Ultrastructural Changes in Apical Meristem Cells of Apple Flower Buds Associated with Dormancy and Cold Tolerance

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Summary
Ultrastructural changes in apical meristem cells of flower buds of apple (Malus domestica Borkh.) were studied in relation to variations in dormancy and cold tolerance. During rest from late October to early December, the cold tolerance of the flower buds increased as the air temperature decreased. At this stage, microvacuolation and an increase in the volume of the cytoplasmic organelles involved in protein synthesis, such as cisterna-like endoplasmic reticulum (ER), polyosomes, dictyosomes, and vesicles. Protein-lipid bodies were also abundant, and organelles resembling ribosomes were attached to their surface. The plastids were located near the nucleus. Simultaneously, in the plastids, a prolamellar body, characteristic of etioplasts developed, and large starch granules disappeared. In mid-December when rest was broken, cisterna-like ER was replaced by vesicular ER. In mid-January, when maximum cold-tolerance was achieved, the microvacuolation was completed. In early February, 7-8 weeks after the breaking of rest, plastid initials formed from the mature plastids (etioplasts) by budding and subsequent division by constriction. During deacclimation from late March to early May, an increase in vacuole volume and reduction in cytoplasmic volume occurred in the cells, while organelles involved in protein synthesis became apparent. Also, the spatial relationship between the nucleus and etioplasts disappeared as the latter were replaced by plastids. Mitochondria were always present in the winter.

Key Words: breaking of rest, cold acclimation, Malus domestica Borkh., microvacuolation, plastid proliferation.

Introduction
In cold regions, deciduous fruit trees, including apple, are subject to extremely low temperatures that markedly affect tree survival and limit productivity. To cope with low temperatures, deciduous fruit trees enter a state of rest or endodormancy and adjust both metabolically and structurally, with a resultant increase in cold tolerance. Ultrastructural changes in the cortical cells of woody species during adaptation to cold conditions occur in cambial cells of Pinus strobus L. (Strivastava and O’Brien, 1966), phloem parenchymal cells of Robinia pseudoacacia L. (Pomeroy and Siminovitch, 1971), cortical cells of Morus bombycis Koidz. (Niki and Sakai, 1981) and those of Populus euramericana (Sagisaka et al., 1990). Prominent ultrastructural changes in cortical cells of apple twigs during cold acclimation, such as microvacuolation, appearance of organelles involved in protein synthesis of endoplasmic reticulum (ER), dictyosomes and vesicles, starch hydrolysis in the plastids, and the enrichment of protein-lipid bodies (PLBs), whereas during deacclimation, significant reversal in ultrastructure, such as fusion of vacuoles, have been noted (Kuroda and Sagisaka, 1993).

Ultrastructural changes in the cortical cells of woody species occur following the breaking of resting stage of dormancy. For example, presumed precursors of plastids, the plastid initials, forms in the cortical cells of poplar and apple twigs after breaking of rest (Sagisaka and Kuroda, 1991; Kuroda and Sagisaka, 1993), while mitochondria proliferate (Sagisaka et al., 1989). To date, however, there have been no reports on ultrastructural changes in the flower bud cells of deciduous fruit trees during adaptation to cold conditions. This study was undertaken to relate ultrastructural changes in apical meristem cells of apple flower buds to seasonal cycles of dormancy and cold tolerance.

Materials and Methods

Plant materials

Flower buds on two-year-old twigs from mature ‘McIntosh’ apple trees, Malus domestica Borkh., grown at the Hokkaido National Agricultural Experiment Sta-
tion (Sapporo), situated at 43°3' N and 141°4' E, were used.

**Evaluation of dormancy status**

The twigs with flower buds were collected from late September to early March at approximately 2 weeks intervals. Five twigs, 20 cm long, were excised and the cut end immersed in water in a greenhouse kept at 25/20 ± 3°C (day/night). After 2 weeks, bud break (green tip stage) was recorded and the degree of rest was expressed as the percentage of bud break. Rest was considered terminated when 50% of the buds reached green tip stage. Approximately 20 flower buds were assessed on each sampling date.

**Evaluation of cold tolerance**

The twigs with flower buds were collected from late September to mid-May at approximately 4-week intervals. Five twigs, 15 cm long, were enclosed in polyethylene bags and frozen at -5°C. After 2 hr, the frozen twigs were cooled in steps of 5°C at hourly intervals. After standing at selected temperatures for 18 hr, the frozen samples were thawed in the air at 5°C and returned to the above greenhouse for 2 weeks. Injury due to freezing was evaluated visually by examining the extent of browning of the inner parts of the flower buds. Cold tolerance was expressed as the minimum temperature at which the flower buds survived freezing without injury. Approximately 15 flower buds were examined on each sampling date.

**Electron microscopy**

Flower buds were collected from late October to early May at approximately 2–3 weeks intervals. The apical meristem (approximately 1.0 mm³) of the flower buds was immediately fixed in 2.5% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.3), then postfixed in 1% osmium tetroxide. Dehydration was achieved by passage through a graded ethanol series and completed by immersion in propylene oxide. Tissue blocks were embedded in Epon 812 and sectioned on a Reichart ultramicrotome. The sections on grids were stained sequentially with uranyl acetate and lead citrate, and examined with a JEM-1200EX electron microscope.

**Results**

**Dormancy status and cold tolerance in apple flower buds**

The changes in percentage bud breaking and cold tolerance of flower buds from apple trees (Fig. 1) reveal that in October, bud break decreased from 68% to 0% within 3 weeks, indicating the apple flower buds go into the resting stage rapidly. The maximum rest was attained in late October immediately after fruit harvest and continued until the beginning of December. Subsequently, buds are released from rest rapidly; i.e. bud break increased from 0% to >56% in 3 weeks, and completely broken by mid-December.

The flower buds survived freezing at -5°C in late September and their cold tolerance increased steadily as the air temperature decreased, reaching -15°C in late October and -25°C in early December. In early January, 3 weeks after the breaking of rest, cold tolerance reached a maximal level of -30°C. Cold tolerance remained unchanged until late February when it started to decrease in March. It then decreased gradually as the ambient air temperature increased, reaching -5°C in early May immediately after bud break and -3°C on 15 May when flower buds started to show color.

**Ultrastructural changes in apical meristem cells of apple flower buds**

The apical meristem cells of apple flower buds in late October (Fig. 2A) reveal that the cell was occupied by relatively large vacuoles. In the cytoplasm, cisterna-like endoplasmic reticulum (ER), mitochondria, protein-lipid bodies (PLBs), and plastids containing large starch granules and plastoglobuli were present.

In mid-November (Fig. 2B), when the flower buds were tolerant to -20°C, cisterna-like ER, dictyosomes and numerous polysomes were visible in the cytoplasm. The cisterna-like ER was studded with ribosomes and the dictyosomes were active in the production of vesicles. Mitochondria and PLBs were also seen. Furthermore, at this stage (Fig. 2C), organelles resembling ribosomes (polysomes) were attached to the surface of the PLBs or were in close proximity. Plastids no longer contained as many large starch granules and their thylakoid membrane systems became sparse.

In late November (Fig. 2D), the plastids were located.
Fig. 2. Photomicrographs derived from electron microscopy of apical meristem cells of apple flower buds. Key to the labeling for the figures. CW, cell wall; D, dictyosome; ER, endoplasmic reticulum; La, prolamellar body; M, mitochondrion; N, nucleus; Nu, nucleolus; P, plastid; Pg, plastoglobuli; PLB, protein-lipid body; Ps, polysome; S, starch granule; V, vacuole. Scale bars represent 1 μm in all figures. A: A late October sample. The cell contains some large vacuoles (V), plastids (P) with large starch granules (S) and plastoglobuli (Pg), protein-lipid bodies (PLB), cisterna-like endoplasmic reticulum (ER), and mitochondria (M). B: A mid-November sample. Polysomes (Ps), cisterna-like endoplasmic reticulum (ER), and dictyosomes (D) producing vesicles are visible. C: Also a mid-November sample. Organelles resembling ribosomes are seen attached to the surface of protein-lipid bodies (PLB) and no large starch granules (S) are present in the plastids (P). D: A late November sample. Plastids (P) are located near the nucleus (N) and possess a few starch granules and a prolamellar body (La). E: A mid-December sample. Vesicular endoplasmic reticulum (ER), polysomes (Ps) and dictyosomes (D) producing vesicles are visible. Plastid (P) contains a prolamellar body (La) with a few thylakoid membranes. F: A mid-January sample. The cell is highly vacuolated. The general appearance is the same as that of the cells sampled in mid-December.
Fig. 2. Photomicrographs derived from electron microscopy of apical meristem cells of apple flower buds. Key to the labeling for the figures. CW, cell wall; D, dictyosome; ER, endoplasmic reticulum; La, prolamellar body; M, mitochondrion; N, nucleus; Nu, nucleolus; P, plastid; Pg, plastoglobuli; PLB, protein–lipid body; Ps, polysome; S, starch granule; V, vacuole. Scale bars represent 1 μm in all figures. G: An early February sample. Dividing plastids (P) (arrows) and organelle (*) that resembles a plastid initial are visible near the nucleus (N). Plastids (P) contain many plastoglobuli (Pg) and a prolamellar body (La). H: A late February sample. A budding plastid (P) with many plastoglobuli (Pg) and a prolamellar body (La), and plastid initials (*) dividing by constriction (arrows) are seen. Vacuoles (V) contain osmiophilic materials. I: A late March sample. Fission of the plastids (P) by constriction (arrow) and plastid initials (*) are seen near the nucleus (N). Vacuoles (V) contain osmiophilic materials. Vesicular endoplasmic reticulum (ER), polysome (Ps) and dictyosomes (D) producing vesicles are present. J: An early April sample. Vacuoles (V) become larger and plastids (P) are dispersed in the cytoplasm. In the plastids (P), prolamellar bodies have disappeared and starch granules (S) have reappeared. K: A mid-April sample. Starch granules (S) in the plastids (P) become larger. In the cytoplasm, vesicular endoplasmic reticulum (ER), mitochondrion (M) and protein–lipid bodies (PLB) are visible. L: An early May sample. The majority of the vacuoles (V) are large, starch granules have disappeared again from the plastids (P), and mitochondria (M) are abundant.
near the nucleus. In the plastids, a prolamellar body, characteristic of etioplasts developed and large starch granules disappeared. Also, the plastids possessed a few thylakoid membranes and plastoglobuli. PLBs and mitochondria were abundant.

In mid-December (Fig. 2E), when rest was released, vacuoles were small and in the cytoplasm, cisterna-like ER became scarce, being replaced by copious vesicular ER. Simultaneously, numerous polysomes became conspicuous in the cytoplasm and the dictyosomes were active again in the production of vesicles. In the plastid (etioplast), a prolamellar body was present, together with a few thylakoid membranes and plastoglobuli; PLBs and mitochondria were present.

In mid-January (Fig. 2F), when cold tolerance was maximal, microvacuolation of the cytoplasm was completed. The plastids (etioplasts) contained a small starch granule and plastoglobuli, with a few thylakoid membranes and a prolamellar body. In the cytoplasm, vesicular ER, dictyosome-generated vesicles, mitochondria, and PLBs were visible as in cells sampled in mid-December.

In early February (Fig. 2G), at continued maximal cold tolerance, ultrastructural features that may be related to the formation of plastid initials (Sagisaka and Kuroda, 1991) became visible. The formation of plastid initials from mature plastids (etioplasts) by budding and subsequent division by constriction (Petitt, 1976) occurred (Fig. 2H). Plastoglobuli were frequently observed in these bodies, but no lamellar structures were detected. At this stage, plastoglobuli in the plastids (etioplasts) tended to increase. The vacuoles again formed in the cytoplasm and contained amorphous stainable materials. Vesicular ER and mitochondria were abundant as were some PLBs.

In late March (Fig. 2I), when cold tolerance decreased to -20 °C, the cell continued to form plastid initials from mature plastids (etioplasts) and vesicles from dictyosomes were produced again as in cells of mid-January. In the cytoplasm, vesicular ER, polysomes, mitochondria, and PLBs were present and the vacuoles contained amorphous stainable materials.

In early April (Fig. 2J), when cold tolerance decreased to -10 °C, the vacuoles enlarged and the plastids that had been concentrated around the nucleus became randomly dispersed in the cytoplasm. In the plastids, the prolamellar body disappeared and small numbers of starch granules reappeared; PLBs and mitochondria were abundant.

In mid-April (Fig. 2K), starch granules in the plastids grew large, the vesicular ER, mitochondria, and PLBs in the cytoplasm were present.

In early May (Fig. 2L), when cold tolerance decreased to -5 °C, the cell contained large vacuoles, while plastids differentiated thylakoid membranes devoid of starch granules. In the cytoplasm, considerable numbers of mitochondria, vesicular ER, and PLBs were present.

**Discussion**

*Relationship between dormancy status and cold tolerance in apple flower buds*

The relationship between dormancy and cold tolerance was proposed as the degree growth stage (°GS) model in temperate woody perennials by Fuchigami et al. (1981). According to this model, cold tolerance increases rapidly after the onset of rest. Maximum cold tolerance remains during deepening of the rest stage; deacclimation occurs between maximum rest and the end of rest. However, in our study, the cold tolerance increased gradually with decreasing temperature after the onset of rest. Also, the maximum cold tolerance was achieved within 3 weeks after the breaking of rest and remained so during the coldest period; deacclimation started in March. The difference between the °GS model and our results may be attributed to the differences in plant species and climatic conditions.

*Ultrastructural changes in apical meristem cells of apple flower buds during dormancy and cold tolerance development*

During deep rest from late October to early December, cold tolerance of the flower buds increased gradually as the air temperature decreased. At this stage, the most striking changes in the ultrastructure in the apical meristem cells of flower buds were microvacuolation and an increase in the volume of the cytoplasm. Similar ultrastructural changes have been observed in the cortical cells of several species of trees (Srivastava and O'Brien, 1966; Pomeroy and Siminovitch, 1971; Niki and Sakai, 1981; Sagisaka et al., 1990; Kuroda and Sagisaka, 1993). The vacuoles become smaller because vacuolar water is absorbed by hydrophilic compounds in the cytoplasm which makes the vacuolar content more concentrated and bound water more difficult to freeze (Chen and Gusta, 1978). The cellular events associated with microvacuolation may, therefore, be a mechanism of protection against intracellular freezing. In addition, the increase in the volume of the cytoplasm is a mechanism of protection against distortion of the protoplasm caused by freezing-induced dehydration of cells (Levitt, 1972). Thus, it appears that microvacuolation and increases in the volume of the cytoplasm are involved in the mechanism of cold acclimation.

In mid-November, organelles that are involved in protein synthesis such as polysomes, cisterna-like ER, dictyosomes, and vesicles generated from the dictyosomes (Warren, 1985) were abundant and the synthesized proteins were destined to become cytosolic and membranous or secretory components in the cells. Similar observations were found in cortical cells of apple (Kuroda and Sagisaka, 1993) and poplar (Sagisaka et al., 1990). Physiological and molecular studies have shown that the onset of tolerance to cold is dependent on
altered gene expression (Thomashow, 1990), and therefore may depend on the synthesis of new proteins such as bark storage proteins (BSPs) (Coleman et al., 1991), antifreeze proteins (Griffith et al., 1993) and COR (cold-regulated) proteins (Thomashow et al., 1993). On the other hand, Lang and Tao (1991) found that the polypeptide of 61 kD increased in peach floral buds during early endormancy. Therefore, the occurrence of organelles involved in protein synthesis during maximum rest may be related to the synthesis of proteins that induce the development of cold acclimation and/or rest.

By late November, the plastids aggregated around the nucleus, and remained so until late March when the cold tolerance began to decrease. Similar profiles were found in cortical cells of apple (Kuroda and Sagisaka, 1993), poplar (Sagisaka et al., 1990) and black locust (Pomeroy and Siminovitch, 1971). These results indicate that a close spatial relationship between nucleus and plastids is a general feature of wintering cells with high cold tolerance. The disappearance of starch granules in the plastids was found to be associated with the timing of cold acclimation as in cortical cells of several woody species (Pomeroy and Siminovitch, 1971; Niki and Sakai, 1981; Sagisaka et al., 1990; Kuroda and Sagisaka, 1993). These observations suggest that starch hydrolyzes to soluble sugars during the hardening process. The protective effect of soluble sugars against freezing injury has been reviewed by Levitt (1972). At this stage, the plastids contain a prolamellar body, characteristic of the etioplast, and remain unaltered until late March. The prolamellar body represents a very efficient way of creating a large membrane surface area within a small volume (Burgess, 1985). However, the physiological significance of the etioplasts in cold adaptation is unclear.

We previously found that PLBs were abundant in the cortical cells of apple twigs during cold acclimation (Kuroda and Sagisaka, 1993). Hatano (1978) reported that numerous lipid bodies, resembling PLBs were observed along the cell wall of Chlorella, related to the development of frost hardiness. In our study, PLBs were abundant during cold acclimation, indicating that PLBs are closely related to the hardening process. Moreover, organelles resembling ribosomes were seen to be attached to the surface of the PLBs in mid-November at maximum rest (Fig. 2C). Since PLBs are rich in lipids and unsaturated fatty acids (Sagisaka et al., 1990), PLBs may be temporary sites for the synthesis of enzymes involved in lipid synthesis. More direct enzymatic studies are needed to further this point.

In mid-December when rest was broken, cistern-like ER was replaced by vesicular ER. These different forms of the ER represent seasonal modifications; vesicular ER may be the site of protein synthesis that take place under cold conditions. Simultaneously, numerous polysomes, along with dictyosomes and mitochondria, became conspicuous in the cytoplasm. Therefore, the organelles involved in proteins synthesis at this stage may participate in synthesis of proteins related to the quiescent or ecodormancy. The report of Muthalif and Rowland (1994) that the levels of three major polypeptides of 65, 60, and 14 kD increase in blueberry floral buds when the chilling requirement of buds is almost satisfied, supports our contention.

The completion of microvacuolation was found to coincide with the timing of the acquisition of the maximum cold-tolerance. This indicates that complete microvacuolation may be involved in the mechanism for the attainment of the maximum cold tolerance to avoid death by freezing. Moreover, in our study, the cold tolerance reached the maximum level immediately after the breaking of rest, which suggest that the completion of microvacuolation may be regulated by the physiological and biochemical events that occur immediately after the breaking of rest.

In early February, 7–8 weeks after the breaking of rest, the formation of plastid initials from mature plastids (etioplasts) occurred through budding and subsequent division by constriction. Approximately 60 days after the breaking of rest in late March, the cells contained plastid initials. This formation of plastid initials after the breaking of rest occurs annually in the cortical cells of poplar (Sagisaka and Kuroda, 1991) and apple (Kuroda and Sagisaka, 1993). Hence, the satisfaction of the low temperature requirement is a prerequisite for the formation of plastid initials (Sagisaka, 1992), a common and physiologically programmed process in woody plants. With apple the production of normal flowers seems to depend, not only on a satisfactory cool periods, but also on formation of plastid initials.

Evidence that most proteins in plastids are encoded in the nucleus, synthesized on cytoplasmic ribosomes and transported to the plastids, has been reviewed by Mullet (1988). The close spatial relationship between the nucleus and plastids may signify that the biogenesis of plastid initials is controlled by nuclear genes. The sequence of reactions involved the formation of plastid initials and related processes are in the gene(s) that is/are probably unexpressed by low temperatures.

The structural profiles of cells during deacclimation that started in March were the reverse of changes occurring during cold acclimation; for example, increases in the volume of vacuoles, reduction of the volume of the cytoplasm, dispersal of etioplasts from around the nucleus, replacement of etioplasts by plastids, etc. Similar observations were reported in the cortical cells of poplar (Sagisaka et al., 1990) and apple (Kuroda and Sagisaka, 1993). In particular, increases in the volume of vacuoles and reduction of the volume of the cytoplasm are likely to be involved in the mechanism of deacclimation because they are related to susceptibility to the stress-producing effects of the dehydration that occurs during freezing.
Vacuoles are thought to serve as storage and lytic compartments (Matile, 1978). Sagisaka et al. (1990) reported that, in the post-resting stage, the lytic activity of vacuoles begins in the cortical cells of poplar. In our study, the vacuoles in late March contained amorphous stable materials that were counterstained with uranyl acetate and lead citrate, indicating that lytic activity of vacuoles occurs at the deacclimation stage. Therefore, at the deacclimation stage, the enlarging vacuoles may participate in the reconstitution of cells required for the onset of regrowth in spring. Likewise, the dispersal of etioplasts from around the nucleus, the replacement of etioplasts by plastids which reaccumulate starch, and the reappearance of organelles involved in protein synthesis signal that the cells are prepared for another growth cycle.

The proliferation of mitochondria after the breaking of rest was not observed but they were omnipresent during winter. This finding is in agreement with the observation that the flower buds of apple contained higher levels of activities of enzymes involved in electron transport during wintering (Kuroda et al., 1991). The ATP-generating system may be needed not only for the maintenance of the nucleolus but also at lower temperatures but also for the energy-requiring processes, such as formation of plastid initials and protein synthesis.

In conclusion, our results suggest that ultrastructural changes in the apical meristem cells of apple flower buds can be divided into three stages: 1) increase in volume of cytoplasm containing organelles involved in protein synthesis and PLBs, microvacuolation, aggregation of plastids around a nucleus, transition from plastid to etioplast, and starch hydrolysis in plastids; these changes are related to the development of cold acclimation and/or rest; 2) completion of microvacuolation and transition from cisterna-like ER to vesicular ER that develop immediately after breaking of rest which are related to the maximum hardiness and/or quiescent; and 3) plastid proliferation, increase in vacuole volume, reduction in cytoplasm volume, reappearance of organelles involved in protein synthesis, disappearance of spatial relationship between nucleus and etioplasts, and replacement of etioplast by plastid, which are related to deacclimation and/or regrowth in spring.

Literature Cited


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休眠および耐凍性と関連したリンゴ花芽生長点細胞の細胞内微細構造変化

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摘 要

休眠および耐凍性と関連したリンゴ花芽生長点細胞の細胞内微細構造変化について調査した。10月下旬から12月上旬にかけての自発休眠期を通じて、花芽の耐凍性は外気温の低下に伴って次第に増加した。この耐凍性増大過程において、細胞構造は液胞の小胞化が進み、細胞質に満ちた状態となった。細胞質内では、蛋白質含有に関連する小胞状の小胞体（ER）、空胞、小胞を放出しているのような小胞体が観察された。またプロテイン・リピドボディーも多く、それらの表面にはリポソーム様粒子の付着が観察された。プラスチドは核の周りに位置するようになり、同時にラメラボディーを含んだエチオプラストに代わり、デンプン粒が消失した。自発休眠が完了した12月中旬には小管状ERが小胞状のERに代わり、耐凍性が最大値に達した1月中旬には液胞の小胞化が完了した。自発休眠完了後、7-8週間が経過した2月上旬には、エチオプラストからの出芽によるプラスチドの分裂が観察された。3月下旬から5月上旬までに耐凍性が減少過程においては、液胞容量の増加と細胞質の減少が進む一方で、蛋白質含有に関連するオルガネラが頻繁に観察された。また、核とエチオプラストの集合関係がみられなくなった。エチオプラストはプラスチドに代わった。なお、ミトコンドリアは越冬を通じて常に観察することができた。

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