Synthesizing Starch: Roles for Rugosus5 and Dull1

One feature that has undoubtedly contributed to the success of higher plants is their ability to set aside excess fixed carbon, either transiently or in specialized storage organs. Starch is the most prevalent carbon storage compound in plants. Its transient deposition in leaf chloroplasts helps to dampen diurnal fluxes in energy input from sunlight, whereas longer term storage in the form of starch granules, which accumulate in specialized plastids termed amyloplasts, plays an important role in nourishing germinating embryos and supporting vegetative propagation.

Starch is also critically important for people. It constitutes the major source of calories in the human diet, and there is profound interest in exploring the possibility that starch biosynthetic pathways could be manipulated to improve the quantity or quality of starch produced by plants. However, it will be difficult to achieve these goals without a comprehensive understanding of both starch biosynthesis and the links between starch chemistry and starch granule structure.

That’s not to say that we know nothing about these processes. In fact, the four enzymatic activities involved in starch synthesis—ADP-glucose pyrophosphorylase, starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (DBE)—have been defined in several plant species and some of the corresponding genes have been cloned (for reviews, see Martin and Smith, 1995; Ball et al., 1996; Nakamura, 1996; Smith et al., 1997).

We also are beginning to learn how these activities cooperate to generate the two polymers that make up the majority of the starch granule: amylose, which is a predominantly linear polymer of glucose units linked by α(1,4) bonds, and amylopectin, usually the more abundant of the two, in which the α(1,4) bonds are interspersed with α(1,6) linkages that generate branches in the growing glucan chains. The frequency and distribution of these branches define several chemically distinct forms of amylopectin.

More importantly, amylopectin branching patterns may help to establish the organization of starch polymers in the developing granule, providing an important link between the activities of starch biosynthetic enzymes and the structure of the starch that is produced (Ball et al., 1996). For example, it has been proposed that as an amylopectin chain grows, it undergoes successive elongation, branching, and debranching steps that generate an ordered structure which alternates between zones of more branched and less branched amylopectin. Longer amylopectin molecules may act as a kind of cement to hold together several adjacent zones.

The periodicity of this alternating structure is about 9 nm in all organisms that have been examined, suggesting that it in turn reflects a carefully orchestrated (and well conserved) series of biochemical activities (Ball et al., 1996). Higher order double helical structures, which may help to maximize the packing efficiency of starch, are also evident in some starches, and there appears to be a correlation between the organization of these structures and the formation of the alternating concentric layers of crystalline and more amorphous starch that can be seen in many starch granules (for a recent review of starch granule structure, see Gallant et al., 1997).

It is also clear that the branching patterns in amylopectin are under genetic control and are not random. Mutations in various SSs and SBEs can cause variation in the arrangement of shorter and longer branches and in the ratio of amylose to amylopectin. These changes can have profound effects on the shape of starch granules and on the physical properties of the starch (Martin and Smith, 1995; Nelson and Pan, 1995; Smith et al., 1997).

To generate this level of organization, the four enzymatic activities mentioned above must be controlled to a high degree of precision. Indeed, biochemical studies in many plant species point to a complex and highly regulated pathway. There are several isoforms of each enzymatic activity, and the extent to which each individual isozyme directs a specific step on the pathway can vary depending on the rate of sugar import into the plastid, the developmental timing of its expression, its location within a growing starch granule (i.e., whether the enzyme is on the surface of a granule or embedded within it), and the environmental conditions (see, e.g., Tomlinson et al., 1998). Gene dosage and genetic background can also exert profound effects on starch synthesis (see, e.g., Nelson and Pan, 1995; Singletary et al., 1997).

Despite this complexity, a combination of biochemical and genetic approaches is beginning to tease out the links between specific genes, individual starch biosynthetic activities, and the chemical nature of starch. For example, analyses of waxy loci in the cereals have helped to determine that a granule-bound isozyme of SS (GBSS) is largely responsible for the synthesis of amylose in storage organs.

Because the lack of this GBSS activity has little effect on the amount of starch or the basic structure of the starch granule, these analyses also suggest that soluble SSs are primarily responsible for amylopectin synthesis. However, with at least three biochemically distinguishable soluble SS activities present in most plant species, the specific roles of individual SSs in amylopectin synthesis...
have been much more difficult to elucidate. In particular, we do not know whether distinct isoforms of soluble SSs contribute to the synthesis of specific forms of amylopectin and/or to specific amylopectin branching patterns.

Two papers in this issue of THE PLANT CELL make major contributions to our understanding of the role of individual soluble SSs in amylopectin synthesis and the manner in which these enzymes interact with other components of the starch biosynthetic pathway. Each article follows a different route to reach its conclusions: on pages 399–412, Gao et al. begin with the endosperm starch-defective dull1 (du1) mutant of maize and use their knowledge of the starch biosynthesis phenotypes provoked by this mutation to identify and characterize the SS encoded by the du1 gene. And on pages 413–426, Craig et al. work from the opposite direction, determining that the rugosus5 (rug5) mutant of pea has a defect in a previously identified SS gene that is active in the pea embryo.

As Gao et al. describe, although mutations in the du1 gene have little effect on the morphology of starch granules in the maize endosperm, a great deal of previous work points to a role for DU1 in starch synthesis (for a review, see Nelson and Pan, 1995). For example, in addition to the eponymous dull luster of the mutant kernels, du1 mutant endosperm has slightly lower total carbohydrate than does that of the wild type. Moreover, the relative amylose content of starch in du1 mutant kernels is significantly higher than it is in wild-type kernels. However, it is the effect of mutations in du1 on amylopectin structure that most interests the authors. Approximately 15% of the starch in the du1 mutant endosperm is thought to consist of abnormally highly branched amylopectin chains (so called “intermediate material”; Wang et al., 1993a, 1993b).

Taken together with the biochemical characterization of the du1 mutants (Boyer and Priess, 1981), these defects in starch synthesis suggest that mutations in du1 negatively affect the activity of a soluble SS and/or that of an SBE. To elucidate the molecular basis for the phenotypes and to determine whether either of these possibilities is supported by additional data, Gao et al. set about cloning du1 by screening for transposon-tagged du1 mutants (a strategy that has paid off in the isolation of genes encoding other components of the starch biosynthetic pathway; for reviews, see Nelson and Pan, 1995; Smith et al., 1997). The authors were able to identify six such mutants in their screen, and in a series of genetic and molecular experiments, they provide solid evidence that du1 encodes a soluble SS.

Gao et al. offer several lines of reasoning to support this contention. First, the predicted mass of the DU1 protein is just a little higher than that defined biochemically for the larger of the two major soluble SSs that have been described in maize endosperm (Mu et al., 1994; the small difference can be accounted for by a putative transit peptide in the deduced amino acid sequence of DU1). Second, du1, which appears to be a single-copy gene, is not expressed in leaves, but it is expressed throughout the early stages of starch synthesis in the endosperm. Third, the du1 mutant with the most severe phenotypes carries a Mutator insertion within the predicted DU1 coding region. However, it is the similarity between the deduced amino acid sequences of DU1 and that of the major soluble SS in potato (termed SSIII; Abel et al., 1996; Marshall et al., 1996) that perhaps offers the most compelling evidence of a role for DU1 in starch synthesis. The C-terminal regions of SSIII and DU1 are both related to a domain that, on the basis of its conservation in other plant SSs and in the analogous (and possibly ancestral) bacterial glycogen synthases, is thought to mediate \( \alpha(1,4) \)-glucosyltransferase activity.

Despite this similarity in the presumed SS active site region, it is the central domain of DU1 that is most similar to the corresponding region of potato SSIII. This region is evidently unrelated to other starch or glycogen synthases and Gao et al. propose that it may define a domain that carries out aspects of starch synthesis that are specific to this subclass of soluble SS.

Following this line of reasoning a little further, Gao et al. go on to suggest that the 770-amino acid N-terminal extension of the DU1 protein, which has no extensive similarities to other SSs, may encode functions specifically related to the role of DU1 in maize. This region of DU1 does include a hierarchical repeat structure with an internal sequence that is similar to a sequence conserved in SBEs from a number of plant species. Although crystallographic and enzymatic studies of SBEs show that this conserved sequence is located in the active site of these enzymes, it is difficult to evaluate the significance of the related repeats in DU1. One possibility raised by Gao et al. is that they form a “flexible” N-terminal domain that may be involved in mediating interactions with other starch biosynthetic enzymes.

Despite these strong indications that du1 encodes the major soluble SS activity in maize endosperm, Gao et al. point out a number of incongruities that warrant continued investigation of the effects of mutations in du1 on other starch biosynthetic enzymes. Most importantly, the overall starch content of du1 mutant kernels is only slightly lower than that of wild-type kernels. This suggests either that there are other SS activities yet to be identified and characterized in maize endosperm or that the du1 alleles identified to date are sufficiently leaky (despite the indications of Gao et al.’s expression data) to allow starch synthesis.

A second striking point that Gao et al.’s work highlights is that mutations in the du1 gene also lead to apparent decreases in SBE activity in maize en-
dosperm (Boyer and Priess, 1981). That mutations in one starch biosynthetic gene should have a significant effect on the activity of another is rather unusual and suggests that distinct steps in the biosynthetic pathway may be coordinately regulated. With DNA probes for the major soluble SS and a number of other starch biosynthetic enzymes now available, the question of whether these biochemical changes are brought about by alterations in gene expression can be evaluated more extensively in a variety of maize mutant backgrounds.

Maize endosperm is by no means the only plant tissue in which the synthesis of storage starch has been investigated in detail. Another important experimental system for evaluating this pathway is the pea embryo, and several starch biosynthetic activities have been identified in this tissue as well. However, many of the same questions posed above regarding interactions among starch biosynthetic activities and the genes that encode them must also be addressed in pea. For example, a pea cDNA that appears on the basis of its sequence and expression characteristics to encode a soluble SS that is active in the pea embryo was cloned some time ago (Dry et al., 1992). However, without mutations in the corresponding gene, it has been difficult to determine precisely what the role of this SS may be.

In the second paper in this issue to focus on soluble SSs, Craig et al. continue their use of a “wrinkled seed” screen to identify genes encoding enzymes involved in starch synthesis in the pea embryo (the wrinkled seed phenotype is often associated with reduced rates of starch accumulation). The authors discovered that mutations at the rug5 locus resulted in aberrant starch granule morphology and reasoned that the mutant plants may have a specific defect in a soluble SS.

Craig et al. confirmed this hypothesis by testing protein extracts from rug5 mutants with an antibody raised against the SSII form of SS from pea embryos and by linkage analysis with the cDNAs identified earlier by Dry et al. (1992), showing that the SSII protein is absent from some of the mutants and that the SSII cDNAs map to the same position as the mutations at the rug5 locus. Moreover, Craig et al. demonstrate that one of the severe rug5 alleles, rug5-a, is caused by the introduction of a premature stop codon that probably leads to the production of a truncated and therefore nonfunctional enzyme.

Craig et al.‘s extensive biochemical analyses show that the effects of mutations in the rug5 gene on the branching characteristics and chain length of amylopectin in the pea embryo are superficially similar to those provoked by mutations in the maize du1 gene. Indeed, their analysis of starch structure in the rug5 mutant embryos points to a marked decrease in the number of amylopectin chains of intermediate length (the so-called B2 and B3 chains) and an increase in the numbers of very short chains.

In this paper, the authors suggest that the SSII activity that is missing in rug5 embryos may be responsible primarily for the synthesis of the B2 and B3 chains in normal amylopectin and that in its absence, larger numbers of shorter chains are formed. Craig et al. go on to postulate that this shift in the length distribution of glucan chains may compromise the ability of the amylopectin in rug5 mutants to form double helices and hence to pack in the concentric layers that determine, at least in part, starch granule morphology.

Like Gao et al., Craig et al. also confront some apparent anomalies in their data. For example, although the rug5-a allele is a suspected null (Craig et al. are unable to immunologically detect the corresponding protein in pea embryos), soluble SS activity is reduced by only a third in the early stages of seed development and is apparently the same as that in wild-type seeds at later stages.

To explain these observations, Craig et al. suggest that other SS activities may increase to accommodate the lack of the SS encoded by rug5 (which usually is responsible for 60% of the soluble SS activity in wild-type embryos). Because their data indicate that an SSIII-like SS that is present in both the wild-type and rug5 mutant embryos is apparently not responsible for this “compensating” SS activity, the authors conclude that a third soluble SS, which remains to be identified, must be present in the pea embryo.

Another factor complicating interpretations of the effects of the rug5 mutation on amylopectin structure is that rug5 mutant embryos exhibit higher GBSS activity than do wild-type embryos. Craig et al. suggest that this increase may account for the greater abundance of very long glucan chains in the amylopectin of these embryos relative to the amount of this class of amylopectin chains in the starch of wild-type pea embryos.

Through their analyses of the effects of the rug5 and du1 mutations on starch synthesis, the authors of these two papers have generated valuable information on the roles played by soluble SSs in starch biosynthesis and the manner in which these enzymes interact with other components of the starch biosynthetic pathway during pea embryo and maize endosperm development. Together with ongoing investigations of starch synthesis in Chlamydomonas, which appears to possess several genes and activities that are related to those involved in starch synthesis in higher plants (see, e.g., Mouille et al., 1996; Buléon et al., 1997), continued analyses of du1 and rug5 and the enzymatic activities they encode should lead to a more comprehensive understanding of the ways in which the activities of specific starch biosynthetic enzymes can affect starch branching patterns and the morphology of starch granules.

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Are the Hypervariable Regions of S RNases Sufficient for Allele-Specific Recognition of Pollen?

In a recent research article published in THE PLANT CELL ("Hypervariable Domains of Self-Incompatibility RNases Mediate Allele-Specific Pollen Recognition"), Matton et al. (1997) show that the pollen recognition function of the Solanum chacoense S<sub>11</sub> RNase can be converted to that of the S<sub>13</sub> RNase by replacing the two hypervariable (HV) regions of S<sub>11</sub> RNase with those of S<sub>13</sub> RNase. From these results, Matton et al. conclude that, "one allelic form of the S RNase molecule can be converted into another by modification of the HV domains alone and that allelic
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