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Starch mobilization in leaves

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Abstract

Starch mobilization is well understood in cereal endosperms, but both the pathway and the regulation of the process are poorly characterized in other types of plant organs. *Arabidopsis* leaves offer the opportunity for rapid progress in this area, because of the genomic resources available in this species and the ease with which starch synthesis and degradation can be monitored and manipulated. Progress in understanding three aspects of starch degradation is described: the role of disproportionating enzyme, the importance of phosphorolytic degradation, and new evidence about the involvement of a starch-phosphorylating enzyme in the degradative process. Major areas requiring further research are outlined.

Key words: Amylase, *Arabidopsis*, chloroplast, disproportionating enzyme, malto-oligosaccharide, starch, starch degradation, starch phosphorylase, starch phosphorylation.

Introduction

Interest in the process of starch degradation dates back several millennia, to the discovery that germinating cereal seeds produce sugars that can be fermented to alcohol. In the last century, the commercial and social importance of beverages derived in this way fuelled detailed scientific investigation of the nature and control of starch degradation in the endosperm, much of it funded directly by the beer and whisky industries. The process is understood in great depth at a biochemical and molecular level (Ritchie *et al*., 2000; Lovegrove and Hooley, 2000).

Unfortunately, the process of starch degradation in cereal endosperm does not serve as a model for this process in other plants. Most plant cells synthesize and degrade starch at some point in their development—as a major function in storage organs such as tubers, roots and embryos, as a transient phase in meristem and organ development, and on a diurnal basis in leaves. In all of these cases starch degradation differs from that in cereal endosperm in one very important respect. Endosperm is acellular at the time of starch degradation whereas degradation in all other circumstances occurs within living cells and, usually, within the plastids of those cells. The endosperm can be regarded simply as a bag of starch, into which hydrolytic enzymes are released during germination. The glucose produced by the combined actions of these enzymes is taken up by the embryo across the scutellum. There is little evidence that the process is subject to any of the fine controls that operate on metabolic pathways in living cells. By contrast, starch degradation within living cells provides carbon directly for the synthesis of sucrose and for growth and maintenance processes within the same cell. The pathway must be regulated in a manner that reflects its integration with these other metabolic processes.

Relatively little is known about either the nature or the regulation of starch degradation in organs other than cereal endosperm. Multiple forms of several different starch-degrading enzymes have been found in almost all of the organs studied in any detail. These include endo- and exo-amylases (α- and β-amylases, respectively), glucosidases, debranching enzymes, starch phosphorylase, and disproportionating enzyme (Trethewey and Smith, 2000). For most of these enzymes, some of the forms are located outside the plastid: the function is not known for such forms in cells in which starch degradation is plastidial.

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This complexity means that there are several possible pathways for starch degradation in most cells. Possible routes for degradation using enzymes known to occur in many plastids are illustrated in Fig. 1. Full understanding of the regulation of degradation cannot be achieved until the pathway itself is better defined. Understanding the regulation of the process is further complicated by the fact that many starch-degrading enzymes are present in both starch-degrading and starch-synthesizing cells. Most have no obvious regulatory properties that would prevent their activity during starch synthesis, and it is technically very difficult to assess whether or not significant degradation is occurring during net starch accumulation. In fact, recent models to explain the synthesis of amylopectin suggest that glucan degradation may be a necessary part of the process (Zeeman *et al*., 1998*a*; Myers *et al*., 2000; Smith, 2001).

The *Arabidopsis* leaf as a model starch-degrading organ

Arabidopsis thaliana potentially provides a model system in which many of the difficulties in understanding starch degradation can be eliminated. First, all of the genes encoding putative starch-degrading enzymes can be discovered from the genome sequence, and knock-out mutants for each can potentially be obtained (Thorneycroft *et al*., 2001). Second, populations of mutated plants can be screened for impaired ability to degrade starch simply by staining decolored leaves with iodine at the end of the night (Caspar *et al*., 1991; Zeeman *et al*., 1998*b*). Genomic resources allow the mutated gene to be identified with relative ease. Third, the diurnal cycle of starch synthesis and degradation in leaves allows starch degradation to be studied alongside starch synthesis in a short space of time. The processes can be tightly controlled and easily manipulated by changing environmental conditions, and followed in detail via pulse-chase experiments with ^{14}C-labelled carbon dioxide (Nielsen *et al*., 2002; Zeeman *et al*., 2002).

Whether the degradation of starch in leaves proceeds in the same manner and is regulated in the same way as that in other, non-photosynthetic starch-storing organs is not yet clear. Leaf starch is remarkably similar in composition and structure to that of other organs (Zeeman *et al*., 2002), and the suite of starch-degrading enzymes in the leaf contains representatives of all of the classes commonly found in non-photosynthetic tissues (Lin *et al*., 1988; Lin and Preiss, 1988; Caspar *et al*., 1989, 1991; Zeeman *et al*.,

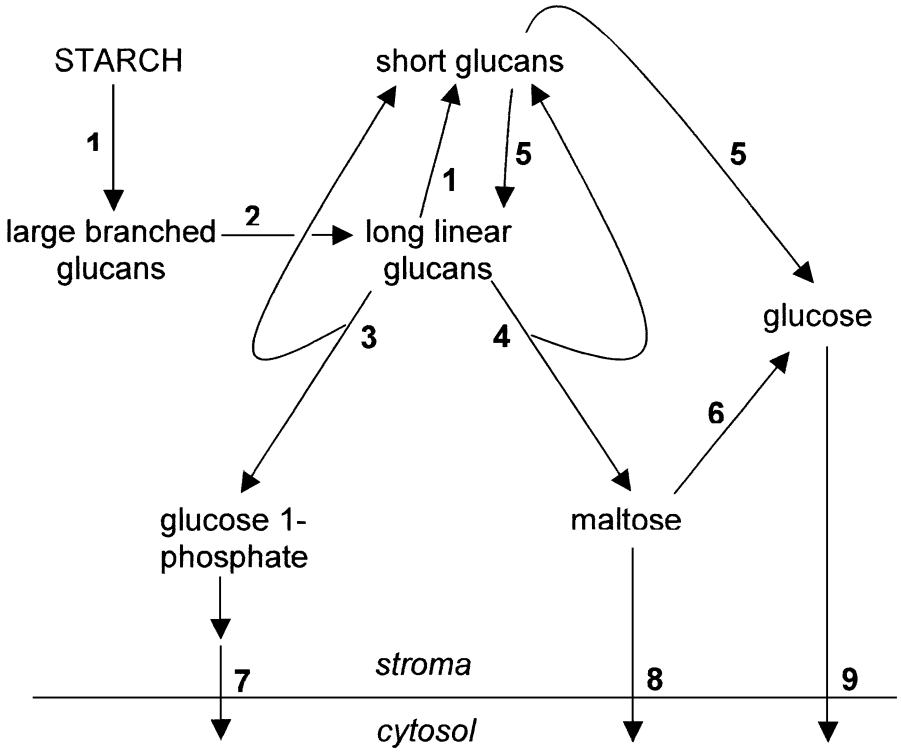

Fig. 1. Possible pathways of starch mobilization in a plastid. Reactions catalysed by enzymes known to occur in starch-containing plastids. Note that not all possible reactions are indicated: the actions of β-amylase, starch phosphorylase and disproportionating enzyme are shown only on linear glucans, but these enzymes can also act on the outer chains of branched glucans provided that these are of sufficient length. In other words, the action of debranching enzymes does not necessarily precede the actions of these exo-acting degradative enzymes. 1, α-amylase; 2, debranching enzymes (isoamylases and limit-dextrinase); 3, starch phosphorylase; 4, β-amylase; 5, disproportionating enzyme; 6, α-glucosidase; 7, triose-phosphate translocator; 8, hypothetical maltose translocator; 9, glucose translocator.

1998*a*). It remains possible, however, that the nature and regulation of the pathway vary considerably from one cell type to another. Differences might be expected between the mesophyll cell of a leaf and, for example, a germinating pea cotyledon and a cell type adjacent to a root meristem. In the pea cotyledon, plastidial membranes have disappeared and starch degradation occurs in the cytosol (Bain and Mercer, 1966; van Berkel *et al*., 1991). The major fate of the carbon released from starch is the synthesis of sucrose for export to the developing embryonic axis. In a cell adjacent to a meristem, starch can potentially act as a 'carbon buffer' balancing demand for carbon skeletons and energy within the cell with the rate of import of carbon as sucrose. It is likely that there is a fine balance between synthesis and degradation, perhaps shifting on a diurnal basis. However, whether or not starch degradation in the *Arabidopsis* leaf cell is representative of that elsewhere, it is accessible to many types of experimental approach that are simply not possible in other cell types and species. The aim is to understand the process in the leaf cell to provide a baseline from which aspects of degradation in other cell types may be investigated.

In this article, three examples of recent progress in understanding starch degradation in *Arabidopsis* leaves are discussed. The first two illustrate the use of a gene-targeted approach to isolate knock-out mutants, enabling analysis of the roles of enzymes of previously unknown importance. The third illustrates the value of conventional phenotypic mutant screens in discovering new components of the starch degradation pathway.

Disproportionating enzyme

Disproportionating enzyme (D-enzyme) catalyses the transfer of α-1,4 linked oligosaccharides from the end of one glucan chain to the end of another. The shortest substrate on which it can act is maltotriose (Fig. 2), but it can also act on very large glucan molecules. *In vitro* it will act on both amylose and amylopectin, and can catalyse the formation of large circular molecules from both of these substrates (Takaha *et al*., 1996, 1998*a*). In spite of its widespread occurrence in plants, its role was unknown until recently. Two speculations had been put forward. First, it might create longer glucans from short malto-oligosaccharides produced by other enzymes during starch degradation. The degradation of glucans released from the starch granule by α-amylase and debranching enzymes is believed to be catalysed by phosphorylase and β-amylase (Fig. 1). These enzymes act preferentially on longer glucans and have no action on maltotriose. By creating longer glucans from short malto-oligosaccharides, D-enzyme would increase the efficiency of glucan degradation by phosphorylase and β-amylase (Takaha *et al*., 1993). Second, D-enzyme might be involved in starch synthesis, acting to recover malto-oligosaccharides produced during the maturation of amylopectin molecules. One model of amylopectin synthesis proposes that a 'pre-amylopectin' molecule is acted on by a debranching enzyme to create the mature amylopectin molecule. The malto-oligosaccharides released by this debranching process could be linked back onto pre-amylopectin molecules by the action of D-enzyme (Myers *et al*., 2000). This proposal for the role of D-enzyme arose from the characterization of a mutant of the unicellular green alga *Chlamydomonas* that lacks D-enzyme activity. The *STA11* mutant has reduced amounts of starch with altered amylopectin structure, and accumulates malto-oligosaccharides (Colleoni *et al*., 1999).

Attempts to discover the role of D-enzyme in higher plants have been made previously by generating transgenic potatoes in which its activity was severely reduced. In spite of reductions in activity in some lines of up to 99%, no effect on starch synthesis, composition and structure in the tuber was observed. However, sprouting of tubers was altered, indicating that D-enzyme may be involved in starch degradation (Takaha *et al*., 1998*b*). To provide more definitive information, an *Arabidopsis* knockout mutant with a T-DNA insertion in the D-enzyme gene was selected. The mutant lacked detectable D-enzyme protein. Several different means of assaying D-enzyme failed to detect activity, indicating that it was reduced to less than 1% of that of wild-type plants (Critchley *et al*., 2001).

Mutant plants grown under a 12 h light regime had reduced rates of both starch synthesis and degradation. It was reasoned that this could reflect either a direct requirement of D-enzyme for both processes or a direct requirement for one of the processes with secondary, knock-on effects on the other process. To distinguish between these possibilities, wild-type and mutant plants were kept in the dark for 36 h to eliminate all starch and

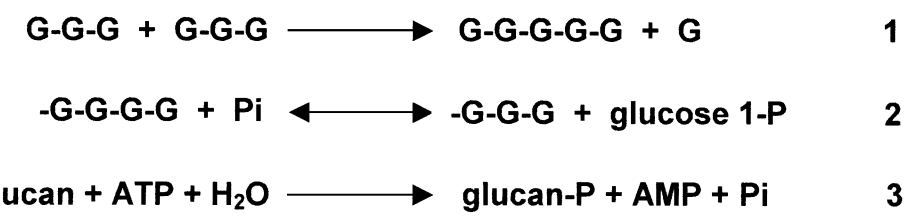

Fig. 2. Reactions catalysed by disproportionating enzyme (1) starch phosphorylase (2) and R1 protein (3). Pi is inorganic phosphate.

starch degradation products. Analysis of starch accumulation and structure during subsequent growth in the light showed that D-enzyme is not necessary for normal starch synthesis in *Arabidopsis* leaves (Critchley *et al*., 2001).

Starch degradation in plants lacking D-enzyme was accompanied by the accumulation of malto-oligosaccharides to levels about 15 times greater than those of wild-type plants. Almost all of this accumulation was as maltotriose: maltotriose levels started to rise immediately at the onset of the dark period and reached very high levels during the night. Malto-oligosaccharide levels then fell to near-normal during the first few hours of the light period (Critchley *et al*., 2001).

These data are entirely consistent with the idea that D-enzyme is necessary for normal starch degradation. It metabolizes glucans that are too short for the actions of β-amylase and phosphorylase, creating longer chains on which these enzymes can act.

Starch phosphorylase

Starch phosphorylase catalyses the phosphorolysis of a linear glucan chain to yield glucose 1-phosphate (Fig. 2). The reaction is potentially reversible under physiological conditions, and the enzyme was formerly supposed to be responsible for starch synthesis. This idea was largely rejected following recognition of the importance of starch synthases and the discovery that levels of glucose 1-phosphate and phosphate in the plastid strongly favour degradation rather than synthesis of glucans via phosphorylase (Preiss and Levi, 1979, 1982). Nonetheless, strong evidence of an important role for phosphorylase in starch degradation is lacking. In spite of the presence of isoforms in both the plastid and the cytosol in essentially all starch-containing cells thus far examined, during both starch synthesis and starch degradation, antisense experiments in potato have failed to reveal a major role for the enzyme (Duwenig, 1997; Duwenig *et al*., 1997; Kossmann and Lloyd, 2000). Early speculation that phosphorolysis was the major or only route of glucan degradation in chloroplasts arose because of difficulty in detecting chloroplastic amylase activities against a background of high extra-chloroplastic activity (Stitt *et al*., 1978). It is now clear that many chloroplasts and other starch-containing plastids possess both α- and β-amylases in addition to phosphorylase (da Silva *et al*., 1997; Lao *et al*., 1999), and are able to degrade starch hydrolytically as well as phosphorolytically (Schleucher *et al*., 1998).

The most plausible explanation for the existence of both phosphorolytic and hydrolytic capacities in chloroplasts is that their products are directed towards different metabolic pathways in the cell. Foyer, Stitt and colleagues speculated that glucose produced via hydrolytic degradation was exported from the plastid and used as a substrate for sucrose synthesis, whereas glucose 1-phosphate produced via phosphorylase was converted to triose phosphates and exported via the triose phosphate translocator for use in the respiratory and biosynthetic metabolism of the leaf cell (Stitt *et al*., 1985).

To gain definitive information about the importance of plastidial phosphorylase in *Arabidopsis* leaves, a knockout mutant with a T-DNA insertion in the single gene encoding this isoform was selected. It has been confirmed that plastidial activity is missing but cytosolic activity is unaffected in the mutant, using native gels that separate the two forms of the enzyme. Assays also confirm a large decrease in total activity of the enzyme in the mutant under conditions that favour activity of the plastidial rather than the cytosolic isoforms. However, thus far it has not been possible to detect any general alteration in starch and sugar metabolism in mutant plants. Changes in starch, sucrose and hexoses over 24 h in plants under a 12 h light regime are essentially identical in wild-type and mutant plants. These results suggest that hydrolysis rather than phosphorolysis may be the major route of starch degradation in the chloroplast. Although they do not rule out the scheme put forward by Foyer, Stitt and colleagues, they show that phosphorolysis is not necessary for normal metabolism in most leaf cells under these growth conditions.

Although there is no general impact of loss of phosphorylase on leaf carbohydrate metabolism, the mutant plants display a minor phenotype that indicates an essential role for the enzyme under some circumstances. The edges of some leaves develop necrotic lesions, accompanied by very large accumulations of starch in adjacent cells. This may indicate that phosphorolysis is necessary to supply carbon to particular metabolic pathways under particular environmental/developmental conditions. Further investigation of this phenotype is in progress.

The R1 protein

Investigations in potato in the last five years have revealed a new and hitherto unsuspected aspect of the pathway of starch degradation. A previously unknown protein, named R1, found in the matrix of starch granules from potato tubers was shown via antisense experiments to be necessary both for normal rates of starch degradation in leaves and tubers and for phosphorylation of the amylopectin component of starch (Lorberth *et al*., 1998). The protein has subsequently been shown to be a starch–water dikinase (Fig. 2), responsible for the low level of phosphorylation of amylopectin observed in most starches (Ritte *et al*., 2002). About 1 in 300 glucosyl residues in potato starch are normally phosphorylated on the six- or three-position: in cereal and leaf starches the level of phosphorylation is considerably lower than this (Blennow *et al*., 2000). The association between the level of R1 protein and the rate of starch degradation suggested that activity of a key starch-

degrading enzyme required association either with phosphate groups on amylopectin or glucans derived from it, or with the R1 protein itself.

The precise relationship between the R1 protein, amylopectin phosphorylation and starch degradation is still not understood. However, the possibility of rapid progress is offered by the recent discovery that the starch accumulating *sex1* mutant of *Arabidopsis* is defective in the R1 protein. The *sex1* mutant was originally isolated in an iodine-based screen for mutant plants unable to degrade starch in the dark (Caspar *et al*., 1991). Levels of starch in the leaves of *sex1* plants grown under a 12 h light regime are about five times higher than the maximum levels in wild-type plants, and show less diurnal variation (Trethewey and ap Rees, 1994). Initial work indicated that capacity for exchange of glucose across the chloroplast envelope was impaired in the mutant, and it was suggested to lack a glucose transporter (Trethewey and ap Rees, 1994). Later cloning of the major glucose transporter of the chloroplast envelope showed, however, that the gene encoding this protein is on chromosome 5 whereas the *sex1* mutation maps to chromosome 1 (Weber *et al*., 2000).

Map-based cloning approaches have recently shown that the gene at the *sex1* locus encodes the *Arabidopsis* homologue of the R1 protein (Yu *et al*., 2001). As expected, the starch of *sex1* mutants was found to have a drastically reduced phosphate content: four out of the six mutant alleles so far discovered give rise to a phosphate level that is 10% or less of that of wild-type plants. There was a general correlation between the level of reduction of phosphate in amylopectin and the extent of starch accumulation in the leaves. The discovery of an *Arabidopsis* R1 mutant will now allow this new aspect of the regulation of starch degradation to be fully explored.

Current questions about starch degradation

As described above, progress made possible by the use of *Arabidopsis* mutants has revealed (a) a critical role for disproportionating enzyme, (b) the importance of the hydrolytic rather than the phosphorolytic pathway under 'normal' *Arabidopsis* growth conditions, and (c) the requirement for R1 protein and/or its product, phosphorylated glucosyl residues of amylopectin, for starch degradation. Several critical questions about the pathway remain to be answered. These include

(1) The nature of the enzyme responsible for the primary attack on the starch granule. Progress towards identification of the enzyme has been made through characterization of the *sex4* mutant, a plant that, like the *sex1* mutant, has a severely reduced capacity for starch degradation. Native gels revealed that a specific amylolytic activity is missing or severely reduced in *sex4* leaves (Zeeman *et al*., 1998*b*). Further work on this amylase is in progress.

(2) The nature of the debranching enzyme responsible for cleaving α-1,6 linkages during starch degradation. Four debranching enzyme genes have been identified in the *Arabidopsis* genome, three encoding isoamylases and one encoding a limit dextrinase. A mutant lacking one of the isoamylases was isolated from an iodine-based screen of a mutant population (the *dbe1* mutant), and was shown to have highly abnormal glucan synthesis. Chloroplasts accumulated a small amount of starch, but also accumulated large amounts of phytoglycogen, a highly-branched non-crystalline glucan. Both starch and phytoglycogen were degraded in a 12 h dark period, indicating that the isoamylase affected by the mutation is not necessary for glucan degradation (Zeeman *et al*., 1998*a*). Which of the remaining three debranching enzymes fulfils this function is under investigation.

(3) The identity of the β-amylases that degrade linear glucans. At least three β-amylases encoded in the *Arabidopsis* genome are predicted to have plastidial target peptides. Their roles in starch degradation in the chloroplast are not known.

(4) The fate of the products of β-amylase. β-amylase catalyses the release of the disaccharide maltose from the non-reducing ends of linear glucan chains. Maltose is presumed either to be hydrolysed to glucose via a maltase (α-glucosidase) inside the chloroplast and exported to the cytosol via the glucose translocator, or exported as maltose and hydrolysed by a cytosolic maltase (Rost *et al*., 1996). The locations and roles of most of the α-glucosidases encoded in the *Arabidopsis* genome are not yet known, and a maltose translocator protein has not yet been described. A maltose-accumulating mutant of *Arabidopsis* has been identified using a novel screen for plants with abnormal levels of malto-oligosaccharides: identification of the mutation is in progress and is expected to shed light on the fate of β-amylase products during starch degradation.

(5) Regulation of the degradative process. Pulse-chase experiments with ^{14}C carbon dioxide confirm that little or no starch degradation occurs in the light in *Arabidopsis* leaves, but the process starts immediately upon the onset of darkness (Zeeman *et al*., 2002). Although transcript levels of several starch-degrading enzymes have been shown to vary on a diurnal basis and in response to the circadian clock (Harmer *et al*., 2000), assays and native gels that reveal activity of these enzymes have failed to show major differences between light and dark (our own data). It has not been possible to confirm earlier reports of a diurnally-varying amylase activity from *Arabidopsis* leaves (Kakefuda and Preiss, 1997), although this is still under investigation. Perhaps the most important piece of information needed in order to understand the regulation of degradation is the nature of the enzyme that catalyses the primary attack on the granule (see (1) above). The key to regulation may lie in the properties of this enzyme, and the

nature of associations it forms with its substrates and, possibly, the R1 protein.

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