dense material (EDM-p) at the site of periplasm. Comparative observations of EDM-p accumulation induced by infiltration of bacterial cells were summarized as shown in Table 2 (cited from the previous reports^{14,15)}). The accumulation of EDM-p in rice cells was considered to be related to rice defensive reactions to infiltrated bacteria as reported previously¹⁵⁾. It might be possible to conclude that frequency of EDM-p appearance in host cells and its amount was closely correlated to the degree of incompatibility. The relationship between non-biotic particle, Polystyrene Latex, and rice parenchymatous tissue was considered to be absolute incompatibility. Many workers have reported the accumulation or deposition of various materials such as electron dense fibrils or vesicular structures inside plant cells located closely to incompatible bacteria^{1-3,9-11)}. EDM-p was also considered to be one of the materials produced by rice cells during incompatible interactions. Golgi bodies and vesicles frequently observed nearby EDM-p might be associated with exclusion of EDM-p.

The results obtained in the present investigation suggested that the relationships between

Table 2. Accumulation of electron dense material (EDM-p) at periplasmic spaces of rice cells adjacent to infiltrated various bacteria

Phenomena	<i>X. campestris</i> pv.				<i>P. fluorescens</i>	Polystyrene Latex
	<i>oryzicola</i>	<i>oryzae</i>	<i>citri</i>	<i>campestris</i>		
Appearance of EDM-p	- a)	+	+	+	+	-
Time required for EDM-p appearance after infiltration	-	7 d b)	10 h-3 d	10 h-2 d	1 d	-
Frequency of EDM-p appearance	-	+	++	++	++	-
Amount of EDM-p	-	+	++	++	+	-
Bacterial cells showing morphological abnormality at the site adjacent to EDM-p	-	+	+	+	+	-

a) + : positive response (degree: ++ > +), - : negative response.

b) d : days, h : hours.

rice leaf parenchymatous tissue and various kinds of bacteria different in pathogenicity were hardly defined in a clear-cut manner either compatible or incompatible, supporting our conclusion reported previously^{14,15}). The relationships were complicated in an ultrastructural viewpoint showing many intermediate grades between absolute compatibility and absolute incompatibility. The degree of a total compatibility might be composed of many individual compatible or incompatible interactions.

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和 文 摘 要

津野和宣・脇本 哲: イネ葉柔組織と注入細菌との相互反応の初期段階にみられる微細構造の変化

イネ条斑細菌病菌, イネ白葉枯病菌, アブラナ科野菜黒腐病菌, および *Pseudomonas fluorescens* をイネ葉柔組織の細胞間隙に注入した際, 宿主-細菌相互反応の結果としておこる微細構造の早期における変化を電子顕微鏡で観察した。すべての供試細菌において, 大部分の菌体は注入後 5 時間までにはイネ細胞の表面に吸着した。イネに病原性のある条斑細菌病菌と白葉枯病菌では, 注入後 10 時間までには菌体近くのイネ細胞の原形質膜を変形させ, 菌体の周囲に液域 (FS) を形成させた。この FS はしだいに拡大し, 2 日後には多くの細胞間隙を満たし, そのなかで細菌の増殖がみられた。FS はイネ細胞の細胞壁と原形質膜が注入細菌に侵されることによるイネ細胞からの漏出液と考えられた。FS はイネに対して病原性を示さないカンキツかいよう病菌およびアブラナ科野菜黒腐病菌の場合にも形成されたが, 形成までに長時間を要し, 形成頻度および拡がりの程度はイネの病原細菌の場合に比較してわずかであった。これらの細菌に接したイネ細胞では, ペリプラズマ領域に高電子密度物質 (EDM-p) が集積し, 膜構造の小胞化が観察された。*P. fluorescens* の場合には, FS の形成はみられず, 一部の菌体はゆるい網状の繊維状物質にとり囲まれ, 近くのイネ細胞では注入 1~2 日後までに EDM-p の集積が認められた。これらの結果から, FS 形成は親和的な現象であり, EDM-p の集積は非親和的な現象と考えられ, 両現象の速度と程度とは宿主細胞と注入細菌との親和性の程度によって異なることが示唆された。

Explanation of plates

Plate I. Ultrastructural features of the interaction between rice leaf parenchymatous tissue and infiltrated cells of *X. campestris* pv. *oryzicola*.

1. Bacterial cells adhered to the surface of rice cells (5 hr after infiltration, $\times 13,200$).
2. Rice cell showing abnormal membrane structures at the site adjacent to bacterial cells (5 hr after infiltration, $\times 20,000$).
3. Rice plasmalemma retracted at the site adjacent to bacterial cells. Amorphous material appeared at periplasmic space of rice cell (10 hr after infiltration, $\times 21,000$).
4. Rice cells adjacent to bacterial cells showing remarkable retraction and fragmentation of cytoplasm (10 hr after infiltration, $\times 25,000$).
5. Fluid sphere (FS) increased in rice intercellular spaces (1 day after infiltration, $\times 6,500$).
6. Small granules appeared inside rice cell wall and at the site between bacterial cell and the rice cells (1 day after infiltration, $\times 32,000$).
7. FS developed markedly, including many bacterial cells in the rice intercellular spaces (2 days after infiltration, $\times 4,400$).
8. Rice cells showing extensive distortion of plasmalemma, proliferation of abnormal vesicles, development of vacuoles and denaturation of chloroplasts (2 days after infiltration, $\times 10,000$).

Plate II. Ultrastructural features of the interaction between rice leaf parenchymatous tissue and infiltrated cells of *X. campestris* pv. *oryzae*.

- 1A. Bacterial cells tightly attached to the rice cell surface (5 hr after infiltration, $\times 22,000$).
- 1B. Unevenly retracted rice plasmalemma at the site adjacent to bacterial cell (5 hr after infiltration, $\times 22,500$).
- 2A. FS induced around bacterial cells in rice intercellular spaces (10 hr after infiltration, $\times 15,600$).
- 2B. Rice plasmalemma adjacent to bacterial cells showing strong distortion (10 hr after infiltration, $\times 13,000$).
3. Enlarged FS containing multiplied bacteria in rice intercellular spaces (1 day after infiltration, $\times 15,900$).
- 4A. Rice cells with developed vacuole, denatured chloroplast and assembled mitochondria (2 days after infiltration, $\times 9,600$).
- 4B. Denatured rice cell wall and plasmalemma adjacent to bacterial cells (2 days after infiltration, $\times 22,700$).

Plate III. Ultrastructural features of the interaction between rice leaf parenchymatous tissue and infiltrated cells of *X. campestris* pv. *citri*.

1. Bacterial cells adhered to the surface of rice cells (5 hr after infiltration, $\times 17,000$).
2. Rice cell showing distortion of plasmalemma and partial retraction and fragmentation of cytoplasm at the site adjacent to bacterial cells (10 hr after infiltration, $\times 25,000$).
3. Electron dense materials (EDM-p) accumulated in rice cell at the site adjacent to bacterial cells (10 hr after infiltration, $\times 32,000$).
4. FS induced in rice intercellular spaces (1 day after infiltration, $\times 2,600$).
5. Bacterial cells in FS showing abnormal shape (1 day after infiltration, $\times 5,400$).
6. Vesicles of various size appeared in rice cytoplasm adjacent to bacterial cells (1 day after infiltration, $\times 7,500$).
7. Network fibrils appeared around bacterial cells in rice intercellular spaces (2 days after infiltration, $\times 13,300$).
8. Fibrils surrounding bacterial cells in rice intercellular spaces (2 days after infiltration, $\times 20,000$).

Plate IV. Ultrastructural features of the interaction between rice leaf parenchymatous tissue and infiltrated cells of *X. campestris* pv. *campestris*.

1. Bacterial cell adhered to the surface of rice cells (5 hr after infiltration, $\times 20,000$).
2. Rice cells showing partial distortion of plasmalemma caused by bacterial attachment (10 hr after infiltration, $\times 33,300$).

3. Rice cell accumulated EDM-p at periplasmic space adjacent to bacterial cell (10 hr after infiltration, $\times 26,700$).
4. Abnormal vesicles of various size appeared in rice cell adjacent to bacterial cells (1 day after infiltration, $\times 20,600$).
5. Fibrils and electron dense materials (EDM-i) appeared in FS surrounding bacteria (2 days after infiltration, $\times 13,300$).
6. Golgi bodies and vesicles assembled near bacterial cell and EDM-p (2 days after infiltration, $\times 20,400$).
7. Much amount of EDM-i surrounding bacterial cells in rice intercellular spaces (2 days after infiltration, $\times 13,000$).
8. Electron dense granules adhered on the surface of bacterial cells disrupted (2 days after infiltration, $\times 12,500$).

Plate V. Ultrastructural features of the interaction between rice leaf parenchymatous tissue and infiltrated cells of *P. fluorescens* (1-5) and Polystyrene Latex (6).

- 1 and 2. Bacterial cells adhered to the surface of rice cells (1: 5 hr after infiltration, $\times 21,000$; 2: 10 hr after infiltration, $\times 21,000$).
3. Network fibrils surrounding bacterial cell in rice intercellular spaces (1 day after infiltration, $\times 12,800$).
4. Bacterial cells adhered to the surface of rice cells in which EDM-p was accumulated at the site adjacent to bacterial cells (2 days after infiltration, $\times 26,700$).
5. EDM-p surrounding bacterial cells in rice intercellular spaces, where EDM-i was accumulated at the site adjacent to bacterial cell (2 days after infiltration, $\times 15,200$).
6. Particles of Polystyrene Latex (arrow) induced no structural changes in rice cell (1 day after infiltration, $\times 24,300$).

Abbreviations used in plates: B, Bacterial cell; EDM-i, Electron dense material accumulated at rice intercellular space; EDM-p, Electron dense material accumulated in rice parenchymatous cell; FS, Fluid sphere; NF, Network fibril; PL, Rice plasmalemma.

Plate I

Plate II

Plate III

Plate IV

Plate V