Structural and Immunocytochemical Characterization of the Synthesis and Accumulation of Starch in Sweet Potato (Ipomoea batatas Lam.) Tuberous Root

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Abstract: The structural changes in the plastid-amylloplast system in the parenchyma cells of sweet potato tuberous roots during thickening were examined by electron microscopy. In the tuberous roots, proplastids and plastids that contain starch granules propagated in young parenchyma cells adjacent to the meristem, but amylloplasts did not in parenchyma cells. It was suggested that the number of amylloplasts in a parenchyma cell is determined by the propagation of the proplastids and plastids. The form of amylloplasts and the number, size and form of starch granules in them were various. Tubular membranes containing the electron-dense substance were formed in plastids and extended from the envelope membranes of plastids to the starch granules. The electron-dense substance also existed around the starch granules. Tubular membranes are converted into membrane-bound inclusion bodies as a result of loading of the electron-dense substance into these tubes in the plastids. The inclusion bodies were also at the periphery of the amylloplasts. In this study, the functions of both tubular membrane and the inclusion were discussed. The localization of starch branching enzyme in tuberous roots was examined by immunogold electron microscopy. The label for branching enzyme was localized predominantly throughout the surface of each starch granule, suggesting that this is the branching for amylopectin synthesis, but not throughout the stroma in the plastid-amylloplast system. Small and round starch granules were often formed at parts of the periphery in the amylloplast. Dense labeling for the enzyme was detected around the granules. The increase of the number of starch granules in an amylloplast is certainly made by means of the formation of new starch granules at the periphery of the amylloplast. It is likely that the new granules are intensively formed there.

Key words: Amyloplast, Electron microscopy, Immunogold electron microscopy, Plastid, Starch branching enzyme, Starch synthesis, Sweet potato.

Sweet potato, Ipomoea batatas Lam., “Satsumaimo” in Japanese, stores large amounts of starch in the parenchyma tissue of tuberous root. The formation and thickening of the tuberous roots are important processes for determining the yield such as starch content (Tsuno and Fujise, 1965). There are many reports about these processes in sweet potato examined by light microscopy. The mechanism involved in determining the yield has been discussed at the organ and tissue levels from the anatomical studies (Togari, 1950; Kokubu, 1973; Wilson and Lowe, 1973; Lowe and Wilson, 1974). However, very little is known about the cellular and subcellular regulatory mechanism. Storage starch is synthesized and accumulated in “the plastid-amylloplast system” (Kawasaki et al., 1997). We have already reported the structural changes of the plastid-amylloplast system during the thickening of storage vegetative organs in Japanese yam (Kawasaki et al., 1997), taro (Kawasaki et al. 1998) and potato (Kawasaki et al., 1999).

Although some investigators observed the starch granules in the tuberous root of sweet potato (Jane et al., 1994), the plastids and amylloplasts have been poorly characterized.

It is generally accepted that the synthesis of starch is mediated by 4 classes of enzymes, i.e., ADP-glucose pyrophosphorylase, starch synthase, starch branching enzyme (BE) and starch debranching enzyme (Smith et al., 1997, Myers et al., 2000). BE hydrolizes an α-1,4 bond inside a glucan chain and subsequently links the severed segment to an acceptor chain via an α-1,6 bond (Barker et al., 1950; Borovsky et al., 1976). The enzyme also contributes to amyllopectin synthesis by forming a non-reducing terminal as glucan acceptor from ADP-glucose for α-1,4-glucan elongation during the branching reaction. Therefore, it is important to determine the localization of BE to understand how amyllopectin is synthesized at a subcellular level. An immunogold labeling study on potato tuber, which forms simple starch

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Abbreviations: A, amylloplast; BE, starch branching enzyme; BSA, bovine serum albumin; M, mitochondrion; MI, membrane-bounded inclusion body; P, plastid; PBS, phosphate buffered saline; PP, plastid; SG, starch granule; TM, tubular membrane; V, vacuole.
granules, showed that BE is concentrated at the interface of starch granules and stroma in amyloplasts (Kram et al., 1993). However, there is little knowledge about the localization of BE in amyloplasts that form compound starch granules.

To understand the mechanism of determining yield and quality of food crops, especially at a subcellular level, we need to clarify the mechanism of the synthesis and accumulation of storage starch in the plastid-amyloplast system. The aim of this study is to elucidate the mechanism of synthesis and accumulation of starch in thickening tuberous roots of sweet potato that forms compound starch granules. We examined the structural changes in the plastid-amyloplast system in the thickening tuberous roots using both scanning and transmission electron microscopes. We also investigated the localization of BE by immunogold labeling and electron microscopy.

Materials and Methods

1. Plant materials

Sweet potato, *Ipomoea batatas* Lam., variety “Beniazuma” was used in this experiment. Plants were planted on the experimental fields of Ibaraki University at Ami, Japan in June 1996. The tuberous roots were sampled at 14-day intervals during the developmental phase and at the harvest time.

2. Electron microscopy

Storage parenchyma tissues in the tuberous roots were fixed for scanning and transmission electron microscopies. The small blocks from the tuberous roots were obtained by cutting with a razor blade. The blocks were fixed in 0.05 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde and 1% paraformaldehyde at 4°C for 2 hr and then at 20°C for 2 hr. The samples were washed with 0.1 M sodium cacodylate buffer, and post-fixed in 0.15 M sodium cacodylate buffer containing 1% osmium tetroxide at 4°C for 8 hr.

For scanning electron microscopy, the fixed samples were immersed in 15, 30 and 50% dimethyl sulfoxide successively each for 30 min. With a freeze-cracking apparatus (TF-2, Eiko, Tokyo), the samples in droplets of dimethyl sulfoxide were frozen on the metal plate chilled with liquid nitrogen and cracked into two pieces with a razor blade and a hammer. After thawing, the samples were rinsed in distilled water. The samples were transferred to 0.15 M sodium cacodylate buffer containing 1% osmium tetroxide at 20°C for 2 hr, and then in 2% tannic acid solution at 20°C for 1.5 hr. After the samples were dehydrated through a graded series of ethyl alcohol, permeated by t-butyl alcohol and then dried with a vacuum freeze-drying apparatus (JFD-300, Japan Electron Optics Laboratory, Tokyo). The dried samples were attached to the specimen stubs, and coated with platinum so that the cut surface was exposed. The specimens were viewed at 5 kV with a scanning electron microscope (JSM 6301F, Japan Electron Optics Laboratory, Tokyo).

For transmission electron microscopy, the fixed samples were dehydrated through a graded series of ethyl alcohol and permeated by propylene oxide. The samples were then embedded in Spurr’s resin and polymerized at 70°C for 24 hr. Ultrathin sections (80 to 90 nm in thickness) were cut with a glass knife on a Reichert ultramicrotome (Ultracut S, Reichert) and placed on 300 mesh copper grids. The sections were stained with 6% uranyl acetate for 7 min followed by 2% lead citrate for 10 min. Then the sections were viewed at 100 kV with a transmission electron microscope (JEM 1010, Japan Electron Optics Laboratory, Tokyo).

3. Protein A-immunogold electron microscopy

Small blocks of the tuberous roots were fixed in 0.05M sodium phosphate (pH 6.8) containing 3% glutaraldehyde for 3 hr on ice, and were then washed in the buffer. They were dehydrated in 30% ethanol on ice and then in increasing concentrations of ethanol at −20°C. They were gradually infiltrated with Lowicryl K4M resin at −20°C. The resin was polymerized under ultraviolet light at −20°C. Ultrathin sections were placed on 300 mesh nickel grids coated with Formvar.

An antiserum raised specifically against starch branching enzyme II of sweet potato (Nakayama and Nakamura, 1994) was used for localization of the branching enzyme. The sections were incubated in phosphate buffered saline (PBS) consisting of 10 mM sodium phosphate (pH 7.2), 150 mM NaCl and 0.1% Tween 20 and 0.5% bovine serum albumin (BSA) for 20 min. Then they were incubated in antiserum diluted with 0.5% BSA in PBS, or non-immune serum as a control. The grids were then incubated in a 1:40 dilution of a suspension of 15 nm Protein A-colloidal gold particles (EY Lab. Inc., San Mateo, CA, USA) for 30 min. After washes with PBS and distilled water, the sections were stained with 6% uranyl acetate for 7 min followed by 2% lead citrate for 10 min. The sections were viewed at 100 kV with a transmission electron microscope.

Results

1. Structural changes in the plastid-amyloplast system of parenchyma cells in thickening tuberous roots

Fig. 1 shows proplastids in young parenchyma cells that are adjacent to the meristem. Several proplastids were often propagating in young parenchyma cells. Proplastids became plastids accumulating starch granules but did not yet have the characteristics of amyloplasts. These plastids also propagated in young parenchyma cells. In plastids forming starch granules, tubular membranes containing the electron-dense substance, were formed (Fig. 2). Many tubular membranes extended from the envelope membranes of plastids to the starch granules. An electron-dense substance (arrows),
which is like the substance in the tubular membranes, was also observed near the surface of starch granules (Fig. 2). With the formation of starch granules, the tubular membranes are converted into membrane-bound inclusion bodies, as a result of loading of electron-dense substance into the tubes in plastids (Fig. 2 and 3). Plastids accumulated a large quantity of starch granules, so that they can be regarded as amyloplasts (Fig. 3 and 4). In amyloplasts of thickening tuberous roots, several membrane-bound inclusion bodies were also recognized at parts of the periphery (Fig. 4).

Figs. 5 and 6 show amyloplasts in the thickening tuberous roots, which were cracked by the freeze-cracking method. The presence of cracked amyloplasts revealed that the number of starch granules per amyloplast and their size and form are various. Small and round starch granules (* in Fig. 5) were often observed at a part of the periphery in amyloplasts. On the cracked surface of starch granules, a depression that had been reported as “cavity” by Hall and Sayre (1970) was observed. This cavity occurred in the center of polygonal starch granules (arrow in Fig. 5), whereas in half capsule-like starch granules, it was observed near the base rather than the center (arrow in Fig. 6). Fig. 7 shows amyloplasts in the parenchyma cells of the tuberous root at harvest time. At this time, there were many amyloplasts that were about 20 to 50 μm in length of the major axis (Fig. 7). These amyloplasts showed various forms, and the individual contained about 2 to 25 starch granules. The size and form of starch granules also varied with the amyloplast.

2. Immunogold localization of branching enzyme

As shown in Fig. 8, no substantial signals due to non-specific and negligible labeling with colloidal gold were shown when the sections of the tuberous roots were incubated in non-immune serum. Figs. 9 to 12 show immunogold labeling for BE in the parenchyma cells of thickening tuberous roots. The labels of BE were observed along the border between starch granules and stroma in plastids (Figs. 9 and 10). Fig 11 shows the internal part of the amyloplast. Between starch granules in amyloplasts, the labels were also found along the surface of starch granules (Fig. 11). Fig. 12 shows a concentrated labeling around a small starch granule (* in Fig. 12) forming in the periphery of the amyloplast, together with labeling along the surface of large starch granules.

Discussion

This study clarified the structural change of the plastid-amyloplast system during the thickening of the tuberous root of sweet potato. In the thickening tuberous roots, the propagation of proplastids and plastids was observed, while propagation of amyloplasts was not. In potato and eddo, however, the propagation was observed even in large amyloplasts during the thickening growth of these tubers (Kawasaki et al., 1998, 1999). Our observation indicates that the number of amyloplasts in sweet potato tuberous roots depends on the propagation

Explanations of figures

Figs. 1 to 4 show the internal parts of the parenchyma cells in thickening tuberous roots at 42 days after transplanting observed by transmission electron microscopy. (Bars for Figs. 1 and 2=0.2 μm) (Bars for Figs. 3 and 4=1 μm)

Fig. 1. Proplastids in a young parenchyma cell adjacent to meristem.

Fig. 2. A part of a plastid forming starch granules. Note some tubular membranes extended adjacent of starch granules. An electron-dense substance (arrows), which is the same as the substance in the tubers, is also adjacent to the granule surface.

Fig. 3. A plastid accumulating starch granules and converting into an amyloplast. Note that the formation of several membrane-bound inclusion bodies initiated along with that of starch granules.

Fig. 4. An amyloplast containing membrane-bound inclusion bodies at several parts of the periphery.

Figs. 5 and 6 show the amyloplasts in thickening tuberous roots at 84 days after transplanting, and Fig. 7 shows the amyloplasts at harvest time, revealed by scanning electron microscopy. (Bars for Figs. 5 to 7=10 μm)

Fig. 5. Amyloplasts cracked by the freeze-cracking method. A cavity (arrow) is seen at the center of a polygonal starch granule. A small starch granule (*) is formed at the periphery in the amyloplast.

Fig. 6. An amyloplast cracked by the freeze-cracking method. It includes half capsule-like starch granules, in which a cavity (arrow) occurs at the base, but not in the center.

Fig. 7. Amyloplasts showing various forms in a parenchyma cell.

Figs. 8 to 12. Immunogold electron micrographs. (Bars for Figs. 8 and 12=1 μm) (Bar for Fig. 9=0.5 μm) (Bars for Figs. 10 and 11=0.2 μm)

Fig. 8. Immunogold labeling with non-immune serum of a parenchyma cell in thickening tuberous root.

Figs. 9 to 11. Immunogold labeling with antiserum to branching enzyme of parenchyma cells in thickening tuberous root at 70 days after transplanting. Arrows show branching enzyme-specific label.

Fig. 12. Immunogold labeling with antiserum to branching enzyme of parenchyma cells in thickening tuberous root at 84 days after transplanting. Arrows and a star (*) show branching enzyme-specific label and a new starch granule developing at the periphery of the amyloplast, respectively.
of proplastids and plastids. The observation of cracked amyloplasts in the thickening tuberous roots demonstrated that small and round starch granules are often present in parts of the periphery of the amyloplast. This suggests that new starch granules are successively formed.

The form of amyloplasts and the form, size and number of starch granules in them are varied. The amyllose content of the starch granules in the potato tuber, that contained single starch granules, is twice as high as that of starch granules in eddo corn, which contains compound granules (Sugimoto et al., 1987; Agbor and Richard, 1990). Whether the composition of starch granules in each amyloplast differs depending on the number of starch granules in amyloplasts or not is a subject of interest. This study revealed that the position of the cavity in the starch granules differ between the polygonal granules and half capsule-like granules. It is suggested that the cavity represents the starting point of the formation of starch granules, and is related to the phenomena of the swelling of the granule (Hall and Sayre, 1970). The polygonal starch granules are formed concentrically, while the half capsule-like granules would be formed more intensively in regions between starch granules rather than in other regions around the granule surface in an amyloplast.

In the plastids synthesizing starch granules, tubular membranes containing electron–dense substance were found. With the progress of the synthesis of starch granules, the tubular membranes are converted into membrane–bound inclusion bodies, as a result of loading of electron–dense substance into the tubes. These inclusion bodies were also observed in the amyloplasts, despite the fact that they have not been reported in the amyloplasts containing compound starch granules. Casadora and Rascio (1977) reported also that tubular membranes with electron–dense contents are converted into the membrane–bounded inclusion bodies during the maturation of plastids.

It is considered that membrane–bound inclusion bodies have a storage function (Marinos, 1967; Newcomb, 1967), and alternatively play a role in the building up of thylakoid system (Stetler and Laetsch, 1969; Cran and Possingham, 1974). However, there are few studies about the inclusion bodies of amyloplasts (Kram et al., 1993; Kawasaki et al., 1997, 1999). Kram et al. (1993) reported that label for BE was observed in the inclusion bodies in potato tubers by immunogold electron microscopy. They suggested that the inclusion bodies serve as a storage compartment and might actually be involved in starch synthesis. Kawasaki et al. (1997, 1999) suggested that the inclusion bodies are involved in storage of BE and supply of the enzyme to the site where starch granules are synthesized. In the present study, it was observed that many tubular membranes are extended to the starch granules. The electron–dense substances were also observed near the surface of the starch granules.

We also found that the label for BE occurs at the surface of starch granules in plastids and amyloplasts, although could not clarify whether the label for BE was specifically in the inclusion bodies. In general, electron–dense substances are correlated with the site where protein is concentrated on transmission electron microscopy with uranyl acetate and lead citrate staining. Judging from our results and those from the previous reports, it is suggested that the tubular membranes and the inclusion bodies are involved in the transportation of BE and the storage of the enzyme respectively. More information and extensive analysis are required to clarify their functions.

Kram et al. (1993) indicated that the label for BE occurs specifically in the interface between stroma and starch granules in the amyloplast of potato tubers. In this study, the localization of BE leads to a conclusion that the actual branching for synthesis of amyllopectin would take place predominantly throughout the surface of each starch granule, but not throughout the stroma. This result is consistent with the presumption by Badenhuizen and Dutton (1956) that amyllopectin is synthesized at the surface of starch granules in accord with growth of the granules. In addition, the label for BE was also concentrated around small starch granules that were present in parts of the periphery in amyloplasts. The increase of the number of starch granules in an amyloplast is certainly made by means of the intensive formation of new starch granules at the periphery of the amyloplast. It is likely that the new granules are intensively formed there.

Amylopectin molecules are ordered in that the branching points are clustered along the molecule (Robin et al., 1974). The molecules are radially oriented in the starch granule with its non-reducing end pointing toward the granule surface. The glucan chains inside the clusters form double helices, which in turn are arranged in a semi-crystalline matrix (Imbert et al., 1991). Such a highly ordered structure would not be formed by precipitation of amyllopectin molecules onto the surface of the starch granules. It is more likely that the formation of the highly ordered structure of amyllopectin in starch granules is the direct result of adding and arranging glucose units to the granule surface by starch synthesizing enzymes (Kram et al., 1993). Based on the present results, we propose that the structure of amyllopectin is formed at the surface of each starch granule by direct enzyme functions of the plastid–amyloplast system containing compound starch granules.

In this study, the propagation pattern of the plastid–amyloplast system and formative pattern of starch granule at the organelle level were revealed. It seems an effective step to improve the propagation pattern of the plastid–amyloplast system and formative pattern of starch granule at the organelle level in order to improve the yield and quality of starch in sweet potato. For further understanding of the mechanism of starch syn-
thesis in sweet potato tuberous root, it would be necessary to examine not only the localization of BE in detail, but also that of other enzymes such as granule-bound starch synthase and starch debranching enzyme in the plastid-amyloplast system.

References


*In Japanese with English summary.

**In Japanese with English abstract.