Latency- and Defense-Related Ultrastructural Characteristics of Apple Fruit Tissues Infected with *Botryosphaeria dothidea*

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ABSTRACT

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Apple fruit tissues infected with *Botryosphaeria dothidea* were examined by transmission electron microscopy using susceptible cv. Fuji and resistant cv. Jonathan. Immature (green) and mature (red) fruits of cv. Fuji with restricted or expanding lesions were also examined to reveal subcellular characteristics related with latent and restricted disease development. In infected susceptible mature fruits, cytoplasmic degeneration and organelle disruption commonly occurred, accompanying cell wall dissolution around invading hyphae. Cell wall dissolution around invading hyphae in subepidermis was rare in immature, red halo-symptomed cv. Fuji and resistant cv. Jonathan fruits. In infected im-

mature fruits of cv. Fuji, presumably at the latent state of disease development, cellular degeneration was less severe, and invading hyphae contained prominent microbody-lipid globule complexes or the deposition of thin electron-dense outer layer around cell wall of intercellular hyphae. Both mature fruits with red halos and resistant apple fruits formed cell wall protuberances at the outside of cell walls. In addition, electron-dense extramural layers were formed in the resistant apple fruits. Aberrant hyphal structures such as intrahyphal hyphae were found only in resistant fruit tissues, indicating the physiologically altered fungal growth. These ultrastructural changes of host tissues and fungal hyphae may reflect the pathogenesis of apple white rot under varying conditions of apple fruits.

Additional keywords: glyoxysomes, latent infection, *Malus domestica*, wall appositions.

White rot is one of the most destructive diseases of apple (*Malus domestica* Borkh.) in the world (33). The disease results in fruit rot and extensive cankers on limbs, eventually resulting in tree death. The causal organism is an ascomycetous fungus *Botryosphaeria dothidea* Ces. & de Not. (synonymous with *B. ribis* Grossenb. & Dug.), anamorph *Fusicoccum aesculi* Corda (33). *B. dothidea* is found in most tropical and warm temperate regions as a pathogen of many economically important crops and woody plants (23). The fungus has been reported to enter apple fruits through natural openings, such as lenticels or surface cracks, or wounds possibly caused by insects, and to form dome-shaped appressoria on the fruit surface (14).

The main inoculum of *B. dothidea* is conidia, which are found in orchards throughout the growing season of apple trees (15,34). Typical soft and light-colored rot symptoms appear mostly on mature red fruits and may expand throughout whole fruits. Symptoms usually become noticeable 6 to 8 weeks before harvest (33). However, fruits can be infected by conidia within 7 weeks after petal fall (26). Latent infection should undergo during the immature stage of apple fruits. The latent infection is related with several host factors such as phenolic compounds, soluble sugars, pH, fruit acids, and amino acids in apple fruits (12,17,32). It is generally accepted that fungitoxic compounds in immature fruits, including phytoalexins and benzoic acid, and fruit substrates unfavorable to fungal growth are responsible for suppressing fungal invasion and delaying symptom appearance in immature fruits (1,17,30).

Different levels of resistance to the white rot have been reported among various apple cultivars (16). Restriction of disease development in the field was found in susceptible apple fruits as well as resistant ones. In susceptible mature fruits, small circular lesions surrounded by red halos are occasionally found on naturally infected apple fruits with *B. dothidea* (33). The lesion surrounded by the halo usually cannot keep expanding, and become harder than other parts of the fruit, which may be a defense-related response. In resistant fruits, the initial morphological characteristics of lenticels or parenchyma tissues beneath the epidermis, and phenolic compounds of apple fruits were suggested to be basic features of resistance. However, it is unclear whether any subcellular defense mechanisms are involved in resistance to the white rot.

Initial symptoms of the disease are circular brown lesions on the fruit surface, but symptom development differs among susceptible and resistant apple fruits. Histopathological studies have been conducted on *B. dothidea* in several hosts by light microscopy (2,11,29). However, subcellular aspects of host responses to fungal infection and their relation to latency and resistance have been little studied in apple fruits. Therefore, this study was conducted to examine the ultrastructural characteristics of latency of symptom expression and differences in disease resistance between apple cultivars. Mature and immature susceptible fruits, red halo-symptomed susceptible fruits (with restricted symptom development), and resistant fruits were compared in the subcellular modifications of the infected apple fruit tissues.

MATERIALS AND METHODS

Inoculum preparation. An isolate of *B. dothidea* (BD-3) was obtained from a naturally infected apple fruit showing typical white rot symptoms. The isolate was grown on freshly prepared

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acidified (pH 4 to 5) potato-sucrose agar amended with streptomycin sulfate at 100 µg/ml to inhibit bacterial contamination. Barley grain media were prepared for inoculum production as previously reported (13). Briefly stated, 20 g of barley (*Hordeum vulgare* L.) grains were immersed with 12 ml of distilled water in 250-ml Erlenmeyer flasks overnight. They were autoclaved at the pressure of 2 kgf/cm² for 1 h, which was repeated on the following day. The media were inoculated with two 5-mm diameter mycelial plugs of *B. dothidea*, and incubated at 25°C under continuous fluorescent light for 1 week. The flasks were occasionally shaken to prevent caking of the grains. Pycnidia were produced on the barley grains 3 days after inoculation. Two weeks after inoculation, conidia were dislodged by flooding the inoculated barley grains with sterilized distilled water. The suspension was filtered through two layers of cheesecloth to remove mycelial fragments. The inoculum concentration was adjusted to 1.0×10^5 conidia per ml using a hemacytometer.

Preparation of infected fruits. Four types of apple fruits infected with *B. dothidea* were prepared, respectively, as follows; red fruits (80-mm diameter) of cv. Fuji as susceptible mature fruits (sample 1), green fruits (25-mm diameter) of cv. Fuji as susceptible immature fruits (sample 2), mature cv. Fuji fruits with red halo symptoms (sample 3), and red fruits (65-mm diameter) of cv. Jonathan as resistant mature fruits (sample 4). Infected tissues from samples 1, 2, and 4 were prepared by artificial inoculation. The fruits were washed with mild detergent to remove dirt, insect honeydew, and pesticide residues. Prior to inoculation, the fruits were surface-sterilized with 0.1% sodium hypochlorite solution for 2 min, rinsed with sterilized distilled water, and allowed to air dry for 30 min. Four to six lenticel regions were circled on each fruit with a marker pen for point inoculations. Drops (approximately 20 µl per drop) of the conidial suspension were placed on each inoculation site of the fruits with an adjustable micropipette. Immediately after inoculation, the fruits were placed in a polystyrene box (32 × 43 × 8 cm) lined with moist paper towels, and the box was placed in an incubator at 25°C under continuous darkness for 2 weeks. The tissues of brown spot symptoms around the inoculation points were used for specimen preparation for transmission electron microscopy.

For sample 3, because no such symptoms were induced by the artificial inoculation, naturally infected red fruits of cv. Fuji (susceptible mature fruits) showing red halos were collected from an apple orchard at Suwon, Korea. The red halo tissues were processed for transmission electron microscopy. Naturally infected red fruits of cv. Fuji showing rapidly expanding lesions were also harvested to compare the subcellular characteristics with those of red halos.

Specimen preparation for transmission electron microscopy. Squares of brown lesions of samples 1, 2, and 4, and red halos of sample 3 (each 1 × 1 mm with approximately 1-mm thickness of underlying tissues) were excised with a sterile razor blade. Uninoculated healthy parts of apple fruits were also excised as a control. Specimens were fixed with modified Karnovsky's fixative consisting of 2% (vol/vol) glutaraldehyde and 2% (vol/vol) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) overnight, and washed three times with the same buffer for 10 min each. They were postfixed with 1% (wt/vol) osmium tetroxide in the same buffer at 4°C for 2 h, and washed twice with distilled water. The postfixed specimens were en bloc stained with 0.5% uranyl acetate at 4°C overnight. They were dehydrated in a graded ethanol series (30, 50, 70, 80, 95, and 100%), and three times in 100% ethanol for 10 min each. The specimens were further treated with propylene oxide as a transitional fluid two times for 30 min, respectively, and embedded in Spurr's medium. Transverse sections of the fruit subepidermis were made by a diamond knife with an ultramicrotome (MT-X, RMC Inc., Tucson, AZ). Ultrathin sections (approximately 70 nm thick) were mounted on copper grids, and double-stained with 2% (wt/vol) uranyl acetate and with

Reynolds' lead citrate for 7 min, respectively. Sections were examined with a transmission electron microscope (JEM-1010, JEOL Ltd., Tokyo) operated at an accelerating voltage of 80 kV. At least three embedded blocks were made for each type of fruit, and more than 10 ultrathin sections per block were examined under the electron microscope.

RESULTS

Cytopathology of susceptible mature fruits (sample 1). The outer flesh of apple fruit derived from the floral tube is composed of cuticular layers, epidermis, subepidermal collenchyma or hypodermis, and inner ground parenchyma (7). In the transmission electron microscopy of healthy apple fruit tissue, the subepidermal collenchyma, which is the main region colonized by the fungus before full symptom development, exhibited thick-walled collenchyma-like cells with slight secondary wall thickenings and with little intercellular spaces (data not shown). The functional cells contained intact cell organelles such as nucleus, mitochondria, and endoplasmic reticulum. There was no indication of cytoplasmic degradation. However, subepidermal cells of mature cv. Fuji fruits infected with *B. dothidea* were degenerated, showing extensive fungal invasion in the lenticel (Fig. 1A). Some hyphae were located above or on subepidermal cells of the lenticel, whereas others grew intercellularly or intramurally (within cell walls) in subepidermal regions. Electron-lucent areas were formed in host cell walls adjacent to the invading hyphae, indicating the dissolution of the cell walls. Disrupted fibrillar matrix from the cell wall was exhibited around the invading hyphae in middle lamella (Fig. 1B). Cellular degradation was noted in the lenticel region that was invaded extensively by the hyphae, characterized by indiscernible cell organelles such as nucleus and plastids and necrotized cytoplasm (Fig. 1C). The shape of the degraded cell was markedly distorted compared with that of the uninoculated control fruit cells. Intracellular occupation of hyphae was sometimes observed, and the infected cells were degenerated, showing degraded cytoplasm with no discernible cell organelles (Fig. 1D). Electron-dense materials accumulated along the periphery of the central vacuoles.

Subcellular features of the invading hyphae in the mature susceptible fruits exhibited cells with electron-lucent wall, prominent lipid globules (Fig. 1A and C) except a few hyphae located in the middle lamella (Fig. 1B), and dense cytoplasm sometimes containing vacuoles (Fig. 1D). No microbodies or structural aberrations of hyphae were observed in infected susceptible mature fruits.

Cytopathology of susceptible immature fruits (sample 2). Hyphae were commonly found between and inside the subepidermal cells of immature cv. Fuji fruit tissues infected with the fungus 2 weeks after inoculation (Fig. 2). By intercellular or intracellular hyphal invasion, the collenchymatic cells were damaged as indicated by the necrotic cytoplasm containing degenerated cell organelles including nucleus and plastid (Fig. 2A and B) as in susceptible mature fruits. However, unlike the mature fruits, dissolution of the host cell walls was rarely found in the vicinity of hyphae (Fig. 2A).

The cellular features of the invading hyphae were different from those in the subepidermal tissues of the mature fruits. The hyphae outside of host cell walls had electron-lucent cell walls, lipid globules, and dense cytoplasm as in sample 1, but the cell wall was covered with electron-dense materials forming a layer (Fig. 2A). On the other hand, no electron-dense layer was formed around the cell walls of intracellular hyphae; lipid globules and microbodies were abundant and placed in close association with each other, forming microbody-lipid globule complexes (Fig. 2B and C). These complexes were frequently found in inter- and intracellular hyphae in the infected susceptible immature fruits. The microbodies contained one or two electron-dense crystalline inclusions.

Cytopathology of susceptible mature fruits with red halos (sample 3). The major difference in the subepidermal cellular responses of red halo regions from those of brown lesions in samples 1 and 2 was the formation of cell wall protuberances adjacent to invading hyphae (Fig. 3A and B). They were extruded outward into wide or narrow intercellular spaces, sometimes occluding intercellular spaces, in which the hyphae appeared to be hindered in

growth, showing the depletion of cytoplasmic contents. However, in the susceptible mature fruits showing rapid symptom expansion, no cell wall protuberances were formed around the invading hyphae in the subepidermal fruit tissues (Fig. 3C). No cytoplasmic depletion of the invading hyphae was noted. As in sample 1, dissolution of the cell wall and middle lamella was exhibited as indicated by the electron-lucent areas around the invading hyphae.

Fig. 1. Transmission electron micrographs of inoculated susceptible mature apple fruits with *Botryosphaeria dothidea*. **A**, Lenticel colonization by intercellular or intramural hyphae (H). Dissolution of cell wall (CW) of subepidermal cells indicated by electron-lucent area (asterisk) adjacent to intercellular or intramural hyphae. CV = host central vacuole; L = lipid globule; Bar = 2 μ m. **B**, Dissolution of middle lamella by an invading hypha (H). Note host cell wall (CW) modification (asterisk) around the hypha. Bar = 1 μ m. **C**, Host cell degradation in the vicinity of a hypha (H). The host cell exhibits electron-dense cytoplasm apparently due to the condensation of cytoplasmic contents. N = nucleus; P = plastid; Bar = 2 μ m. **D**, Intracellular growth of hyphae. Arrows indicate accumulation of osmiophilic materials in host central vacuole (CV). V = fungal vacuole; W = fungal cell wall; Bar = 2 μ m.

Cytopathology of resistant mature fruits (sample 4). Hyphal penetration was observed in the epidermal and subepidermal tissues of the resistant mature fruits. Infected epidermal cells were necrotic and degenerated, containing intracellular hyphae (data not shown). In the subepidermal tissues, prominent cell wall protuberances were formed locally or over a wide area of host cell wall

adjacent to intercellular hyphae (Fig. 4A) as in the red halo regions of the susceptible mature fruits. The wall protuberances appeared to block the way for the fungal growth by extended wall outgrowth, by surrounding the invading hypha (Fig. 4A) or by occluding intercellular space with wall bridges formed from the neighboring cell walls (Fig. 4B). The outer parts of the wall pro-

Fig. 2. Transmission electron micrographs of inoculated susceptible immature apple fruits with *Botryosphaeria dothidea*. **A**, Subepidermal cells with intercellular hyphae (H). The host cytoplasm is electron-dense (probably necrotizing). The hyphae are in contact with host cell walls (CW), having electron-dense outer layer (OL) around the fungal cell wall (W). CV = host central vacuole; P = plastid; Bar = 2 μ m. **B**, Subepidermal cells colonized by intracellular hyphae (H). Host cell cytoplasm appears to be necrotizing. N = nucleus; Bar = 3 μ m. **C**, Higher magnification of the rectangle of Figure 2B, showing topographical association of microbodies (Mb) and lipid globules (L) in the intracellular hypha. Crystalline inclusions are found in microbodies. V = fungal vacuole; W = fungal cell wall; Bar = 0.5 μ m.

tubercles were electron-dense, whereas the inner parts were electron-lucent as normal plant cell wall (Fig. 4A). In addition, electron-dense extramural layers often extended over a wide area of host cell walls that were in the vicinity of intercellular hyphae.

In the resistant fruits, cell wall dissolution (electron-lucent area) around invading hyphae was rarely observed (Fig. 4B), and the infected subepidermal cells were not so extensively destroyed as in the susceptible fruits.

Fig. 3. Transmission electron micrographs of naturally infected apple fruits showing **A and B**, red halos around white rot lesions and **C**, rapidly expanding white rot lesions. **A**, Occlusion of intercellular space (IS) by a cell wall protuberance (WP). A hypha (H) entrapped in the occluded intercellular space exhibits depletion of cytoplasmic contents. Bar = 1 μ m. **B**, Cell wall protuberances (WP) adjacent to hyphae on the outer side of host cell wall (CW). Bar = 3 μ m. **C**, Tissue with a rapidly expanding lesion, showing dissolution of cell wall (CW) indicated by electron-lucent areas (asterisks). CV = host central vacuole; Bar = 2 μ m.

Invading hyphae in the resistant mature fruits were not much different from those in the susceptible fruits; both typically had electron-lucent cell wall and lipid globules. Microbodies associated with lipid globules were occasionally found in hyphal cytoplasm. However, a characteristic hyphal structure was often found in the resistant fruits, termed as intrahyphal hypha that was surrounded by and delimited by electron-lucent cell wall of the enclosing hypha (Fig. 4C). In some instances, a hypha contained several intrahyphal hyphae, and an intrahyphal hypha also contained another intrahyphal hypha (Fig. 4D).

DISCUSSION

The ultrastructural features of apple fruit tissues infected with *B. dothidea* in this study indicated that the fungus was definitely a facultative parasite that colonizes dying or dead tissues for nutritional uptake. The death of infected fruit cells was commonly found regardless of susceptible or resistant and mature or immature states of apple fruits, although the degree of cellular degeneration varied depending on the states of fruits examined. The cell death itself may not delay or stop the growth of the necrotrophic

Fig. 4. Transmission electron micrographs of inoculated resistant mature apple fruits with *Botryosphaeria dothidea*. **A**, Formation of cell wall protuberances (WP) and extramural layers (EL) on the outside of host cell wall (CW). CV = host central vacuole; H = hypha; P = plastid; Bar = 2 μ m. **B**, Occlusion of intercellular space by forming a bridge with two cell wall protuberances (WP) from neighboring host cell walls (CW). Bar = 1 μ m. **C**, Longitudinal section of an intrahyphal hypha (HH) in an intercellular hypha (H). Note a septum within an intrahyphal hypha. Bar = 2 μ m. **D**, Transverse section of intrahyphal hyphae (HH) in an intercellular hypha. Note an intrahyphal hypha (asterisk) in an intrahyphal hypha. Bar = 1 μ m.

fungus. Our findings suggest that some biochemical phenomena rather than mere cell death may govern the fungal growth in the different fruit states, modifying ultrastructural characteristics of the hyphae as well as host cells, especially host cell walls.

In susceptible mature fruits with expanding rot symptoms, cell degradation and especially cell wall dissolution around the invading hyphae were prominent. Considering the major components of cell wall and middle lamella, the cell wall dissolution may have occurred due to cellulose- and pectin-degrading enzymes produced in situ, which may diffuse extracellularly from the hyphae (9). The splitting of middle lamella, which is related with tissue softening and thus rot symptom development, may be mostly caused by enzymatic removal of pectins. Several pectolytic enzymes such as *exo*-polygalacturonase, *exo*-polymethylgalacturonase, polygalacturonase-*trans*-eliminase, and pectinmethyl-*trans*-eliminase are produced in apple fruits by *B. dothidea* (22, 25), and cell degradation and cell wall modifications are due to the combined action of these enzymes (24). The fungus grew intercellularly, intracellularly, and intramurally in subepidermal tissues of apple fruits. Cell degradation and cell wall dissolution around the various invading hyphae may lead to expansion of rot symptoms in susceptible mature fruits.

Cell wall dissolution around invading hyphae in subepidermal cells was rare in immature, red halo-symptomed cv. Fuji and resistant cv. Jonathan fruits. In immature cv. Fuji fruits, infected host tissues were also damaged as in mature ones, but not severely. However, the most striking difference in the immature fruit tissues from the mature ones was not noted in the structural modifications of host cells but in the morphology of the invading hyphae, which had prominent microbody-lipid globule complexes. The association of microbodies with lipid globules in the hyphae is structurally very similar to that found in plant seeds, where microbodies (glyoxysomes) are involved in lipid utilization via glyoxylate cycle (10). Microbodies in the fungal hyphae are glyoxysomes and their close association with lipid globules and crystalline inclusions also support their identity (10,21). Crystalline inclusions in glyoxysomes are catalase crystals that degrade hydrogen peroxide produced from lipid metabolism or from host defense-related reactions (8,10,27,28). The above aspects suggest that the fungus in the susceptible immature fruits may be in the latent state of disease development. The fungus stores nutritional materials that can be easily used for its growth when placed under favorable conditions. Field observations of the latent infections in immature fruits support the conclusion that the fungal hyphae can survive fungistatic conditions in immature fruits and resume their host invasion at near harvest.

Another structural feature of the hyphal characteristics in the susceptible immature fruits was the deposition of the thin electron-dense outer layer around the cell wall of the intercellular hyphae. This wall layer may be a reflection of retarded fungal growth or a form of dormant state, because such a structural characteristic was found in nongerminated conidia and associated with limited growth of *Cladosporium* spp. within tissues of a resistant host (18,19). In immature apple fruits, subepidermal collenchymatic tissues have no or very little intercellular spaces (7) that can inhibit the intercellular fungal growth. Low soluble sugars, low pH, and high antimicrobial substances such as phenolics and acids in immature apple fruits may also retard the fungal growth (1,12, 17,32). These physicochemical characteristics of immature apple fruits may be responsible for suppressing fungal invasion and delaying symptom appearance, leading to the latent infection.

Cell wall protuberances were invariably formed in the subepidermal tissues of red halo regions of susceptible cv. Fuji fruits and resistant cv. Jonathan fruits in this study. These structures are outgrowth forms of cell walls that are distinct from papillae formed on the inside of host cell walls (6). The wall protuberances probably deter intra- and intercellular fungal invasion by forming structural barriers with thickening of cell walls and occlusion of

intercellular spaces. The fact that the wall protuberances were formed in resistant and susceptible apple fruits only with restricted symptoms suggests that they may not be constitutive or pre-existing but induced by the fungal infection. The formation of wall protuberances is observed in bell pepper fruits (5), scutellum of maize seedlings (24), and also in apple fruits inoculated with other pathogens (6). In addition, electron-dense extramural layers were formed outside of the cell wall protuberances in resistant apple fruit tissues. Their chemical composition was not determined; however, the electron-dense materials may be pectic substances, because the pectic material forms crests or wartlike accumulations in collenchymatic tissues of some plant species (7). The above aspects suggest that their formation may not be induced in a pathogen-specific manner, but may be constitutive depending on apple cultivars or can be induced under certain fruit conditions as defense-related structures.

Intrahyphal hyphae were observed in the intercellular spaces of subepidermal tissues in resistant mature fruits. Because these structures were not found around cell wall protuberances in red halo regions of susceptible fruits, the structural aberrations may not be caused only by the structural defenses. Certain biochemical factors may have been involved in the hyphal morphological modifications. These structures are also formed in other fungi, and are suggested to be survival forms that can be induced under stressed conditions (3,4,20,31). The intrahyphal hyphae may be a form of fungal responses to host resistance to be protected from surrounding stressed environments by the enclosing hyphae.

In conclusion, these ultrastructural changes of host tissues and fungal hyphae may reflect the pathogenesis of apple white rot under varying conditions of apple fruits. The structural differences were noted between mature and immature fruits as well as between susceptible and resistant responses, providing information on latency- and defense-related ultrastructural characteristics of apple fruit tissues and *B. dothidea*.

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