Biosynthesis and Degradation of Starch

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Summary

Higher plants accumulate transitory and storage starches in chloroplasts of illuminated photosynthetic organs and amyloplasts of heterotrophic organs, respectively. In addition to the plastids, the cytosol is also involved in the control of starch biosynthesis. These compartments are metabolically interconnected by means of carriers localized in the envelope membrane of the plastid. Multiple enzymes involved in starch biosynthesis have been identified from the genetic and biochemical analyses of various plant mutants exhibiting phenotypic changes in starch accumulation. Especially, genetic mutants of maize have offered valuable information to elucidate the role and function of various enzymes in starch biosynthesis. With the involvement of genetic manipulation techniques in the last decade, it has became theoretically possible to manipulate and create plants with novel, useful, and powerful property of starch.

We summarize the knowledge on plant starch metabolism and introduce the developments on the procedure and regulation of starch biosynthesis and on the mechanism of energy recovery through degradation of starch.

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Starch biosynthesis

Sucrose and starch biosynthesis

Arrival of sucrose into terminal sink organs is a dynamic process that can take either a symplastic and/or apoplastic route depending on the type of organ and developmental state (Patrick, 1997). In either case, the overall ability of the heterotrophic organ to attract photoassimilates is strongly dependent on the capacity of individual cells to import, metabolize and store sucrose, ultimately determining plant productivity and crop yield (Ho, 1988).

In organs where apoplastic sucrose unloading occurs, different routes can be envisaged for the subsequent uptake into storage cells: (a) hydrolysis of sucrose by an apoplastic invertase and subsequent uptake of glucose and fructose, and/or (b) import of sucrose by plasmalemma bound carriers. In addition, sucrose can also be taken up by endocytosis and transported to the central vacuole (Etxeberria *et al*., 2005) Subsequent conversion of internalized sucrose to starch involves a series of enzymatic reactions wherein sucrose synthase predominantly controls sucrose breakdown and production of a C6 starch precursor molecule entering the amyloplast (Baroja-Fernández *et al*., 2003). Results obtained from sycamore cultured cells, potato tuber slices and developing barley endosperms treated with potent endocytic inhibitors suggest the presence of an important pool of apoplastic sucrose produced by endocytosis prior to its subsequent conversion into starch in heterotrophic organsn of both mono- and di-cotyldonous plants (Baroja-Fernández *et al*., 2006)

Pathway of starch biosynthesis

Genetic and biochemical data demonstrate that starch biosynthesis is mediated by at least four enzymes: ADP-glucose pyrophosphorylase (ADP: α-D-glucose-1-phosphate adenyltransferase, AGPase, EC 2.7.7.27), starch synthase (ADP-glucose:(1,4)-α-D-glucan 4-α-D-glucosyltransferase, SS, EC 2.4.1.21 and 2.4.1.242), starch-branching enzyme ((1,4)-α-D-glucan:(1,4)-α-D-glucan 6-α-D-((1,4)-α-D-glucano)-transferase SBE, EC 2.4.1.18), and starch-debranching enzyme (DBE; EC 3.2.1.68 for isoamylase-type and EC 3.2.1.41 for pullulanase-type). In starch biosynthesis, ADP-glucose is the glucose donor for α-glucan elongation and is formed from ATP and glucose 1-phosphate (G1P) by AGPase. SS catalyzes the transfer of glucose moiety from ADP-glucose to the non-reducing end of an α-glucan and is responsible for the elongation of amylose and amylopectin molecules. SBE catalyzes hydrolysis of an α-1,4-linkage and the subsequent transfer of an α-1,4 glucan to form an α-1,6 branching point. Hence, the catalytic actions of these enzymes synthesize non-branched and branched glucans; the sequential actions by SS, theoretically synthesize non-branched glucans and a cooperative actions of SS and SBE produces branched glucans. In fact, the analysis of glucans synthesized by *E. coli* cells co-expressing maize SSs and SBEs suggested that the function of SS and SBE is important for determining the structure of α-glucan, but the *in vivo* synthesis of amylose and amylopectin is not so simple. *Sugary-1* (*su1*) mutants isolated form maize and rice have the reduced starch content and accumulation of water-soluble and highly branched phytoglycogens. Since the *su1* loci encoded the genes for

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isoamylases, the formation of the highly ordered amylopectin *in vivo* has been believed to require the actions of as well SSs and SBEs as of isoamylases. For more detailes reviews on starch biosynthesis are recommended (e.g. Smith, 1999; Kavakli *et al*., 2000; Kossmann and Lloyd, 2000; Myers *et al*., 2000; Nakamura, 2002; Ball and Morell, 2003). Although participation of various players in plant starch biosynthsis has been demonstrated, much remains to be elucidated about the regulation of each of these players, the interactions between the players and the influence of environment, finally.

Regulation of AGPase

Function of small and large subunits

Plant AGPase is a heterotetrameric enzyme composed of a pair of small and large subunits that are encoded by distinct genes (Smith-White and Preiss, 1992). Comparison of amino acid sequences of AGPases from various plant species showed that there are (i) about 90% identity among small subunits, (ii) 50-70% identity among large subunits, and (iii) 40-50% identity between small and large subunits, suggesting that both subunit genes were diverged from a common ancestral gene (Ballicora *et al*., 2005). Both subunits have evolved differentially with a different function. Small subunits have kept the catalytic ability, while large subunits have acquired the modulatory function without catalytic ability by discarding the critical residues for catalysis.

Most plant AGPases are allosterically regulated by small effector molecules. Analysis of potato tuber AGPase expressed in *E. coli* cells indicated that both subunits are required for the optimal enzyme activity but have non-equivalent roles in enzyme function (Iglesias *et al*., 1993). The large subunit plays more of regulatory role while the small subunit has both catalytic and regulatory functions (Kavakli *et al*., 2002; Frueauf *et al*., 2003; Cross *et al*., 2004). In the absence of large subunits, small subunits are able to self-assemble to yield a homotetrameric enzyme that still shows the catalytic ability and regulatory properties (Ballicora *et al*., 1995).

Allosteric and redox regulation of AGPase

AGPase is a key regulatory enzyme of plant starch biosynthesis as it controls carbon flux *via* the allosteric and redox regulation. The importance of AGPase activity in starch metabolism is readily seen in genetic mutants defective in small or large subunit such as the *shrunken2* and *brittle2* of maize (Giroux and Hannah, 1994) and the *adg1* and *adg2* of *Arabidopsis* (Wang *et al*., 1997 and 1998). Seeds from the maize mutants have a high sugar content and the decreased starch levels, and leaves from the *Arabidopsis* mutants accumulate a low or no transitory starch. A similar situation can be observed by antisense expression of AGPase gene (Müller-Röber *et al*., 1992).

The plant AGPases are allosterically activated by 3-phosphoglycerate (3-PGA) and inhibited by orthophosphate (Pi) (Sivak and Preiss, 1998). The increased 3-PGA levels by carbon fixation and decreased Pi levels by photophosphorylation during light period activate AGPase activity and promote the biosynthesis of transitory starch, while the decreased 3-PGA and phosphate metabolites during dark period inactivate AGPase activity and suppress starch biosynthesis. Likewise leaf enzymes, AGPases from potato tubers and maize and rice endosperms are also allosterically regulated by 3-PGA and Pi (Sowokinos and Preiss, 1982; Plaxton and Preiss, 1987; Sikka *et al*., 2001). In contrast, AGPases from barley and wheat endosperms and pea and bean embryos show a little sensitive or insensitive to 3-PGA and Pi (Ballicora et al., 2004). It is completely unclear, but in maize mutant with AGPase allosteric mutation of decreased sensitivity to Pi, the seed weights have 15% increase of normal seeds, suggesting the allosteric regulation of AGPases is important for storage starch synthesis in maize (Giroux *et al*., 1996).

Recently, the crystallographic structure of potato tuber AGPase was elucidated (Jin et al., 2005). The three-dimensional structure was of an inactive form of homotetrameric enzyme, but provided some insight into the conformational change by the binding of allosteric effectors. Site-directed mutagenesis study indicated that the mutations in the ATP binding site reduce the affinity for not only ATP but also G1P and 3-PGA. Interestingly, the ATP binding site on the structural model locates between the allosteric effector binding site and oscillatory structure, suggesting that the ATP binding to large subunit functions like an allosteric effector and causes a conformational change. Structural analysis of a heterotetramer will lead to better understanding of the allosteric regulatory property.

Redox regulation is an essential system for maintaining the homeostasis of the reduction/oxidation state in a cell. The ferredoxin-thioredoxin system plays a central role for light-dependent regulation of some chloroplastic enzymes. Reduced thioredoxin reduces the disulfide bonds of various target enzymes and consequently their enzyme activities are modulated (Buchanan and Balmer, 2005). Potato tuber AGPase has an intermolecular disulfide bridge between the cysteine residues of the two small subunits. Reduction of the disulfide bond is achieved by thioredoxin due to the increases in affinity for ATP and in sensitivity to 3-PGA (Ballicora *et al*., 2000). Recent studies showed that sugars provide the redox activation of potato tuber AGPase *via* two signaling pathways involving in a sucrose non-fermenting (SNF)-related kinase and hexokinase (Tiessen *et al*., 2002 and 2003). Likewise, leaf AGPase of *Arabidopsis* was also found to be subject to redox activation by not only light but also sugars including trehalose 6-phosphate (Hendriks *et al*., 2003; Kolbe *et al*., 2005; Lunn *et al*., 2006). The cysteine residue is conserved in small subunits of leaf AGPases except for the monocot endosperm forms that are cytosolic enzymes (See the following section, “Subcellular localization of AGPases”). While some issues, such as identification of AGPase activating thioredoxin isoforms and elucidation of sugar-signaling pathways for redox regulation, still remain to be solved, the results obtained to date suggest that plant leaf AGPases are redox-

regulated *via* light- and sugar-dependent signals and the reduction leads to the conformational change for effective binding of allosteric effectors.

Subcellular localization of plant AGPases in non-photosynthetic tissues

Currently, at least two distinct types of AGPases, cytosolic and plastidial forms, are believed to be in cereal endosperms **(Fig. 1)** (Beckles *et al*., 2001; James *et al*., 2003). Thorbjørnsen et al. (1996) indicated that an alternative splicing of the first exon for the barley AGPase small subunit gene gives rise to two distinct transcripts whose products locate in the plastid and cytosol in the barley endosperm. Similar observations are obtained from the small subunit genes for wheat and rice AGPases (Burton *et al*., 2002a; Ohdan *et al*., 2005). Unlike these plants, maize utilizes two different genes for AGPase small subunits of cytosolic and plastidic forms (Hannah *et al*., 2001).

The regulation of the cytosolic AGPases is not fully understood. As explained above, conformational change by reduction of the cysteine residues is required for the effective allosteric regulation of AGPase in leaves. However, the cysteine residue involved in the redox regulation is not conserved in the cytosolic AGPase small subunits of cereal endosperms. Because AGPases from barley and wheat endosperms are insensitive to allosteric effectors, these enzymes are likely not regulated by redox. In contrast, rice and maize cytosolic AGPase activities are affected by the allosteric molecules. This suggests that these AGPases are allosterically regulated without the reduction or that another cysteine residue engages with the redox regulation.

ADP-glucose translocator

The occurrence of extraplastidial AGPases implies that ADP-glucose formed in cytosol needs to be transferred to plastids *via* a carrier protein. Endosperms of the maize *brittle1 (bt1)* mutant display a low starch phenotype, since they accumulate 20% of the normal starch content. In addition, this mutant accumulates high levels of ADP-glucose (10-fold higher than wild-type), strongly suggesting that BT1 is an ADP-glucose translocator. The deduced amino acid sequence showed the BT1 protein shares homology with a mitochondrial adenine nucleotide transporter (Shannon *et al*., 1998). Recently ADP-glucose transporter from wheat endosperm amyloplast has been shown to transport ADP-glucose from cytosol in exchange for ADP (Bowsher *et al*., 2007). A comprehensive study on gene expression for putative rice plastidic translocators revealed that one (*OsBT1-1*) of three BT1 homolog genes is exclusively expressed in the seeds, and the other two are predominantly in photosynthetic tissues, indicating the possibility that OsBT1-1 is responsible for the transport of ADP-glucose from the cytosol to amyloplasts in rice endosperms (Toyota *et al*., 2006). Interestingly, a homolog of BT1 is also found in non-cereal plants such as potato and *Arabidopsis*. Although the physiological advantage of synthesizing ADP-glucose in cytosol of cereal endosperms is not fully understood, the mechanism is speculated to be responsible for the carbon partitioning from sucrose into starch without the utilization of hexose phosphates to other metabolisms (Beckles *et al*., 2001; James *et al*., 2003).

ADP-glucose producing sucrose synthase

Sucrose synthase (UDP-D-glucose:D-fructose 2-glucosyl-

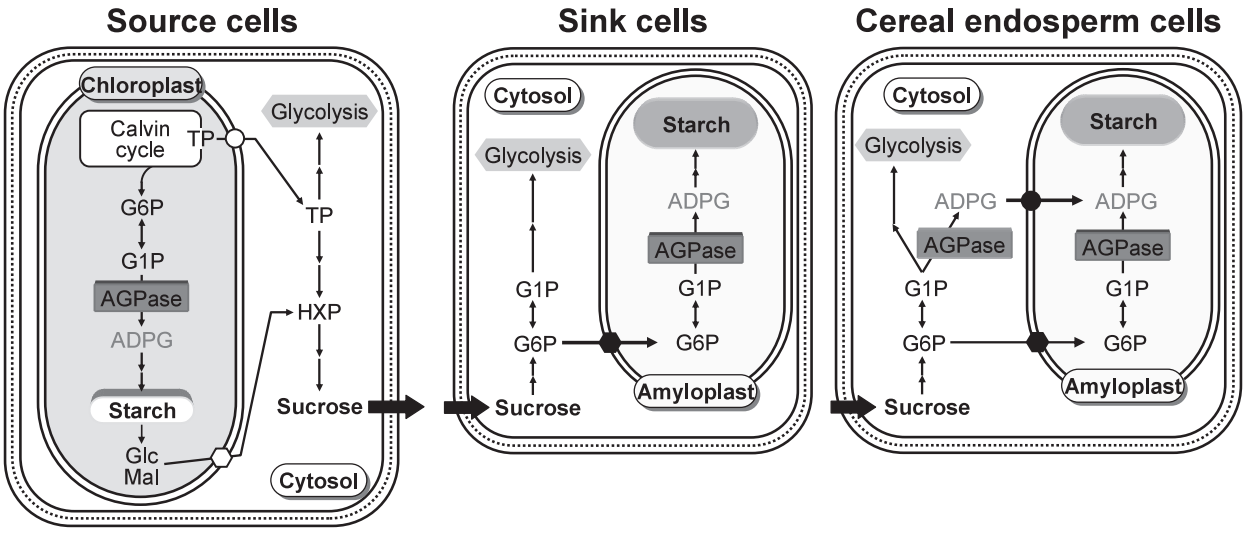

Fig.1 Sucrose metabolism and starch biosynthesis in plant cells.

TP, triose phosphate; HXP, hexose phosphate; G1P and G6P, glucose 1-phosphate and glucose 6-phosphate; Glc, glucose; Mal, maltose; ADPG, ADP-glucose; AGPase, ADP-glucose pyrophsophorylase. Open and closed circles, TP and ADPG transporter; open hexagon, hexose and maltose transporter; closed hexagon, HXP transporter.

transferase, SUS, EC 2.4.1.13) is a highly regulated enzyme (Alexander and Morris, 2006) composed of subunits with a molecular size of about 90 kDa (Nguyen-Quoc *et al*., 1990) that catalyzes the reversible conversion of sucrose and UDP to UDP-glucose and fructose. Different isoforms of SUS are temporally and spatially expressed in distinct parts of the plant. In maize for instance, SUS-SH1 is predominant in developing kernels, while SUS1 is highly expressed in the leaf elongation zone, internode cortex tissue, and etiolated shoots and roots. SUS2 is present in all tissues, but particularly abundant in kernels at various pollination stages. Furthermore, SUS1 and SUS-SH1, but not SUS2, are associated with membranes *in vivo*. The isoform complex of SUS1/SUS2 might have roles in both cytosolic and membrane-associated sucrose metabolism (Duncan *et al*., 2006). In rice leaf tissues, RSUS1 (SUS-SH1) was localized in the mesophyll while RSUS2 (SUS1) was in both the phloem and the mesophyll. RSUS2 was ubiquitously expressed in the developing seed tissues. In contrast, RSUS1 was present in the aleurone layers of developing seeds, and RSUS3 (SUS2) was localized predominantly in the endosperm (Wang *et al*., 1999).

SUS is thought to be the major determinant of sink strength in starch storing organs (Zrenner *et al*., 1995; Chourey *et al*., 1998). Although UDP is the most preferred nucleotide diphosphate for the enzyme reaction of SUS, several studies have revealed that ADP serves as an effective acceptor substrate to form ADP-glucose (Murata *et al*., 1966; Delmer, 1972; Su, 1995; Baroja-Fernández *et al*., 2003). Baroja-Fernández *et al*. (2003) demonstrated that maximal activities of ADP-glucose synthesizing SUS are several fold higher than those of AGPase in developing barley seeds. Furthermore, in the S-112 SUS antisensed and starch deficient potato tuber (Zrenner *et al*., 1995), the contents of ADP-glucose and UDP-glucose were shown to be 35% and 30% of these measured in wild-type plants, whereas both G1P and G6P contents were normal as compared with the wild-type. On the other hand, SUS-overexpressed potato leaves exhibited a marked increase of both ADP-glucose and starch (Muñoz et al., 2005). Although some controversy still remains (Neuhaus *et al*., 2005; Baroja-Fernández *et al*., 2005), these observations suggested that SUS catalyzes the *de novo* production of ADP-glucose linked to starch biosynthesis in both sink and source tissues.

ADP-glucose hydrolytic enzymes

ADP-glucose pyrophosphatase (AGPPase) catalyzes the hydrolytic breakdown of ADP-glucose to G1P and AMP (Rodríguez-López *et al*., 2000). This activity also occurs in *E. coli* and is catalyzed by the enzyme designated as adenosine diphosphate sugar pyrophosphatase (ASPP) (Moreno-Bruna *et al*., 2001). Sequence analysis revealed that ASPP is a member of the ubiquitously distributed group of nucleotide pyrophosphatases designated as "nudix" hydrolases. The Nudix hydrolases constitute a family of metal-requiring phosphoanhydrases that catalyze the hydrolytic breakdown of nucleotide diphosphates linked to some other moiety such as a phosphate, sugar or nucleoside (Bessman *et al*., 1996). They possess a conserved $GX_5EX_7REUXEEXGU$ motif where U is usually isoleucine, leucine or valine. ASPP activity in *E. coli* is inversely correlated with the intracellular glycogen content. Furthermore, ASPP-overexpressing cells display a glycogen-less phenotype (Morán-Zorzano *et al*., 2007). These strongly suggest that ASPP plays a role in preventing carbon flow toward glycogen biosynthesis in bacteria. Recently, Nudix hydrolases have been identified and characterized in *Arabidopsis* and potato plants that catalyze the hydrolytic breakdown of ADP-glucose (Muñoz *et al*., 2006). Leaves of transgenic plants overexpressing plant ASPPs displayed a starch-deficient phenotype, indicating that plant ASPPs have access to a sizable pool of ADP-glucose linked to starch biosynthesis.

Members of other ADP-glucose hydrolytic enzymes (designated as nucleotide pyrophosphatase/phosphodiestrase (NPP)) were purified and characterized from several plant species (Rodríguez-López *et al*., 2000; Nanjo *et al*., 2006). NPPs were glycoproteins, however, one of them (NPP1) was shown to occur in the plastidial compartment (Nanjo *et al*., 2006). Rice genome possesses six NPP encoding genes (Mitsui unpublished data). The physiological functions of NPPs are still unknown.

Biosynthesis of amylose and amylopectin

Starch synthases

Starch synthase (SS) is responsible for the elongation of α-1,4-glucan chains of starch molecules. Multiple SS isozymes have been found in various plant species and classified into five classes (GBSSI, SSI, SSII, SSIII, and SSIV) based on their primary sequences (Ball and Morell, 2003). Each SS isozyme has a distinct enzymatic property and a different role in the starch biosynthesis, and the physiological role of GBSSI members is understood than that of other SS members. In 2005, Nomenclature Committee of the International Union of Biochemistry and Molecular Biology assigned a new enzyme code (EC 2.4.1.242) to the members for GBSSI class because of the specificity of glucosyl donors and the physiological role for amylose biosynthesis.

GBSSI (granule-bound starch synthase I) is encoded by the *waxy (wx)* allele. Genetic mutants lacking GBSSI (e.g. Shure *et al*., 1983; Hovenkamp-Hermelink *et al*., 1987; Denyer *et al*., 1995) and transgenic plants with expression of an antisense RNA eliminating GBSSI (Shimada *et al*., 1993; Kuipers *et al*., 1994) synthesize starch without amylose or with a reduced amylose content. These observations show that GBSSI, which is exclusively bound to starch granules, is responsible for the synthesis of amylose (Denyer *et al*., 2001). In addition, GBSSI is also involved in amylopectin synthesis (Myers *et al*., 2000). Some experiments in which ADP-glucose was supplied to the isolated starch granules have indicated that GBSSI can transfer a glucose residue to the extra-long chains in the amylopectin fraction (Baba *et al*., 1987; Denyer *et al*., 1996). Moreover, the mutant of *Chlamydomonas* lacking

GBSSI activity generates amylose-free starch with the altered structure of amylopectin (Delrue *et al*., 1992). Hence, GBSSI contributes to synthesizing amylose and amylopectin, although it is still unclear how the synthesis of amylose by GBSSI is accomplished and how the GBSSI protein is entirely associated with starch granules.

The nature of GBSSI enzyme is not fully understood, because it is difficult to prepare the native enzyme from starch granules. Several attempts to solubilize an active GBSSI protein from starch granules have been unsuccessful. Maize GBSSI solubilized from endosperm starch granules by α-amylase showed the low specific activity compared with GBSSI within granules (Macdonald and Preiss, 1985). Recentry the recombinant potato GBSSI was prepared by using *E. coli* and the some properties was shown *in vitro* (Denyer *et al*., 1999; Edwards *et al*., 1999a). Potato GBSSI under the granule-bound state showed high affinity against malto-oligosaccharide (MOS) and added glucose residues by a processive manner (Denyer *et al*., 1996), whereas the recombinant enzyme extended MOSs by a distributive manner *in vitro* with a low affinity against the substrate. However, the presence of amylopectin not only stimulated the elongation reaction of MOSs by the recombinant GBSSI, but also shifted the manner from a distributive to a processive mode. It was also shown that the effector response by amylopectin was highly activated in the presence of a higher amylopectin concentration. From these results, a hypothesis was proposed for MOS-primed amylose synthesis in plants: amylose can be synthesized from MOS by GBSSI in the amylopectin matrix because GBSSI has MOS elongating activity even in the semicrystalline in the presence of a high concentration of amylopectin, while the other enzymes, particularly branching enzymes and/or starch synthases except GBSSI can not act in such conditions (Zeeman *et al*., 2002a). An alternative model was also proposed for amylopectin primed amylose synthesis from *in vitro* experiments using starch granules of *Chlamydomonas* (van de Wal *et al*., 1998; Ball *et al*., 1998). In this model, it was hypothesized that GBSSI can use amylopectin chains as substrates for amylose synthesis, and the elongated chains can be cleaved and released by GBSSI itself or by an unidentified glucan hydrolase. When amylopectin was used as a primer, recombinant kidney bean GBSSI caused elongation of amylopectin chains processively, but did not release the elongated chains (Ito *et al*., unpublished data), suggesting that GBSSI can be responsible for the formation of extra-long chains but cannot hydrolyze the elongated chains *in vitro*. Although the amylopectin priming model might be an innovative idea, it is useful for the identification of protein(s) with a hydrolase activity.

In contrast to GBSSI members, other SS members contribute to amylopectin synthesis and might not directly participate in amylose synthesis. Despite the simple enzymatic reaction of SS isozymes, the elongation of each chain for amylopectin construction is very daedal because of the occurrence of the heterogeneous substrates and various SS and SBE isozymes. GBSSI isozyme(s) is believed to be included in all higher plants that synthesize starches with amylose, whereas other SS members are likely distributed in different combinations according to plant species. For example, SSI and SSII in maize endosperm (Macdonald and Preiss, 1985; Cao *et al*., 2000), SSI in rice seed (Baba *et al*., 1993), SSII in pea embryo (Edwards *et al*., 1996), and SSII and SSIII in potato tuber (Marshall *et al*., 1996) are showed predominant activity for soluble starch synthases.

In the rice mutant that is deficient in SSI isozyme by the insertion of retrotransposon, the endosperm amylopectin exhibited a decrease in chains with the degree of polymerization (DP) 8 to 12 and an increase in chains with DP 6-7 and 16-19 (Fujita *et al*., 2006). Likewise, the amylopectin in the *Arabidopsis* mutant lacking the SSI isozyme showed a decrease in short chains with DP 8-12 (Delvallé *et al*., 2005). From these results, SSI isozyme is believed to be involved in the synthesis of DP 8-12 chains from short outer chains of the A or B1 chain in amylopectin. Analysis of pea *rug5* mutants with SSII activities eliminated indicated that the amylopectin had altered branching patterns with decreased amounts of intermediate-sized glucans and increased amounts of short-chain glucans (Craig *et al*., 1998). Similar results were observed in amylopectin from barley mutants lacking starch synthase IIa activity (Morell *et al*., 2003) and antisense transgenic potato plants with reduced SSII activity (Edwards *et al*., 1999b; Lloyd *et al*., 1999). These results suggest that SSII isozymes play a role in the synthesis or maintenance of intermediate-sized glucan chains in amylopectin.

Transgenic potato tuber expressing antisense RNA for SSIII have an altered amylopectin with increased proportion of extra long chains, which are synthesized by GBSSI isozyme (Edwards *et al*., 1999b; Lloyd *et al*., 1999), and therefore result in fissuring of starch granules (Edwards *et al*., 1999b; Fulton *et al*., 2002). A similar increase in the amount of longer length chains in amylopectin is observed in *Arabidopsis* mutants lacking the SSIII isozyme, but the transitory starch granules from the mutants have almost the same morphology as those from wild type (Zhang *et al*., 2005). Maize *dull1* mutants that are deficient in SSIII isozyme (Gao *et al*., 1998) synthesize amylopectin with increased branching frequency, but no fissure was observed in the starch granules (Shannon and Garwood, 1984; Wang *et al*., 1993). The morphological differences in starch granules from these plants seem to be attributed to the distinct contribution of SSIII isozyme in plant species: SSIII is the major SS isozyme in potato tuber, but not in maize endosperm and *Arabidopsis* leaves. Interestingly, reduction or elimination of SSIII activity in these plants resulted in a higher phosphate content of the starches (Lloyd *et al*., 1999; Zhang *et al*., 2005). Although the biochemical reason is unclear, the higher phosphate level appears to be involved in the synthesis of excess extra-long chains (Abel *et al*., 1996; Zhang *et al*., 2005). Despite analysis of these mutants and transgenic plants, the detailed role of the SSIII isozyme for starch biosynthesis remains to be

elucidated. Because the occurrence of the SSIV isozyme in several plants has been shown by actually quite recent genome projects, there is very little information on the isozyme. However, from more recent analysis of SSIV deficient mutants of *Arabidopsis*, the priming of starch granule formation was predicted as a possible function of SSIV isozyme (Roldán *et al*., 2007).

Genetic mutants and/or transgenic plants with eliminated or reduced activity of one or more SS isozyme(s) has made a great contribution to understanding of the roles of the isozyme(s). However, it is difficult to comprehend the precise function of each SS isozyme because of the pleiotropic effects of the absence or reduction of SSIII activity on other starch biosynthetic enzymes (Singletary *et al*., 1997; Edwards *et al*., 1999b). An alternative approach to understanding the role of each SS isozyme in starch synthesis is to investigate the biochemical properties of each SS isozyme. However, preparation of each native SS isozyme from plant materials has been difficult due to low abundance, multiple isozymes, and instability. By recent progress in genetic engineering, several recombinant SS enzymes have been expressed in *E. coli* and characterized (Imparl-Radosevich *et al*., 1998 and 1999; Knight *et al*., 1998; Edwards *et al*., 1999a; Commuri and Keeling, 2001; Isono *et al*., 2003; Senoura *et al*., 2004). Combining the results from *in vitro* enzymatic properties and *in vivo* analysis of genetic mutants and transgenic plants might provide insight into the roles of SS isozymes and amylopectin biosynthesis. All the SS isozymes are composed of an N-domain and C-domain. The C-domain is a catalytic domain and is highly conserved in all SS members, whereas no significant similarity is found in the N-domains between different classes. The N-domain of SSIII members functions as a glucan-binding domain (Machovic and Janecek, 2006; Palopoli *et al*., 2006; Senoura *et al*., 2007), while the N-domain of SSI and SSII members appear to have no significant function in their enzymatic catalysis (Imparl-Radosevich *et al*., 1998; Edwards *et al*., 1999a; Commuri and Keeling, 2001). In contrast to the N-domain, the C-domain of SS isozymes also share high similarity to bacterial glycogen synthases at their primary sequence levels. The three-dimensional structure of *Agrobacterium tumefaciens* glycogen synthase (AtGS) was recently solved (Buschiazzo *et al*., 2004). Interestingly, the active site architecture of AtGS is strikingly similar to those of human glycogen phosphorylase (Rath *et al*., 2000) and *E. coli* maltodextrin phosphorylase (Watson *et al*., 1997), indicating that AtGS, and probably plant SS isozymes, share a common catalytic mechanism to glycogen and maltodextrin phosphorylases.

Starch branching enzymes

Starch branching enzymes (SBE) make α-1,6 branch points in starch molecules. Higher plants have multiple SBE isozymes that are classified into two families A and B based on their primary sequences (Burton *et al*., 1995). Members of the two families display distinct enzymatic properties such as substrate preferences and chain transfer patterns: family A SBEs show a lower affinity for amylose than family B SBEs and preferentially catalyze the transfer of chains shorter than those catalyzed by the latter (Takeda *et al*., 1993). The SBE gene families also show different temporal patterns during endosperm or seed development. In most instances, family A and B SBE transcripts accumulate in the middle and late stages respectively of seed development (Mizuno *et al*., 1993; Burton *et al*., 1995; Gao *et al*., 1996; Hamada *et al*., 2001). Thus multiple forms of SBE with different expression patterns may synthesize variegated amylopectin with chains of different lengths or branched points.

SBE has an important role in determining the amylopectin structure of starch. The importance of this enzyme activity in starch biosynthesis is readily seen in genetic mutants defective in this enzyme activity. Some mutants lacking SBE isozyme activity have been identified as *amylose-extender (ae)* mutants of maize (Boyer and Preiss, 1981; Kim *et al*., 1998) and rice (Mizuno *et al*., 1993; Nishi *et al*., 2001). These *ae* loci correspond to the *rugosas* locus represented wrinkled phenotypes (first described by Gregor Mendel) of pea (Bhattacharyya *et al*., 1990). All the loci encode family A SBE isozyme, and seeds from these mutants accumulate a higher proportion of amylose and its amylopectin component is loosely branched. Similar results have been also observed in transgenic potato plants with reduced family A SBE activity (Jobling *et al*., 1999). In contrast to the ae mutants, in transgenic or mutant plants with reduced or eliminated family B SBE activity, only a moderate effect on starch structure was observed (Blauth *et al*., 2002; Satoh *et al*., 2003; Yao *et al*., 2004).

SBEs together with SSs synergistically contribute to amylopectin biosynthesis. When the maize SBEI and SBEII were simultaneously expressed in branching-enzyme-deficient *E. coli*, the structure of polymers produced were greatly different from that of amylopectin (Guan *et al*., 1995). Similar observations were made when different combinations of maize SSs and SBEs were co-expressed in bacterial cells (Guan and Keeling, 1998). These observations indicate that the function of SS and SBE is essential for amylopectin biosynthesis but that other factors are also required for the formation of semicrystalline amylopectin (Smith, 1999).

Plant SBEs and bacterial glycogen-branching enzymes (GBEs) are members of the α-amylase family (glycoside hydrolase family 13; GH13; Kuriki and Imanaka, 1999). The alignment of primary sequences, the secondary structure prediction, and the three-dimensional structures of the GH13 members have shown that SBEs contain three domains: an amino-terminal (N) domain, a carboxyl-terminal (C) domain, and a central catalytic $(\beta/\alpha)_8$ barrel domain (Abad *et al*., 2002). Because the barrel domains of the SBEs of the families A and B share a significant homology with each other, it is assumed that their distinct enzymatic characteristics are largely attributable to the difference in their N- and C-domains. Indeed, analyses of several chimeric enzymes between families A and B SBEs have indicated that the both domains are important for the determination of substrate

preference, catalytic capacity, and chain length transfer (Kuriki *et al*., 1997; Hong *et al*., 2001; Ito *et al*., 2004; Hamada *et al*., 2007). In addition, analyses by site-directed mutagenesis and chemical modification have revealed important amino acid residues for branching activity in plant SBEs and bacterial branching enzymes (Sivak and Preiss, 1998). It has recently been demonstrated that protein phosphorylation-dephosphorylation regulates wheat SBE activity and the formation of a complex between SBEs and starch phosphorylase (Tetlow *et al*., 2004). This study also indicated that SBEs and starch phosphorylase are phosphorylated in plastids. It is important to shed light on the post-transcriptional regulation of each enzyme, as well as to analyze enzymatic properties and transgenic plants and genetic mutants to understand the precise physiological roles of SBE isozymes *in vivo*.

Starch-debranching enzymes

Nowadays, it is recognized that the formation of the highly ordered amylopectin structure *in vivo* requires a cooperation of not only SSs and SBEs but also starch-debranching enzymes (DBEs). DBEs catalyze a hydrolysis of α-(1,6) glucosidic linkages of polyglucans. In higher plants, two types of DBEs with distinct substrate specificities have been identified (Doehlert and Knutson, 1991): pullulanase-type and isoamylase-type. Pullulanase-type DBEs prefer pullulan and β-limit dextrins to amylopectin and glycogen as a substrate, whereas isoamylase-type DBEs hydrolyze the α-(1,6) linkages of amylopectin and glycogen, but do not act on pullulan. The isoamylase-type DBEs are also divided into three isoforms (ISA1, ISA2 and ISA3) based on the primary sequences (Hussain *et al*., 2003; Wattebled *et al*., 2005).

The *sugary-1 (su1)* mutants from maize and rice show the reduced starch content and accumulation of water-soluble polysaccharides with highly branched polysaccharides (phytoglycogens) in endosperms (Pan and Nelson, 1984; Nakamura *et al*., 1996). Although it was demonstrated subsequently that the *su1* loci in these mutants encode isoamylase-type DBEs (James *et al*., 1995; Fujita *et al*., 1999; Kubo *et al*., 1999), there is a decrease of both isoamylase- and pullulanase-type DBE activities in these mutants (Nakamura *et al*., 1996; Rahman *et al*., 1998; Beatty *et al*., 1999). The accumulation of phytoglycogen has been observed in not only mutant plants such as *Arabidopsis dbe1* (Zeeman *et al*., 1998a and 1998b), barley *notch-2* (Burton *et al*., 2002b), and *Chlamidomonas sta7* (Mouille *et al*., 1996), but also antisense transgenic potato tubers (Bustos *et al*., 2004) and *Arabidopsis* (Delatte *et al*., 2005). In these plants, however, the activity of pullulanase-type DBE is at the wild-type level. Therefore, the precise cause of the reduced pullulanase-type DBE activity in the maize and rice *sugary-1* mutants is still unexplained. The maize mutant without pullulanase-type DBE accumulates branched malto-oligosaccharides in leaves (Dinges *et al*., 2003). In addition, analysis of the maize and *Arabidopsis* double mutants deficient in both isoamylase- and pullulanase-type DBEs indicated that pullulanase-type DBE can partially compensate the function of isoamylase-type DBE in the endosperm and leaves (Dinges *et al*., 2003; Wattebled *et al*., 2005). These results suggest that pullulanase-type DBEs also function in both starch degradation and synthesis as well as isoamylase-type DBEs.

Two models have been proposed to explain the function of isoamylase-type DBE in starch biosynthesis. In the first model, the glucan-trimming model, which was proposed from the analysis of *Chlamidomonas sta7* mutant (Mouille *et al*., 1996), isoamylase-type DBE plays a direct role in the synthesis of amylopectin and trims the highly branched glucans (pre-amylopectin) that are synthesized by SSs and SBEs, to produce mature amylopectin (Ball *et al*., 1996; Myers *et al*., 2000). In the second model, the water-soluble polysaccharide clearing model, which was proposed on the basis of analyzing *Arabidopsis dbe1* mutants (Zeeman *et al*., 1998a and 1998b), isoamylase-type DBE plays an indirect role on the synthesis of amylopectin as it functions to prevent the formation of soluble branched glucans in plastids. However, the conclusive evidence that either model is correct has not been provided.

Like SBEs, both types of DBEs are also members of α-amylase family and contain $(\beta/\alpha)_8$ barrel structure, which is responsible for catalysis. Apart from the catalytic domain, isoamylase-type DBE has an N-terminal and a C-terminal domains, and pullulanase-type DBE has an N-terminal domain. However, the functions of these domains are not established. Recent studies have shown that ISA2 has no enzymatic activity due to lack of catalytic residues and that ISA1 and ISA2 form a heteromultimeric complex (Hussain *et al*., 2003; Bustos *et al*., 2004; Delatte *et al*., 2005; Utsumi and Nakamura, 2006). Although two distinct isoamylase forms, the ISA1 homo-oligomer and ISA1/2 hetero-oligomer, exist in rice endosperm, kinetic analysis suggested that the hetero-oligomer has a predominant role in amylopectin synthesis (Utsumi and Nakamura, 2006). The occurrence of ISA1/2 complex is also supported by the facts that several mutant plants with reduced isoamylase-type DBE activity have the mutation in the *ISA1* or *ISA2* gene and that *Arabidopsis* mutants in the *ISA1* and *ISA2* display identical phenotypes (Delatte *et al*., 2005). In addition, it was recently shown that ISA1/2 complex controls starch granule initiation because the barley mutant and antisense potato with reduced ISA1 or ISA2 level accumulate large numbers of small granules not seen in wild type plants (Burton *et al*., 2002b; Bustos *et al*., 2004). In contrast to ISA1/2 complex, ISA3 appears to be a monomeric enzyme (Hussain *et al*., 2003; Takashima *et al*., 2007). The *Arabidopsis* mutants deficient in ISA3 has a starch-excess phenotype in leaves and have an altered structure of amylopectin with increases in very short chains, suggesting that ISA3 plays a role in starch degradation and probably removes the short chains on the surface of starch granule (Wattebled *et al*., 2005; Delatte *et al*., 2006).

Biological starch degradation

The accumulation and subsequent utilization of reserve starches are fundamental processes of living plant cells. Photoautotrophic plants obviously rely upon carbohydrate reserves when the photosynthetic system is not operating, for example, at night. In the germination stage, particularly in cereal seeds, growth and metabolic activities entirely depend on the utilization of storage starches until the photosynthetic machinery has been developed. As mentioned above, two types of starches are distinguishable in plants: transitory and reserve starches. The physiological aspects of these starches are quite different, but both types of starches have a similar granule structure with semi-crystalline arrays. The semi-crystalline structure of the starch granule with an internal lamellar structure, called "growth rings", is quite stable and also relatively resistant to enzymatic attacks. α-Amylase, which can act on raw starch, is considered to play the main role in starch degradation including the initial attack on the starch granules. However, recent investigation suggested that the pathway for starch degradation differs with the organs and species.

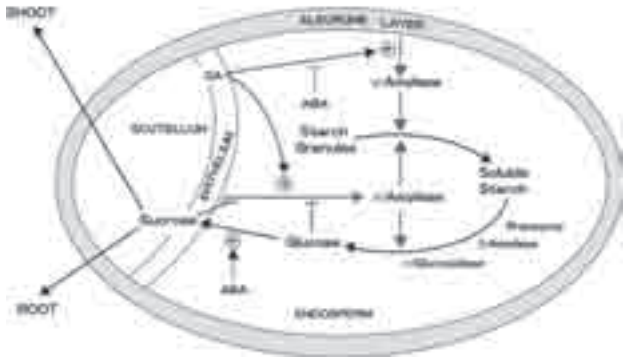

Fig.2 Starch degradation in cereal storage organ.

Starch-sucrose conversion in germinating rice seed. Reserved starch is degraded into glucose by amylolytic enzyme, mainly α-amylases, which are secreted from the scutellar epithelium and the aleurone layer. Glucose is absorbed into the scutellum, and then converted to sucrose. The starch-sucrose conversion is coordinated in balance of the signal strength of gibberellin, abscisic acid, and metabolic sugars.

Degradation of reserved starch in germinating cereal seeds

The hydrolytic process of reserve starch has been studied extensively in the endosperm of germinating cereal seeds. It is generally accepted that α-amylases play the pivotal role for breakdown of starch in the storage organ endosperm including the degradation initiation, since α-amylase is the most predominant amylolytic enzyme that can attack intact starch granules (Yamamoto, 1988; Beck and Ziegler, 1989). In the germinating seeds, α-amylases are secreted to the starchy endosperm from the scutellar epithelium at the early stage of germination and from the aleurone layer at the later stage, where these directly bind and degrade the starch granules in dead cell amyloplasts (Akazawa *et al*., 1988). The regulation of starch-sucrose conversion in germinating rice seeds is summarized in **Fig. 2**.

The starch granules are initially digested and destroyed by α-amylase to soluble starch. The complete degradation of soluble starches proceeds with the concerted action of α-amylase, debranching enzyme (R-enzyme), β-amylase, and α-glucosidase. The glucose produced in the endosperm is taken up and converted to sucrose in the scutellum, then the sucrose is provided to the growing tissues, such as the young shoot and root tissues.

Enzymic characteristics of α-amylase

Plant α-amylases (EC 3.2.1.1) randomly hydrolyze α-(1,4)-glucosidic linkages in glucan polymers. α-Amylases are known to be Ca^{2+}-containing metalloenzymes. Ca^{2+} is essential for the expression of enzymatic activity of α-amylase. They have a wide optimum temperature range from 26 to 70℃. The optimum pH for enzyme reaction is 5.3 to 6.0.

α-Amylases are composed of a single polypeptide with a molecular size ranging from 40 to 50 kDa (Yamamoto, 1988; Mitsunaga et al., 2004). α-Amylases in cereal are well-known polymorphic enzymes. Barley α-amylase isoforms are distinguished as Amy1 and Amy2 with different isoelectric points (pI) and are referred to as the low-pI and the high-pI isozyme, respectively (Jacobsen and Higgins, 1982). Despite a sequence identity of 80%, Amy1 and Amy2 are significantly different in their physico-chemical and enzymatic properties (Jones and Jacobsen, 1991; Rodenburg *et al*., 1994 and 2000; Jensen *et al*., 2003). In rice, ten distinct α-amylase genes have been cloned and sequenced, and more than 20 native α-amylase isoforms have been identified and characterized. Rice α-amylase isoforms are also classified into two classes, AmyI and AmyII, based on their epitope structures (Mitsui *et al*., 1996), oligosaccharide degradation patterns (Terashima *et al*., 1996), and optimum temperatures for enzymatic activity (Mitsunaga *et al*., 2004; Nanjo *et al*., 2004a). All deduced amino acid sequences of the above α-amylase isoforms contain signal sequences characteristic for the translocation of endoplasmic reticulum (ER) membrane (O'Neill *et al*., 1990).

Both barley α-amylase isoforms, Amy1 and Amy2, have been crystallized and their three-dimensional structures were determined (Kadziola *et al*., 1994 and 1998; Robert *et al*., 2005). The α-amylase peptide chain folds into three domains: domain A, the large central domain of $(\beta/\alpha)_8$-barrels; domain B, a protruding, irregular-structured loop domain; domain C, the C-terminal domain mainly forming β-sheet. There exist three oligosaccharide binding sites, that is, the active site and the starch granule-binding surface site at the domain A-B, and the "sugar tongs" binding site at the domain C. Three Ca^{2+}-binding sites locate at the loop domain B.

Functional expression of α-amylase isoforms

The starch degradation linked to the germination and subsequent growth is finely regulated by the plant hormone gibberellin **(Fig. 2)**. The functional expression of α-amylase in germinating cereal seeds is precisely controlled with gibberellin at both the transcriptional and translational levels. The promoter analyses of α-amylase genes have revealed the elements involved in gibberellin-regulated gene expression (Skriver *et al*., 1991). Some conserved box sequences responsible for gibberellin-response have been defined, though there exists little sequence homology in the promoter regions of cereal α-amylases (Mitsui and Itoh, 1997). These conserved elements are called gibberellin-response complexes (GARCs), and it is likely that each cereal α-amylase gene has individual components of GARCs (Gómez-Cadenas *et al*., 2001). In general, the most important cis-acting element in GARCs is a TAACAAA motif that is common to all gibberellin-responsive genes and the sequence is designated as gibberellin-response element (GARE) (Huang *et al*., 1990). The sequence of GARE was found to have homology to the animal *Myb* proto-oncogene consensus binding sites, which led to the identification of Myb-related *trans*-acting factor (GAMYB) from barley aleurone cells (Gubler *et al*., 1995). GAMYB specifically binds to GARE. GA induces the expression of *GAMYB* prior to the induction of α-amylase. From several lines of evidence, GAMYB is hypothesized as a central GA-regulated *trans*-acting factor, which is located at the downstream of GA signal transduction leading to the activation of α-amylase gene expression in aleurone cells. Further investigation revealed that a Mak-like kinase, KGM (kinase associated with GAMYB) operates as a repressor of GAMYB in barley aleurone (Woodger *et al*., 2003). Furthermore, a number of regulators of gibberellin signaling, such as DELLA proteins and protein turnover complex (SCF), etc., were identified and characterized through the mutational analyses. Recently, Ueguchi-Tanaka *et al*. (2005) proposed that GIBBERELLIN INSENSITIVE DWARF 1 (GID1) is a soluble receptor for gibberellin.

α-Amylase isoform I-1 and II-4 that are predominant isoforms in rice seedlings exhibited distinct differences in their gene structure and mode of expression. The most predominant isoform α-amylase I-1 encoded by *RAmy1A* is gibberellin-inducible, and is actively expressed in the scutellar epithelium and the aleurone layer and in calli derived from the embryo. Moreover, the enzyme isoform is the best-characterized secretory glycoprotein bearing typical *N*-linked oligosaccharide chains, including both high-mannose and modified type oligosaccharides (Hayashi *et al*., 1990; Lecommandeur *et al*., 1990): it is synthesized at the rough ER and transported to the plasma membrane through the Golgi complex. α-Amylase II-4 encoded by *RAmy3D* is also expressed and secreted from the calli, the scutellar epithelium and the aleurone layer. α-Amylase II-4 in the aleurone layer showed temporal and spatial expression essentially identical to α-amylase I-1, although the expression was distinguishable in the embryo tissues at the early stage of germination. Rice α-amylase gene *RAmy3D* lacks GARE in the promoter region (Huang *et al*., 1990), though the gibberellin-responsive expression of α-amylase II-4 in the aleurone was also similar to that of α-amylase I-1 whose gene contains GARE. As expected, gibberellin did not increase the level of α-amylase II-4 mRNA, indicating that post-transcriptional enhancement of α-amylase II-4 expression did occur in the aleurone (Nanjo *et al*., 2004b). Metabolic sugars suppress the gene expression of *RAmy3D*. Recent studies have shown that multiple *cis*-elements and trans-acting factors are involved in this response (Chen *et al*., 2006). Metabolic sugars also regulate the formation of α-amylase II-4 in rice cells at the post-transcriptional steps, i.e., mRNA turnover (Chan and Yu, 1998), protein synthesis, intracellular transport and turnover (Mitsui *et al*., 1999a). Both gibberellin and sugar starvation signals raise the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{cyt}$ markedly (Bush, 1996; Mitsui *et al*., 1999b). The expression and secretion of α-amylases in cereal cells require an appropriately high $[Ca^{2+}]_{cyt}$ (Bethke *et al*., 1997).

Seed germination and seedling growth in a transgenic rice line with suppressed expression of α-amylase I-1 markedly delayed in comparison with those of the wild-type (Asatsuma *et al*., 2005). These findings indicated that α-amylase must be the main contributor in the amylolytic degradation of starch. Thus, the breakdown of reserve starch in cereal seeds is mainly controlled by the expression of α-amylase coordinated with multiple factors, such as hormones, sugars, Ca^{2+}, and so on.

DBE, β-amylase, and α-glucosidase

As described above, debranching enzymes (DBE) catalyze the hydrolysis of α-(1,6)-linkages in glucan polymers. The activity of pullulanase-type DBE was shown to be substantially expressed in germinating rice endosperm (Iwaki and Fuwa, 1981; Daussant *et al*., 1983), and the limit dextrinase-type increased in germinating barley seeds in response to gibberellin (Kristensen *et al*., 1998). These circumstantial evidences indicate that DBEs perhaps involve the hydrolysis of α-(1,6) linkages during starch degradation in the germination stage.

β-Amylase (EC 3.2.1.2) catalyzes the liberation of maltose from non-reduced ends of starch-related α-(1,4)-glucans. β-Amylases in soybean, sweet potato and cereals have been studied energetically. The optimum pH for enzyme reaction is 5.0 to 6.0. The molecular sizes of enzymes range from 50 to 60 kDa, except the sweet potato β-amylase which shows to be 220 kDa protein composed of four subunits (Yamamoto, 1988). Crystal structures of β-amylases were solved in soybean (Mikami *et al*., 1993; Adachi *et al*., 1998), sweet potato (Cheong *et al*., 1995) and barley (Mikami *et al*., 1999). The proteins consist of a core with an $(\alpha/\beta)_8$ supersecondary structure, plus a smaller globular region formed by long loops (L3, L4, and L5) extending from β-strands β3, β4, and β5. Between the two regions is a cleft that opens into a pocket whose floor contains the postulated catalytic center (Mikami *et al*., 1993).

In germinating cereal endosperm, β-amylase is one of the major amylolytic enzymes. In barley, the β-amylase protein exists in the dry grain, where it is synthesized and accumulated during the period of ripening, and is likely bound to the starch granules (Sopanen and Lauriére, 1989). On the other hand, in rice, the β-amylase enzyme is synthesized *de novo* during seed germination, although it is almost absent in dry seeds (Okamoto and Akazawa, 1980; Yamaguchi *et al*., 1999). Several rice cultivars (Yamaguchi *et al*., 1999; Mitsunaga *et al*., 2001) and inbred lines of mutant in barley (Kreis *et al*., 1987) and rye (Rorat *et al*., 1991) show markedly reduced β-amylase activity if any in the mature seeds. However, no significant growth retardation was observed in the mutant seeds with the β-amylase-deficient phenotype. Probably, β-amylase plays a subordinate role in the breakdown of reserved starch in germinating cereal seeds.

α-Glucosidases (EC 3.2.1.20) belong to a group of exo-glycoside hydrolases, which catalyze the release of α-D-glucose from the non-reduced ends of α-linked glucans (Frandsen and Svensson, 1998). The seeds of rice, corn, buckwheat, barley, and sugar beet contain a relatively large amount of α-glucosidase. The optimum pH for enzyme reaction is 3.6 to 5.0. The molecular sizes of enzymes range from 65-100 kDa (Matsui *et al*., 1978; Chiba *et al*., 1979). A couple of research groups have reported that barley, millet and rice α-glucosidases are capable of degrading the native starch granules *in vitro* (Sun and Henson, 1990; Yamazaki *et al*., 2005; Nakai *et al*., 2006). However, the information about the physiological aspects of α-glucosidase is limited. There is controversy over the contribution efficiency of exo-type amylase toward the degradation initiation of starch granules yet.

Degradation of reserved starch in potato tubers

The other typical reserve starch is observed in storage tubers. In contrast to the starch degradation in the endosperm of germinating cereal seeds, the breakdown of tuber starch occurs within living cells. A series of starch-degrading enzyme activities have been detected in potato tubers during sprouting (Davies, 1990) and cold-induced sweetening (Nielsen *et al*., 1997), including α-amylase, isoamylase, β-amylase, α-glucosidase, and phosphorylase, but the nature of the attack on the starch granule is much less clear in comparison with that in the germinating cereal endosperm.

Plant starch excepting cereal storage starch is extensively phosphorylated, and in potato tubers, starch contains about 1 monoesterified phosphate group in 150 to 600 glucosyl residues (Blennow *et al*., 2002; Tabata *et al*., 1975). The phosphate groups were bound at the C-6 and C-3 positions of the glucose units (Hizukuri *et al*., 1970; Tabata and Hizukuri, 1971). Recently, it has been identified and characterized the starch phosphorylating enzyme, glucan, water dikinase (EC 2.7.9.4, GWD), which catalyzes the transfer of β-phosphate of ATP to either the C6 or C3 position of glucosyl residues within amylopectin (Ritte *et al*., 2002; Mikkelsen *et al*., 2004). Several lines of evidence strongly suggested the importance of starch phosphorylation in starch turnover (Lorberth *et al*., 1998; Yu *et al*., 2001). Transgenic potatoes in which GWD was greatly reduced by an antisense construct had very low levels of phosphate in tuber starch, and exhibited a starch excess phenotype in leaves and a reduction in cold-induced sweetening in tuber (Lorberth *et al*., 1998).

As described above, DBEs are involved in both starch synthesis and degradation processes. It has been predicted that in the *Arabidopsis* genome there are three genes encoding isoamylase-like proteins (ISA1, ISA2, and ISA3) and a single gene encoding limit dextrinase (Delatte *et al*., 2006). Three distinct isoamylase isoforms (StISA1, StISA2, and StISA3) have been also identified in potato (Hussain *et al*., 2003). StISA1 and StISA2 were convinced of importance in controlling starch granule initiation (Bustos *et al*., 2004). On the other hand, StISA3 had a markedly high activity on β-limit amylopectin *in vitro* (Hussain *et al*., 2003), suggesting that this isoform is a candidate for the debranching enzyme with a specific role in starch degradation. The degradation pathway of soluble glucans derived from starch in tuber is totally obscure. Cytosolic glucan phosphorylase (Duwenig *et al*., 1997) and transglycosidase (Lloyd *et al*., 2004), which are possibly involved in starch degradation in leaves, may not be necessary for tuber starch degradation.

Degradation of assimilatory starch in Arabidopsis leaves

Plants fix air CO_2 to sugar by the photosynthetic machinery during the day, and a portion of the synthesized sugar is accumulated as assimilatory starch in their leaves to utilize the energy source for the coming night. The degradation of assimilatory leaf starch differs topographically from that of cereal storage organs: it occurs in chloroplasts in living cells, a situation rather similar to that of the intact amyloplasts of seed cotyledons and storage tubers. Recent investigations employing transgenic and mutant plants of *Arabidopsis* revealed that β-amylase rather than phosphorylase and endoamylase seems to be important in leaf starch mobilization. Several excellent review articles are available on this subject (Zeeman *et al*. 2004, Smith *et al*. 2005, Lloyd *et al*. 2005).

The starch in chloroplasts forms granules organizing semi-crystalline arrays and the structure of leaf chloroplastic starch is similar to that of crop storage starch in many respects (Zeeman *et al*., 2004). However, it is still uncertain whether the transitory starch granules are surrounded by an outer pasty layer (Beck, 1985). Whereas starch granules isolated from (spinach) chloroplasts could not be degraded by the glucan-metabolizing enzymes except α-amylase (Steup *et al*., 1983), the reported pasty appearance could not be caught by scanning electron microscopy of starch granules prepared using gentle aqueous extraction techniques (Zeeman *et al*., 2002b). The structural difference of chloroplastic starch

granules from reserve starch seems to be reasonable, when considering its diurnal change between net synthesis and degradation in contrast to the situation of reserve starch.

In *Arabidopsis* leaves, approximately 1 in 2,000 glucosyl residues of the starch is phosphorylated (Yu *et al*., 2001). Mutations of the *GWD* gene, which prevent the expression of the enzyme protein or alter its dikinase domain, dramatically decreased both the amount of starch phosphorylation and the rate of starch degradation. Consequently, the amounts of starch accumulated in mature leaves of mutants (*starch excess 1 or sex1*) increased to seven times those in the wild-type (Casper *et al*., 1991; Yu *et al*., 2001). A second type of GWD, phosphoglucan, water dikinase (PWD) was recently found in Arabidopsis (Baunsgaard *et al*., 2005; Kötting *et al*., 2005). PWD catalyzes the same reaction as GWD, but the enzyme phosphorylates only starch granules phosphorylated by GWD and the phosphate group is conjugated to the C3 rather than the C6 position of glucosyl residues of amylopectin. Knockout mutants lacking PWD exhibited a starch excess phenotype. These findings indicate that in leaves, starch turnover requires a close collaboration of GWD and PWD. An explanation is that the phosphate groups influence the packing of the glucose polymers within the granule and hence the susceptibility of the granule surface to attack by enzyme (Blennow *et al*., 2002; Zeeman *et al*., 2004).

As mentioned above, the initial step of starch granule degradation is to destroy the surface semi-crystalline structure. One of the candidates for initial attacking enzyme is α-amylase. In *Arabidopsis* genome, three proteins are predicted to be α-amylases. Triple mutants lacking all three α-amylases, however, had normal rates of starch degradation in *Arabidopsis* leaves at night (Yu *et al*., 2005), indicating that the absence of α-amylase can be compensated for by another amylolytic enzyme. Recently, both isoamylase and limit dextrinase have been shown to contribute to the starch breakdown in *Arabidopsis* leaves. Mutants of ISA3 (*Atiso3*) had more leaf starch and a slower rate of starch breakdown than wild-type plants. Mutants of limit dextrinase (*Atlda*) were indistinguishable from the wild-type. However, the *Atiso3/Atlda* double mutant exhibited a more severe starch-excess phenotype and a slower rate of starch degradation than *Atiso3* single mutants. In the *Atiso3/Atlda* double mutant, the activity of chloroplastic α-amylase and the amount of soluble glucans were increased. These results strongly indicated that ISA3 is the major debranching enzyme involved in starch degradation and may act directly on the granule surface in *Arabidopsis* leaves (Delatte *et al*. 2006). In addition, there exists the pathway of starch degradation by α-amylase, although the endoamylase may not play a major role for the starch degradation under normal circumstances in *Arabidopsis* leaves.

Several lines of evidence strongly suggested that, following the action of debranching enzymes, β-amylase is responsible for degradation of linear glucans in the *Arabidopsis* chloroplasts. Four of the nine genes encoding β-amylase contained putative transit peptides that would target them to the chloroplast (Lloyd *et al*., 2005), and one of these proteins has been shown to be in the chloroplast (Lao *et al*., 1999). *Arabidopsis* RNAi lines with lower expression of the chloroplastic β-amylase exhibited a starch-excess phenotype, and a dramatic decrease in maltose accumulation upon cold shock (Kaplan and Guy, 2005). Increase in maltose level at night in leaves of *Arabidopsis* was observed, suggesting that it is perhaps an intermediate of starch catabolism (Chia *et al*., 2004; Weise *et al*., 2004 and 2005). Mutations of a maltose transporter gene (*mex1*) caused accumulation of both starch and maltose in leaves. Maltose levels were at least 40 times those of wild-type leaves, showing that maltose produced during starch degradation is exported to the cytosol via a specific maltose transporter MEX1. It is consistent with a block in the metabolism of maltose produced by β-amylolytic degradation of starch (Niittylä *et al*., 2004).

The disproportionating enzyme (D-enzyme), DPE1, is also involved in the catabolism of linear maltooligosaccharides. DPE1 is a (1,4)-α-D-glucan: (1,4)-α-D-glucan, 4-α-D-glucanotransferase (EC 2.4.1.25) which can catalyze a wide range of reactions, transferring 1,4-α bonds from one donor glucan molecule to another. The smallest donor and acceptor molecules are maltotriose and glucose, respectively, though the enzyme can use large glucans as donors and acceptors. The preferred substrate is maltotriose, which is converted to glucose and maltopentaose (Jones and Whelan, 1969). In *Arabidopsis*, a single gene encodes DPE1 that predicted to be chloroplastic. Knockout mutants of DPE1 have been isolated and characterized in *Arabidopsis*, showing that at night the rate of starch degradation in the mutant decreased in comparison with the wild-type and appreciable amounts of malto-oligosaccharide, exclusively maltotriose, accumulated in the mutant leaves (Critchley *et al*., 2001). In addition, these results provide evidence that β-amylase rather than glucan phosphorylase is responsible for the metabolism of linear glucans. β-Amylase can hydrolyze maltotetraose to two maltoses and maltopentaose to maltose and maltotriose, but not or hardly act maltotriose (Chapman *et al*., 1972), while the phosphorylase enzyme can catalyze the reaction of maltopentaose to maltotetraose and glucose-1-phosphate and maltotetraose is the end product of phosphorolytic maltodextrin degradation (Setup and Schächtele, 1981). Accumulation of maltotriose in mutants lacking DPE1 thus suggested that glucan phosphorylase weakly or scarcely contributes to the degradation of linear glucans. Actually, the plastidial phosphorylase *(PHS1)*-destroyed *Arabidopsis* was the normal rate for starch degradation in leaves (Zeeman *et al*. 2004).

Both lines of evidence obtained by biochemical and knockout mutant studies have revealed that in *Arabidopsis*, most of the carbon from starch degradation is exported as maltose (Niittylä *et al*., 2004; Weise *et al*., 2004 and 2005). Export of triose-phosphate and glucose may not be essential for normal starch degradation (Häusler *et al*., 1998; Weber *et al*., 2000; Yu *et al*., 2001). Maltose exported from the chloroplasts to the cytosol is metabolized *via* a

transglycosylation reaction. A cytosolic transglycosidase (DPE2), that is similar in sequence to the *E. coli* enzyme amylomaltase, catalyzes the release of one of the glucosyl moieties of maltose and the transfer of the other to a glucan acceptor (Lloyd *et al*., 2004).

Mutations in the *DPE2* gene led to a massive accumulation of maltose, excess starch, reduced the rate of growth and slightly pale appearance (Chia *et al*., 2004; Lu and Sharkey *et al*., 2004). However, the nature of the endogenous acceptor is still obscure. One candidate is a specific type of soluble arabinogalactan detected in leaves of some plants (Yang and Steup, 1990; Fettke *et al*., 2004). This heteroglycan is a good substrate *in vitro* for the cytosolic glucan phosphorylase (PHS2) (Matheson and Richardson, 1976; Duwenig *et al*., 1997). It is possible, hence, that glucosyl moieties from maltose are transferred to the heteroglycan by DPE2 and released again as G1P by PHS2 in cytosol. The final product glucose is used for the synthesis of sucrose and for cellular metabolism. The recently proposed pathway of starch degradation in *Arabidopsis* leaves at night is summarized in **Fig. 3A**.

Like starch biosynthesis, starch degradation in *Arabidopsis* leaves is highly regulated. The starch in leaves is synthesized during the day, by and little or no starch degradation occurs. After the onset of darkness, the rate of starch degradation increases to a maximum within the first a couple of hours (Zeeman *et al*., 1999). On the other hand, mutations and environmental changes that reduce the starch accumulation during the day cause a decline in the rate of its consumption at night (Lin *et al*., 1988). However, the information about the mechanisms for switching-on and subsequent control of the starch degradation is limited. One possibility for the diurnal control of starch degradation in leaves is the circadian clock regulation at the transcriptional level (Harmer *et al*., 2000). Indeed, transcripts encoding essential enzymes for starch degradation, such as GWD, PWD, DPE1, DPE2 and ISA3, showed a coordinated decline in the light followed by rapid accumulation in the dark. The other starch degradation-related enzymes including a plastdial α-amylase AMY3, PHS1 and PHS2 were also similarly cycled. Quite surprisingly however, the protein amounts of most important enzymes, β-amylases and MEX1, do not change essentially through the diurnal cycle (Smith *et al*., 2004). The facts that the levels of enzymes of starch degradation did not change in parallel with gene expression strongly suggested the importance of posttranslational regulation of this process (Grennan, 2006). Possible factor candidates operated in the posttranslational regulation are pH shift, malto-oligosaccharide level, redox potential, and protein phosphorylation (Kerk *et al*., 2006; Niittylä *et al*., 2006). The pH in the stroma shifts from 8 to 7 during the transition from light to dark. This pH decrease is thought to enhance activity of starch-degrading enzymes (Stitt and Heldt, 1981). The *dpe1*, *dpe2*, and *mex1* mutants accumulate abnormally high levels of maltose or maltotriose. Malto-oligosaccharides inhibit α-amylase, probably by competing with granular starch for a starch-binding domain necessary for attack on the granule (Witt and Sauter, 1996). Maltose inhibits some β-amylases at a high concentration (Lizotte *et al*., 1990). Chloroplastic thioredoxin is reduced during photosynthesis by electrons from PSI, transferred *via* ferredoxin. Many enzymes interacting with thioredoxin were reported, including a spinach β-amylase (Balmer *et al*., 2003 and 2006). The results that *starch excess 4 (sex4)* mutants, which have strongly reduced rates of starch metabolism, lack a protein predicted to be a dual specificity protein phosphatase indicated that protein phosphorylation may be involved in

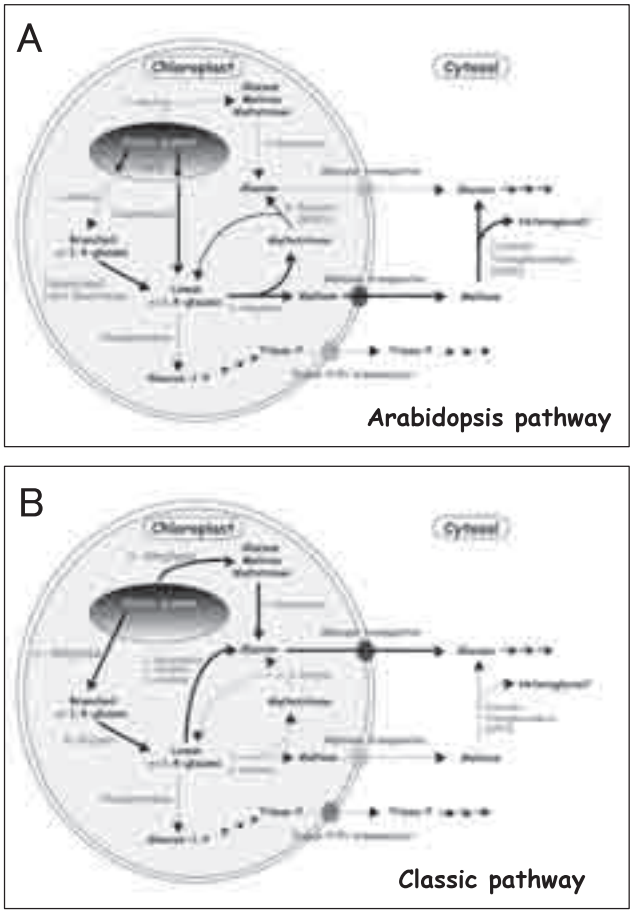

Fig.3 Different pathways of starch degradation in plant living cells.

(A) Arabidopsis pathway: GWD, PWD, isoamylase3, β-amylase and DPE1 mainly contribute the starch degradation in chloroplasts. The produced maltose is export to cytosol through maltose transporter MEX1. Maltose is further metabolized by DPE2 in cytosol. (B) Classical pathway: α-Amylase degrades starch granule to branched glucan, maltotriose, maltose and glucose. Branched glucan and maltooligosaccharides are further hydrolyzed to glucose by the concerted action of amlyases and R-enzyme. The resulting glucose is exported to cytosol via glucose transporter, and then entered to the usual metabolic pathway.

the control of starch metabolism in *Arabidopsis* leaves (Niittylä *et al*., 2006).

Starch degradation in cereal living cells

There is little information on the degradation mechanism of transitory starch in living cells in the seedling, leaf sheath and culm of cereal plants. However, the results obtained from studies on transgenic rice plants with suppressed expression and overexpression of α-amylase isoform I-1 (AmyI-1) (Asatsuma *et al*., 2005 and 2006) evoked an idea that endoamylase mainly contributes to the mechanism of starch breakdown (**Fig. 3B**, Perez *et al*., 1971). that is distinguishable from that in *Arabidopsis* leaves (Grennan, 2006).

Accompanied with the suppressed expression of AmyI-1, seed germination and subsequent seedling growth were markedly delayed in comparison with those in the wild-type plants. The slow germination seems to be due to the reduction of carbon source supply by the amylolytic breakdown of reserve starch in the endosperm. As expected, the growth retardation was overcome by supplementation of sugars, indicating that the phenotype is resulted from the inactivation of AmyI-1 expression. Interestingly, a significant increase of starch accumulation in the young leaf tissues was observed under sugar-supplemented condition. In contrast, the starch content of leaves harvested immediately after sunset was lower in the plants overexpressing AmyI-1. If AmyI-1 is involved in the starch metabolism in living cells, it must be in the plastids. The plastidial localization of AmyI-1 was confirmed through immunocytochemical and cell fractionation analyses, and expression and targeting of AmyI-1 fused to green fluorescence protein (GFP) in re-differentiated green cells (Asatsuma *et al*., 2005). α-Amylase probably plays a significant role in starch degradation. This idea may not be limited to cereals. The correlation between the increase in α-amylase activity and starch degradation has been observed in cotyledons during germination of starch-storing legume (Yomo and Varner, 1973; Tarrágo and Nicolás, 1976) and pea seeds (Juliano and Varner, 1969). In addition, during development of the spadix of cuckoo-pint (*Arum maculatum*) prior to thermogenesis, dramatic increase of the activity of endoamylase and appearance of oligosaccharides is consistent with the idea that the enzyme activity is responsible for granule degradation (Bulpin and ap Rees, 1978).

In *Arabidopsis* leaves, the plastidic β-amylase and maltose transporter without doubt play essential roles in normal starch degradation. By contrast, positive evidence for involvement of β-amylase in starch degradation in cereal living cells has not been reported yet. The β-amylase activity-deficient rice cultivars and inbred lines of mutant barley and rye exhibited normal growth and development. (Kreis *et al*., 1987; Rorat *et al*., 1991; Yamaguchi *et al*., 1999; Mitsunaga *et al*., 2001) Recent expression profiling studies of starch-metabolism-related plastidic translocator genes in both photosynthetic and non-photosynthetic organs of rice showed that the plastidic glucose transporter was substantially expressed in both source and sink organs, while the expression of the maltose transporter was obviously low (Toyota *et al*., 2006). These circumstantial evidences appear to indicate that the contribution of plastidic α-amylase and glucose transporter to starch degradation are physiologically important rather than the set of β-amylase and maltose transporter.

Novel targeting pathway of plastid proteins

All plastids in a particular plant species contain multiple copies of the same relatively small genome. However, most of the proteins existing in these organelles are encoded by the genes of nuclear genome, since the plastid genome has limited coding potential. Nuclear-encoded plastidial proteins are usually synthesized in the cytosol and posttranslationally imported into the organelle. In most cases, precursor proteins are synthesized with an NH_2-terminal pre-sequence called transit peptide. The transit peptide is necessary for and also sufficient for plastidial targeting and translocation initiation. Upon import, the transit peptide is proteolytically removed by a stromal processing peptidase and the mature protein attains the proper conformation. The transit peptide is recognized on the chloroplast surface by receptors, which are integral subunits of the Toc (translocon at the outer envelope of chloroplast) complex, and the import across the inner envelope is facilitated by the Tic (translocon at the inner envelope of chloroplast) complex (Soll and Schleiff, 2004; Kessler and Schnell, 2006). Several enzymes involved in starch metabolism, such as α-amylase (AMY3: Yu et al. 2005), β-amylase (BMY8: Lao *et al*., 1999), GWD (Yu *et al*., 2001), PWD (Kötting *et al*., 2005), pullulanase (LDA1: Renz *et al*., 1998), isoamylase (ISA1,2,3: Hussain *et al*., 2003), glucan phosphorylase (PHS1, Schächtele and Steup, 1986), possessed the typical N-terminal extensions acting as plastid-addressing domains in the precursor proteins, and were actually targeted to the plastids. This strongly suggests that these proteins are translocated to the plastid stroma through the canonical import machinery from the cytosol (**Fig. 4**). However, recent investigations have demonstrated that some glycoproteins including AmyI-1 and NPP1 are localized in the plastids (Asatsuma et al., 2005; Nanjo *et al*., 2006).

One carbonic anhydrase isoform (CAH1) in Arabidopsis was shown to be glycosylated and localized in the chloroplast (Villarejo *et al*., 2005). The nature of plastid targeting of CAH1-GFP fusion protein was investigated in transiently transformed *Arabidopsis* protoplasts. In the cells treated with brefeldin A (BFA), which blocks in the secretory system of eukaryotic cells (Ritzenthaler *et al*., 2002), accumulation of CAH1-GFP in the ER and Golgi-like structures was observed. Moreover, CAH1-GFP was redistributed to the chloroplast after removal of BFA (Villarejo *et al*., 2005). BFA also prevented NPP1-GFP accumulation in the chloropoasts of rice cells, and GFP fluorescence was distributed in the ER network (Nanjo et al., 2006). These experimental results

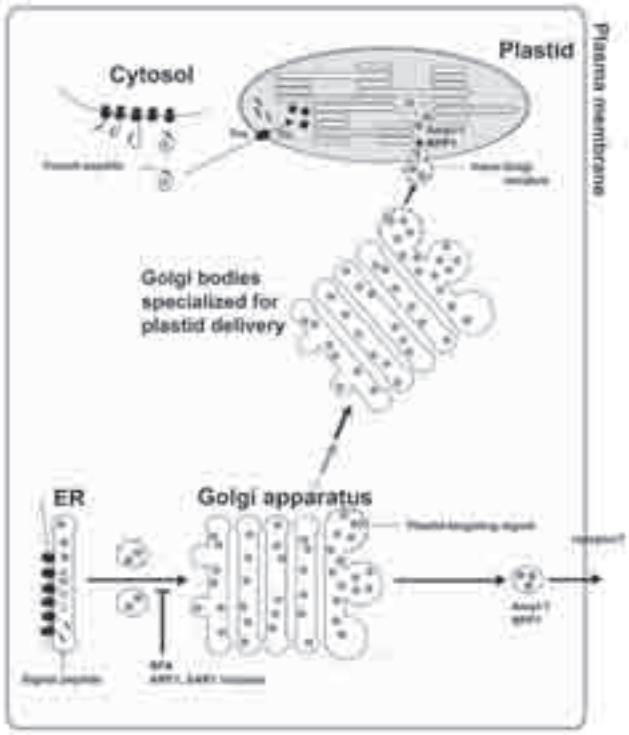

Fig.4 Plastid targeting pathway of proteins involved in starch metabolism.

Plastid targeting pathway of proteins involved in starch metabolism. Novel pathway. Rice AmyI-1 and NPP1 are transported from the Golgi apparatus to the plastid through the secretory pathway.

indicate that the ER-to-Golgi traffic is necessary for the plastid targeting of glycoproteins.

If there exists a traffic route from the Golgi apparatus to the plastid, a possible communication between these two organelles might go on. To test this idea, three-dimensional time-lapse and quantitative examinations were carried out by using high-performance conforcal laser scanning microscopic system. When fluorescent transe-Golgi marker (ST-mRFP) and plastid marker (WxTP-GFP) were simultaneously expressed together with AmyI-1 in onion epidermal cells, significant merging of ST-mRFP with GFP-labelled plastids was observed. A statistical analysis indicated that a large portion of ST-mRFP was incorporated into the plastids in the cells. Moreover, the time-lapse scanning revealed the occasional close contact of the Golgi and the plastids, and the presence of small membrane vesicles derived from the trans-Golgi on the surface of the plastid, which did not stay long on the envelope membranes, and that were finally located inside the plastid (Kitajima *et al*., 2009).

A hypothetical model for the plastid targeting of enzyme glycoproteins *via* secretory pathway is summarized in **Fig. 4**. The plastid-destined glycoproteins are synthesized in the ER lumen and transported to the Golgi apparatus in a way susceptible to BFA. In the Golgi apparatus, the glycoproteins are sorted and redirected to the plastids by as yet unknown receptor. The Golgi bodies with cargo move to the plastid, and the Golgi-derived vesicles fuse with the envelope membrane of the plastid. The fused membrane and cargo are imported inside the stroma by a mechanism totally unknown.

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デンプン生合成と分解

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要　約

高等植物は、光合成器官である葉緑体および従属栄養器官であるアミロプラストにそれぞれ一過性または貯蔵性デンプンを蓄積する。デンプン生合成にはプラスチドに加え細胞質もまた制御に関与している。これらのコンパートメントはプラスチド膜に局在するキャリアーによって代謝的につながっている。デンプン蓄積の表現型が異なるさまざまな変異体植物の遺伝学的および生化学的解析により、デンプン生合成に関与する複数の酵素が同定されてきた。特に、トウモロコシの変異体はデンプン生合成における様々な酵素の機能と役割を決める情報を提供してきた。さらに、近年の遺伝子操作技術にともない、新規かつ有用なデンプンの性質を持つ植物体の作製および操作が可能になっている。

我々は植物のデンプン代謝についての知見を概説するとともに、デンプン生合成の制御とその手法の発達およびデンプン分解によるエネルギー回収のメカニズムを紹介する。

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