Seasonal Ultrastructural Changes in Xylem Parenchyma Cells of Apple Twigs in Relation to Cold Hardiness

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Summary

Ultrastructural changes in xylem parenchyma cells of apple twigs were studied in relation to seasonal fluctuation in cold hardness. During active growth from mid-June to mid-July, the most striking changes were cell wall thickening and the formation of pits. In the cytoplasm, the appearance of rough endoplasmic reticulum (ER), polysomes, dictyosomes, and vesicles indicate that these organelles participate in cell wall thickening and the formation of pits. In early August immediately after cessation of shoot elongation, starch granules in the plastids began to increase in size and number; protein-lipid bodies appeared. During cold acclimation from late September to mid-October, the vacuoles shrank in size, and the cell became full of plastids containing large starch granules. In early November, unidentified organelles that were similar in size to plastoglobuli appeared near the thylakoid membranes in plastids. In mid-January when cold hardness reached a maximal level, small vacuoles were dispersed throughout the cytoplasm. In late February, the formation of plastid initials occurred, while plasmodesmata formed in the pits. During deacclimation from late March to mid-April, the vacuoles enlarged, starch granules in the plastids decreased in size, and unidentified organelles reappeared in the plastids. At the onset of flowering in mid-May, starch granules in the plastids increased in size again. Moreover, during a one-year cycle, the rough ER was prominent along the cell wall, while mitochondria appeared to be adjacent or attached to the surface of the plastids. Based on these results, the relationship between ultrastructures in xylem parenchyma cells of the apple twigs and cold hardness, especially the deep supercooling characteristic is discussed.

Key Words: apple twig, cold hardness, deep supercooling, ultrastructural change, xylem parenchyma cell.

Introduction

Fruit trees in the temperate zone are exposed to low temperature stresses. Trees that survive subfreezing temperatures do so by several means. Two of the most important ones are: 1) tolerating cell dehydration caused by extracellular freezing and 2) avoiding freezing by deep supercooling. The defense strategy to such freezing not only differs among the species, but also between the organization of tissue within the individual. For example, the cortical tissues in twigs of the apple, pear, and peach possess the ability to tolerate the presence of extracellular ice, whereas living xylem tissues avoid low-temperature stress by deep supercooling (Quamme et al., 1972, 1973; Rajashekar and Burke, 1978). How xylem tissues overwinter by deep supercooling is not clear, but the establishment and cultivation of fruit species in northern regions are limited chiefly by this adaptive characteristics (George et al., 1974; Quamme, 1976).

During a deep supercooling event, water is retained within living xylem tissue despite the presence of extracellular ice. Additionally, the cellular water remains in a liquid phase at very low temperatures by remaining isolated from the nucleating effect of extracellular ice. For deep supercooling to occur, a barrier must exist that allows the cell to remain isolated from the effects of the presence of extracellular ice. This barrier is attributed to the cell wall (George and Burke, 1977). Furthermore, Wisniewski et al. (1991) showed that this phenomenon is dependent on the porosity or permeability of the pit membrane and the amorphous layer on the membrane of the parenchyma cells. However, little is known about the seasonal ultrastructural changes of parenchyma cells in xylem tissue that allow deep supercooling to occur.

The freezing event, referred to as the low temperature exotherm (LTE), corresponds to the freezing of deep, supercooled water; this LTE is correlated with injury to living xylem tissues (George and Burke, 1977; Quamme et al., 1972, 1973). Moreover, the initiation temperature
of LTE in hardy species occurs at lower temperatures than the less hardy ones (Kaku and Iwaya, 1978); it rises as cold hardness decreases (George and Burke, 1977; Kaku and Iwaya, 1978; Quanme et al., 1972). Thus, deep supercooling in living xylem tissues is closely related to their cold hardness.

In this study, ultrastructural changes in xylem parenchyma cells of apple twigs were studied in relation to seasonal fluctuation in cold hardness. Special attention was focused on the relationship between ultrastructures and the deep supercooling mechanism in xylem parenchyma cells.

Materials and Methods

Plant materials

One-year-old twigs from mature apple trees, Malus domestica Borkh. ‘McIntosh’, grown at the National Agricultural Research Center for Hokkaido Region (Sapporo), situated at 43° 3’ N and 141° 4’ E, were used.

Electron microscopy

The twigs were collected from mid-June, 1987 to mid-May, 1988, and those portions located from the middle to two-thirds the distance towards the apex end were fixed for SEM on the same day. Xylem tissues of the twig were cut into small pieces of about 1.0 mm³. The samples were initially fixed in 25% glutaraldehyde in 0.05 M potassium phosphate buffer, pH 7.3, and then were postfixed in 1% osmium tetroxide (pH 7.3). Dehydration was effected in a graded ethanol series and completed by immersion in propylene oxide. The blocks were embedded in Epon 812 and sectioned on a Reichart ultramicrotome. The sections on the grids were stained sequentially with uranyl acetate and lead citrate, and then they were examined under a JEM-1200EX electron microscope.

Evaluation of cold hardness

The twigs were collected periodically from late September, 1987 to mid-May, 1988. Four twigs, 15 cm long, were enclosed in polyethylene bags and frozen at -5°C. After 2 h, they were cooled in steps of 5°C at hourly intervals to successively lower temperatures. After standing at selected test temperatures for 18 h, the frozen twigs were thawed in the air at 5°C. After thawing, the lower parts of the twigs were kept in water in a green house for two weeks. Injury due to freezing was evaluated visually from browning of the xylem tissues. Cold hardness was expressed as the minimum temperature at which the xylem tissues survived freezing without injury.

Results

Seasonal cycle in cold hardness of xylem tissues of apple twigs

In 1987, the bud-breaking of the apple started on April 26. The shoots elongated at a high rate from June to July, but their growth had virtually halted by early August. In late September, the xylem tissues of twigs survived freezing -5°C. Subsequently, cold hardness of the xylem tissues increased steadily as air temperature decreased, reaching -10°C in mid-October immediately after fruit harvest, and -20°C in mid-November when the leaves were shed. In early January, 1988, cold hardness reached a maximal level at -30°C. The maximal hardness remained unchanged until late February. Thereafter, cold hardness of the xylem tissues decreased gradually as the air temperature increased, reaching -10°C in late April at the onset of bud-break, and -5°C in mid-May at the time when flower buds started to show color (Fig. 1).

Ultrastructural changes in xylem parenchyma cells of apple twigs

The seasonal ultrastructural changes in xylem parenchyma cells of apple twigs (Fig. 2) show that in mid-June (Fig. 2-1) when the shoots were elongating at a high rate, the xylem parenchyma cell has large vacuoles. Within the thin layer of cytoplasm along the cell wall within which the endoplasmic reticulum studded with ribosomes (rough ER), dictyosomes, and plastids, containing starch granules existed. Mitochondria were attached to the surface of the plastid.

In mid-July (Fig. 2-2) when shoot elongation ceased, cold hardness of the xylem tissues increased (with decreasing air temperature). In late October, the cold hardness reached to a maximal level (Fig. 1). Cold hardness started to decrease from mid-November, reaching -5°C at the end of December. In April, cold hardness started to increase as air temperature increased (Fig. 1). The seasonal cycle of cold hardness (○) of the xylem tissues of apple twigs is shown in Fig. 1.

Fig. 1. Seasonal cycle in cold hardness (○) of the xylem tissues of apple twigs. F.H, Fruit harvest; D, Defoliation; B.B, Bud breaking; F.S, Flowering start; T, Air temperature.
Fig. 2. Electron micrograph of xylem parenchyma cells in apple twigs. 1: A portion of a xylem parenchyma cell in mid-June, 2: In mid-July, 3: In early August, 4: In late September, 5a and 5b: In mid-October, 6: In early November, 7: In mid-December, 8: In mid-January, 9a and 9b: In late February, 10: In late March, 11: In mid-April, 12: In mid-May. Asterisks (*) in 5a, 9b and 10 show the pl. Arrows in 5b show the intercellular spaces. Arrows in 6 and 10 show the unidentified organelle. Pentagram (☆) and arrow in 9a show the plastid initial and constriction, respectively. CW, cell wall; D, dictyosome; ER, endoplasmic reticulum; M, mitochondria; N, nucleus; Nu, nucleolus; P, plastid; Pp, plastoglobuli; PLB, protein-lipid body; Ps, polysome; S, starch granule; V, vacuole; Ve, vesicle. Scale markers indicate 1 μm.
the cell walls thickened as a result of the formation of the secondary wall; simultaneously, wall structures known as pits (data not shown). Starch granules in the plastids, which had appeared in mid-June, were no longer visible. Dictyosomes were found and numerous vesicles were present in the cytoplasm. The interior of these vesicles contained an electron dense material, and therefore, they appeared to be coated (Pearse, 1980; Ryser, 1979; Van Der Valk and Fowke, 1981). Polysomes were abundant and the rough ER was seen near the cell wall. Mitochondria were attached to the surface of the plastids.
In early August (Fig. 2.-3) when vegetative growth visually ceased, the cell walls underwent marked thickening, and the vacuoles began to decrease in size. The plastids accumulated large starch granules and the rough ER became prominent along the cell wall. PLBs appeared in the cytoplasm, but dictyosomes, polyosomes, and mitochondria were sparse.

In late September (Fig. 2.4) when the xylem became hardy to $-5^\circ C$, vacuoles shrank in size, such that most of the cytoplasm was now occupied by plastids that contained large starch granules and a large nucleus. PLBs and mitochondria were frequently seen.

In mid-October during the harvest period when the xylem became hardy to $-10^\circ C$, the cells were filled with plastids that contained large starch granules. Pits were seen in the thickened cell wall (Fig. 2.5a), and in a transverse section (Fig. 2.5b), intercellular spaces were observed at the junctions of three cells.

In early November (Fig. 2.6) immediately before defoliation occurred, the xylem cells survived freezing at $-15^\circ C$, and the vacuoles became even smaller. In the plastids that contained large starch granules, unidentified organelles and plastoglobuli appeared near the thylakoid membrane. The unidentified organelles were similar in size to plastoglobuli but differed morphologically in that their ultrastructural profiles were darkly stained. Mitochondria had attached to the surface of the plastids, whereas PLBs and small vacuoles were apparent in the cytoplasm.

In mid-December (Fig. 2.7) when the xylem cells withstood $-25^\circ C$, plastids that contained large starch granules and a few plastoglobuli had undergone sharp amoeba-like changes. Mitochondria were seen near the periphery of the plastid, and rough ERs were found along the cell wall.

In mid-January (Fig. 2.8) when cold hardiness became the maximum, small vacuoles contained a small amount of stained amorphous material were dispersed throughout the cytoplasm. The same profiles of vacuoles were seen until mid-April. Large starch granules in the plastids tended to decrease in size, while a few plastoglobuli appeared in the stroma. Rough ER and mitochondria were present, but PLBs were sparse.

In late February (Fig. 2.9a) when the maximal cold hardiness remained unchanged, plastids were located near the nucleus, and the formation of plastid initials from mature plastids as a result of constriction of mature plastids and their subsequent pinching off of new initials (Kuroda and Sagisaka, 1993, 2001) occurred. Mitochondria were located near the surface of the plastids, and the rough ER and PLBs were still present in the cytoplasm. The cavity of pits became deep from the secondary wall thickening; in the pit, plasmodesmata could be clearly seen (Fig. 2.9b).

In late March (Fig. 2.10) when cold hardiness began to decrease, unidentified organelles that had been observed in early November were seen again in the plastids. They seemed to be discharged from the point part of the thylakoid membrane and inflated into membrane sacs. Also, plastoglobuli were visible near the thylakoid membrane. Mitochondria were located near the surface of plastids and the nucleus, the rough ER was found along the cell wall, and plasmodesmata were present in the pits.

In mid-April (Fig. 2.11) immediately before bud sprouting when cold hardiness decreased to $-15^\circ C$, starch granules in the plastids decreased in size and the plastids were no longer engorged with starch granules as they had been in mid-October. Dictyosomes reappeared and mitochondria were located near the surface of the plastids. Vacuoles increased in size and number.

In mid-May (Fig. 2.12) at the onset of flowering, cold hardiness decreased to $-5^\circ C$, and the vacuoles became enlarged. In the cytoplasm, the rough ER was present and starch granules in the plastids were increasing in size. Mitochondria that were attached to the surface of plastid were present, and PLBs were no longer electron-dense.

**Discussion**

During the active growth from mid-June to mid-July, the most striking changes were the cell wall thickening and the formation of pits by secondary wall synthesis. At this stage, organelles involved in protein synthesis, such as the rough ER, polyosomes, and dictyosomes (Warren, 1985) were abundant. Vesicles that resemble dictyosome-associated coated vesicles (Pearse, 1980; Ryser, 1979; Van Der Valk and Fowke, 1981) could be seen, indicating that these organelles were contributing to secondary wall synthesis through protein sorting.

The pit is formed in the pit field in the primary wall, through which plasmodesmata connects adjacent cells and serves as important channels of communication. Plasmodesmata are blocked by a desmotubule, and the desmotubule is connected on both sides to the rough ER (Burgess, 1985). At the start of secondary wall deposition, thickening of the wall is restricted to the region of pit field. Vesicles, resembling dictyosome-associated coated vesicles, contribute wall components, whereas rough ER inhibits this process (Burgess, 1985). Therefore, it is possible that the ER takes part in pit formation through the obstruction of secondary wall thickening. In this study, the rough ER was seen in the region along the cell wall during a one-year cycle. This may be related to the above-mentioned assumption.

As described in the introduction, cold hardiness in xylem tissues is closely related to their deep supercooling ability (George and Burke, 1977; Kaku and Iwaya, 1978; Quamme et al., 1972). Therefore, it can be considered that the seasonal changes in cold hardiness of the xylem tissues of apple twigs observed in this study is a reflection of their deep supercooling characteristic.

For deep supercooling to occur, a barrier must exist...
that allows the cell to remain isolated from the effects of the presence of extracellular ice. This barrier is attributed to the cell wall (George and Burke, 1977). Current studies suggest that a restricted pore size (< 60 to 100 nm) in the cell wall plays a major role in forming barriers to water movement and ice crystal penetration that allow deep supercooling to occur (Ashworth and Abeles, 1984; George, 1983; George and Burke, 1977). Namely, water within small diameter pores will freeze at lower temperatures than bulk water, and it has been suggested that small diameter pores within the cell wall could inhibit the loss of cellular water and the propagation of ice. Wisniewski and Ashworth (1985) suggested that pits correspond to cell-wall microcapillaries. Indeed, the pit is porous and filled with plasmodesmata; the diameter of the pores is between 30 to 60 nm (Burgess, 1985). The deep supercooling phenomenon in the xylem tissues may be dependent on the porosity or permeability of the pit membrane.

As noted above, plasmodesmata are blocked by a desmotubule that is connected to the rough ER of adjacent cells (Burgess, 1985). Therefore, cell-wall microcapillaries that are related to deep supercooling are not plasmodesmata, but are desmotubules. The ER net of pits may possibly play the role of the barrier that allows the cell to remain isolated from the effects of extracellular ice. However, it is noteworthy that the pits are present in xylem parenchyma cells of non-deep supercooling poplar (Asada et al., 1988).

From early August when growth virtually halted, starch granules in the plastids increased in size and number, and by mid-October the interior of the wintering cells was occupied by plastids that contained large starch granules, indicating that one function of the xylem parenchyma cells of the perennials is to store starch.

In general, starch granules in the plastids disappear during cold acclimation (Kuroda and Sagisaka, 1993, 2001; Pomeroy and Siminovitch, 1971; Sagisaka et al., 1990). The disappearance of starch granules from the plastids indicates that starch is hydrolyzed to soluble sugars, which can osmotically protect cells against freezing injury (Levitt, 1972). In xylem parenchyma cells of the apple, however, the accumulated starch within plastids persisted during the winter. The presence of plastids that contain large starch granules decreases the water content in the cells. This leads to a concentration increase of the intracellular solution and a depression of the homogeneous ice nucleation temperature. Therefore, accumulated starch in xylem parenchyma cells of the apple twigs may serve to promote deep supercooling.

In some tissues that undergo deep supercooling, intercellular spaces are absent (Ashworth and Abeles, 1984). However, in xylem parenchyma tissues of apple twigs in mid-October, intercellular spaces were observed between adjacent cells. Wintering apple twigs contain large amounts of sorbitol, a sugar alcohol that is an effective cryoprotectant (Sakai and Yoshida, 1968); the sorbitol is stored within the intercellular spaces (Williams and Rease, 1974). Thus, it is conceivable that the intercellular spaces play an important role as a mechanism that maintains deep supercooling of xylem parenchyma cells of the apple twigs.

Asada et al. (1988) reported that from October to November the main component of the xylem parenchyma cells of non-supercooling poplar were PLBs that exhibit peroxidative activities (Sagisaka and Asada, 1986) and contain lipids and unsaturated fatty acids (Sagisaka et al., 1990). In xylem parenchyma cells of the apple twigs, however, PLBs were scarce at the same stage. This difference might be structural one between species that survive the winter by deep supercooling and those that do not.

In cortical and flower bud cells of apple trees (Kuroda and Sagisaka, 1993, 2001), the ultimate form of ER in winter is vesicular, but the ER form in the xylem parenchyma cells of the apple twigs was rough ER, studded with ribosomes throughout the year. Since the response of the ER may be directly related to the selective synthesis of proteins required for adjustment to a cold environment (Tomashow, 1990), differences in the ER form may be important in determining a tissue's adaptability to freezing.

In this study, the formation of plastid initials from mature plastids was observed in late February when the maximal hardness remained unchanged. At this stage, plastids aggregated around the nucleus and mitochondria are attached to the surface of the plastids. A similar situation was seen in the cortical cells of poplar and apple twigs (Kuroda and Sagisaka, 1993; Sagisaka, 1991; Sagisaka and Kuroda, 1991) and apical cells of apple flower buds (Kuroda and Sagisaka, 2001). The biogenesis of plastid initials is probably controlled by nuclear genes and ergastic compounds in mitochondria. The formation of plastid initials in mid-winter is a general feature of woody plants and may reflect the early stage of the onset of regrowth after wintering.

In early November of cold acclimation process and late March of deacclimation process, the plastids contained unidentified organelles in the stroma near the thylakoid membrane. These unidentified organelles were similar in size to plastoglobuli, but they contained no osmiophilic material. They also seemed to be discharged from the edges of the thylakoid membrane and inflated into membrane sacs. A more detailed examination is necessary although these unidentified organelles may possibly be the precursors of plastoglobuli. The physiological significance of the unidentified organelles in cold adaptation is unclear.

During deacclimation from late March to mid-April, starch granules in the plastids decreased in size. However, this decrease does not seem to be a general phenomenon since cortical cells show a reverse behavior.
(Kuroda and Sagisaka, 1993, 2001; Pomeroy and Siminovitch, 1971; Sagisaka et al., 1990). The xylem forms a long-distance transport system for water in plants, and the water columns freeze in the winter (Lybeck, 1959). In spring, the dissolved air forms bubbles when the ice melts, but the air in the ice remains dissolved. This bubble formation disrupts the continuity of the water columns in the spring. In diffuse-porous trees such as the apple, metabolic forces produce positive pressures in early spring, which serve to refill some of the conducting tissue (Zimmermann, 1964). The ability to repair the water-conducting system is undoubtedly one of the key factors responsible for the geographical distribution of tall species of trees in the northern latitudes where winters are severe. It is possible that the decrease in numbers of starch granules in the plastids in spring may participate in the generation of such metabolic forces as an energy source.

A supply of ATP appears to be a prerequisite for the generation of metabolic forces. Mitochondria appeared to be attached to the surface of plastids or to be very close to them during the winter, indicating that high-energy compounds formed as a result of heterotrophic metabolism might be provided by the mitochondria. However, the mitochondrial electron transport system serves as a univalent pathway for the reduction of oxygen and production of active oxygen (Azzi et al., 1975; Cadenas et al., 1977). Since active oxygen harm cells, the removal of active oxygen is necessary if plants are to maintain their metabolic integrity (Elstner, 1982).

Indeed, appreciable levels of metabolic activities of peroxide scavenging systems that require glutathione and ascorbate have been found in xylem tissues of wintering apple twigs (Kuroda et al., 1990). In this way, xylem parenchyma cells of apple twigs are able to continue the overwintering stage of their metabolism.

Literature Cited


リンゴ枝条部柔細胞における耐凍性変動と細胞内微細構造変化

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摘 要

リンゴ枝条部柔細胞における耐凍性変動と細胞内微細構造変化の関係を調査した。新梢成長がおおむね6月中旬から7月中旬にかけて、最も顕著な変化は細胞壁の肥大と壁孔の形成であった。細胞質内には粗面小胞体、レチオーム、カルテオームおよび被覆小胞体がある。これらは細胞を内小器官が細胞壁肥大と壁孔形成に関与していることが示唆された。新梢成長停止直前の8月下旬には、プラスタチド内のデンプン粒が数と大きさを増し、プロテイン・リビドボディーがみられた。9月下旬から10月中旬の耐凍性増大期には、液胞の小液化を進み、細胞は大きくデンプン粒を含んだプラスタチドで充満するようになった。11月上旬には、プラスタチドのチラコイド膜近傍において、プラストグロブリと大ささ

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