Light-Induced Degradation of Starch Granules in Turions of *Spirodela polyrhiza* Studied by Electron Microscopy

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*Spirodela polyrhiza* forms turions, starch-storing perennial organs. The light-induced process of starch degradation starts with an erosion of the surface of starch grains. The grain size decreases over a period of red irradiation and the surface becomes rougher. The existence of funnel-shaped erosion structures demonstrates that starch degradation is also possible inside the grains. Neither etioplasts nor clues as to their transition into chloroplasts were found in the storage tissue by transmission electron microscopy. Juvenile chloroplasts always contained the starch grains which remained from amyloplasts. No chloroplasts were found which developed independently of starch grains. Amyloplasts are therefore the only source of chloroplasts in the cells of irradiated turions. The intactness of amyloplast envelope membranes could not be directly proved by electron microscopy. However, the light-induced transition of amyloplasts into chloroplasts provides indirect evidence for the integrity of the envelope membranes throughout the whole process. The starch grains are sequestered from the cytosolic enzymes, and only plastid-localized enzymes, which have access to the starch grains, can carry out starch degradation. In this respect the turion system resembles transitory starch degradation as known from Arabidopsis leaves. On the other hand, with α-amylase playing the dominant role, it resembles the mechanism operating in the endosperm of cereals. Thus, turions appear to possess a unique system of starch degradation in plants combining elements from both known starch-storing systems.

**Keywords:** Electron microscopy • *Spirodela polyrhiza* • Starch degradation • Starch grains • Storage starch • Turion.

**Abbreviations:** SEM, scanning electron microscopy; TEM, transmission electron microscopy.

**Introduction**

Starch is the major form of carbon storage in plants. Interest in the nature and control of starch degradation began a long time ago because the germinating cereal caryopses, like those of barley or maize, produce sugars that can be fermented into alcohol. Like the biosynthesis of starch and the formation of granules, starch degradation is a complex process (Smith et al. 2004, Zeeman et al. 2007a, Zeeman et al. 2007b, Fettke et al. 2009). Elucidation of the starch-degrading pathway is complicated by the fact that many of the enzymes involved exist as multiple isoforms localized both within plastids and, extraplastidically, in the cytosol (Smith et al. 2004, Zeeman et al. 2007a). Moreover, while the phosphorylation of starch has been proved to be essential for its degradation (Lorbert et al. 1998), the related enzymology has been determined only recently (Lloyd et al. 2005, Ritte et al. 2006, Kötting et al. 2009).

Two contrasting models have been postulated for starch degradation, one for the storage system of cereals and the other for transitory starch.

First, the process of starch degradation is well understood in the endosperm of cereals (Ritchie et al. 2000). Because the plastidic membranes lose their integrity in the process of seed maturation, all enzymes, regardless of their original localization, have access to starch granules. α-Amylase plays a major role in the initial degradation in this system (Ziegler 1995, Asatsuma et al. 2005). Both the synthesis and degradation of storage starch in caryopses takes place within the annual cycle, i.e. synthesis proceeds towards the end of the vegetation season and degradation at the beginning of the next vegetation season.

There can also be a diurnal pattern, with starch typically synthesized during the day and degraded during the night (Smith et al. 2004, Zeeman et al. 2007b). This transitory starch is localized within chloroplasts in leaves, and intact plastidic membranes prevent it from being attacked by
Degradation of storage starch

Extraplastidic enzymes. Starch degradation has been shown to be undisturbed in an Arabidopsis thaliana mutant lacking plastidic α-amylase (Yu et al. 2005). It has therefore been concluded that α-amylase is not necessary for the degradation of transitory starch in Arabidopsis leaves under normal conditions (Zeeman et al. 2007a, Zeeman et al. 2007b). There are two essential steps in transitory starch degradation. These are phosphorylation by glucan–water dikinase (Reimann et al. 2004, Ritte et al. 2006) along with phosphoglucan–water dikinase (Ritte et al. 2006), and dephosphorylation, e.g. with SEX4 (Köting et al. 2009, Comparot-Moss et al. 2010). Whereas starch phosphorylation is known in many plant species, starch dephosphorylation has been investigated only in A. thaliana. Enzymatic starch phosphorylation has been shown to initiate the phase transition of glucans from a crystalline to a less ordered, hydrated state (Hejazi et al. 2009). It has been suggested that this mechanism makes leaf starch accessible to β-amylases and, to a lesser degree, to isoamylase ISA3 (Zeeman et al. 2007a, Zeeman et al. 2007b, Fulton et al. 2008; cf. Fettke et al. 2009).

Thus, there is little in common between the two systems considered as models for starch degradation in plants. Membrane breakdown in the cereal storage system gives all enzymes access to starch granules, with a dominating role for α-amylase. Conversely, membranes remain intact in the case of transitory leaf starch, with a dominant role for plastidic β-amylase. Similarly, in a transgenic potato with the repressed disproportionating enzyme (stDPE2; 4-α-glucanotransferase), starch degradation was almost completely inhibited in leaves (transitory starch) but undisturbed in tubers (storage starch) (Lloyd et al. 2004). From a knowledge of whether the relevant plastidic membranes (chloroplasts or amyloplasts) remain intact throughout the process of starch degradation it is possible to answer the question of whether only plastidic or both plastidic and extraplastidic enzymes have access to starch granules and play a role in the metabolic process.

In this paper we focus on yet another interesting system of starch degradation operating in Spirodela polyrhiza. This aquatic duckweed propagates itself from year to year by forming turions, which are vegetative organs of perennation (Landolt and Kandeler 1987, Appenroth et al. 1989, Appenroth and Nickel 2010). Formed in the late summer, turions germinate in the following spring. Starch is the main storage compound accumulated in turions throughout winter (Henssen 1954, Doelger et al. 1997). Its degradation products support the fast growth of a newly formed frond following turion germination in spring (Ley et al. 1997).

Two sections through a turion are shown in Fig. 1. Two frond primordia in pockets are shown in the upper section (Fig. 1B), while the storage tissue is shown in the lower section (Fig. 1C). The storage tissue is filled with starch granules (magnified in Fig. 1D). Following germination (i.e. the emergence of the frond primordia from the pockets), starch is degraded, and the low molecular weight carbohydrates that are produced fuel the fast growth of the newly formed fronds. This allows S. polyrhiza to follow the strategy of space occupation (r-strategy of population dynamics) which results in a rapid spread of the plant on the water surface in spring (Appenroth 2008). In order to understand the underlying mechanism of fast starch degradation we investigated whether starch is degraded only by surface erosion or, additionally, inside the granules.

The starch breakdown is controlled by light absorbed by the phytochrome, requiring exposure to red light. In this particular case, a single pulse of red light is not sufficient; continuous red irradiation or repeated pulses of red light over a period of several days are necessary (Appenroth and Gabrys 2001, Appenroth and Gabrys 2003). The irradiation of dark-adapted turions with continuous red light results in starch degradation, which progresses at an approximately constant rate for several days after a lag phase of about 24 h (Doelger et al. 1997). This is the only instance of higher plant starch degradation known to
be mediated by phytochrome. Turions are formed in late summer while starch degradation proceeds in spring. Thus, biosynthesis and degradation are well separated in time and the starch storage is clearly subject to an annual cycle—as in the model system caryopsis.

In a previous report α-amylase associated with the surface of starch granules was shown to play a crucial role in the regulation of starch degradation in the turions of *S. polyrhiza* (Reimann et al. 2007, Appenroth and Ziegler 2008). In this respect, turions also resemble the endosperm system in car- yopses. Two potential scenarios for the light induction of starch degradation in turions have been discussed (Reimann et al. 2007). According to one, amyloplast envelope membranes lose their integrity, allowing the originally extraplastidic en-
ymes to penetrate into the amyloplasts. Alternatively, irradi-
ation eliminates an inhibitor of starch-associated en-
zymes to penetrate into the amyloplasts. According to this model, the greening of etiolated turions results from the transformation of etioplasts into chloroplasts or, alternatively, from amyloplasts to chloroplasts. The transition from amyloplasts to chloroplasts would provide indirect evidence that the outer plastidic membranes remained intact because chloroplasts cannot develop from damaged amyloplasts.

The following questions are addressed in the present paper (i) Are starch granules mainly degraded by surface erosion (thus removing the original binding sites for proteins) or is degradation from the inside also possible? (ii) Is the greening of etiolated turions based on the transformation of etioplasts into chloroplasts, or are amyloplasts the source of newly formed chloroplasts? (iii) Are starch granules located within intact plastidic membranes or do extraplastidic enzymes have access to starch?

**Results and Discussion**

**Influence of starch degradation on size distribution of grains**

In order to investigate the degradation of starch granules in the storage tissue of *S. polyrhiza*, turions were irradiated for 24, 48, 72 or 96 h, or they were kept in the dark. Starch granules were isolated and their sizes were measured using scanning electron microscopy (SEM) scans. The photographs in **Fig. 2A and B** show typical examples of measured granules from a non-irradiated tissue and from one irradiated for 96 h. **Fig. 2C** shows the size distribution of starch granules for differently irradiated turions. The area (μm²) of the granule projections was measured using SEM pictures, and relative distributions within 10 μm² intervals were calculated for dark control samples and for those irradiated for 24, 48, 72 or 96 h.

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ificant change in size distribution detected: the fraction of the smallest grains rose abruptly to >50% and no grains with cross-sections >50 μm² were recorded. This distribution did not change substantially after the next 24 h of irradiation. Thus, SEM investigations demonstrated that the granules became smaller due to light-induced starch degradation.

**Influence of starch degradation on the grain surface**

Apart from this reduction in size, red irradiation of turions also affected the surface of starch granules. The first changes in the roughness of the surface were detectable as early as within 24 h
of irradiation, as shown by SEM (Fig. 3). At this time, the overall size of the granules was still unchanged (Fig. 2C). Accordingly, no change in starch content was detected by chemical analysis after 24 h red irradiation (Doelger et al. 1997, Ley et al. 1997). On the other hand, the strongest α-amylase association with the starch surface and the strongest phosphorylation of that surface were recorded for this irradiation time in previous studies (Reimann et al. 2004, Reimann et al. 2007). Thus, the initial 24 h of red irradiation can be considered a preparatory phase for starch degradation in turions. In this period the starch surface becomes phosphorylated by two enzymes, glucan–water dikinase (Reimann et al. 2002) and, most probably, phosphoglucan–water dikinase (Ritte et al. 2006). During the subsequent phase of actual degradation the surface becomes evidently rougher, with granules being heavily damaged (Fig. 3, cf. 1cR and 2cR/3cR). In this phase (starting after 24 h of irradiation) both the phosphate content and the amount of bound α-amylase decrease (Reimann et al. 2007). Evidently, the enhanced roughness reflects the fact that the phosphorylated glucose moieties and the binding sites for proteins are removed from the granule surface during starch degradation. These results confirm the model for starch degradation in turions as presented by Reimann et al. (2004) and Appenroth and Ziegler (2008) on the basis of biochemical analyses.

**Starch degradation inside grains**

Cracks observed after 72 h of constant irradiation suggested that degradation could also proceed inside the granules. An example of such a crack, marked with an arrowhead in Fig. 3, 3cR, most probably represents the opening of an internal channel at the surface (as if we looked into the channel); the smaller fissures below are signs of superficial erosion. Because SEM is unsuitable for investigating this effect in detail, additional experiments were carried out using the freeze-fracture technique. The image of a fractured granule in Fig. 4 provides evidence that this internal degradation started even earlier than assumed on the basis of SEM analysis alone: after 48 h several distinct radial-oriented channels were detected close to the surface (marked with arrowheads in Fig. 4B). These funnel-shaped structures were generated during electron microscopy preparation when liquid inclusions, formed in the starch body due to the activity of starch-degrading enzymes, were frozen. Dark control samples did not contain such areas (Fig. 4A). Thus, freeze-fracture microscopy provided evidence that the degradation observed initially at the surface is not the only mechanism. Instead, starch degradation also proceeded inside the starch granules, which might be the reason for the high speed of degradation following the germination of turions. In summary, two different mechanisms of starch grain degradation exist in turions, and the related processes operate in parallel. This answers the first question raised in the Introduction.

**Intactness of amyloplast membranes as investigated by TEM**

TEM of the control, dark-adapted turions (Fig. 5A, B) shows storage cells densely filled with starch granules in accordance with a previous chemical analysis (Appenroth and Gabrys 2001) and with the results of SEM (Fig. 1). Some starch grains can be seen inside epidermal cells although they are much smaller and less numerous than those in the main storage tissue (Fig. 5A, inset). It should be noted that epidermal cells normally do not contain starch in terrestrial plants.

Although some outlines can be seen (several examples are marked with arrowheads in Figs. 5 and 6B), we were unable to show intact membranes surrounding the granules. This could be due to the strong structural non-homogeneity of the storage tissue between areas of high density (starch granules) and lower density (cytoplasm) which made its preparation difficult. Thus, the TEM of the control samples does not provide unequivocal information as to whether the starch-storing plastids are undamaged.

**Changes in the tissue upon illumination and the transition from amyloplasts to chloroplasts**

After 48 h of constant illumination the starch granules are smaller and less numerous (Fig. 6A). Two different subpopulations of starch granules can be distinguished: one with an electron-dense material lining the surface (marked with black arrowheads), and a second with radial ‘channels’ sometimes crossing the whole granule (marked with white arrowheads). Distinct clusters of 2–3 granules can be detected (Fig. 6B). We assume that this image represents an early stage of chloroplast
**Fig. 4** Electron micrograph (freeze-etching technique) of starch granules isolated from (A) a control, dark-stored turion, (B) a turion irradiated for 48 h with continuous red light. Arrowheads indicate erosion channels caused by starch degradation. Bars = 1 μm.

**Fig. 5** Transmission electron micrographs of the storage tissue of dark-adapted turions. Arrowheads mark the remains of membranes surrounding starch grains. A, bar = 10 μm; inset: small starch grains in an epidermal cell. B, bar = 3 μm.

**Fig. 6** Transmission electron micrographs of the storage tissue of turions after 48 h irradiation with continuous red light. (A) Starch grains belonging to two subpopulations are marked with: (1) black arrowheads—an electron-dense material lining the surface, and (2) white arrowheads—radial channels; bar 10 = μm. (B) Arrowheads mark the remains of membranes surrounding starch grains; bar = 2 μm.
organization. More advanced stages are demonstrated in Fig. 7A and B in tissue illuminated for 96 h. Numerous lipid-rich droplets present all over the cells at the early stage disappeared in the course of subsequent chloroplast development (compare Figs. 6A and 7A). On the other hand, large groups of mitochondria became visible in the storage tissue under development (Fig. 7B, inset). It is noteworthy that mitochondria were hardly detectable in the dark controls. Both observations, the disappearance of lipid-rich droplets and the presence of numerous mitochondria, are evidence for high metabolic activity in the cells illuminated for 96 h.

The juvenile chloroplasts developed after 96 h of permanent illumination always contained the starch granules which remained. Two typical examples of developing chloroplast structures are given in Fig. 7A and B. Note the commencement of grana formation at this stage (Fig. 7A, inset). To date no indication has been obtained as to the origin of chloroplasts in the greening turion tissue. One possibility is that they develop from etioplasts; another that they are transformed amyloplasts. The process of greening has been investigated in etiolated turions subjected to continuous red light, but the fluorescence microscopy used in that study (Appenroth and Bergfeld 1993) could not provide any answer to this problem. In the few hundred cells analyzed in the present study, not a single chloroplast was found to develop independently of starch granules. The degradation of amyloplast starch granules and the development of the chloroplast thylakoid system were observed to proceed simultaneously. An analysis of all TEM images of the irradiated tissue points to amyloplasts as the sole source of chloroplasts in the storage tissue of S. polyrhiza turions. This answers the second question.

As mentioned above, TEM did not provide unequivocal evidence as to whether the amyloplast membranes of the control samples were completely intact. However, indirect evidence is provided by the observation that each chloroplast developed from an amyloplast which had intact envelope membranes surrounding one or several starch granules. This is clearly demonstrated in Fig. 7A and B. Thus, the membrane must have been intact throughout the whole process and only plastidic enzymes had access to starch. Apart from the starch-phosphorylating enzymes glucan- and phosphogluco- water dikinases, this could also be possible for α-amylase, β-amylase, a disproportionating enzyme, a debranching enzyme and α-glucosidase, all of which have plastidic isoforms (Zeeman et al. 2007a). This answers the third question.

As already mentioned in the Introduction, light might possibly start the degradation process (i) by disturbing the integrity of amyloplast membranes and permitting the access of extraplastidic enzymes to the granule, or (ii) by removing or inactivating an inhibitor of starch-associated α-amylase (cf. Reimann et al. 2007). The present results are consistent with the assumption that the membranes are intact, because only that would guarantee the normal development of the photosynthetic apparatus from amyloplasts. Thus, our results do not support the first mentioned possibility.

**Mechanism of starch degradation in turions**

There are two distinct models which describe starch degradation, one referring to storage starch in caryopses, and the second to transitory starch in leaves. In the endosperm of germinating caryopses starch granules are not sequestered by any membranes, which results in the unrestricted access of α-amylases (Ritchie et al. 2000). In contrast, transitory starch is protected by the inner chloroplast membrane from the cytoplasmic enzymes which represent the bulk starch-degrading activity in a leaf mesophyll cell (Smith et al. 2003, Lu and Sharkey 2006). In that respect the turion system is similar to the mechanism of transitory starch degradation. As shown recently, α-amylases do not play an essential role in the degradation of transitory starch in the chloroplasts of A. thaliana (Yu et al. 2005). In contrast, these enzymes have been shown to play a decisive role in starch degradation in turions, similar to the caryopsis system (Reimann et al. 2007, cf. Köting et al. 2009). Thus, the system operating in turions appears to combine elements from both described models.
Materials and Methods

Plant material and irradiation

Etiolated turions of the duckweed *S. polyrhiza* (L.) Schleiden, clone Sj, were produced under phosphate-limiting conditions as described by Appenroth et al. (1996). The turions were harvested and kept for 28 d in darkness at 5 ± 1°C to break dormancy. The turions were then transferred into a fresh nutrient medium (Appenroth et al. 1996) and kept for 72 h at 25 ± 0.1°C in darkness to adapt to the new temperature. At this point the non-dormant and ‘dark-adapted’ turions were subjected to experimental irradiation at 25°C and sampling. Turions were either kept in darkness (‘Dark’) or irradiated for 24, 48, 72 or 96 h. All irradiations were done with continuous red light (658 nm, half-band width 25 nm) at a fluence rate of 12 μmol m⁻² s⁻¹ (details of the light sources are given in Reimann et al. 2002). Irrespective of the storage time in the dark at 25°C, starch grains did not show any changes (data not shown). The isolation of starch granules has been described previously (Reimann et al. 2002).

Scanning electron microscopy

Turions or isolated starch granules were fixed in 2% (v/v) glutaraldehyde in a 0.05 M cacodylate buffer at pH 7.2 at room temperature for 4 h, rinsed in the same buffer and post-fixed in 1% (w/v) osmium tetroxide at room temperature for 1 h. Following thorough rinsing, the material was dehydrated in a graded acetone series with a final passage in 100% (v/v) acetone (details of the light sources are given in Reimann et al. 2002). The isolation of starch granules has been described previously (Reimann et al. 2002).

Measuring the cross-sections

The granule cross-sections were analyzed using negatives obtained from SEM. All the SEM images were taken at a magnification of ×3,500. The negatives were scanned in a NIKON Eclipse 2000 microscope using an Evolution VF camera (Media Cybernetics). Between 60 and 170 granules were analyzed for each irradiation time. The analysis was carried out using Image-Pro Plus 6.0 software. The area was determined in triplicate for each granule and the averages were taken for analysis.

Freeze-fracture electron microscopy

Isolated starch granules were dehydrated in a graded acetone series with a final concentration of 70% (v/v) sucrose and stored until use. After rehydration in purified water the samples were sandwiched between thin copper profiles and quick-frozen in liquid propane cooled in liquid nitrogen (cooling rate approximately 5,000 K s⁻¹). Fracture, etching (−120°C, 30 s) and replication were carried out with a BAF 400 T freezing-fracture device (Balzers) using a double-replica stage and electron gun evaporators. Pt (C) was deposited on the etched fracture faces under a 35° angle to a controlled thickness of about 2 nm. The replicas were cleaned in 70% (w/v) sulfuric acid, washed three times with purified water, placed on uncoated copper grids, and examined in an EM 900 electron microscope (Zeiss, Jena, Germany).

Transmission electron microscopy

Samples of turions (control and irradiated for 48 or 96 h) were immersed in an ice-cold fixative containing 1.5% (v/v) glutaraldehyde and 0.5% (w/v) paraformaldehyde in a 0.165 M phosphate buffer (pH 7.2) for 5 h at room temperature under constant stirring. The tissues were then rinsed in four changes of phosphate buffer and left overnight at 4°C. The next day, the turions were post-fixed in 1% osmium tetroxide (w/v) in a 0.1 M phosphate buffer (pH 7.2) for 1.5 h at room temperature, washed briefly in distilled water, dehydrated in grades of ethanol, cleared in propylene oxide and embedded in Poly-Bed 812 epoxy resin. Semi-thin sections were then cut on an LKB ultramicrotome II with glass knives, stained in a mixture of toluidine blue and azure II in a borax solution and examined by light microscopy. Ultra-thin sections were cut with a diamond knife, then mounted on copper grids and contrasted with uranyl acetate and lead citrate. The sections (15–20 for each irradiation time) were finally examined in a JEOL 100 S-X transmission electron microscope. All the cells (and grains) come from the central part of the storage tissue. We did not find differences with cells from the outer regions of the storage tissue.

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References


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