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Electron Microscopic Observation of Plastid Containing Taxol-like Substances in Callus Cells of *Taxus cuspidata* var. Nana

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Abstract: Starch grains in callus cells of *Taxus cuspidata* var. nana were clearly detectable when the vesicles containing Taxol-like substances in the cells were qualitatively stained with iodine-potassium iodide reagent solution. Moreover, from observations made under a transmission electron microscope, the vesicles containing starch grains were demonstrated to be plastids wrapped with a double membrane. The plastids were divided roughly into proplastids, leucoplasts and amyloplasts. The proplastid had mainly formed around the nucleus in the juvenile callus cells right after the cell division of 1.5 to 2.5-month-old callus cells. Then, the leucoplast that developed from the proplast moved from the nucleus to the vicinity of the vacuole. Furthermore, the leucoplast in 2.5-month-old mature callus cells was scattered around the large-hypertrophied vacuole. The leucoplast containing 1 to 2 starch grains was observed in the plastid of 1.5-month-old juvenile callus cells. Moreover, numerous amyloplasts containing 3 to 5 starch grains were observed in the 2.5 to 3-month-old mature and aged callus cells. The amyloplast began to invaginate into the tonoplast in the aged and browning callus cells and finally it floated in the vacuole.

Key words: Callus cell, Taxol-like vesicle, plastid, transmission electron microscope, *Taxus cuspidata* var. nana, proplastid, leucoplastid, amyloplast

INTRODUCTION

Taxol, a most promising anticancer agent, is produced in the plant cells of trees of the genus *Taxus*. Fornalè *et al.*[1] reported that taxol was mostly extracted from the bark of trees of the genus *Taxus*, however, the yield was very low. Therefore, much research on the production of taxol using synthetic[2-4] semi-synthetic[5-7] and tissue culture[1,8,9] methods has been conducted. However, there is no report about morphological functions concerning the formation of taxol in plant cells[10]. In a previous report, we applied histochemical techniques for clarification of the biosynthesis of taxol in callus cells of *Taxus cuspidata* var. nana[10]. We found that vesicles which were scattered in the cells showed a positive color reaction to three kinds of taxol detection agent[11,12] and an immunological reaction[13] and found that taxol-like substances existed in the vesicles[10]. Furthermore, the number of vesicles scattered in the callus cells increased with the cultivation period. In contrast, the number of vesicles decreased with the browning and aging of the cells.

To begin with, the vesicles were formed around the nucleus in juvenile callus cells of *T. cuspidata* var. nana. However, in the mature callus cells, the vesicles were greatly hypertrophied and scattered around the vacuole. Then, in the senescent callus cells, a pattern of incorporation of the vesicles into the vacuole was recognized[10]. The present study have tried to investigate in detail the formation and development of vesicles in 1.5, 2.5 and 3-month-old callus cells under the transmission electron microscope. Various intracellular organelles around the nucleus in the juvenile callus cells were observed during the development of vesicles. Furthermore, the development and structural characteristics of vesicles containing taxol-like substances near the vacuole were intended to investigated in the mature callus cells. For the qualitative analysis of vesicles containing taxol-like substances, histochemical techniques were applied to the juvenile and mature callus cells. The development of vesicles containing taxol-like substances was examined on the fusion and hypertrophy of single and/or multiple vesicles. It is also intended to clarify in detail the invagination

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process of vesicles containing taxol-like substances into the vacuole in mature, senescent and browning callus cells under the transmission electron microscope. Several new findings were made concerning the formation and development of vesicles containing taxol-like substances in callus cells of *Taxus cuspidata* var. nana.

MATERIALS AND METHODS

This research project was carried out in the Laboratory of Chemistry and Biotechnology for Utilization of Forest Resources, Faculty of Agriculture, Ehime University from 2003 to 2004. The explants were obtained from young stem sections of Kyaraboku, *Taxus cuspidata* var. nana, in Matsuyama city, Ehime prefecture, Japan.

Explants and methods of callus cultivation: The explants were sterilized by soaking in 70% ethanol for a few minutes followed by immersion in sodium hypochlorite (1% active chlorine) for 20 to 30 min with gentle stirring and three washes with sterile water. Needle segments of the stem were then removed and transferred into a sterilized medium. The explants were kept on solid Gamborg's B5 medium[14] with 4.0 mg L^{-1} of 2, 4-D, 0.5 mg L^{-1} of kinetin, 1.0 g L^{-1} of casamino acid and 15 g L^{-1} of polyvinyl pyrrolidone. Cultivation was maintained at 25°C in the dark for 1.5, 2.5 and 3 months.

Observation of the callus cells with a transmission electron microscope: For transmission electron microscopy, samples of 1.5, 2.5 and 3-month-old fresh and browning callus cells were cut into 2 mm cubes with a feather knife. Each sample was pre-fixed immediately in Karnovsky's solution[15] and post-fixed with osmium tetraoxide solution. The specimens were dehydrated in a graded ethanol series and embedded in epoxy resin. The appropriate parts were selected through the observation of semi-thin sections under a light microscope. The ultra-thin sections of the selected portions were cut and double stained with uranyl acetate and lead citrate. The sections were observed under a HITACHI H-7100 transmission electron microscope at 100 kV and photographed.

Detection of callus cells with iodine-potassium iodide reagent: To clarify the presence of starch grains in the plastid, 1.5, 2.5 and 3-month-old callus cells were visualized using Iodine-potassium iodide reagent solution[16]. Fresh samples of callus cells were cut into 2 mm cubes with a feather knife. Each sample was pre-fixed immediately in Karnovsky's solution[15] and then post-fixed with osmium tetraoxide solution. The specimens were dehydrated in a graded ethanol series and embedded in epoxy resin. Sections (0.5 μm) were cut with a Sorval UT-1000 ultramicrotome. Each section was put on a slide glass. Two drops of chemical reagent were added to each section. Each stained section was covered with cover glass and observed under an Olympus, BH-2 light microscope and photographed.

RESULTS

Formation and determination of proplastids in young callus cells: To clarify the formation and distribution of taxol-like vesicles in callus cells of *T. cuspidata* var. nana, 1.5-month-old juvenile callus cells were observed under the transmission electron microscope (Fig. 1). Chromosomes were scattered all over the nucleoplasm and the signal for the prophase of cell division was observed in comparatively young callus cells (Fig. 1, N). In such cells, each plastid wrapped with a double membrane was scattered around the nucleus (Fig. 1, 7 and 8). Figure 8 shows a Proplastid (PP) clustered around the nucleus. The proplastid was a club or irregular shape, with an average size of 1.9×3.7 μm. The proplastid was surrounded by an exosporium consisting of a double membrane and the matrix had a slightly higher electron density (Fig. 9, PP). Moreover, Lamellas (La) surrounded by a double membrane were clearly observed in the matrixes of the organelles (Fig. 9, La). Therefore, these small organs were identified as proplastids. On the other hand, a large number of Vesicles (Ve) standing in a line surrounded the exosporium of the proplastid in the matrixes of *T. cuspidata* var. nana callus cells (Fig. 9, Ve).

Development of leucoplasts from proplastids in mature callus cells: In 1.5-month-old juvenile callus cells, substances with an extraordinarily low electron density were observed in the proplastid near the nucleus (Fig. 1). Therefore, the detection of substances with iodine-potassium iodide reagent solution was conducted. The substances showed a strong brown color reaction with dextrin-type starch (Table 1 and Fig. 12-15). Consequently, the grains in the plastid were identified as starch grains containing dextrin-type starch (Table 1 and Fig. 12-15). On the other hand, starch grains in the vacuole showed a strong purple color reaction with amylose-type starch (Table 1 and Fig. 15). The change in the area of starch grains per callus cell during the cultivation period is shown in Fig. 16.

Many organelles of mitochondria, the rough-surfaced endoplasmic reticulum (rough ER), microsomes, Golgi bodies and so forth were recognized in the 2.5-month-old young and mature callus cells. In the cells, a leucoplast

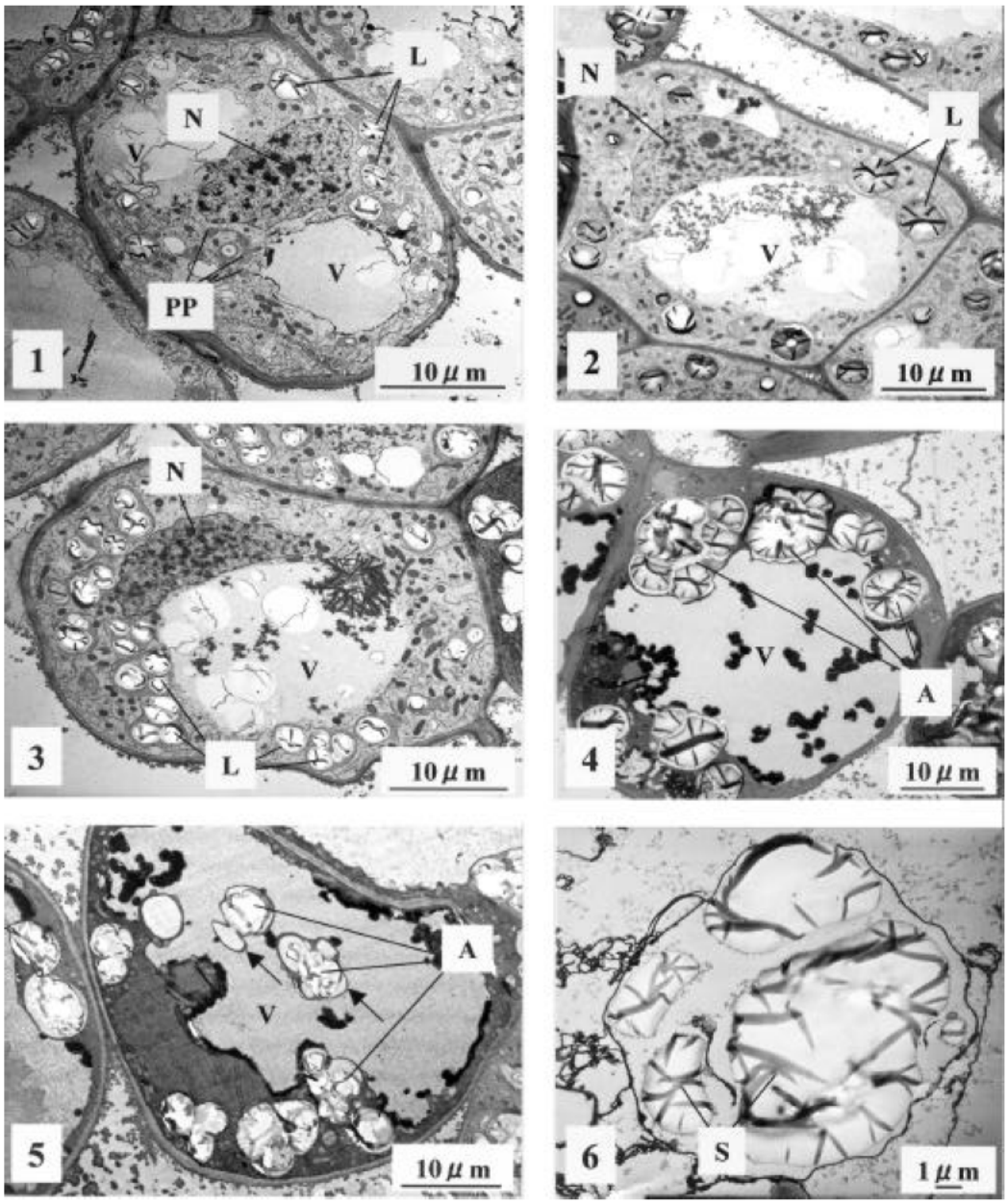

Fig. 1-6: Formation, development, localization and decomposition of plastids in callus cells of *Taxus cuspidata* var. nana.

1: In young callus cells, Proplastids (PP) and Leucoplasts (L) were formed around the Nucleus (N).
2: In young to mature callus cells, Leucoplasts (L) moved from the nucleus to the Vacuole (V).
3: In mature cells, the Vacuole (V) was enlarged greatly and Leucoplasts (L) localized around the vacuole.
4: Amyloplasts (A) were developed from leucoplasts in old cells. (Leucoplasts grew remarkably in old cells.) Amyloplasts (A) began to be incorporated into the Vacuole (V).
5: When the cell aged more, amyloplast (A) floated in the vacuole (arrows).
6: Finally, amyloplast collapsed. However, Starch grains (S) were observed

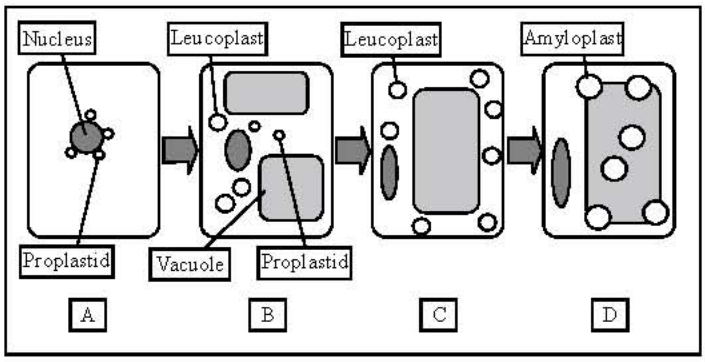

Fig. 7: Schematic features of Fig. 1-6 on development of plastids.
A: Firstly, proplastids were formed around the nucleus in young callus cells.
B: Secondly, proplastids and leucoplasts began to separate from the nucleus.
C: Thirdly, leucoplasts were localized around the vacuole.
D: Finally, amyloplasts began to be incorporated into the vacuole and then floated in the vacuole in old cells

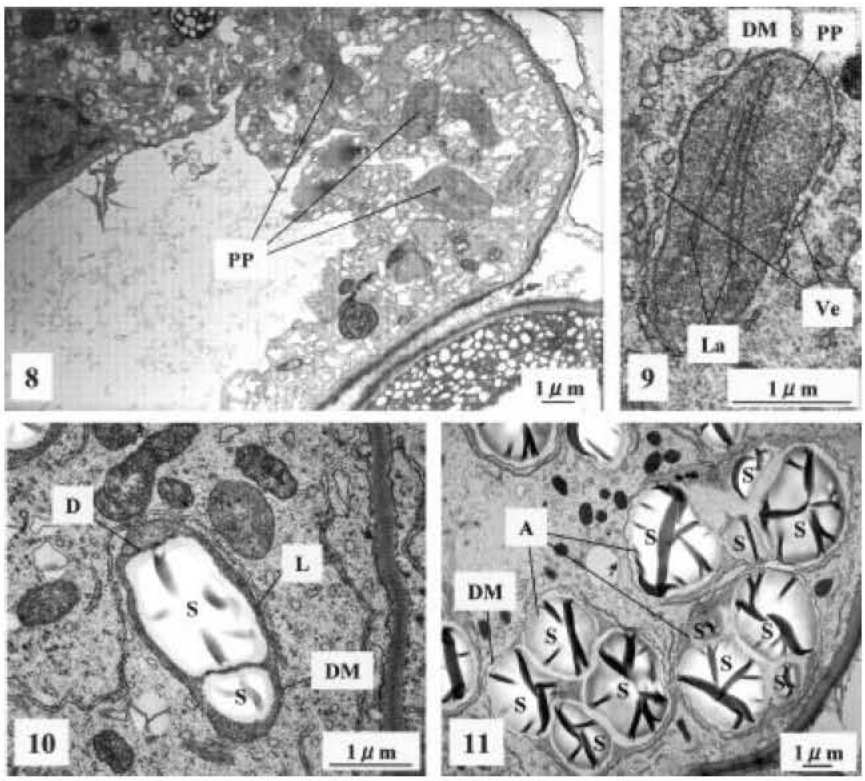

Fig. 8-11: Proplastids (PP), leucoplasts (L) and amyloplasts (A) in callus cells of *Taxus cuspidata* var. nana.
8: The proplastids (PP) before storing the starch grain was observed in young callus cells.
9: These proplastids (PP) were surrounded by a double membrane (DM) and two lamellas (La) were observed clearly and a large number of Vesicles (Ve) stood a line was surrownded by near the exporium of proplastid.
10: The Leucoplasts (L) stored several starch grains (S) wrapped with Double memblane (D) and Leucoplast (L) also wrapped with a double memblane (DM).
11: The amyloplasts (A) stored large number o starch grains (S). These grains did not wrapped with a memblane. However, Amyloplasts (A) were wrapped with a double memblane (DM)

Table 1: Iodstarch reaction of starch grains in callus cells of *T. cuspidata* var. nana

The callus cells	Reaction	Diameter	Color
Young	+	1.0 μm	Brown
Mature	++	23. μm	Brown
Mature to old	++	2.0 μm	Brown
Old	+++	4.3 μm	Deep brown and purple

Notes: The callus cells were cultivated at 25°C in the dark for 1.5, 2.5 and 3 months. The callus cells were fixed with epoxy resins and semi-thin sections were made with an ultra microtome. Then the sections were stained with Iodine-potassium iodide reagent solution. The sections were observed under the light microscope. + positive reaction, ++ intensively positive reaction, +++ extremely positive reaction

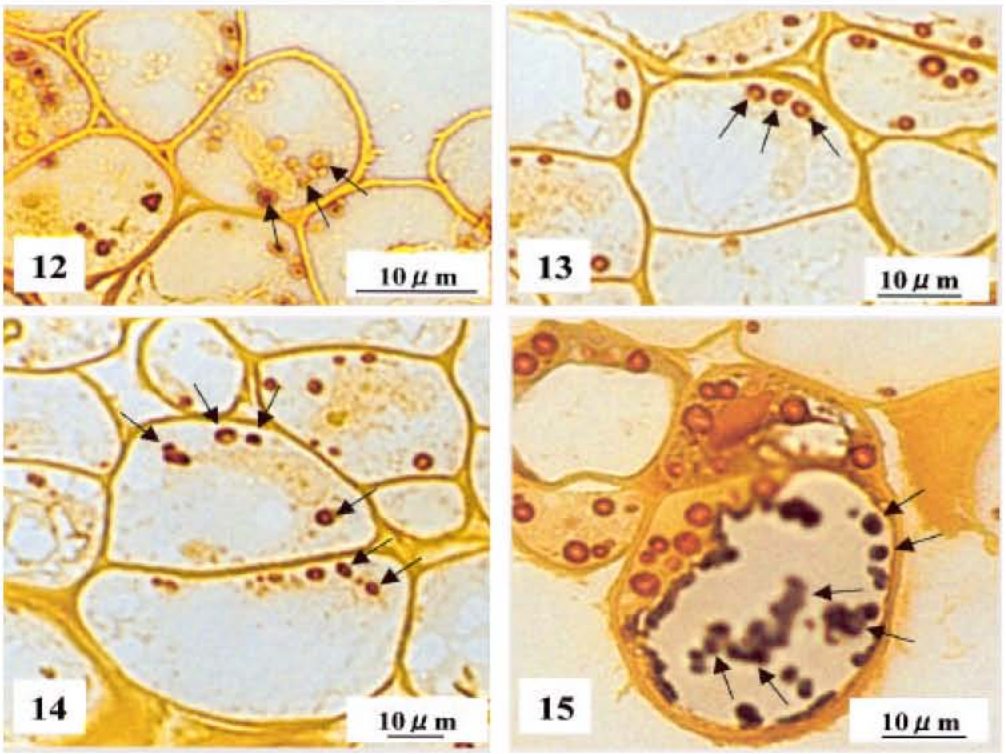

Fig. 12-15: Longitudinal features of callus cells of *T. cuspidata* var. nana stained with Iodine-potassium iodide reagent solution.
12: Young callus cells revealed several starch grains (arrows) near the nucleus.
13: Mature callus cells showed several starch grains (arrows) between the nucleus and the vacuole.
14: Mature to Old callus cells had several starch grains (arrows) around the vacuole.
15: Starch grains began to be incorporated into the vacuole (arrows) in old callus cells

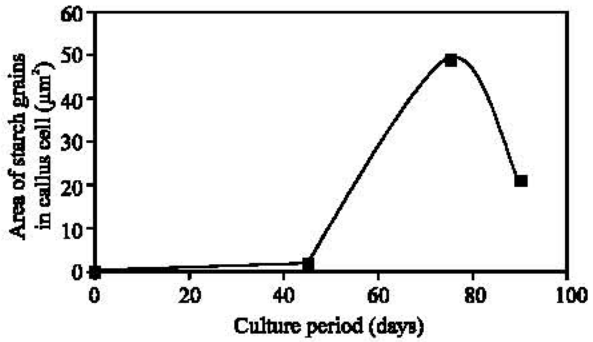

Fig. 16: Effect of culture period on the development of starch grains in a callus cell of *T. cuspidata* var. nana. Notes: The area of starch grains is the total area of starch grains observed in the sections (0.5 μm) in a callus cell under the light microscope

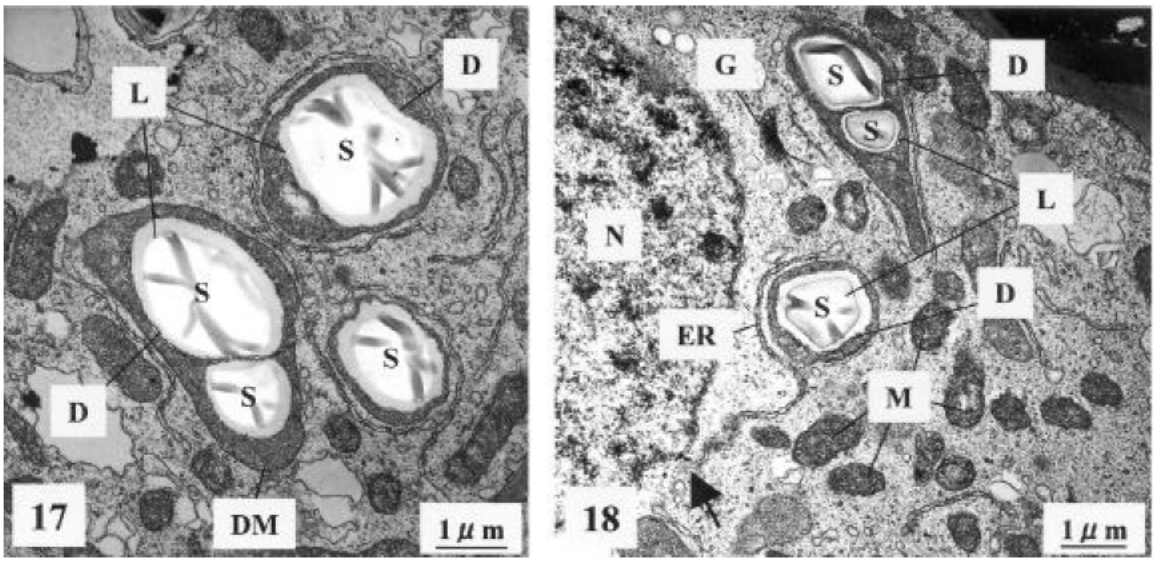

Fig. 17-18: Young Leucoplasts (L) and Leucoplasts (L) in mature callus cells.
17: In mature callus cells, the young Leucoplast (L) included some starch grains (S) wrapped with a Double memblane (D). The leucoplast was wrapped with a double membrane (DM). Moreover, it was surrounded by the rough ER.
18: A number of Mitochondoria (M), rough ER (ER) and Golgi body (G) existed around the leucoplast in mature callus cells. Starch grains (S) wrapped with a Double memblane (D) were observed in leucoplast. In addition, rough ER (ER) was connected to the nucleus membrane (arrow)

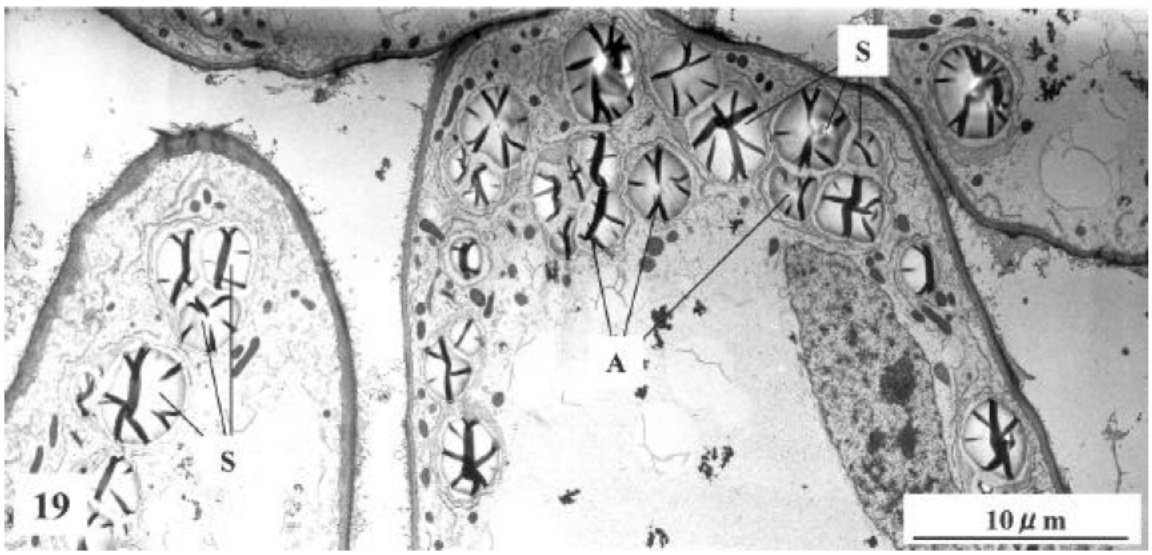

Fig. 19: Accumulation and expansion of starch grains of Amyloplast (A) in mature callus cells.
The starch grains increased in size 4 to 5-fold and became large size of Amyloplast (A) with no lamella. The Amyloplast (A) contained several starch grains (S)

containing taxol-like substances began to separate from the nucleus (Fig. 2 and 7). The hypertrophied leucoplast began to show a squeezing pattern (Fig. 10). Furthermore, a large number of leucoplasts were present around the vacuole when the callus aged and the vacuole in the cells enlarged (Fig. 3 and 7). In the 3-month-old mature callus cells, the diameter of the leucoplast around the nucleous was 3.6x5.0 μm and the starch grains in the leucoplast showed a intense purple and brown color reaction with iodine-potassium iodide reagent solution (Table 1 and Fig. 15). Moreover, when the starch grains were observed with the transmission electron microscope, a squeezing pattern was clearly revealed (Fig. 4 and 11). Furthermore, taxol-like vesicles in the mature callus cells were observed with the transmission electron microscope (Fig. 17 and 18).

Figure 17 shows that young leucoplasts exist both around the nucleus and near the vacuole. Each of them was wrapped with a double membrane and had a lamella with a double membrane, with high electron density in the matrix. Moreover, the leucoplast including the substances showed the squeezing pattern with a low electron density. As shown in Fig. 17, the substances with the squeezing pattern were detected clearly as starch grains by the iodine-potassium iodide color reaction (Table 1, Fig. 10 and 12-15). Therefore, taxol-like vesicles were identified as leucoplasts. Furthermore, most leucoplasts in cells were surrounded by a rough ER (Fig. 17 and 18).

Development of amyloplasts from leucoplasts in mature callus cells: In considerably enlarged 2.5 and 3-month-old mature callus cells, various organelles localized to the narrow space between enlarged vacuoles and cell walls (Fig. 3). Figure 3 shows a leucoplast enlarged greatly around the vacuoles. As shown in Fig.10, the leucoplast stored several starch grains which wrapped with a double membrane and the leucoplast was also wrapped with an exosporium of double membrane. Moreover, in the aged callus cells, an amyloplast incorporated into the vacuoles through invagination of the tonoplast was also observed when the callus cells aged (Fig. 4 and 5). As the substances having single and/or plural grains with low electron density in the leucoplast had a positive color reaction with iodine-potassium iodide reagent solution, they were identified as starch grains. In addition, the starch grains increased in size 4 to 5-fold and became large particles with no lamella (Fig. 19). Such large particles were wrapped with a double membrane and contained several starch grains. Therefore, the particle was identified as amyloplast.

Decomposition of various organs in senescence callus cells: The callus cells cultured for 3 months began to brown (Fig. 4 and 5). Those cells suddenly became more electron dense in both cytoplasm and various organelles. However, substances with a high electron density were precipitated in the vacuole and a number of large and small amyloplasts and organelles flowed into the vacuole. Figure 6 shows that the necrosis caused plasmolysis and separation from the cell walls and then the plasma membrane began to macerate. The exosporium of the amyloplast collapsed and then the starch grains floated in the cells.

DISCUSSION

To examine the process of formation and characteristics of taxol-like vesicles in callus cells of *T. cuspidata* var. nana, the micro structure of 1.5-month-old juvenile callus cells was observed with the transmission electron microscope. We found a large number of organelles wrapped with a double membrane containing substrates with a slightly higher electron density and having an internal lamellar system (Fig. 8 and 9), whose external size was 1.9×3.7 μm, around the nucleus in the callus cells right before and after the cell division. These were club-like and small spherical shapes. Moreover, the organelle had the same characteristics as those published by Toyama *et al.*[17]. Therefore, the organelle was identified a proplastid.

Regarding the formation of the proplastid in the cell, it is said that the proplastid is recognized first as a spherical particle and then formed from the plastid[18]. But, we can not observe the initial formation of the plastid, a precursor of the proplastid. However, the presence of proplastids in the cells right after cell division was not confirmed when a large number of proplastids formed around the nucleus. The results obtained above show that the formation of the proplastid in juvenile callus cells is not caused by division of the plastid. In short, the origin of the proplastid is considered to be the nucleus. In addition, we observed several lamella in the proplastid (Fig. 9). This lamellar system coincided with the initial formation pattern of the internal lamellar system reported by Frey-Wyssling *et al.*[18]. That is to say, these proplastids differentiated into chloroplasts in the light. However, these proplastids are considered to differentiate into leucoplasts in the dark.

Figure 9 shows that a large number of vesicles align parallel around the proplastid. However, no plastid was completely surrounded by the rough ER and a large number of mitochondria were localized near the plastid as shown in Fig. 17 and 18. Furthermore, the pattern in which the rough ER connected to the nuclear membrane was observed as shown in Fig. 18 (arrow). In short, the

leucoplast containing starch grains was surrounded by the rough ER and the ER connected with the nuclear membrane and large numbers of mitochondria and Golgi bodies existed around the leucoplast. Such morphological characteristics seems to suggest that the level of metabolic activity is considerably high.

According to Fornalè *et al.*[1], when [^{14}C]-taxol was added to the cell suspension cultures of *Taxus baccata*, 20% was incorporated in the cell walls and 80% into the cells. The results obtained by Fornalè *et al.*[1], our previous results[10] and this result also suggest that taxol and related compounds are accumulated in the callus cells. Moreover, taxol is an alkaline secondary metabolite called alkaloids. Most alkaloids are synthesized from amino acids and alkaloids are mostly stored in a protonated form in the vacuole for an acidic environment[19]. There is some possibility that taxol may exist in a protonated form.

In the case of berberine, an isoquinoline-type alkaloid, a study on the biogenetic enzymes for the formation of berberine has been conducted in comparative detail[20,21]. In the biogenetic pathway of berberine, tyrosine to (S)-reticulin are formed by enzymes in the cytoplasm. It was also found that the biosyntheses of (S)-reticulin to berberine is conducted by four kinds of enzyme existing in the microsome[20,22]. Furthermore, Amann *et al.*[20] reported that berberine synthesized in the microsome is transported to the vacuole. From the presence or absence of many organs and arrangement around the nucleus shown here and our previous results[10], it is considered that the plastid is highly responsible for the biosynthesis of taxol in the 2.5-month-old callus cells of *T. cuspidata* var. nana.

In a previous report[10], taxol-like vesicles scattered around the nucleus showed a strong positive reaction to histochemical techniques and with the immunoassay of taxol in the 1.5-month-old callus cells of *T. cuspidata* var. nana. Therefore, taxol is considered to exist somewhere in the proplastid containing starch grains around the nucleus in 1.5-month-old juvenile callus cells.

To examine the hypertrophy of the taxol-like vesicles observed under a light microscope in a previous paper[10], the callus cells were observed with the transmission electron microscope. In 2.5-month-old mature callus cells, taxol-like vesicles had moved from around the nucleus to around the enlarged vacuole and were greatly hypertrophied there and the squeezing pattern in the vesicles was clearly recognized. The size of taxol-like vesicles reached 3.6×5.0 μm and the vesicles showed a strong positive reaction with iodine-potassium iodide reagent solution. Therefore, the substances having the squeezing pattern in the vesicles were identified as starch grains. Then, when those vesicles were observed under the transmission electron microscope, the vesicles wrapped with a double membrane and which had substrates with a high electron density and with clear internal lamellar system were recognized. Moreover, the vesicles contained several large starch grains with the squeezing pattern. The morphological characteristics of leucoplasts corresponded to the theory of Frey-Wyssling *et al.*[18] that the leucoplast has a few lamellar, but not thylakoid structure.

In addition, most of the leucoplast was surrounded by the rough ER in the 2.5 and 3-month-old mature callus cells of *T. cuspidata* var. nana. Given this finding, the leucoplast is considered to have considerably greater metabolic activity as described in the leucoplast containing starch grains.

From the light microscopic observation of the 2.5 and 3-month-old callus cells, the incorporation of taxol-like vesicles into vacuole was reported in the previous study[10]. The incorporation of the leucoplast into the vacuole was clearly observed when the senescence callus cells were observed under the transmission electron microscope. It was observed that the amyloplast was incorporated into the vacuole through invagination of the tonoplast. When the amyloplast started to be incorporated into the enlarged vacuole through invagination of the leucoplast around the vacuole, the number of starch grains in the leucoplast began to increase : in some cases, five to six large starch grains per section were observed in the leucoplast. When those leucoplast were observed at a high magnification, the leucoplast was found to be hypertrophied in large vesicles without an internal lamella system (Fig. 11 and 17). Such large vesicles were wrapped with a double membrane and contained several starch grains. Therefore, the vesicles were identified as amyloplasts (Fig. 11).

From the morphological characteristics of taxol-like vesicles from juvenile to mature and senescent callus cells of *T. cuspidata* var. nana, it was revealed that the proplastid differentiated into the leucoplast and then the amyloplast. As a result, it became clear that the starch grains in those organs developed from simple to compound grains (Fig. 19). Notably, the hypertrophy and development of starch grains in the leucoplast and amyloplast of mature and old callus cells corresponded to that reported by Okada *et al.*[10]. They reported that the amount of taxol in each callus cell increased rapidly after 2.5 months cultivation and decreased suddenly after 3 months cultivation. The increase in taxol content in each callus cell was accompanied by an increase in the number of leucoplasts and amyloplasts in each period and moreover, 4 to 6 large starch grains in the amyloplast were

recognized during the culture (Fig. 19). Therefore, from the observations made under the transmission electron microscope, it is considered that the biosynthesis of taxol in the callus cells is closely related to the increase in the number of proplastids, leucoplasts and amyloplasts. Furthermore, in the 3-month-old senescence callus cells, the amyloplast was incorporated into the vacuole through invagination of the tonoplast and then floated in the vacuole on the degradation of the exosporium of the amyloplast. After that, the starch grains in the amyloplast began to diminish when the amyloplast was begun to be released into the cells via degradation of the tonoplast. We already reported that the taxol content of the callus cells dramatically decreased after the culture for 3 months[10]. Figure 16 shows the change in the area of starch grains per callus cell during the cultivation period. The area increased rapidly during the cultivation for 1.5 to 2.5 months and reached a maximum when the cultivation was conducted for 2.5 months and then decreased. From the results obtained here and in the previous paper[10], it was revealed that the decrease in taxol content was deeply correlated with the diminution of starch grains in the cells. In the present research, it was found that the enlargement of starch grains was concerned with the hypertrophy of simple grain as well as the hypertrophy and development of several grains in the leucoplast and amyloplast of callus cells (Fig. 3 and 4). The number of starch grains increases with the culture period.

According to Heldt[19], the glucose molecules in starch are connected to each other by glycosidic linkages. The aldehyde group in the glucose molecule escaped from oxidation by the linkage except terminal glucose molecule. In this way, a long glycosidic chain was formed and a side chain was also formed by glycosidic linkage. It is said that the starch molecule becomes much larger by making a connection with the glucose moiety[19]. The results obtained here on the enlargement of starch grains by the hypertrophy and development of single grain in the plastid supported the theory of Heldt[19].

The plastid, namely the chloroplast and leucoplast, is the only organelle which can form starch in plants[19]. According to Heldt, for the accumulation of carbohydrates as an energy store for the growth of the next generation, plants store carbohydrates in the form of oligosaccharides having comparatively low molecular weights and polysaccharides, especially in the form of starch and fructan. In addition, glucose is comparatively unstable in order to automatically oxidize the aldehyde group into the corresponding carboxyl group. So, glucose is not adequate as a storage form of carbohydrates. Moreover, there is a limit on storage as the monosaccharides in the cells for the sake of osmotic pressure. It is said that it is possible to store an extraordinarily large amount of glucose molecule in the cell by conversion of glucose into starch without changing the osmotic pressure of cell sap[19].

We consider that the leucoplast is surrounded by the rough ER in order to supply the fatty acids synthesized in the plastid to the ER membrane and to facilitate the biosynthesis of fatty acids (Fig. 10, 17 and 18). Furthermore, mitochondria exist around the leucoplast (Fig. 10, 17 and 18). One of the functions of mitochondria is to form ATP necessary for the glutamine synthetase reaction and the ATP is incorporated into the leucoplast via a translocator by exchange of ADP[19]. There is some glutamate synthase which can utilize directly NADPH and NADH in the leucoplast[19].

In the leucoplast having no chlorophyll, the reducing agents for nitrite reduction are supplied by the oxidative pentose phosphate pathway and amides such as glutamic acid and 2-oxoglutaric acid and so forth are formed by ammonia[19,23,24]. There is a good possibility that the leucoplast forms taxol, since amide side chain, phenylisoserine also exists in the chemical structure of taxol. Furthermore, isoprenoids are necessary for the biosynthesis of terpenoids and isoprenoids are usually synthesized by the mevalonate pathway through isopentenyl pyrophosphate as a precursor of isoprenoid in the plastid. However, it is also shown that the biosynthesis of isopentenyl pyrophosphate is conducted by another route in the cytoplasm. Since the biosynthesis of terpenoids in the plastid has been clarified[19,25], the possibility of the biosynthesis of taxol in the proplastid, leucoplast and amyloplast is extremely high. To confirm the conclusion established by the results obtained here and in the previous report[10], it is necessary to extract taxol from the proplastid, leucoplast and amyloplast in callus cells of *T. cuspidata* var. nana and detect it by instrumental analysis.

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