Cytological Alterations Associated with Flame Chlorosis, a Novel Viruslike Disease of Barley, Wheat, and Oat

J. Chong and S. Haber

Research scientists, Agriculture Canada, Research Station, 195 Dafoe Road, Winnipeg, Manitoba, Canada, R3T 2M9.
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ABSTRACT

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The cytological alterations associated with flame chlorosis were similar for the three known hosts, barley, wheat, and oat. The first detectable changes in affected mesophyll cells were the appearance of double-membrane-bounded vesicles, which contained densely staining fibrils, along the periphery of the chloroplasts and mitochondria. Affected chloroplasts became swollen, rounded, and clumped in groups; they became extensively vesiculated mainly in the periplastidial spaces between the chloroplast membranes. Whorls of membranous structures occurred in a majority of affected chloroplasts. Mitochondria also were altered; the most striking features were hypertrophy and extensive ramification of cytoplasmic channels through the organelles. Vesiculation occurred extensively in the perimitochondrial spaces around these invaginations. Affected mitochondria were closely associated with endoplasmic reticulum. Five different types of inclusion bodies, consisting of electron-dense amorphous material, were observed. No virus particles were observed in any of the affected cells.

Additional keywords: root tissues, ultrastructure, vascular tissues.

Flame chlorosis is a soil-transmitted, viruslike disease of cereals (16,17). It was first observed in barley in western Manitoba, Canada, in 1985 (17) and in wheat and oat in 1988 and 1989, respectively (15). Flame chlorosis has steadily intensified in western Manitoba and has spread to other areas of the province (16). To date, it has caused only isolated yield losses in barley, the most severely affected of the known hosts (16). However, the recent emergence and continued spread and intensification of flame chlorosis are a potential concern for cereal production in Manitoba.

The striking visual symptoms are similar in the three hosts and, to the experienced observer, characteristic of the disease (16). Analysis of the patterns of double-stranded (ds) RNA bands, visualized by ethidium bromide staining in agarose gel electrophoresis (15,17), and Northern and dot blot analyses (18) also indicate that dsRNAs isolated from diseased barley, wheat, and oat are similar to each other but unlike those reported for any known diseases of cereals. A preliminary study of the cytopathology of symptomatic barley did not find evidence of infection of leaves by fungi, bacteria, or fastidious prokaryotic plant pathogens. The affected mesophyll cells are characterized by massive vesiculation in chloroplasts and mitochondria (17). Although the cytopathic effects of flame chlorosis are consistent with infection by a virus or viruslike agent, no viruslike particles have been observed in the affected cells. Moreover, repeated attempts to isolate virus particles from diseased tissue have failed (15,18; S. Haber, *unpublished*). If putative virus particles exist but are indistinguishable from ribosomes in ultrathin sections, it may be possible to induce the formation of paracrystalline arrays under conditions of wilting (31,37) or plasmolysis (19,23).

In this investigation, our objectives were to detail the cytological alterations in cells of barley, wheat, and oat affected by flame chlorosis and to determine if viruslike particles are detectable in the affected cells under stress conditions known to enhance the detection of small isometric viruslike particles.

MATERIALS AND METHODS

Maintenance of flame chlorosis-affected plants. Plants with characteristic symptoms were taken from the field, transplanted into 15-cm fiber pots of field soil, and kept cool (<20 C) during transport to the laboratory. To facilitate recovery from transplant stress and ensure that saprophytic fungi would not later damage them (as usually happens with the symptomatic plants left in the field), we transferred transplants to growth cabinets and sprayed them 1–2 days later with the foliar fungicide, Morestan, at 1.0 g/L (active ingredient, chinomethionate; supplied by Chem-Agro Ltd., Mississauga, Canada). Growth cabinets (Model EG-15, Conviron, Winnipeg, Canada) were maintained on a 14-h light (approx. 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 10-h dark regime; the day temperature was 18 C and the night temperature was 15 C. When transplants produced new growth, they were freed from soil, and their roots were washed free of field soil by running them under water for 2–3 h. Transplants were then repotted in steam-sterilized soil.

The repotted plants were maintained in growth cabinets. To delay senescence and promote tillering, we continually trimmed emerging heads. Barley, wheat, and oat tissues with flame chlorosis symptoms were propagated from cultured transplants by teasing newly emerging tillers away from the main plant for repotting into steam-sterilized soil. After 4–6 wk, the cycle was repeated. These clones of original field plants affected by flame chlorosis were the tissue source for most of the electron microscopy studies; other sources were diseased plants freshly transplanted from the field. Healthy plants (controls) were similarly propagated to verify that symptoms and cytological alterations were not attributable to plant culturing methods.

Electron microscopy. Leaf disks (1.5 mm diameter) were sampled from chlorotic and adjacent green areas of symptomatic leaves of barley, wheat, and oat, and from healthy controls. For root tissues, 2-mm-long segments were cut from diseased barley roots near the tip regions. Leaf and root samples were fixed (11) in 1.5% (v/v) glutaraldehyde in 0.025 M phosphate buffer, pH 6.8, at room temperature for 2 h, and then in 6% (v/v) glutaraldehyde in the same buffer at 4 C for 16 h. The samples were postfixed with 2% OsO_4 in phosphate buffer, dehydrated, and embedded

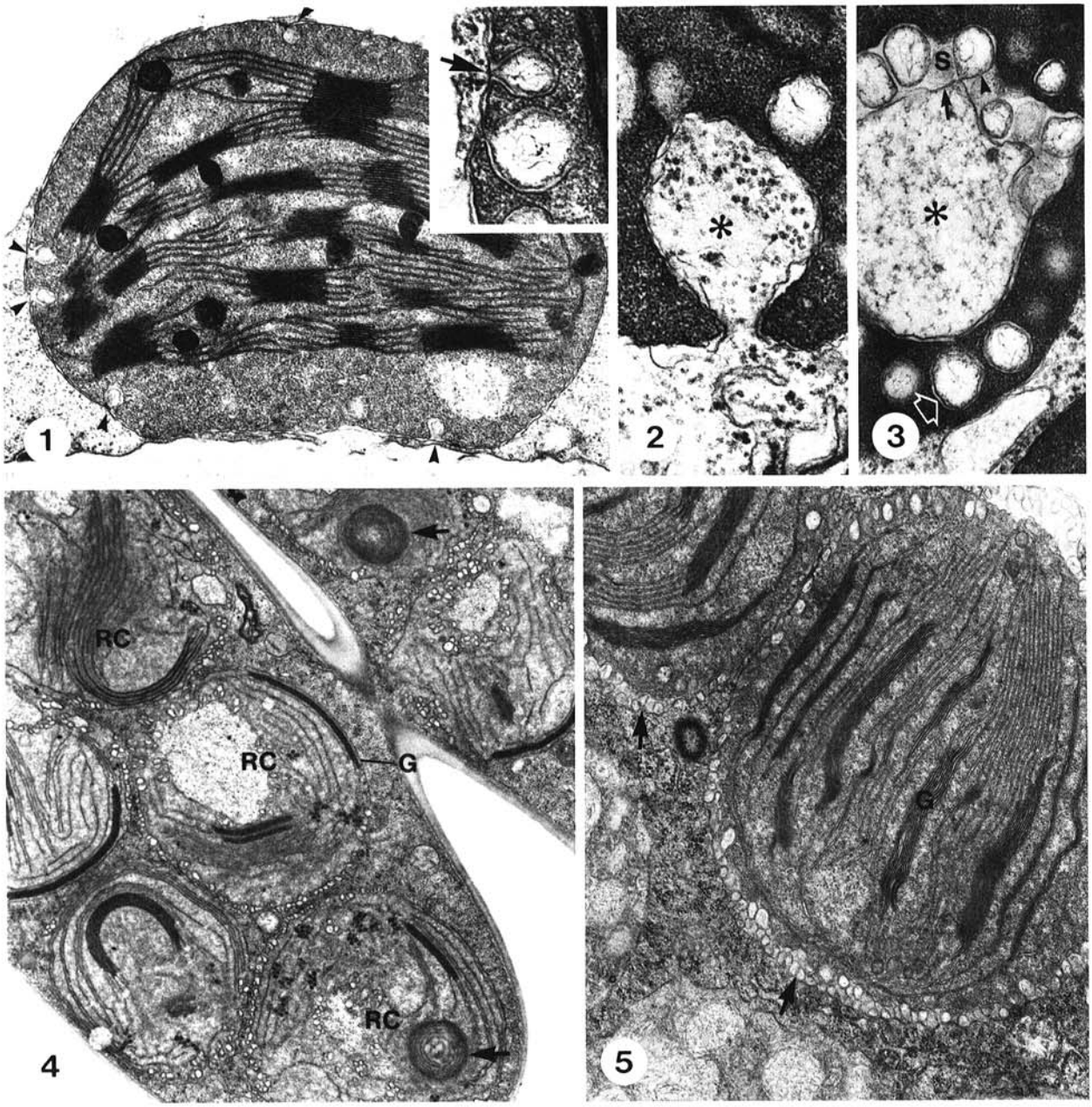
in Epon-Araldite (J. B. EM Services Inc., Quebec, Canada) or Spurr-embedding medium (SPI Supplies, Toronto, Canada). Ultrathin sections were mounted on Formvar and carbon-coated 100-mesh or single-slot copper grids and stained with 5% uranyl acetate in 50% aqueous ethanol for 10 min and in lead citrate for 10 min. The sections were examined with a Philips EM-420 transmission electron microscope.

Plasmolysis or wilting treatment. The conditions used for plasmolysis were as described (19,23). Leaf disks (1.5 mm diameter) were excised from healthy barley leaves and from chlorotic areas of symptomatic barley leaves and floated on 40% sucrose in distilled water for 1 or 3 h at room temperature. They were then fixed in glutaraldehyde and processed for electron microscopy as described above.

To induce wilting, we subjected detached leaves of healthy barley and symptomatic barley to the following treatments before fixation with glutaraldehyde: wilting at room temperature (21 C) for 3 h (31) and up to 18 h; heating in an oven at 60 C for 5–7 min; and heating in an oven at 60 C for 5 min, followed by 18 h at room temperature (37). After the wilting treatment, leaf segments were excised from the healthy leaves and from the chlorotic areas of symptomatic leaves and processed for electron microscopy as described above.

RESULTS

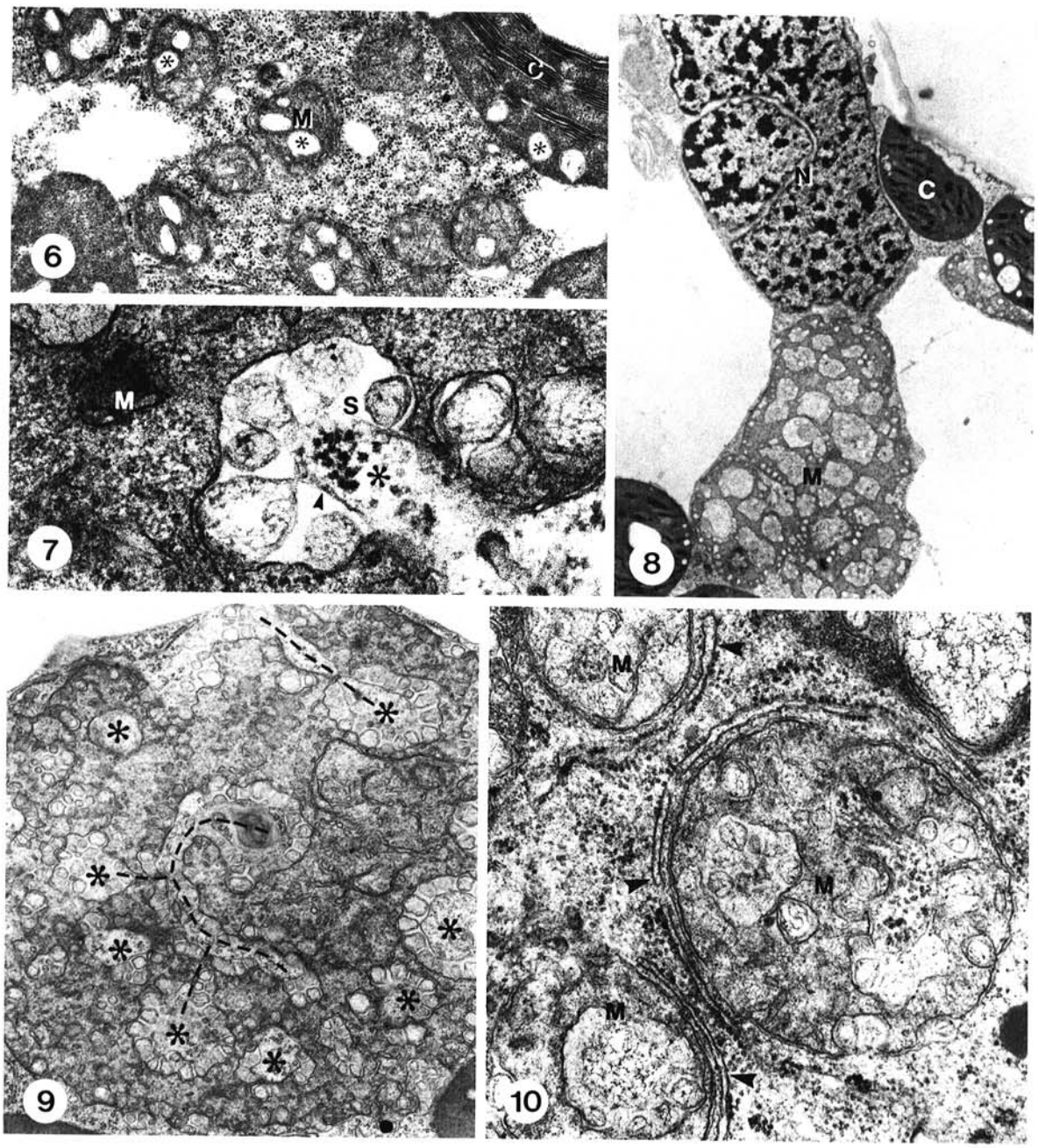
The cloning and culturing of cereal plants did not induce any abnormal cytological changes (not shown). The observed cyto-



Figs. 1–5. Ultrathin sections of mesophyll cells of symptomatic barley (1–3,5) and oat (4) showing chloroplast alterations. **1,** A few scattered vesicles (arrowheads) along the inner periphery. ×17,900. Inset, peripheral vesicles, bounded by a double membrane, contain densely staining fibrils. These membranes are continuous with the chloroplast envelope (arrow). ×33,000. **2,** Chloroplast indented by the surrounding cytoplasm (asterisk). Vesicles around the indented region are visible. ×32,100. **3,** Vesicles occurring singly around the indented cytoplasmic region (asterisk) are bounded by double membranes (open arrow). Those in the periplastidial space (S) are bounded by a single membrane (arrowhead); this membrane is continuous with the outer chloroplast membrane (arrow). ×38,600. **4,** Clumps of swollen and rounded chloroplasts (RC) whose grana (G) and thylakoids are extensively elongated. Whorls of membranous structures (arrows) occur in some RC. ×9,500. **5,** Chloroplast with elongated thylakoids and grana (G) and accumulation of vesicles in the periplastidial spaces of the envelope (arrows). ×21,500.

logical alterations were specifically associated with symptomatic leaves of barley, wheat, and oat for cloned plants and plants freshly transplanted from the field. Affected cells were recognized on the basis of some or all of the following alterations: vesiculation in chloroplasts; swelling, rounding, and clumping of chloroplasts; disruption of grana and thylakoids, which formed large whorls of membranous structures in the chloroplasts; and hypertrophy of mitochondria accompanied by indentations of the surrounding cytoplasm and extensive vesiculation around the indented regions. On the basis of these criteria, affected cells were found in all the main tissues of host leaves, including epidermal, mesophyll, bundle sheath, phloem companion and parenchymal, and xylem parenchymal cells, as well as in the root cells (only barley roots were examined). In green areas of symptomatic leaves, the proportion of cells with cytological alterations and the degree of those alterations were much less than in chlorotic areas. To avoid duplications, we present only representative cases from each host.

Alterations of chloroplasts. In the green areas of symptomatic leaves of barley, wheat, and oat, many of the mesophyll cells appeared normal. However, some mesophyll cells in these areas had a few scattered vesicles along the inner periphery of the chloroplasts, which otherwise appeared normal in shape (Fig. 1). These vesicles (up to about 0.4 μm in diameter) were bounded by a double membrane and contained densely staining fibrils (Fig. 1, inset). In sections median through the vesicles, the contents were open to the cytoplasm through a narrow opening, and the outer and inner membranes of the vesicles were continuous with

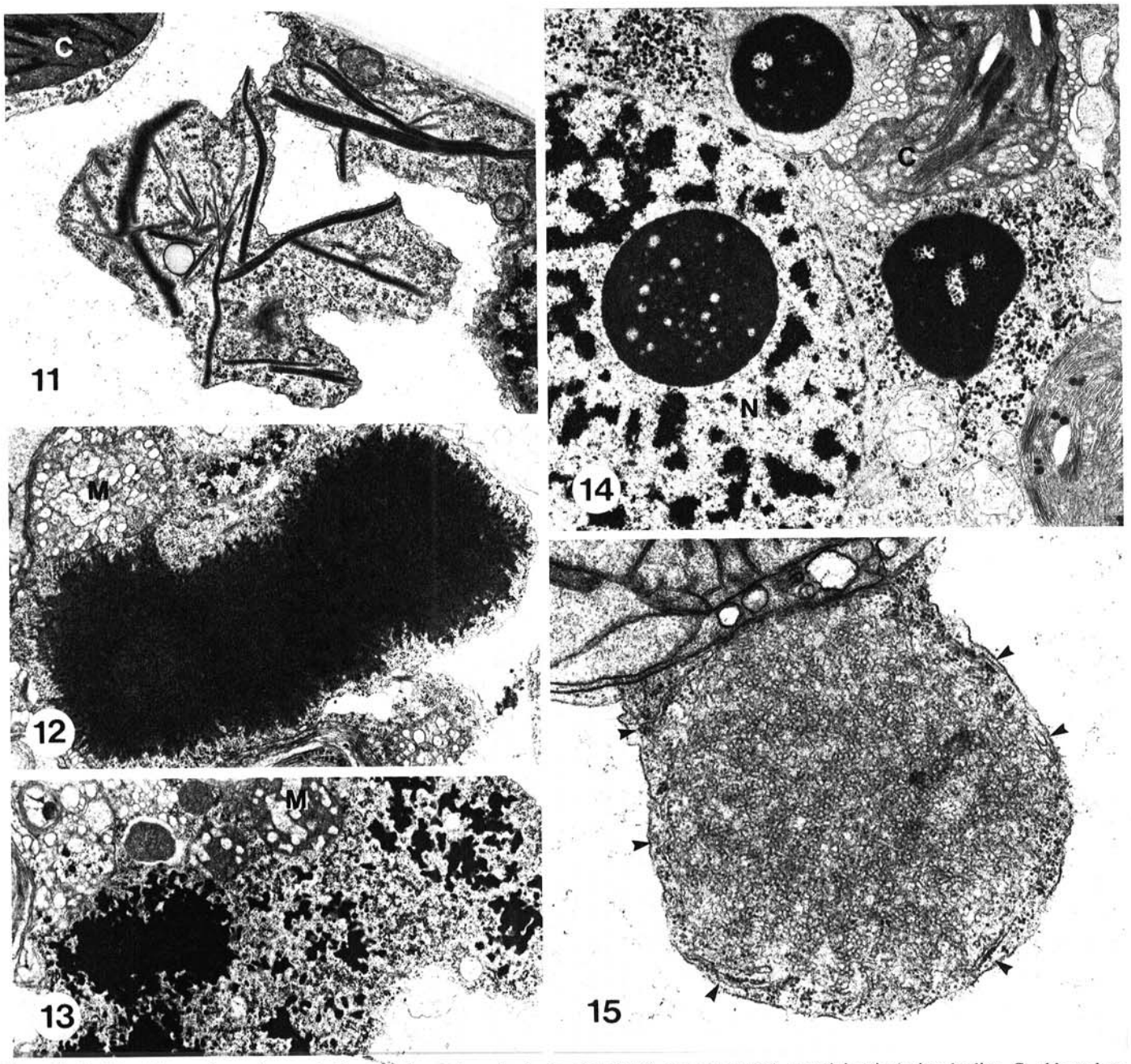


Figs. 6–10. Ultrathin sections of mesophyll cells of symptomatic barley (6) and oat (7–10) showing mitochondrial alterations. **6,** Presence of vesicles (asterisks) bounded by double membranes in normal size mitochondria (M). Part of a chloroplast (C) with scattered peripheral vesicles is shown. ×22,200. **7,** Mitochondrion indented by the surrounding cytoplasm (asterisk). Vesicles in the perimitochondrial space (S) are bounded by single membranes. The membrane of the vesicles is continuous with the outer membrane (arrowhead) of the mitochondrial envelope. ×62,000. **8,** A vesiculate mitochondrion (M) was as large as the lobed nucleus (N). ×5,400. **9,** Hypertrophied mitochondrion with a network of cytoplasmic channels (dashed lines), some of which were seen in cross-sections (asterisks). Extensive vesiculation in the perimitochondrial spaces around the cytoplasm occurred in this organelle. ×16,400. **10,** Close association of endoplasmic reticulum (arrowheads) with several slightly swollen, vesiculate mitochondria (M). ×42,900.

the two chloroplast membranes (Fig. 1, inset). Some of the chloroplasts were indented by the surrounding cytoplasm; vesicles occurred around the periphery of the indented regions (Figs. 2,3). Some of these vesicles occurred singly around the indented regions and appeared to be enclosed by a double membrane (Fig. 3). The other vesicles occurred in clusters in the enlarged periplastidial spaces between the two chloroplast membranes and were bounded by a single membrane (Fig. 3). With some vesicles, the bounding membrane was continuous with the outer membrane of the chloroplast (Fig. 3). Frequently, densely staining fibrils, similar to those in the vesicles, were observed in the cytoplasm of the indented regions (not shown).

From the chlorotic areas of the diseased leaves of barley, wheat, and oat, chloroplasts with various degrees of alterations were commonly observed in mesophyll cells. In many of these cells, the chloroplasts appeared swollen and rounded and occurred in clumps (Fig. 4). Not all chloroplasts in the same cell were affected to the same degree; occasionally rounded and normal shape chloroplasts were observed in the same cells. There were fewer grana in the rounded chloroplasts than in the normal shape chloroplasts (Figs. 4,5). The grana and thylakoids in the rounded chloroplasts were extensively elongated (Figs. 4,5). In other rounded chloroplasts, whorls of membranous structures were present (Fig. 4). Occasionally some rounded chloroplasts contained massive arrays of tubules in the stroma, similar to those observed in cells infected by belladonna mottle virus (26). At advanced states of alterations, there was a massive proliferation of vesicles, but mainly in the periplastidial spaces between the two chloroplast membranes (Fig. 5). The vesicles located at these sites were enclosed by a single membrane. In some infected cells, the outer membrane of the chloroplast was disrupted, and vesicles were found in large numbers in the cytoplasm.

Alterations of mitochondria. With the occurrence of double-membrane-bounded vesicles in the chloroplasts, similar vesicles



Figs. 11–15. Ultrathin sections of mesophyll cells of symptomatic barley (11–13,15) and wheat (14) containing inclusion bodies. C, chloroplast; M, mitochondrion; N, nucleus. **11,** Fibrillar inclusions consisting of long strands of electron-dense, amorphous material. ×12,800. **12,** Large, rectangular, electron-dense body with an irregular border. ×8,600. **13,** Inclusions of irregular masses of electron-dense, amorphous material. ×8,600. **14,** Large electron-dense bodies containing electron-transparent cavities filled with electron-dense granules. ×11,300. **15,** Large circular body of membranous structures. Endoplasmic reticulum surrounds (arrowheads) the body. ×26,000.

also were observed in mitochondria (Fig. 6). The membranes of these vesicles were continuous with the mitochondrial envelope. The affected mitochondria were often indented by the surrounding cytoplasm (Fig. 7). Around the indented regions, vesicles of various sizes were formed in the enlarged perimitochondrial spaces between the two mitochondrial membranes (Fig. 7). The vesicles at these sites were enclosed by a single membrane. In some views, this membrane was continuous with the outer membrane of the mitochondrial envelope (Fig. 7).

Mitochondria of many mesophyll cells in chlorotic areas of diseased leaves were hypertrophied to the point that they were as large as the nucleus (Fig. 8). Mitochondria of healthy mesophyll cells usually were smaller than chloroplasts (e.g., see Fig. 6), which were smaller than nuclei. The hypertrophied mitochondria contained a network of channels (Fig. 9). These channels appeared to be extensions of the indentations noted in Figure 7 and were continuous with the cytoplasm. Extensive vesiculation occurred in the mitochondria but mainly in the perimitochondrial spaces around the indented regions (Fig. 9). As with the vesicles in the periplastidial spaces in the chloroplasts, the vesicles in the perimitochondrial spaces were bounded by a single membrane (Fig. 9). Profiles of the endoplasmic reticulum (ER) were consistently associated with the altered mitochondria (Fig. 10). At advanced states of disorganization, vesicles were commonly observed in the cytoplasm around the altered mitochondria.

Alterations of nuclei. Compared with chloroplasts and mitochondria, nuclei appeared much less affected. The altered nuclei in affected mesophyll cells were highly irregular in shape (Fig. 8), whereas in healthy cells the nuclei were circular (not shown). Inclusion bodies consisting of electron-dense amorphous material commonly were found in the nuclei of affected cells (see below), but there were no inclusions of viruslike particles.

Inclusion bodies. Five morphologically distinct types of inclusion bodies were observed in affected mesophyll cells: type a, fibrillar inclusions of long strands of electron-dense, amorphous material (Fig. 11); type b, large circular to rectangular electron-dense bodies with an irregular border (Fig. 12); type c, inclusions of irregular masses of amorphous, electron-dense material of various sizes and shapes (Fig. 13); type d, large electron-dense bodies containing electron-transparent cavities filled with electron-dense granules (Fig. 14); and type e, large circular bodies of membranous structures, the periphery of which was surrounded by profiles of ER (Fig. 15). Inclusion bodies of types a–d were found in cytoplasm and nuclei, whereas those of type e were observed only in cytoplasm of affected mesophyll cells. Only type a inclusions were present in cells that showed few scattered vesicles in the chloroplasts. The other types were found only in cells that contained chloroplasts and mitochondria at advanced states of alterations.

Vascular tissues. Many phloem parenchymal and companion cells, and xylem parenchymal cells of the symptomatic leaves of barley, wheat, and oat contained plastids and mitochondria showing various levels of alterations as noted above. The vesiculations in these organelles were similar to those in mesophyll chloroplasts and mitochondria. Affected mitochondria became greatly enlarged and were closely associated with profiles of ER. Similarly, inclusion bodies reminiscent of those in affected mesophyll cells were observed in the vascular tissues.

Root tissues. The alterations of plastids and mitochondria in root tissues of barley plants with flame chlorosis symptoms were essentially similar to those of chloroplasts and mitochondria of affected mesophyll cells in terms of vesiculation and indentations of the organelles by the surrounding cytoplasm (Figs. 16,17). However, unlike diseased leaf tissue, no inclusion bodies of amorphous material were found in affected root cells.

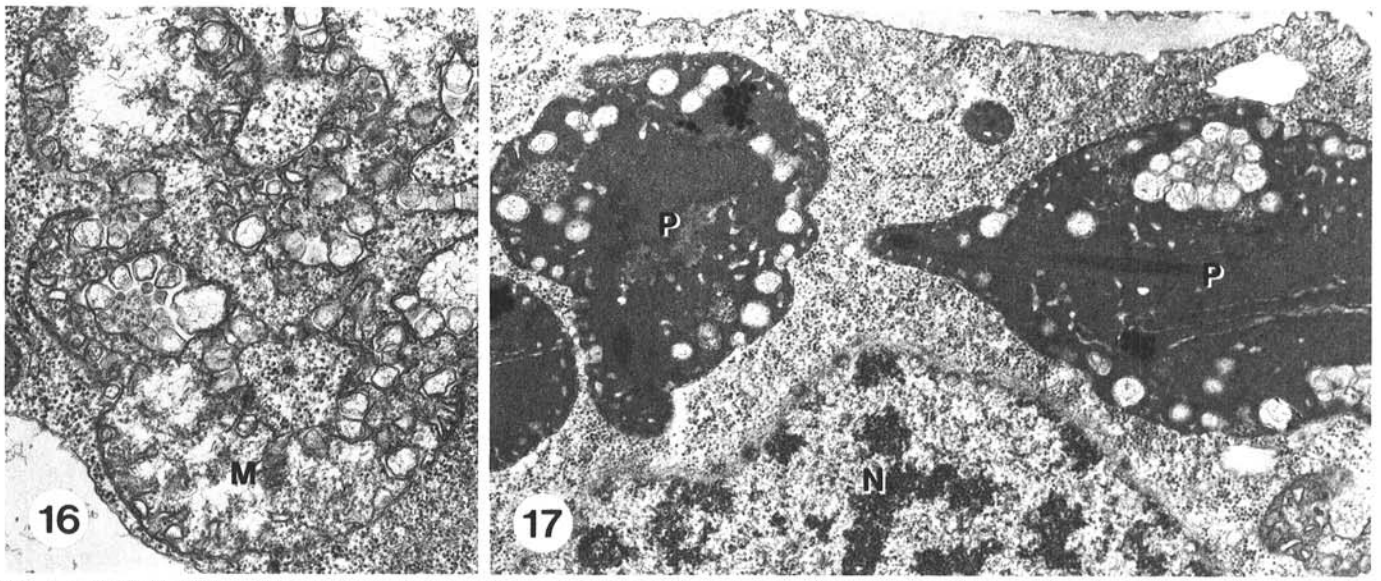
Wilting and plasmolysis experiments. None of the wilting or plasmolysis treatments resulted in the formation of the paracrystalline arrays of particles that would be expected if isometric virus particles were present in infected cells.

DISCUSSION

The most striking cytological changes in cells affected by flame chlorosis were the alterations of chloroplasts and mitochondria. Similar alterations were seen in affected cells of barley, wheat, and oat. Although we did not study the cytological alterations of diseased roots of wheat and oat, similar alterations would likely be observed in the roots of all three hosts.

The diversity of the types of inclusion bodies induced by flame chlorosis in the cytoplasm and nuclei of many of the affected cells is probably not specific to flame chlorosis, because similar inclusions have been observed in other viral infections. The fibrillarlike inclusions are similar to the "star-shaped" inclusion bodies described in Chinese cabbage leaves infected by turnip yellow mosaic virus (5). The type c inclusions are similar to the clumps of densely staining material or "dense granules" reported in many virus infections by members of the bromo-, carmo-, como-, cucumo-, diantho-, luteo-, necro-, tombus- and tospovirus groups (2,3,9,10,13,27,28,30,32,34,36). The type d inclusions in flame chlorosis-affected cells resemble the "satellite bodies" associated with beet mosaic virus infections (29).

The vesiculation induced by flame chlorosis in the altered



Figs. 16 and 17. Ultrathin sections of root parenchymal cells of symptomatic barley. **16,** Hypertrophied mitochondrion (M) with extensive vesiculation. ×22,200. **17,** Two vesiculate plastids (P). N, nucleus. ×18,000.

chloroplasts and mitochondria is extensive. At advanced states of alterations, vesicles also accumulate in the cytoplasm. Similar vesicles, bounded by either single or double membranes, have been reported in cells infected by viruses of the carmo-, clostero-, como-, cucumo-, hordei-, luteo-, poty-, tobamo-, tobra-, tombus-, and tymovirus groups (1,4,8–10,12,13,22,24,26,28,32,34,35) and also in a viruslike disease of the wild grass, *Panicum sabulorum* (14). Of these infections, only Galinsoga mosaic virus (GaMV) (22), clitoria yellow vein virus (CYVV) (26), grapevine Algerian latent virus (GALV), and Neckar river virus (NRV) (34) induce vesiculation in chloroplasts and mitochondria similar to that induced by flame chlorosis. However, other aspects of cytopathology point to important differences between flame chlorosis and these virus infections.

Virus particles are not seen in flame chlorosis-affected cells, even under artificial conditions of wilting or plasmolysis known to aggregate small isometric particles into regular arrays (23,31,37); by contrast, virus particles are readily identified in cells infected by CYVV, GaMV, GALV, and NRV (22,26,34). The absence of viruslike particles in ultrathin sections is consistent with the failure of our repeated attempts to isolate virus particles from tissues with flame chlorosis. In addition, flame chlorosis does not alter the appearance of peroxisomes, whereas both GALV and NRV induce the formation of "multivesicular bodies" from these organelles, a feature typical of many tombusvirus infections (28,34). A further unique feature of flame chlorosis is the extensive ramification of channels of cytoplasm through the vesiculated mitochondria combined with close association of ER with these altered organelles.

Vesiculation is a common cytopathic effect associated with the diverse virus infections noted above. Although the origins of the vesicles seen in these infections differ with respect to the "mother" organelle from which the membranes of the vesicles are derived, all such vesicles contain fibrils reminiscent of nucleic acid material. In several of these infections, the fibrils consist of dsRNA (6,20,21,33), and these vesicles likely have a role in viral RNA replication. With flame chlorosis, sets of seven to eleven disease-specific dsRNAs (approx. 350–3,500 bp) are consistently isolated from diseased leaves of barley, wheat, and oat (15,17). The chlorotic areas of these leaves contain as much as 1% (of dry mass) of these dsRNAs (18), and cells from these areas are extensively vesiculated; the adjacent green areas have undetectable or very low levels of dsRNA (18), and the proportion of cells affected and the extent of vesiculation in these cells are much smaller. This coincidence of macroscopic symptoms, disease-specific dsRNAs, and proliferation of vesicles containing fibrils suggests that the dsRNA is present in the vesicles and is the disease agent or its replicative intermediate.

Our earlier work (17) and this investigation have not found any association of flame chlorosis with fungi, bacteria, or fastidious prokaryotic plant pathogens. A viruslike etiology with absence of viruslike particles might point to a viroid, but the pattern of flame chlorosis dsRNA fragments (15), as well as partial nucleotide sequence information (S. Haber, *unpublished*), indicates no similarity with known viroids. Although the cytopathic effects associated with flame chlorosis are similar in certain aspects to those induced by several viruses given above, they are unlike those described for the known viroids (7) and soilborne viruses that infect small grain cereals (25). This finding, as well as the evidence from symptoms (16) and disease-specific dsRNAs (15,18), is consistent with our conclusion that flame chlorosis is a novel, viruslike disease of barley, wheat, and oat, despite the absence of virus particles.

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