ULTRASTRUCTURE OF STRIPE RUST (*PUCCINIA STRIIFORMIS* f. sp. *TRITICI*) INTERACTING WITH SLOW-RUSTING, HIGHLY RESISTANT, AND SUSCEPTIBLE WHEAT CULTIVARS

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SUMMARY

Interactions between slow-rusting, highly resistant, and susceptible cultivars of common wheat (*Triticum aestivum*) with stripe rust (*Puccinia striiformis* f. sp. *tritici*) were studied at the ultrastructural level. The rust fungus extensively colonized leaves of the susceptible wheat cv. Mingxian 169, but was limited or extremely limited in the leaves of slow-rusting cv. Dongfanghong 3 and of the highly resistant Hybrid 46. Microscopic observations showed that nearly all haustoria and hyphae were inhibited in the nearly immune cultivar, the majority of them were inhibited in the slow-rusting cultivars, but nearly all of them developed normally in the susceptible cultivar. The highly resistant cultivar showed heavily necrotized cells, while in slow-rusting wheat cell necrosis occurred during the process of hyphal extension. The slow-rusting cultivar has the same hypersensitive response characters as the resistant cultivar, but the host cells necrotized later and were fewer in number. This inhibited only in part the fungal growth, which resulted in lesser inhibition and necrosis of the fungus. As in the resistant cultivar, defense structures and materials associated with defense reaction were also produced in slow-rusting wheat, but to a lesser extent. The cytological similarities of slow-rusting with hypersensitive resistance are briefly discussed.

Key words: wheat, *Puccinia striiformis*, slow-rusting cultivars, ultrastructure.

INTRODUCTION

Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*, is one of the most widespread and destructive diseases of wheat (*Triticum aestivum* L.) throughout the world. Although it is generally accepted that growing resistant cultivars is the most economical and environmentally friendly way to control the disease, many cultivars lose their resistance to stripe rust three to five years after growing in the field as new virulent rust strains continually appear (Johnson, 2000; Ma and Shang, 2004). Development of durable resistant cultivars and prolongation of the growing time, therefore, become important tasks. Since the horizontal resistance concept was put forward by Van der Plank (1963), agronomists and plant pathologists have sought to develop novel plant-protection strategies along that line (Boukhatem *et al.*, 2002; Kushwaha *et al.*, 2007; Moldenhauer *et al.*, 2006; Prabhu *et al.*, 1993; Van Kijk *et al.*, 1988). Slow rusting, a form of quantitative resistance that prolongs the latent period of fungal infection and decreases disease severity (Rashid, 1997; Wang *et al.*, 2000), can slow the incidence and development of stripe rust in the field, thus reducing yield losses and being of practical value. Slow rusting is thought to be a non-race-specific resistance that effectively controls the epidemic spread of stripe rust in a stable, sustained, and durable manner (Das *et al.*, 1992).

Slow rusting is a susceptible reaction that delays the appearance of symptoms, reduces their severity, and minimizes crop losses. It does not completely prevent reproduction of the pathogen on the host plant, but retards reproduction rate to such an extent that the pathogen population cannot reach damaging levels before grain ripening. Generally, slow-rusting is identified by the area under disease progress curve (AUDPC) to assess the quantitative disease resistance and related resistant components at the adult plant stage (Das *et al.*, 1993; Gao *et al.*, 2000; Jeger and Viljanen-Rollinson, 2001; Rashid, 1997; Van Kijk *et al.*, 1988). The resistance expression, however, is observed even at the seedling stage (Ma *et al.*, 2002; Mares, 1979; Martin *et al.*, 1977).

Our preliminary histopathological studies with fluorescence microscopy showed that the primary infection process, e.g. germ tube of the urediospore penetrating the leaves and the primary haustoria formation, did not differ among slow-rusting, susceptible (fast-rusting), and hypersensitively resistant wheat cultivars (Ma, 2000). Slow-rusting has the characteristics of the hyper-

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sensitive response, but necrosis of host cells is less extensive, and occurs later (2-3 days) in the infection process, compared with the hypersensitively resistant control. In slow rusting cultivars the host cell necrosis occurs during the process of hyphal extension and is scattered among the colonies or around the hyphae, while in the resistant cultivar, necrosis is present around the penetration sites. Fungal development and host responses, however, were not clearly discenible at the cytological level. Therefore, to reveal the mechanisms of slow-rusting, the ultrastructural characters of host-pathogen interaction in the expression of resistance were examined by transmission electron microscopy in a typical slow-rusting wheat cultivar, inoculated with *P. striiformis*. Highly resistant and susceptible fast-rusting cultivars served as controls.

MATERIALS AND METHODS

Wheat cultivars and fungus. The slow-rusting wheat cultivar studied was Dongfanghong 3. Infection by *P. striiformis* f. sp. *tritici* CY29 was of type 3, (*sensu* Stakman *et al*., 1962) with both seedling and plants in the adult stage showing a susceptible reaction, but with a long-lasting low epidemic spread in the field. Wheat cvs Mingxian 169 and Hybrid 46 were used as susceptible fast-rusting and hypersensitively resistant controls, respectively. Infection induced by CY29 to cv. Mingxian 169 was of type 4, i.e. susceptible reaction. In Hybrid 46 the infection was of type 0, i.e. necrotic symptoms. Seven-day-old seedlings were used for inoculation. The pathogen was maintained on the susceptible cv. Mingxian 169, and fresh urediospores were collected and used for inoculation.

Inoculation and sampling. Freshly collected urediospores of CY29 were applied with a fine paintbrush to the adaxial surface of the first leaf of wheat seedlings. These were kept in a moist chamber for 24 h at 14-16°C in the dark, and then moved to a growth chamber at 14-16°C, with a light intensity of 8,000-10,000 lux and a photoperiod of 16 h light and 8 h dark. Based on preliminary histological observation, five leaves were excised 5 and 7 days after inoculation for processing for electron microscope observations. Two sets of observations were made.

Sample preparation for transmission electron microscopy. Leaf tissues were cut to small pieces (1-2 mm), fixed in 4% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2), rinsed thoroughly with the same buffer and postfixed in 1% osmium tetroxide. After thorough rinsing with phosphate buffer, the samples were dehydrated in a graded acetone series to 100%, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife, mounted on uncoated copper grids, stained with 2% aqueous uranyl acetate and lead citrate, and examined with a JEOL JEM-200EX transmission electron microscope.

RESULTS

P. striiformis colonized extensively the leaves of the susceptible wheat cultivar, producing a large amount of intercellular hyphae and haustoria. By contrast, the leaves of the slow-rusting cultivar were colonized only to a limited extent and, in the highly resistant cultivar, fungal development was markedly restricted, for only a limited number of hyphae and haustoria were seen around the infection sites. The cells of slow-rusting cultivar behaved like those of the resistant one, as they produced defense structures associated with infection as well as hypersensitive responses.

Fungal and host plant cell ultrastructure in susceptible cultivar. In the susceptible cv. Mingxian 169 five days post inoculation (dpi) large amounts of intercellular hyphae were present in the intercellular spaces and many haustoria were produced, nearly all of which developed normally. Fungal hyphae had a well preserved cytology and normal looking mitochondria and nuclei (Fig. 1a). The haustorial mother cells produced infection pegs that penetrated mesophyll cells, inducing the invagination of the plasmalemma and causing plasmolysis. The host cell plasmalemma surrounded the haustorial neck and the outer part of the haustorial body, forming an extrahaustorial cell membrane that separated the protoplasm of the host mesophyll cell from the haustorium, which consisted, typically, of a neck and a body. An electron-opaque band was observed around the haustorial neck (Fig. 1b). No obvious adverse effects were detected in the host cells after haustorium formation, although the nucleus and other organelles often gathered next to the haustorial body (Fig. 1c). The haustorial protoplasm was well preserved and the organelles were regularly arranged. The walls of the haustorial bodies were smooth, the extrahaustorial plasmalemma was continuous and undulated, the extrahaustorial matrix was narrow, with no or just a little material deposited (Fig. 1d). One of the frequent host responses observed was the deposition of electron-dense material, i.e. a collar around the haustorial neck in some of the infected host cells (not shown). At 7 dpi fungal and host plant cells retained a normal appearance.

Fungal and host plant cell ultrastructure in highly resistant cultivar. In the highly resistant cv. Hybrid 46, at all sampling times, only a very limited number of abnormal intercellular hyphae and haustoria were formed. The cytoplasm was electron-dense, the walls thickened,

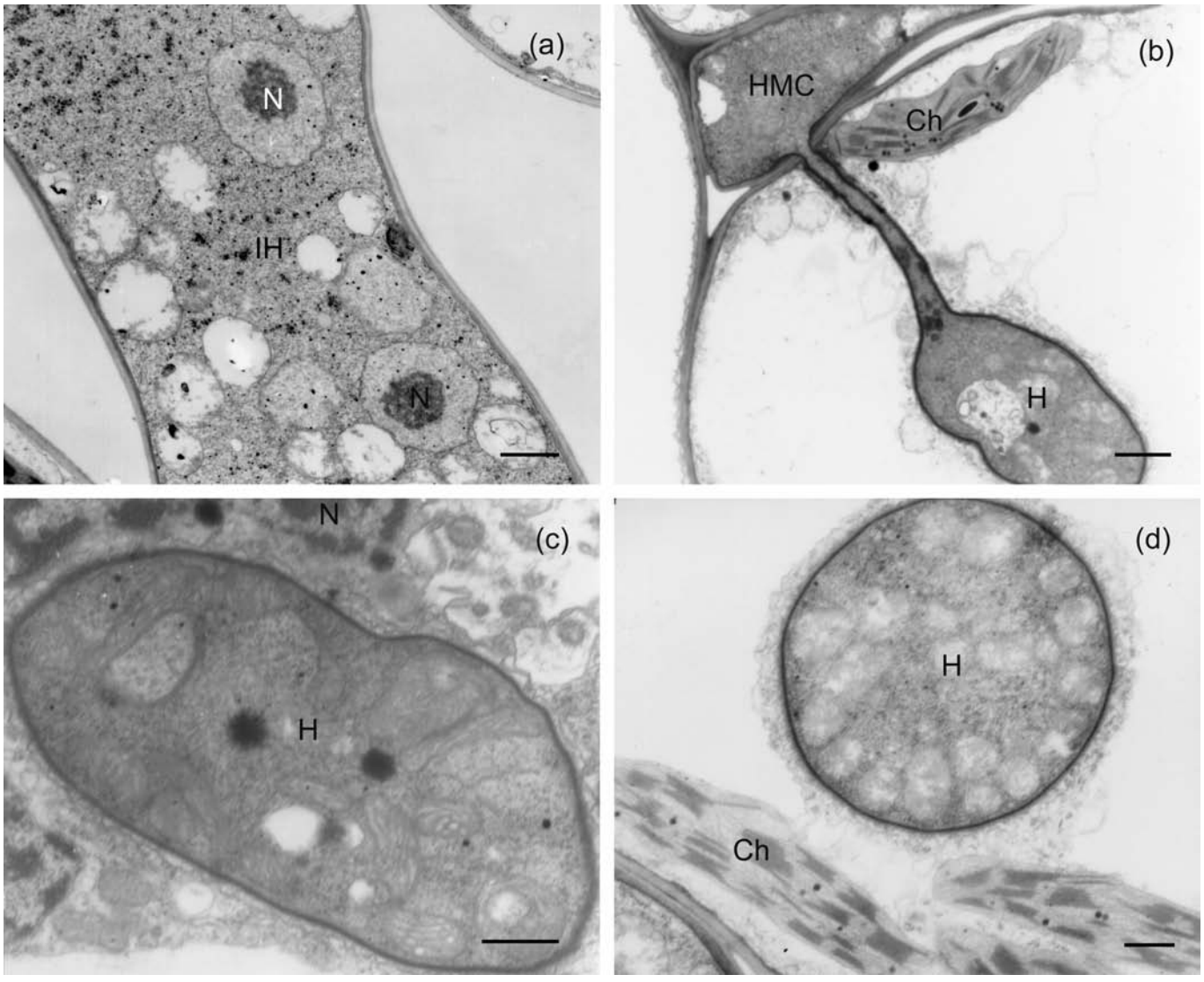

Fig. 1. Fungal development and host cell responses in infected susceptible wheat leaves (Mingxian 169) inoculated with *Puccinia striiformis* on the fifth day after inoculation. a) Intercellular hypha with nuclei. Bar = 1 µm. b) A mature haustorium in the host cell consisting of haustorial neck and haustorial body. Bar = 1 µm. c) Haustorium with host cell nucleus and other organelles alongside the haustorium. Bar = 1 µm. d) Haustorium with smooth extrahaustorial plasmalemma and narrow extrahaustorial matrix. Bar = 0.5 µm. Ch: chloroplast; H: haustorium; HMC: haustorial mother cell; IH: intercellular hypha; N: nucleus.

and organelles like mitochondria were vacuolated (Fig. 2a, b). Many electron-dense granular deposits were present and the hyphal wall appeared also thickened and deeply stained (Fig. 2c). Haustorial mother cells were vacuolated and contained electron-dense material resembling lipids (Fig. 2d). Growth inhibition of hyphae, haustorial mother cells, and haustoria was most severe. Some haustoria were necrotic and completely surrounded by callose (Fig. 2e). The wall of some of them was thickened, the extrahaustorial matrix was inflated by the presence of deposits, and the host organelles around the haustoria were disintegrated into vesicles (Fig. 2f). The alterations shown by hyphae, haustorial mother cells and haustoria were distinctly more severe than in the slow-rusting cultivar.

Fungal cell ultrastructure in the slow-rusting wheat cultivar. In the slow-rusting cultivar 5 dpi a few hyphal cells showed abnormalities. Many small vacuoles appeared in the cytoplasm, mitochondria were vacuolated (Fig. 3a), and many electron-dense granular deposits were present in the cytosol (Fig. 3b). Dark lipid-like materials often appeared around, or within large vacuoles in the haustorial mother cell (Fig. 3c). The haustorial mother cell walls became thickened and electron-dense and lost their ability to produce penetration structures (Fig. 3d). A few haustoria were malformed, walls were thickened, the protoplasm was densely stained and necrotic (Fig. 3e). Some haustorial bodies were unable to expand normally and were malformed. Electron-dense bodies were present in the cytosol, the organelles

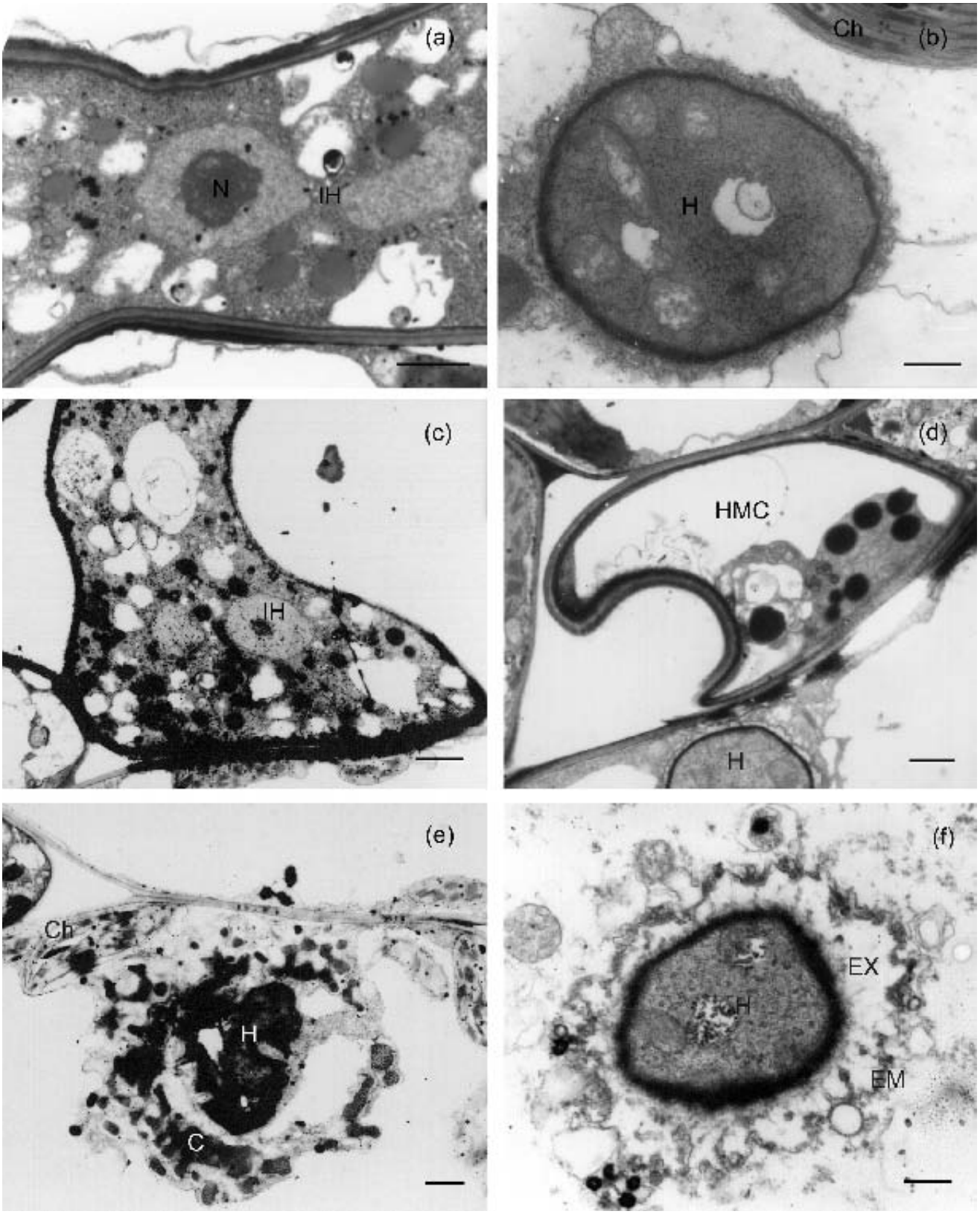

Fig. 2. Fungal development and host cell responses in infected resistant wheat leaves (Hybrid 46) inoculated with *Puccinia striiformis* on the fifth day after inoculation. a) Intercellular hypha with vacuolated mitochondria. Bar = 1 µm. b) A haustorium in the host cell. Bar = 1 µm. c) Hyphal wall thickening and deeply stained. Bar = 1 µm. d) Haustorial mother cell vacuolated with deeply stained lipid material. Bar = 1 µm. e) Haustorium surrounded by callose and necrotized while the host cell still appears living. Bar = 1 µm. f) Extrahaustorial membrane wrinkled, extrahaustorial matrix thickened with electron-dense material deposited, host organelles disintegrated. Bar = 0.5 µm. C: callose; Ch: chloroplast; H: haustorium; HMC: haustorial mother cell; IH: intercellular hypha; EM: extrahaustorial membrane; EX: extrahaustorial matrix; N: nucleus.

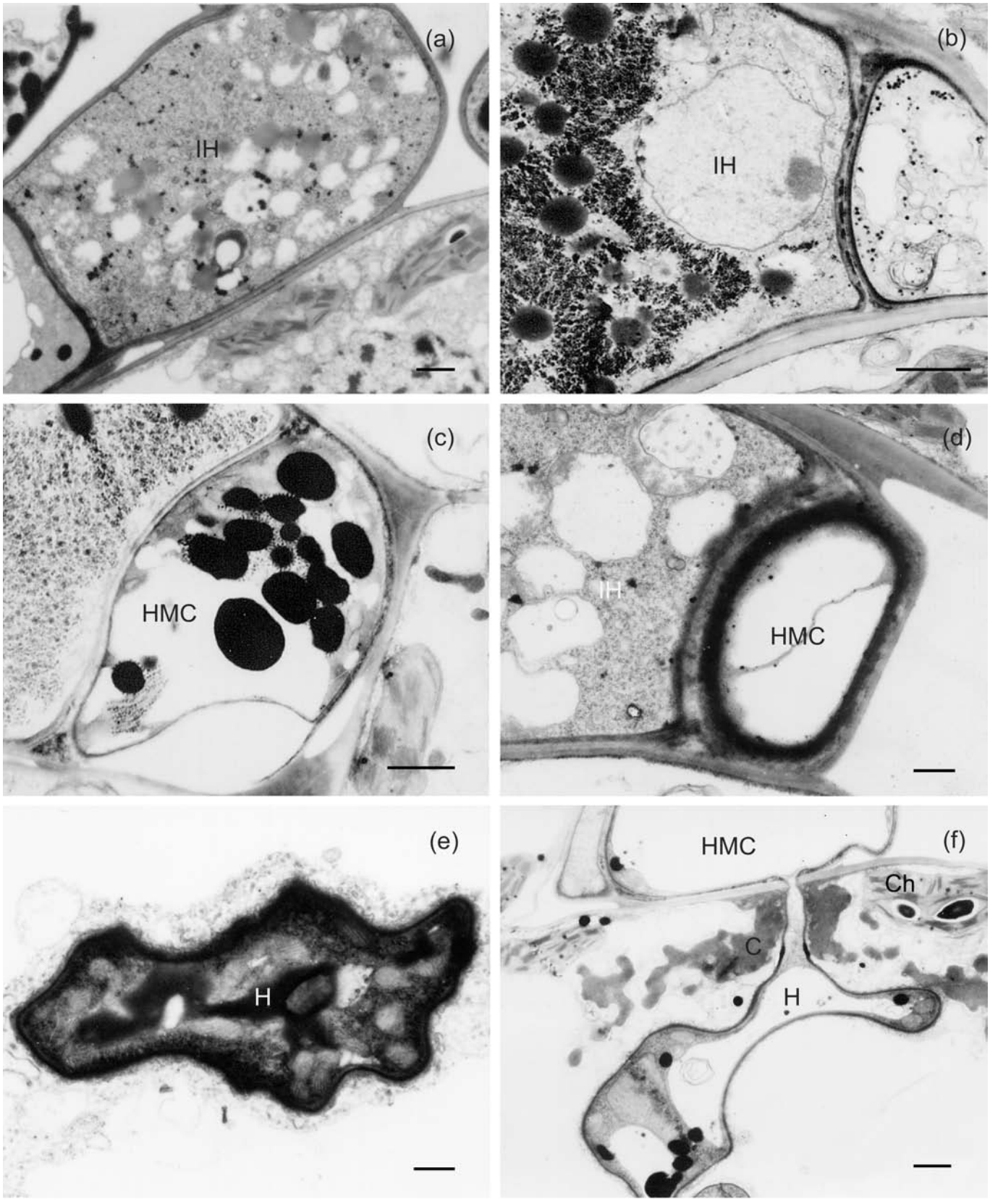

Fig. 3. Fungal development in infected slow-rusting wheat leaves (Dongfanghong 3) inoculated with *Puccinia striiformis* on the fifth day after inoculation. a) Intercellular hypha vesicularized. Bar = 0.5 µm. b) Intercellular hypha vesicularized, disintegrated with deeply stained lipid material. Bar = 1 µm. c) Haustorial mother cell vacuolated with deeply stained lipid material. Bar = 0.5 µm. d) Haustorial mother cell vacuolated with wall thickened. Bar = 0.5 µm. e) Malformed haustorium with wall thickened and electron-dense material. Bar = 1 µm. f) Malformed, vacuolated haustorium with lipid-like material. Bar = 1 µm. C: collar; Ch: chloroplast; H: haustorium; HMC: haustorial mother cell; IH: intercellular hypha.

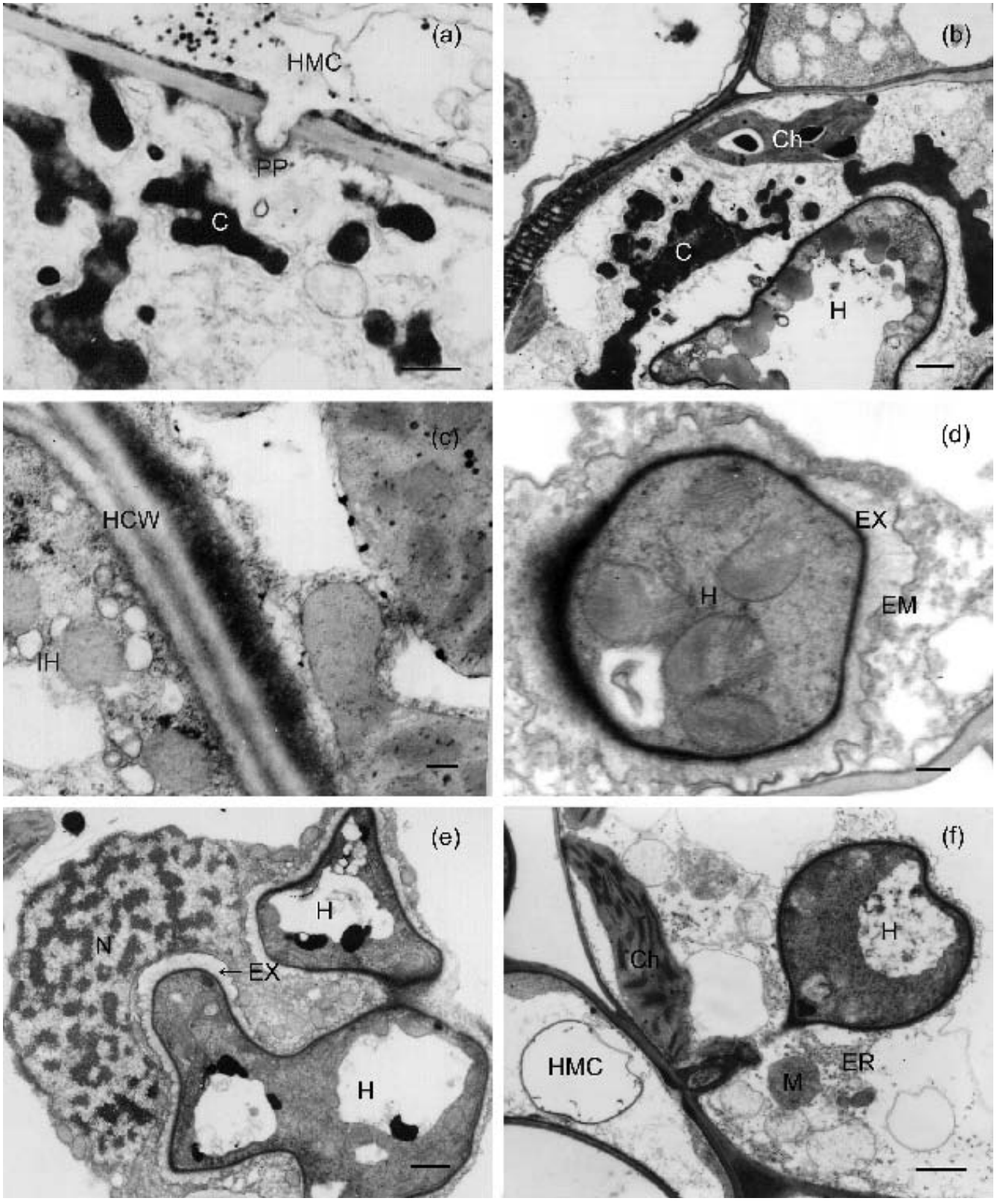

Fig. 4. Host cell responses in infected slow-rusting wheat leaves inoculated with *Puccinia striiformis* on the fifth day after inoculation. a) Penetration peg (PP) penetrating the host mesophyll cell. Bar = 0.5 µm. b) Haustorium (H) surrounded by callose (C), vacuolatized with densely stained lipid-like material. Bar = 1 µm. c) Host cell wall deposition while contacting with intercellular hypha. Bar = 0.2 µm. d) Haustorial wall thickened with extrahaustorial matrix (EX) widened. Bar = 0.5 µm. e) Haustorium with host cell nucleus and other organelles alongside the haustorium. Bar = 0.5 µm. f) Mitochondria and endoplasmic reticula increase in number. Bar = 1 µm. C: callose; Ch: chloroplast; EM: extrahaustorial membrane; ER: endoplasmic reticulum; EX: extrahaustorial matrix; H: haustorium; HCW: host cell wall; HMC: haustorial mother cell; IH: intercellular hypha; M: mitochondria; N: nucleus; PP: penetration peg.

were disorganized, and electron-dense lipid-like materials often accumulated around the vacuoles (Fig. 3f).

Host plant cell ultrastructure of slow-rusting wheat cultivar. When the haustorial mother cells produced the penetration peg to invade the mesophyll cells in the slow-rusting cultivar, host cell walls became thickened and densely stained. At the penetration site, the papillar structure that opposes the invasion of the penetration pegs was rarely produced by the host cell. Following pathogen invasion host cells produced defense structures such as cell wall apposition. At the peg penetration site callose was formed (Fig. 4a). This process occurs equally well in both resistant and susceptible cultivars (Heath, 1982; Ma and Shang, 2002), and in the slow-rusting cultivar we investigated, suggesting that it represents a non-specific response of the host cell to pathogen invasion. Some developing haustoria were surrounded by callose in the slow-rusting and resistant cultivars, which might result in the inhibition of further development (Fig. 4b). Some black granular materials were deposited between the host cell wall and the plasmalemma, but the formation of haustoria seemed largely unaffected. The same situation also appeared while the intercellular hyphae contacted the host cell wall (Fig. 4c). The densely staining cell walls and the production of deposits may play a role in the resistance process by increasing the physical strength of cell walls and restricting the spread of the fungus. Another marked change in haustoria shown by infected leaves of the slow-rusting cultivar was the filling of the extrahaustorial matrix with electron-dense fibrillar and granular materials. The extrahaustorial membrane became wrinkled and perforated (Fig. 4d). In the invaded mesophyll cells, nuclei, chloroplasts, mitochondria, and endoplasmic reticulum were along the periphery of the haustoria (Fig. 4e). Mitochondria and endoplasmic reticulum increased in number (Fig. 4f), a phenomenon observed in all cultivars, regardless of their level of resistance. Mitochondria have a respiratory function in plants, thus an increase of their number may lead to an increase of respiration in the invaded cells, but this does not appear to be a cause of resistance in wheat cultivars.

Owing to the invagination of the plasmalemma, organelles such as chloroplasts, formerly adjacent to cell walls, were forced to move toward the centre of the cell. Whether this may affect the photosynthesis rate of the host cell to any degree remains to be established. There was a small increase of starch grains in some chloroplasts.

At the fifth dpi, a few infected mesophyll cells in the slow-rusting cultivar expressed hypersensitive responses similar to those of the resistant cultivar, but the necrotic cells were markedly less in number, compared with the resistant control cultivar (in which necrotic cells could be observed in all infection sites). In the susceptible cultivar, chloroplasts in the mesophyll cells were elongated, shuttle-like, had a normal looking boundary membrane and the internal membrane system (grana and intergrana lamellae). Mitochondria were tendentially spherical or long-elliptical in shape depending on the cutting angle, with distinctly visible cristae. In the slow rusting cultivar, especially 7 dpi, the mitochondrial cristae in host cells became swollen and deformed, gradually disintegrating from the central part and collapsing. The double membrane became single, was partially disrupted, and eventually became disorganized into vesicles (Fig. 5a). Endoplasmic reticulum was swollen and deformed and partially disrupted. Ribosomes of the rough endoplasmic reticulum were reduced in number. The polymerized ribosomes in the cytoplasm were depolymerized into single units. Chloroplasts were swollen, acquired a spherical shape, showed an increased number of osmiophilic granules and starch grains. Stromata were densely stained, the thylakoid membrane of the grana lamellae was thickened and indistinct, and the grana (thylakoid stacks) appeared to be spherical in shape (Fig. 5b). Thereafter, during the process of chloroplast swelling, the bounding membrane was ruptured; starch grains present in the chloroplasts were gradually reduced both in number and in size and eventually disintegrated. The stroma lamellae were disrupted and the stroma became electron-lucent. The internal membrane system and the very structure of the chloroplasts were heavily deranged so that some of these organelles eventually disintegrated. Likewise, the cytoplasmic membrane and the tonoplast were densely stained, invaginated, folded, and partially fragmented to complete disintegration. The cytoplasm became heavily vacuolated (Fig. 5b). The nuclear membrane was partially ruptured and the organelle no longer displayed the uniform loose structure of the matrix (not shown). The cell wall became structurally loosened and perforated, within which the haustoria disintegrated and necrotized. With the gradual disintegration and disappearance of chloroplasts, cytoplasm, and haustoria, host cells exhibited an empty lumen surrounded by cell walls in the process of disintegrating (Fig. 5c). Necrosis of fungal haustoria preceded that of the host cells (Fig. 5d). Ultrastructural changes occurred earlier in the haustoria than in the intercellular hyphae, like in the resistant cultivar. The death of host cells with haustoria was accompanied by that of the haustoria. In the advanced stage of infection, more necrotic host cells were detected in infected leaves, and more intercellular hyphae were necrotic or collapsed, indicating that fungal development had been inhibited.

DISCUSSION

Resistance of wheat cultivars to *P. striiformis* is usually race-specific, and is accompanied by host cell death around the infection site, known as hypersensitive re-

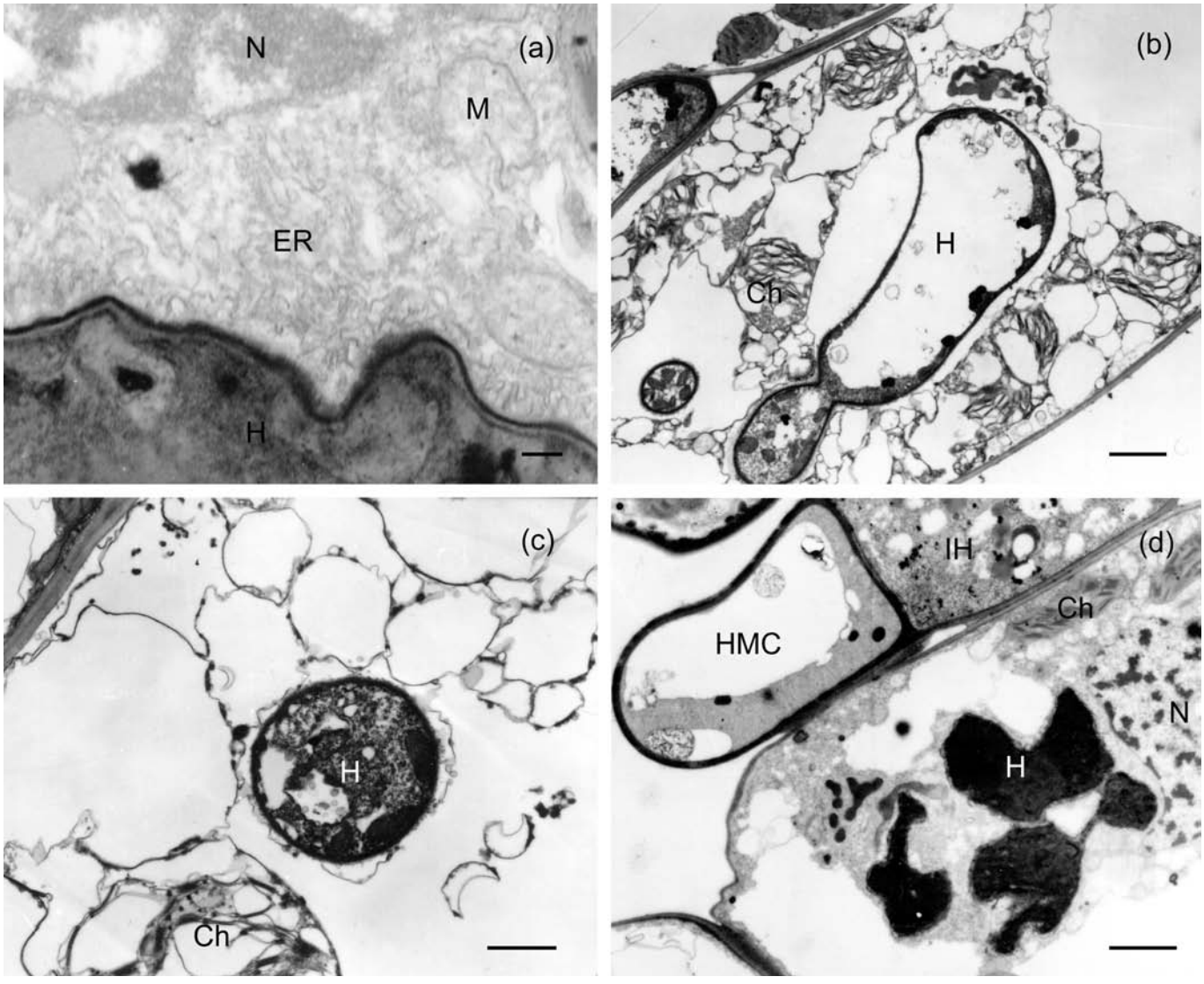

Fig. 5. Host cell responses in infected slow-rusting wheat leaves inoculated with *Puccinia striiformis* on the seventh day after inoculation. a) Mitochondria (M) with membrane disrupted. Bar = 0.2 µm. b) Disintegrated host cell and haustorium with part of chloroplasts (Ch) still visible. Bar = 1 µm. c) Haustorium necrotized with host cell disorganized. Bar = 1 µm. d) Haustorium necrotized while host cell not yet. Bar = 1 µm. Ch: chloroplast; E: endoplasmic reticula; H: haustorium; HMC: haustorial mother cell; IH: intercellular hypha; M: mitochondria; N: nucleus.

sponse (HR). This type of resistance, however, is easy to be broken by novel virulent rust strains that keep emerging. Producing durable resistance has been the goal of much research. The resistance mechanism of slow-rusting is rather complex, and has no unanimous explanation (Wang *et al.*, 2000). Many researchers have observed host cells necrosis in the histopathological study of slow-rusting (Mares and Cousen, 1977; Sun and Zeng, 1987). Compared with the susceptible cultivar, the slow-rusting cultivar we have invstigated is characterized by the slower spread of the fungus, smaller colony size, and less sporulation. Our research indicates that the main mechanism of resistance is the hypersensitive response of the host mesophyll cells. It differs from race-specific resistance mainly in that fewer papillar structures against fungal invasion were present at the fungal penetration sites, and the incidence of necrotic host cells was lower. This only partially restricted fungal spread. Conversely, in the cultivar with race-specific resistance, mesophyll cell necrosis occurred earlier and targeted around the penetration sites where more cells necrotized, thus restricting intercellular hyphal growth. It is therefore reasonable to speculate that the slow-rusting of wheat to stripe rust may derive from a resistance mechanism similar to that underlying race-specific resistance. However, it has a lower intensity and differences of infection type are not distinct enough to be recognized by the naked eyes. The ultrastructural features of infected wheat cells

confirm these findings, which agree with the results of previous studies in which two other slow-rusting cultivars showed a similar behaviour (Ma, 2000).

The hypersensitive response is one of the most typical resistance mechanisms in host plants. Our study has shown that at the ultrastructural level, regardless of slow-rusting or highly resistant status, the cytological processes leading to cell death in the hypersensitive response are essentially the same (see also Heath, 1982; Ma and Shang, 2002). In flax rust, this kind of hypersensitive response of quantitative resistance was referred to as incomplete hypersensitivity (Kowalska and Niks, 1999), which is expressed in the late period of the infection process, and only occurs in some infected cells. Hypersensitive responses, however, are essentially of the same nature for various kinds of resistance (Heath, 1982; Ma and Shang, 2002; Mares and Cousen, 1977).

Haustoria are considered to be sites of exchange of information and nutrients between fungus and host plant (Mendgen *et al.*, 2000; Mendgen and Hahn, 2002). The structural changes of haustoria in slow-rusting and resistant cultivars undoubtedly affect the normal physiological functions of these fungal structures, further influencing hyphal development. Haustorial formation also induces structural changes in the host cell, including cytoskeletal rearrangements, nuclear migration, and chromatin condensation (Heath, 1997; Kobayashi *et al.*, 1994). In the interaction between flax (*Linum usitatissimum*) and its rust pathogen (*Melampsora lini*), R genes and avr genes have been isolated. The avr genes, expressed in haustoria, encode small proteins with functional secretory signal peptides. During haustorial formation, these peptides are secreted into the extrahaustorial matrix and translocated into the host cytoplasm where they are recognized by R gene products (Catanzariti *et al.*, 2006; Dodds *et al.*, 2004). Through ultrastructural research on the slow-rusting and race-specific resistance, it was found that when the haustoria of the stripe rust began to show inhibition or necrosis, the infected host cells were mostly normal, followed by the haustoria and the host cells, all showing necrosis. Similar observations were also made in previous studies on wheat stem rust, cowpea rust and wheat stripe rust (Heath, 1982; Ma and Shang, 2002; Skipp, 1974), further revealing the similarity in resistant mechanism underlying the slow-rusting and the high race-specific resistance. These findings establish a platform for the study of molecular mechanisms involved in the interactions between *P. striiformis* and slow-rusting wheat in future research.

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