Advances in the Understanding of Starch Synthesis in Wheat and Barley

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Abstract: The synthesis of starch in wheat and barley is an important topic for research because of the extensive utility of starch from these crop species in human and animal foods, and in industrial processes. Wheat and barley starches are highly characteristic due to their granular architecture and multi-modal granule size distribution. This granule architecture is important because it defines the ways in which wheat and barley starches behave during food processing. The core starch biosynthetic genes of wheat have been cloned and shown to exist as homeologous sets of genes represented on each of the three wheat genomes. While hexaploidy represents a major impediment to the selection of altered starch phenotypes by phenotypic screening, the availability of methods for identifying the products of homeologous genes from each of the wheat genes has provided methods for the selection of triple null lines from waxy, starch synthase IIa, and branching enzyme I genes. In barley, direct phenotypic selection has resulted in the identification of waxy, amol and SSIIa mutations. In this paper, we review the state of knowledge of starch synthesis in wheat and barley and discuss the relationships between individual genes and their roles in starch biosynthesis.

Key words: ADPglucose pyrophosphorylase, barley, starch branching enzyme, starch debranching enzyme, starch synthase, wheat

Plants need to store carbon in compact dehydrated forms that are efficiently synthesized and yet are readily available to be degraded when the stored carbon is required to fuel physiological processes. Plants have produced two economically useful solutions to this problem, accumulation of starch and lipid. Starch in higher plants is deposited in discrete granular structures in the plastid, present in almost all tissues of the plant, but predominantly in either leaves and storage organs such as seeds or tubers. Starch is composed of a single monomer unit, glucose, connected through either α-1,4 or α-1,6 linkages. Starch is generally considered to be composed of two broad classes of molecules, amylose with a total degree of polymerization of 500 to 5000 and with infrequent α-1,6 linkages (<1%), and amylpectin with a much higher degree of polymerization (50,000 to 500,000) and frequent α-1,6 linkages (5-6%). In wheat and barley, starch in the seed endosperm is packaged into two types of granules, known as “A” and “B” granules. “A” granules are initiated in the first few days after anthesis and reach diameters of 20–25 μm at grain maturity, whereas “B” granules are typically initiated from 12 days after anthesis and reach sizes of up to 10 μm in diameter.11 “A” granules are lenticular in shape and contain a characteristic equatorial groove encircling the granule, whereas “B” granules tend to be essentially spherical in shape and do not contain a clear equatorial groove. Vast numbers of B granules are initiated during endosperm development, so that by grain maturity, greater than 98% of the number of granules in the endosperm are B granules, yet only 20–30% of the starch weight is found in this fraction.11

Starch biosynthesis in higher plants can be considered to include at least 4 central steps, firstly, substrate activation by ADPglucose pyrophosphorylase, secondly, chain elongation by starch synthases; thirdly, chain branching by branching enzymes; and fourthly, chain debranching by debranching enzymes.4 Multiple forms of each of the enzymes catalyzing these reactions are present in plants. Due to the complex nature of the biosynthetic process much of the investigation of starch synthesis has concentrated on model systems such as Chlamydomonas, Arabidopsis and peas. However, because of their economic importance it is essential to be able to understand and to manipulate starch biosynthesis in food crops such as wheat, rice and maize. Although great insight can be obtained from model systems, there are often species-specific properties that can be of considerable practical importance. In this paper, we will discuss the starch biosynthetic process in higher plants with special reference to starch biosynthesis in wheat and barley.

ADPglucose pyrophosphorylase.

The first committed step in starch biosynthesis in wheat and barley endosperm, as in other tissues, depends on the activation of glucose-1-phosphate to form ADPglucose (Fig. 1). Early work on leaf tissues demonstrated that ADPglucose pyrophosphorylase was a plastid located enzyme with complex regulatory properties including allosteric activation by 3-phosphoglycerate and allosteric inhibition by inorganic phosphate.3 However, this situation does not necessarily apply in the endosperm of plants and does not appear to apply in barley. A range of studies have shown that in the cereal endosperm there are both plastidic and cytosolic forms of ADPglucose pyrophosphorylase.18,19 In barley the cytosolic enzyme appears to be insensitive to 3-phosphoglycerate activation yet remains somewhat sensitive to inorganic phosphate inhibition.9

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Higher plant ADPglucose pyrophosphorylases are composed of two subunit types, a large and a small subunit. Evidence from the analysis of the maize shrunken-2 and brittle-2 mutants indicates that cytosolic and amyloplastic enzymes are encoded by separate sets of large and small subunit genes. Mutations associated with ADPglucose pyrophosphorylase genes that reduce starch content (in maize, brittle-2 and shrunken-2) are found to be lesions within the large and small subunit genes respectively that are expressed in the cytoplasm. These results suggest that the plastidic forms are unable to support the flux of carbon required to maintain normal starch biosynthesis and their role in the endosperm is unknown. The plastidic forms may however be of importance in leaf starch biosynthesis in cereals, however this has yet to be unambiguously demonstrated. The cytoplasmic location of ADPglucose pyrophosphorylase and the demonstration of the importance of the cytosolic forms to starch biosynthesis, indicates that an active transport mechanism is required to transfer ADPglucose from the cytosol to the amyloplast and Emes and colleagues have presented preliminary evidence on the characterization of such a transporter.

**Starch synthases.**

The existing chains of amylose or amylopectin are elongated by starch synthases, enzymes that utilize ADPglucose as substrate, transferring the glucosyl moiety to the non-reducing end of the extant polymer, forming a new α-1,4 linkage. This raises a fundamental question in starch biosynthesis that remains to be answered—how are the amylose and amylopectin chains first primed? While there is no clear answer to this question to date, a number of suggestions have been made. Firstly, that a self-glucosylating enzyme such as glycogenin is present that catalyses the synthesis of priming molecules. Secondly, that malto-oligosaccharides released from starch by debranching enzyme and other degradative reactions are available to prime synthesis, and thirdly, that starch synthases themselves are able to prime de novo synthesis through alternative mechanisms. It is fully possible that a combination of these, or alternative mechanisms, is involved.

In plants, there are two general classes of starch synthases. Firstly, those that are exclusively found in the starch granule and are known as granule bound starch synthases (GBSS), and secondly, a class present either in the soluble fraction alone or distributed between the soluble and granular fractions. This second class are denoted simply as the starch synthase class. The genetic and biochemical evidence suggests that the GBSS forms are required for amylose synthesis, and that the remaining starch synthases are principally involved in amylopectin synthesis. Figure 2 shows the relationships between the starch synthase genes identified to date in plants.

Two classes of GBSS are found in higher plants. Firstly, a form of GBSS in the endosperm that is responsible for the synthesis of the amylose present in the endosperm of cereals and the embryo of dicotyledonous seeds. Mutation of the cereal endosperm expressed GBSS gene in a wide range of species leads to the production of a near zero amylose “waxy” starch. This enzyme may also play a minor role in the synthesis of amylopectin. Recently, a second class of GBSS gene has been described, both in wheat and in pea that is expressed in non-endosperm tissue. The pea non-endosperm GBSS (GBSSIII) enzyme has been shown to synthesize a rather different amylose structure to the embryo expressed form. The gene structure has been determined for the

![Fig. 1. Schematic representation of the starch biosynthesis pathway in wheat and barley.](image)

![Fig. 2. Phylogenetic tree constructed following an alignment and distance calculation for deduced amino acid sequences of starch synthase from higher plants, Chlamydomonas and E. coli.](image)
endosperm expressed GBSS of cereals but only a cDNA sequence is available for the wheat non-endosperm form. In barley, the classic "waxy" mutation leading to low amylose does not produce a zero amylose, and it has been shown that this is due to the presence of amylose in a few cell layers at the outer edge of the endosperm. In wheat, waxy types have been generated by the accumulation of mutations in each of the three GBSS genes present in the hexaploid genome. It is important to note that a translocation event has transferred the endosperm expressed GBSS gene from the short arm of chromosome 7B to the short arm of chromosome 4A. It has clearly been shown that the loss of the 4A form of GBSS leads to a clear phenotype, a starch with increased ability to swell in water on heating, a property that is clearly correlated with increased quality of Udon style noodles.

Starch synthase I is expressed at high levels in the endosperm of wheat, barley, rice and maize, and the enzyme demonstrated to be partitioned between the soluble fraction and granule. However, no mutants of SSI are available, and no reports of down regulation of the level of the enzyme by gene suppression technologies have been reported. The role of this enzyme in higher plant starch biosynthesis therefore remains unclear. In wheat, the SSI protein (also known as Sgp-3) is encoded by a gene located on the short arm of chromosome 7, and the homeologous genes are present on chromosomes 7A, 7B and 7D. The structure of the wheat gene is shown in Fig. 3.

In wheat, the SSIIa gene has been shown to encode a group of prominent starch granule proteins known as the Sgp-1 proteins, with molecular weights of 100, 105 and 115 kDa respectively. These proteins are products of homeologous SSIIa genes on the B, A and D genomes respectively. A SSIIa triple null line has been constructed by combining single nulls at the homeologous Sgp-1 loci of the three wheat genomes. The starch characteristics of this Sgp-1 null have been characterized. Briefly, the amylose content is elevated to 35% and the chain length distribution of amylopectin shows a greater frequency of short chains. The gelatinisation temperature is reduced by 10 degrees.

In barley we have characterized a SSIIa mutant generated by treating a barley population with the mutagen sodium azide. In contrast to the wheat Sgp-1 null, the amylose content is about 70%. In common with the wheat Sgp-1 null, the amylopectin chain length distribution shows a greater frequency of short chains and the gelatination temperature is also reduced 10 degrees. The starch crystallinity is altered from the A to the V form. The starch granules are distorted in appearance and the grain is clearly shrunken in appearance. The integral starch granule proteins are reduced greatly in amount and only GBSS was could be detected. Both the wheat and barley SSIIa mutants share one feature that may shed further light on starch biosynthesis. In both mutants, the granules contain strongly reduced amounts of the other granule bound proteins, SSI, BEIIa, BEIlb and GBSS. Figure 4 shows the starch granule proteins present in wild type and SSIIa-deficient wheat and barley. Two possible reasons for this phenomenon are plausible and can not be discriminated between on the basis of the available evidence. Firstly, that the proteins are present in the starch granule in a protein complex and removal of one member of the complex causes the other members of the complex to no longer bind to the granule. An alternative hypothesis is that the starch of the mutants is altered so that these enzymes no longer bind at high affinity to the granule. Further research is required in order to resolve this possibility.

In rice it has been demonstrated that a difference in the amylopectin structure between indica and japonica types can be mapped to chromosome 6. This same map location is obtained for differences in various starch qualities measured by disintegration in alkali (the alk gene) and the

![Fig. 3. Comparison of exon structures of wheat and Arabidopsis starch synthase genes.](image)

The figure shows the exons of each gene, with the exons alternating between open and filled patterns in order to clearly define the exon structure. The intron spaces between exons have been removed as they vary greatly between wheat and Arabidopsis. In each gene pair, the upper gene is wheat and the lower gene is Arabidopsis. The vertical lines indicate the presence of highly conserved motifs in the amino acid sequence.
gelatinisation behaviour (*gel(t)* gene). The gene for SSIIa in rice is also mapped to this locus and it has been concluded that the difference in properties between *indica* and *japonica* cultivars is due to the reduced or absent expression of the SSII gene in *japonica* cultivars. The properties of the starch from *japonica* rice are similar in several respects to the starch from other SSIIa mutants in that chain length distribution is skewed towards short amylopectin chains, and gelatinisation temperature is reduced. The effect on amylose content on rice is not dramatic; however, the situation is complicated by alterations in GBSSI expression between indica and japonica rice.

In maize the starch from the shrunken-2 mutation shows similarities to the starch produced from wheat and barley lines that are lacking SSIIa. The granules are distorted and the average chain length of the branches is reduced. The amylose content is apparently elevated but only to 27% (instead of 18%). The starch shows lower gelatinisation temperature and enthalpy. Definitive proof that a deficiency in SSIIa activity leads to the sugary-2 phenotype in maize is lacking, however, SSIIa is a strong candidate gene for the site of the mutation given the phenotype and map location of the mutation.

The pea *rug 5* mutant is also missing SSII. The result is a shrunken seed with altered branching of amylopectin, and an amylose content of 43–52%. As in the case of wheat and barley, the proportion of short chains in the amylopectin fraction increases.

The comparison of the effect of the SSII mutation across these crops is shown in Table 1. Clearly there are some striking similarities: lowering of the average chain length and the production of distorted granules and the lowering of the gelatinisation temperature. However, there are also important differences. Firstly, the effect on the amylose content appears to vary considerably: from an increase to 70% in the barley SSIIa mutant to an essentially unchanged amylose content in rice. The effect of the loss of SSII on other starch biosynthetic enzymes also varies considerably. In barley and wheat, there is almost complete loss of other granule bound proteins (e.g., SSI, GBSSI, SBEs) and this is also observed in wheat. In contrast in peas there is no effect on GBSSI and the authors have concluded that there is no effect on other enzymes except for a compensatory effect on an unidentified isoform of soluble starch synthase. In contrast we have demonstrated that in the SSIIa mutant in wheat there is an alteration in the distribution of granule-bound and soluble starch biosynthetic enzymes (Kosar-Hashemi personal communication).

These results suggest that in different crops, the synthesis of amylopectin is shared differently between the various starch synthase isoforms and the result of a loss of SSII activity has different effects in different cereals. This is clear from a comparison of pea and wheat or barley: in pea it has been reported that the SSII gene is the major soluble activity in the embryo whereas in the cereals it appears to be relatively minor however, the dramatic effect on phenotype observed in cereals and particularly barley suggests that this apparently minor activity is nevertheless critical for starch granule formation.

The presence of transcripts for SSIIb has been demonstrated in maize endosperm and a number of ESTs with highest homology to the maize SSIIb gene are found in the public databases. However, there has yet to be a report of the demonstration of the presence of the product of the SSIIb protein in any species or tissue, and no mutant or gene suppression experiment has been reported. Therefore, it is not currently possible to define the contribution of SSIIb to cereal starch biosynthesis.

Mutations eliminating SSIII activity have been described in maize (*dal*) and *Chlamydomonas* (*sta3*), and suppression of SSIII expression has been reported from potato using antisense technology. The suppression of SSIII activity in both potato and *Chlamydomonas* had a major impact on the synthesis of amylopectin, resulting in decreased starch synthesis and an amylopectin with reduced chain length distribution. In maize, elimination of SSIII leads to the dull phenotype, a subtle phenotype often only capable of clear selection in backgrounds also containing the waxy mutation. As with SSII, the role of

Table 1. Comparison of amylose contents of SSII mutants from diverse species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Wild type amylose content (%)</th>
<th>SSIIa mutant amylose content (%)</th>
<th>Extent of mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td>25</td>
<td>43–52</td>
<td>Null*</td>
<td>28, 29</td>
</tr>
<tr>
<td>Wheat</td>
<td>25</td>
<td>35</td>
<td>Probable Null**</td>
<td>24</td>
</tr>
<tr>
<td>Barley</td>
<td>25</td>
<td>71</td>
<td>Null*</td>
<td>This paper</td>
</tr>
<tr>
<td>Rice</td>
<td>20</td>
<td>20</td>
<td>Probable Null**</td>
<td>25</td>
</tr>
<tr>
<td>Maize</td>
<td>25</td>
<td>30–35</td>
<td>Unknown</td>
<td>26</td>
</tr>
</tbody>
</table>

*Null mutation demonstrated through molecular analysis. **Sgp-1 proteins can not be detected in the granule and soluble fraction however the molecular basis of the mutations remains to be described in detail. SSIIa protein is lost from the granule and only an apparently inactive enzyme is present in the soluble fraction. It remains to be shown whether or not traces of activity remain.
the enzyme appears to be primarily in amylopectin biosynthesis and any role in amylose biosynthesis is minor. In wheat, an endosperm expressed SSIII gene has been described and the gene shown to be located on chromosome I. The SSIII enzyme is entirely located within the soluble fraction of the wheat endosperm.40 EST sequence databases carry a further sequence from both monocotyledonous and dicotyledonous plants that shows high homology to other starch synthases, and clusters to form a distinct class of gene in phylogenetic analysis, defined as SSIV (Fig. 2). However, there has yet to be a report of the demonstration of the translation of these transcripts to protein and no mutants have been reported that have a lesion in this gene. The role of this gene in cereal starch biosynthesis is therefore a matter of speculation.

**Branching enzymes.**

The comparison of available branching enzyme sequences shows that there are two classes of branching enzymes in plants, designated branching enzyme I (known also as SBE B) and branching enzyme II (also known as SBE A).35,41 The branching enzyme II class is further represented two discrete sets of genes found in monocots, branching enzyme IIA (BEIIa) and branching enzyme IIB (BEIIb).35 The near identical structures of the BEIIa and BEIIb genes from cereals suggests that the additional form of BEII present in monocots arose from a gene duplication event.35 The sequences at the 5’ end of the BEIIa and BEIIb genes diverge and sequence differences in this region may therefore be responsible for the differences in function observed for the two enzymes.35

The branching enzyme isoforms are differentially expressed with respect to temporal patterns of expression and their levels of expression in the differing tissues of the plant. BEI is expressed from mid endosperm development onwards in wheat and maize, and during the latter stages of embryo development in pea.35,37 In contrast, both BEIIa and BEIIb are expressed throughout endosperm in maize and wheat. BEIIb in maize is only found in the endosperm, not in the leaf, whereas BEIIa is present at high expression levels in the leaf. In contrast to the maize situation, in wheat BEIIa levels are higher in endosperm that BEIIb level.35 The different branching enzyme isoforms also differ in their partitioning between granule and soluble fraction. BEI is not present within the starch granule, whereas both BEIIa and BEIIb are present as integral proteins within the granule.35,36

The selection of mutants in maize with shrunken endosperm phenotypes leads to the isolation of different alleles of high amylose containing BEIIb mutants, but not to the isolation of mutants with lesions in either BEIIa or BEI. Data from mutants lacking either BEI or BEIIa is consistent with this result. In both monocots and dicots, down regulation or elimination of BEI activity has minimal effects on starch synthesis and composition in both tubers, and leaves and endosperm, respectively.35,36 In our laboratory we have also investigated the loss of starch branching enzyme I on the starch produced in the wheat grain. In wheat the SBE I locus has been examined from the D genome and is complex, consisting of the expressed gene and two SBE I type genes which are likely pseudogenes. Of these genes, only one (W SBE I-D4) contains a sequence present at the N-terminus of the BEI gene expressed in the wheat endosperm35 indicating that this gene encodes a functional starch branching enzyme I. A number of RNA transcripts from the SBE I locus have been reported;40-42 and it is clear that the transcripts are subject to alternative splicing pathways. In our work, we utilized an immunoblotting technique to identify naturally occurring mutations eliminating the expression of the BEI protein encoded by the 7A, 7B and 7D chromosomes of wheat. The mutations were combined through a standard crossing protocol and two null lines of wheat lacking the SBE I protein encoded by each of the three genomes of wheat were identified. The resultant line was completely lacking in starch branching enzyme I activity. The properties of this starch were analysed. No differences in the appearance or size distribution of the starch granules were seen and the amylose/amylopectin ratios are also unaltered, as is the chain length distribution. An investigation of the viscosity of the starch also was unable to identify consistent statistically significant differences between the genotypes. These observations therefore agree with the results obtained for maize38 where the SBE I gene was inactivated by transposon tagging but no clear phenotype was observed.

A transposable element induced mutation in BEIIa has been described in maize and while there was a clear phenotype in the leaf starch of the mutant,40 there was no clear phenotype in the endosperm, suggesting that BEIIa has a primary role in the synthesis of leaf starch. Data from other species is required to provide a more comprehensive definition of the roles of BEI and BEIIa.

The construction of double and triple mutations for BE genes is proving to be very informative in defining the specific roles of the branching enzyme isoforms. Down regulation of BEII in potato had some effect in increasing amylose content,44,45 however it was only the combination of BEII and BEI down regulation events that lead to very high amylose contents.46 These results suggest that branching enzymes have overlapping specificities and the remaining BE isoforms can provide partial complementation of BE mutations. Elimination of BEI expression in a wild type background has no clear functional impact, suggesting that its role in starch biosynthesis can be carried out by other branching enzymes. It is only when BEII activity is down regulated that a clear additional phenotype is observed when BEI activity is eliminated. This suggests that BEI does not interact with substrates until BEII or BEIIa have acted. The physiological role of BEI remains obscure.

**Debranching enzymes.**

The requirement for debranching enzyme activity in normal starch biosynthesis has been clearly established through the analysis of mutant in a range of species which accumulate phytoglycogen.47 All have been shown to lack isoamylase type debranching enzyme, and many also show reduced expression of pullulanase also. In maize, the *Sugary-1* mutation has been demonstrated to be caused by a lesion in an isoamylase type debranching en-
zyme gene, *SuI*. A similar mutation is known in rice where the mutation maps to a location consistent with a lesion affecting an isoamylase type debranching enzyme. In barley, mutations eliminating isoamylase activity have recently been shown to produce sugary phenotypes. It is probable that the decreases in pullulanase expression are not a direct consequence of the mutation in isoamylase but rather reflect a suppression of expression of this gene brought about through the high sugar levels in the grain. Two mechanisms for explaining the role of isoamylase in starch biosynthesis have been proposed. One proposal is known as the “glucan trimming” mechanism and suggests that isoamylase acts to remove branch points in amyllopectin that interfere with crystallization of amyllopectin, thus promoting the formation of stable amyllopectin structures that build up the granular structure. An alternative proposal holds that isoamylase is required to prevent the competitive synthesis of phytoglycogen in the developing amylloplast which would draw carbon away from starch synthesis and into amyllopectin synthesis. There is currently insufficient evidence to resolve which of the models, if either, is more correct than the other, and further research is clearly required. The wheat and barley isoamylase genes have been sequenced at the cDNA level and the gene shown to be located on chromosome 7.

**Other enzymes with potential roles in starch biosynthesis.**

A range of other enzymes have been proposed to be involved in starch synthesis in plants. Phosphorylase was first thought to be involved in starch biosynthesis, however, natural mutants lacking phosphorylase activity are lacking and therefore it is unclear what role if any the enzyme plays in starch biosynthesis. In cereals, both plastidic and cytosolic forms are present, however, it is not known what role if any a cytosolic phosphorylase could play in starch biosynthesis. Disproportionating enzyme (known as D-enzyme) is an enzyme found in a range of plants that is capable of the disproportionation of oligosaccharides such as maltotriose. In *Chlamydomonas*, the *Sta-11* mutant lacks D-enzyme activity and yet accumulates only very low levels of starch. However, in *Arabidopsis*, a D-enzyme mutant lacking leaf D-enzyme activity accumulated starch, consistent with a role for this enzyme in this tissue, to be in starch degradation. D-enzyme activities have not been reported from cereal endosperm. The role of D-enzyme therefore remains a matter for further research. The self-glucosylating enzyme, glycogenin, has been suggested to be a candidate for the protein that initiates either the formation of the starch granule, or initiates the synthesis of individual starch polymers. However, glycogenin exists in complex gene families in plants and the specific roles of any members of the gene family in cereal starch biosynthesis remains to be proven.

**Starch mutants with an unknown genetic basis.**

In barley, the *amol* mutation was first described in 1968 and the phenotype of barleys containing this mutation is an amylose content of 35–45%. The starch characteristics of the mutant are not characteristic of BEIIb mutations in maize and rice, and the mutation maps to chromosome 7, a map location inconsistent with the mutation being in the structural gene for a branching enzyme. Further research is required to identify the causal gene for this mutation.

**Conclusion.**

The synthesis of starch in wheat and barley involves a set of enzymes that are orthologous to the enzymes present in the endosperm of maize and rice. In wheat, the hexaploid genetic system makes the identification of triple mutants difficult, however, triple mutants are now available for GBSS, SSIIa and BEI. In each case, the triple mutants show phenotypes that are essentially consistent with the phenotypes of mutants in other species with lesions in orthologous genes. In barley, a novel mutation in SSIIa yields an unexpected high amylose starch phenotype that is novel in that the high amylose phenotype is accompanied by a low gelatinisation temperature starch. This result suggests that while the biosynthetic roles of the core starch biosynthesis enzymes in higher plants are conserved, there are important and potentially very useful differences in structure and properties in the starches produced in different species. This indicates that the results from model systems must be extrapolated to crop species with caution and that it is essential to examine the phenotypes of mutations in each core biosynthetic gene in each major starch synthesizing crop. The availability of improved tools for the identification of genes, the isolation of mutants, and the molecular and functional characterization of starches will generate well characterized mutants in wheat and barley that will be valuable future sources of genetic variation for the development of novel, useful, sources of starch.

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