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B-type granule containing protrusions and interconnections between amyloplasts in developing wheat endosperm revealed by transmission electron microscopy and GFP expression

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Abstract

Starch granules in mature wheat endosperm show a bimodal size distribution. The formation of small starch granules in wheat endosperm cells was studied by transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) after expression and targeting of fluorescent protein into amyloplasts. Both techniques demonstrated the presence of protrusions emanating from A-type granules-containing amyloplasts and the presence of B-type starch granules in these evaginations. Moreover, CLSM recordings demonstrated the interconnection of the amyloplasts by these protrusions, suggesting a possible role of these protrusions in interplastid communication.

Key words: Amyloplasts, protrusions, B-type starch granules, CLSM, wheat endosperm.

Introduction

Endosperm of mature wheat and barley contains two major classes of starch granules. Formation of the large lenticular A-type granules initiates about 4–5 d post-anthesis (DPA). About 4 d later the final number of A-type granules is achieved (Briarty *et al*., 1979). Their size reaches up to 45 μm (Briarty *et al*., 1979), depending on cultivar (Dengate and Meredith, 1984) and season (Baruch *et al*., 1979). B-type starch granules are reported to be first initiated between 12–16 DPA (Parker, 1985) and between 16–22 DPA (Briarty *et al*., 1979). At grain maturity B-type granules vary in size between 1 μm and 10 μm and are spherical in shape. The final number of B-type granules per grain is affected by the environmental conditions during grain growth such as temperature (Tester *et al*., 1991; Cochrane *et al*., 1996).

The quality of starch extracted from wheat and barley is greatly influenced by the ratio of A- versus B-type granules. During the brewing process, a significant proportion of the B-type granules from barley is not gelatinized in the mash. Subsequently, this undegraded residue blocks the filter beds in lauter tuns, which results in increased run-off times (Tillett and Bryce, 1993). Therefore, a large number of small granules is unfavourable in the brewing industry. On the other hand, small granules are suitable as a paper coating and also find application in cosmetic products such as face powders (Ellis *et al*., 1998, and references herein). Thus, the production of cultivars containing starch enriched in the A-type granule fractions will improve the quality of the raw material for the brewer, whereas enrichment in the B-type granule fractions is favourable for the paper and cosmetic industry.

In the history of starch research there has been some disagreement about the origin of B-type granules. In 1958 Badenhuizen suggested they arose in mitochondria

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Abbreviations: CLSM, confocal laser scanning microscopy; DPA, days post anthesis; TEM, transmission electron microscopy; GFP, green fluorescent protein; LM, light microscopy; TP, transit peptide; YFP, yellow fluorescent protein.

(Badenhuizen, 1958). Buttrose, investigating B-granule formation in barley and wheat, concluded that the small granules were formed in vesicles budded off from outgrowths of the A-type granule-containing amyloplasts (Buttrose, 1960, 1963). Hughes endorsed this view (Hughes, 1976), but Duffus could not confirm the budding off of B-type granule containing vesicles (Duffus, 1979). Czaja studied granule formation by light microscopy only and concluded that there was no evidence for the formation of vesicles in which B-type granules developed (Czaja, 1982). However, using this method, exclusive evidence cannot be obtained, since protrusions are hardly visible at the light microscopy level. By using electron microscopy (EM), the presence of narrow protrusions between B-type granules and the parent amyloplast has been shown, but evidence for the budding off of B-type amyloplasts was lacking (Parker, 1985).

Since these EM observations were obtained, new technologies have been developed. Green Fluorescent Protein (GFP) of the jellyfish *Aequorea victoria* has been discovered as a powerful reporter enabling visualization of dynamic processes in living cells or organisms (for a review, see Gerdes *et al*., 1996). Modified versions of this reporter protein, such as Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP), are available. These fluorescent proteins can be targeted to specific subcellular organelles like mitochondria (Rizzuto *et al*., 1995; Köhler *et al*., 1997*a*) or plastids (Köhler *et al*., 1997*b*) by including a specific targeting sequence at the amino terminus. Thus, monitoring these proteins by confocal laser scanning microscopy (CLSM) and generation of 3D images is possible.

In order to gain more knowledge about B-type granule formation in fixed and in living endosperm cells, the presence of these small granules and the structure of amyloplasts were studied by EM as well as by CLSM. Here, the presence of B-type granules in protrusions of A-type granule-containing amyloplasts is demonstrated, conclusively confirming some of the earlier observations (Buttrose, 1960, 1963; Parker, 1985). Moreover, the interconnection of amyloplasts by these protrusions is shown using CLSM.

Materials and methods

Plant material and growth conditions

Wheat grains, *Triticum aestivum* L., cv. Minaret, were germinated in pots with a diameter of 6 cm (containing potting compost, perlite and peat in a 1 : 1 : 1, by vol., ratio) in a climate chamber at 20 °C/80% humidity with a 16 h photoperiod. After 1 week the seedlings were transferred to pots with a diameter of 15 cm in a phytotron with day/night temperatures of 15/12 °C, 80% humidity and a 16 h photoperiod. Light intensity at ear level was 24 klx. Ears were tagged when the first stamen appeared and harvested at the desired age, indicated as Days Post-Anthesis (DPA).

Transmission electron microscopy

Transverse sections, about 1.5 mm thick, were cut across the centre of each grain, fixed at room temperature in 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate (pH 7.2) for 16 h and post-fixed at room temperature for 2 h in 1% (w/v) osmium tetroxide in 0.1 M Na-cacodylate (pH 7.2). Samples were dehydrated in a graded ethanol series and infiltrated and embedded in Epon. Serial sections (100 nm) were cut on an ultramicrotome, collected on Formvar-coated grids, stained with uranyl acetate and examined in a Jeol Transmission Electron Microscope 1010 (TEM).

Confocal laser scanning microscopy

A plasmid containing the *gusA* reporter gene under the control of the rice actin promoter (McElroy *et al*., 1990) and the rice *RbcS* terminator (Pactin-GUS) was obtained from Sören Knudsen (McElroy group) of the Carlsberg Research Laboratory, Copenhagen. This construct was derived from plasmid pDM803, which is a pSP72 vector (Promega) containing two expression cassettes. The cassette consisting of the *Bar* selection marker gene under the control of the maize ubiquitin promoter and *nos* terminator was deleted, resulting in the Pactin-GUS plasmid. Pactin-GFP and Pactin-YFP were constructed by replacing the *gusA* coding region from Pactin-GUS by GFP(S65T) (Chiu *et al*., 1996) and YFP (Clontech) coding sequences respectively. *NcoI-NotI* fragments (the *NotI* sites made blunt using the Klenow fragment of DNA polymerase I) from pGFP(S65T) and pEYFP were cloned into the *NcoI* and *SmaI* sites of Pactin-GUS. A DNA fragment encoding the transit peptide (TP) from wheat granule bound starch synthase (Ainsworth *et al*., 1993) was constructed by PCR using genomic DNA from wheat as a template. The primers SP74 5′-CGCG**CCATGG**CGGCTCTG-3′ and SP75 5′-GG**CCATGG**TGGCGCGCACCACCATAGAGAGGCACC-3′ were used to remove an internal *NcoI* site and to introduce *NcoI* sites at both ends of the TP fragment. The PCR fragment was digested with *NcoI*, sequenced, isolated and introduced into the *NcoI* site of the Pactin-GFP and Pactin-YFP plasmids, resulting in Pactin-TP-GFP and Pactin-TP-YFP. The orientation was checked by restriction analysis.

Wheat grains (8–13 DPA) were cut in half longitudinally, perpendicular to the crease and placed on Petri dishes containing Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with 0.8% (w/v) agar, the cut side facing up. Low melting agarose (1%, w/v) containing MS medium was used to stick the grains to the solid medium. Grains were transiently transformed with one of the constructs described above, using the Biorad Biolistic Particle Delivery System-1000/He. The Petri dishes were transferred to a phytotron with a 16 h photoperiod (2 klx) and a temperature of 21 °C. Fluorescent cells were examined 1 d after bombardment in a Leica TCS/SP Confocal Laser Scanning Microscope using an excitation wavelength of 488 nm. Some of the recordings were restored by deconvolution with the Huygens System 2 program (Scientific Volume Imaging, The Netherlands) using an experimentally determined point spread function.

Results

In order to visualize putative protrusions emanating from A-type granule-containing amyloplasts, serial sections of wheat grains ranging from 8–13 DPA were examined by

transmission electron microscopy (TEM). Except for the aleurone layer, protrusions were observed throughout the endosperm from 8 DPA onwards, although in older cells the protrusions were less prominent. B-type granule formation was first visible at 11 DPA in plants grown under the conditions described in the Materials and methods section, in 2–3 cell layers from the sub-aleurone cell layer. Figures 1A and B show two serial sections of a string of B-type granules (Fig. 1A) which at first sight did not seem to be connected to the amyloplast with the A-type starch granule and protrusion (Fig. 1B). However, mounting the two sections together (Fig. 1C) suggested that the B-type granules are present in the protrusion emanating from the parent amyloplast. Figure 1D shows a B-type granule in a protrusion directly connected to an A-type granule-containing amyloplast (13 DPA).

In order to visualize these protrusions and their dynamics in living cells, GFP and YFP constructs were introduced into wheat endosperm of 9–13 DPA by particle bombardment. Bombardments with seeds older than 13 DPA were not successful. Most frequent transformations were obtained in cells 1–3 cell layers from the sub-aleurone layer. The constructs used contained a transit peptide from granule-bound starch synthase which enables the delivery of the fluorescent protein into the stroma of amyloplasts and other plastids. CLSM observations indeed showed targeting of GFP and YFP to the amyloplast stroma (Fig. 2A, B), visualizing starch granules as black areas within the amyloplast. In all cells examined amyloplast protrusions were clearly observed, and in some of these protuberances small B-type granules were visible (Fig. 2B, inlay). Moreover, amyloplasts seemed to be connected to each other (Fig. 2B, arrow). Figures 2C and D show two optical sections (4 μm apart) from the same cell as shown in Fig. 2B. A mount of these sections (Fig. 2E), demonstrated the interconnection of two amyloplasts by a protrusion. Study of the optical sections in between (each 0.20 μm apart) confirmed the physical connection between the amyloplasts (not shown). When a time-lapse recording with intervals of 7 s was performed, movement of the amyloplast protrusions was visible (results not shown at wwwimp.leidenuniv.nl/tno.html).

The protrusions varied in length from 2 μm to 30 μm and ranged in width between 0.5 μm and 1.5 μm, depending on the presence of B-type granules when observed by CLSM. This corresponds with the TEM observations, where the width of the protrusions ranged from 0.35–1.4 μm. The maximum length of the protuberances inferred from serial sections obtained by TEM was 16 μm. Considering the resistance of starch-containing material to sectioning and examination by TEM, the latter is probably an underestimation.

Taken together, results obtained by both TEM and CLSM techniques showed the presence of B-type granules in protrusions emanating from A-type granule-containing amyloplasts. Moreover, these observations showed the interconnection of amyloplasts by these protrusions.

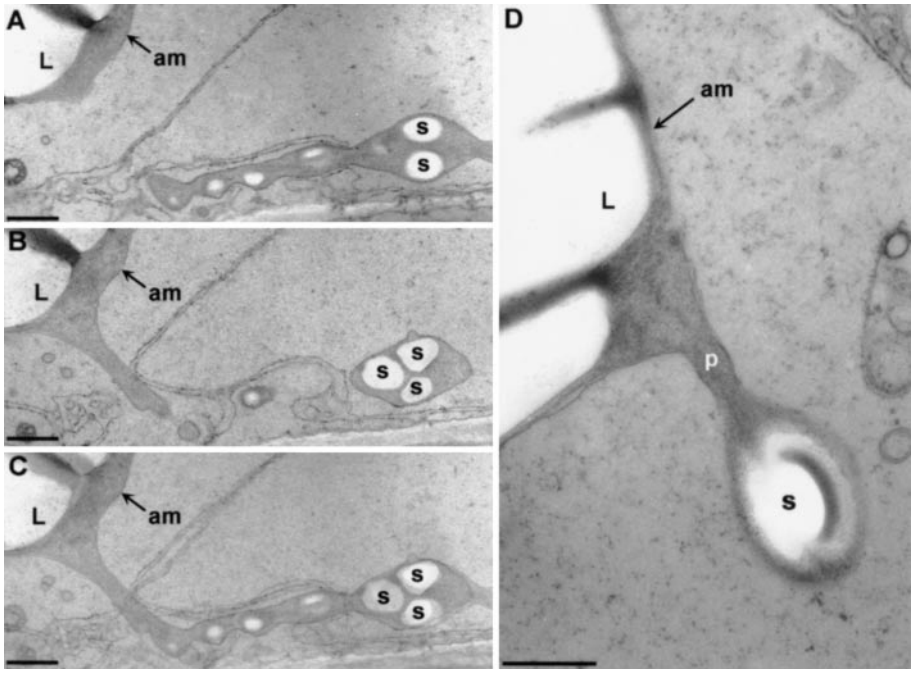

Fig. 1. TEM images of wheat endosperm. (A, B) sequential sections of an amyloplast (am) with A-type (L) and B-type (s) starch granules in 11 DPA endosperm. (C) Overlay of (A) and (B) strongly suggesting that the string of B-type granules is connected to the A-type granule-containing amyloplast. (D) B-type starch granule in a protrusion (p) emanating from the A-type granule-containing amyloplast in 13 DPA endosperm. Bars represent 1 μm.

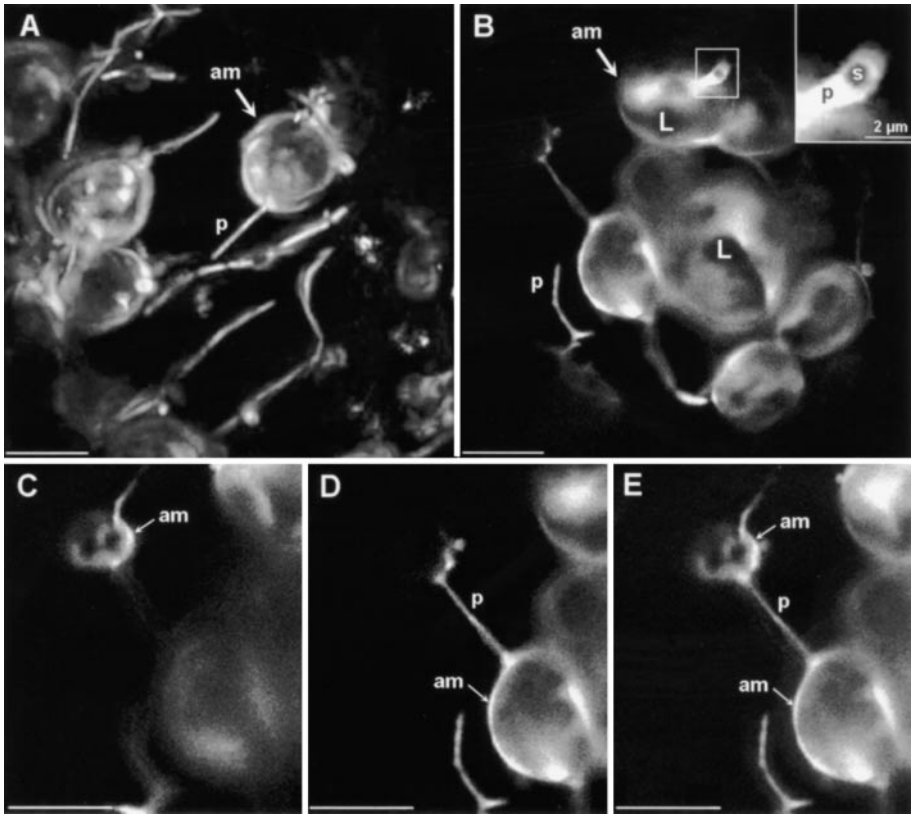

Fig. 2. CLSM images of wheat endosperm bombarded with either the Pactin-TP-GFP or the Pactin-TP-YFP construct. (A) GFP-labelled amyloplasts (am) with protrusions (p) in 13 DPA endosperm. The resolution of this image was computationally enhanced by deconvolution. (B) Optical section of YFP-labelled amyloplasts (am) with A-type (L) and B-type (s) starch granules in 11 DPA endosperm. The inlay shows a protrusion (p) in which a B-type starch granule is present. (C, D) Two optical sections (4 μm apart) of YFP-labelled amyloplasts (am) and a protrusion (p) in 11 DPA endosperm. (E) Overlay of (C) and (D) showing the interconnection of the amyloplasts by the protrusion. Bars represent 10 μm.

Discussion

Amyloplast structure and B-type granule formation in wheat endosperm were studied by TEM and CLSM. Both techniques showed protrusions in which B-type granules were present, confirming some of the earlier results (Buttrose, 1960, 1963; Parker, 1985). B-type granules were first detected in seed of 11 DPA. Buttrose and Parker observed B-type granule initiation about 14 DPA (Buttrose, 1963; Parker, 1985). This difference can be explained considering the wheat cultivar and growth conditions used. Although grains of different ages were analysed, the variation in development within one age was huge. This is likely to be due to the development of the endosperm, the place of the grain in the ear, the sequential order of the ear on the plant, and the labelling procedure.

CLSM enabled the amyloplasts in living cells to be visualized and showed the interconnection of amyloplasts by these protrusions. These observations are consistent with the results from Köhler *et al.* who showed connections between chloroplasts, starting as protrusions emanating from the chloroplasts (Köhler *et al.*, 1997*b*). These connections are 0.35–0.85 μm wide with a maximum length of 15 μm. They demonstrated the exchange of molecules through these protrusions, suggesting the presence of a communication system facilitating the co-ordination of plastid activities.

In all endosperm cells examined by CLSM, protrusions were present. Because only the outer cell layers (1–3 layers from the sub-aleuron layer) were transiently transformed, it is possible that the interplastid connections are a characteristic of young endosperm cells. When older cell layers were examined by TEM, protrusions were less abundant, augmenting the possibility that protrusions are a developmental phenomenon.

Up to now, it has not been possible to visualize a starch granule larger than 20 μm using bombardment labelling and there has been no success in obtaining GFP or YFP expression in grains older than 13 DPA, both possibly due to the increasing amount of starch, impeding delivery of the construct to the nucleus. In order to get an overall view of amyloplast development in older grains, transgenic plants expressing the TP-YFP construct would be useful tools.

It can be concluded that new microscopic techniques such as CLSM in combination with the use of fluorescent protein labelling permit the visualization of protrusions in three dimensions. It has been demonstrated that amyloplasts are interconnected by these protrusions. More research is required to elucidate the exact function of the connections between these plastids.

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