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MINI REVIEW

Genetic Alteration of Starch Functionality in Wheat

S. Rahman*, Z. Li*, I. Batey*, M. P. Cochrane†, R. Appels* and M. Morell*

**CSIRO Division of Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia; †(Retired) Address for correspondence: Scottish Agricultural College, West Mains Road, Edinburgh, Scotland, EH9 3JG U.K.*

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INTRODUCTION

Wheat forms a major part of the diet of many millions of people around the world and factors that affect the nutritional and product quality of products derived from wheat are important from both an economic and social perspective[1]. In 1941, Harris and Sibbitt[2] made the following comments in the introduction to a paper on the comparative baking qualities of starches prepared from different wheat varieties: *'the starch in wheat flour has not received the consideration that it deserves from the standpoint of flour quality. This constituent is normally present in wheat flour in a concentration of at least 70% by weight and it is only reasonable to expect that some effect must be exerted upon baking strength by variations in starch properties due to wheat variety or environmental conditions. . .'*. In the last few years starch has finally begun to receive the attention that it merits. The availability of mutants affecting the starch biosynthetic pathway in a range of diploid species and plants altered by genetic engineering has provided a wealth of information about potential linkages between specific genes and specific functionalities. However, the hexaploid nature of wheat has limited the extent to which natural variation in the key genes of the starch biosynthetic pathway have been identified and combined. In this review, we discuss the synthesis of starch in cereals and the prospects for the genetic manipulation of wheat starch, and assess just how far we have progressed in relating starch structure and properties to end-use performance since Harris and Sibbitt[2] made their prescient comments in 1941.

Wheat starch is composed only of glucose units; the glucose units are linked α-1,4 to form linear chains and branches are formed through the connection of α-1,4 linked chains via α-1,6 linkages. Starch is generally described as containing two broad classes of molecules, amylose and amylopectin, that differ in degree of polymerisation and branch frequency. Amylopectin is a very large molecule with a degree of polymerisation from 10^5 to 10^7 and contains frequent branch points, on average approximately one branch for every 15–20 glucose units. Amylose has a lower degree of polymerisation (10^3 to 10^4) and contains from zero to a few branch points. These differences in amylose and amylopectin are functionally important and are reflected in the variety of applications these polymers find in the food and chemical industries. In hexaploid and durum wheats, amylose content ranges from about 18 to 35%, although waxy wheats containing effectively zero amylose have now been produced[3].

Starch is deposited in granules in the wheat endosperm in amyloplasts[4], specialised starch biosynthetic organelles derived from the same proplastids as chloroplasts, but containing no photosynthetic apparatus[5]. The precise molecular events that occur at the initiation of the starch granule remain obscure. In wheat, granule initiation occurs in two phases, in the period 3–7 days after anthesis, during which time the large 'A' granules are initiated[4]. Granule initiation then appears to cease until mid endosperm development, when a

Corresponding author: S. Rahman. Tel: 61 02 6246 5314; Fax: 61 02 6246 5345; E-mail: s.rahman@pi.csiro.au

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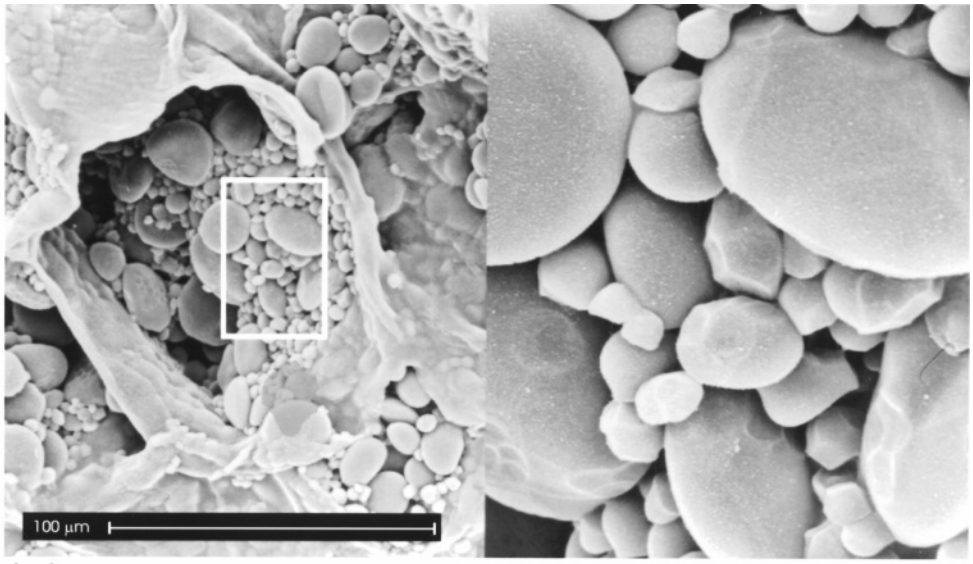

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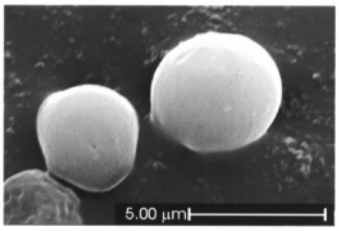

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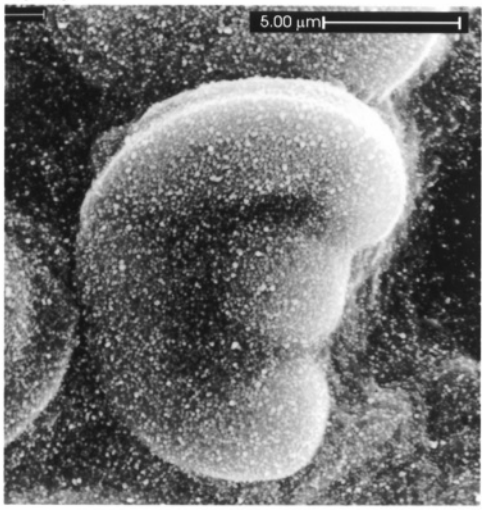

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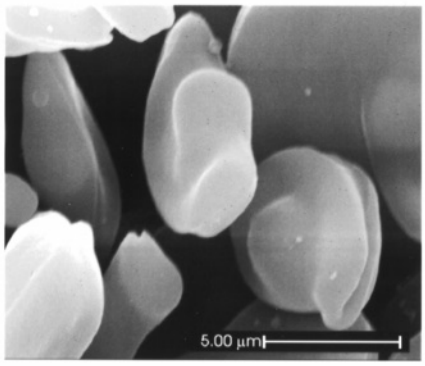

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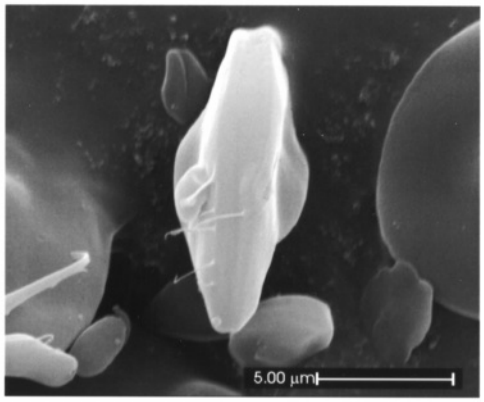

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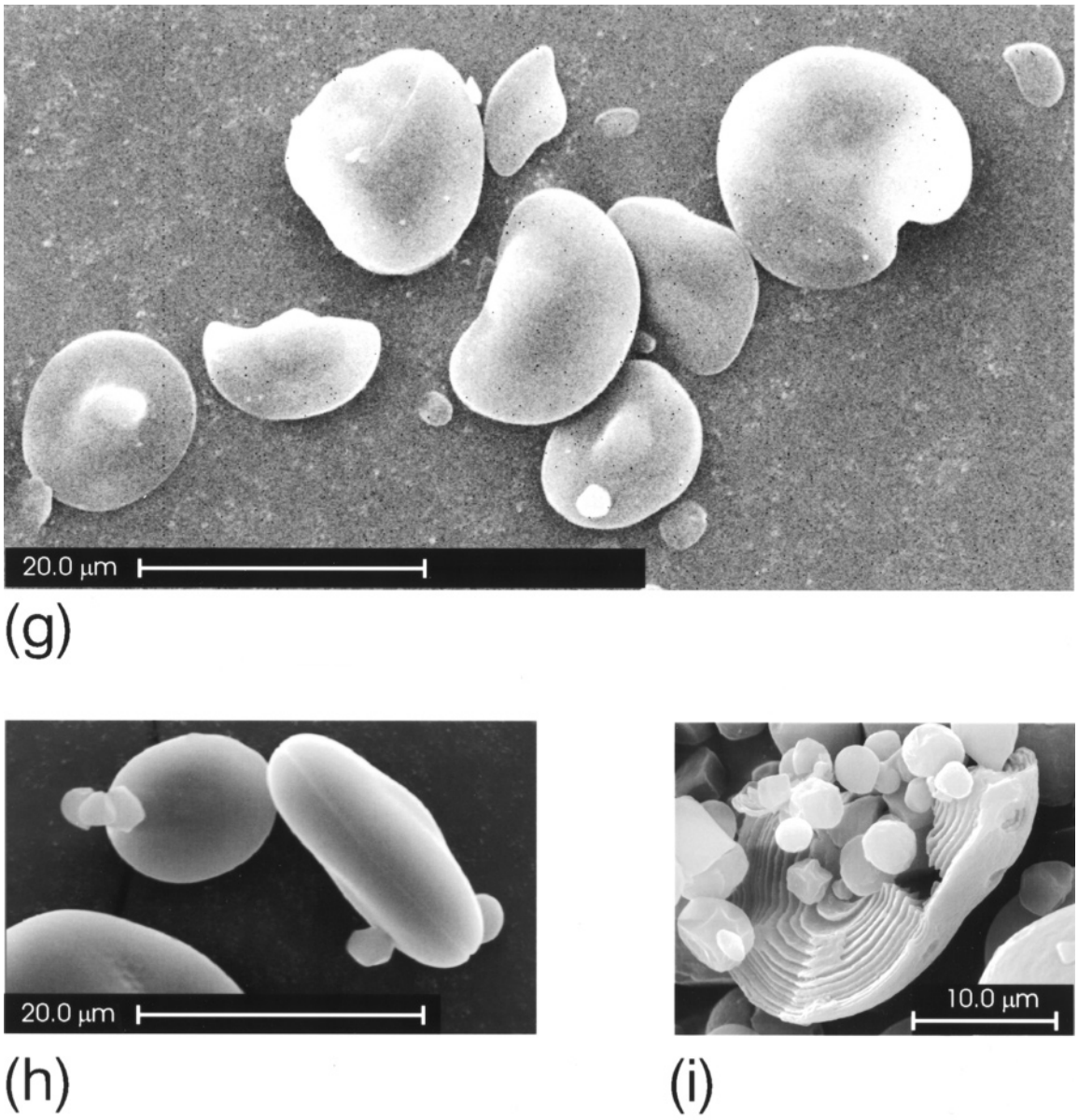

Figure 1 Scanning electron micrographs of wheat starch granules prepared at different times after anthesis. Panels (a) and (b) show a central endosperm region of a transverse section of a mature wheat grain which was prepared for scanning electron microscopy by briefly immersing the cut surface in water to remove soluble components. Panel (b) is a magnified image of the area outlined by a white box in Panel (a). Panels (c) to (h) show granules isolated from endosperm at different stages after anthesis, in days after anthesis; (c) 8 days (d) 12 days (e) 16 days (f) 14 days (g) 14 days (h) mature grain. The starch in panel (i) was extracted from mature wheat grain, fractured by percussive force, and incubated them with 3 U/ml alpha-amylase (from *Bacillus licheniformis*; supplied by Megazyme) for 15 min at room temperature. All samples were sputter coated with gold and SEM pictures were collected on a Hitachi S2250-N Scanning Electron Microscope.

second, much more prolific, period of granule initiation occurs leading to the development of the small 'B' granule populations[4]. A third burst of 'C' granule initiation has also been observed in wheat[6].

Figure 1 panels (a) and (b) show a scanning electron micrograph of a transverse section of a wheat grain in the late stages of development. The packaging of 'A' and 'B' granules into the cell is tight [panel (a)], and the characteristic indentations on the surface of the A and B granules caused by their tight packaging in the granule can be seen in panel (b). In wheat and barley, the A-granule follows a characteristic developmental pathway,

Table I Summary of starch granule properties from several cereals[156]

Starch source	Size range (μm)	Size distribution	Gelatinisation onset temperature °C	Approximate amylose content %
Wheat	3–34	Bi-modal	61	26
Barley	2–35	Tri-modal	57	22
Rice	2–13	Normal	74·5	18
Maize	5–20	Normal	67	28
Waxy maize	4–18	Normal	68	0
Amylomaize	6–15	Normal	68	>70

which has been described in some detail in wheat[7]. The first structures that can be defined as starch granules are spherical granules of about 0·5–1 μm diameter, and these granules continue to grow radially until 2–4 μm in diameter [Fig. 1, panel (c)]. A-granules develop a bulbous protuberance that develops into an apposition plate that progressively extends around the granule [Fig. 1 panels (d) and (e)], eventually encircling the granule [Fig. 1 panels (f) and (g)]. The rim of the equatorial plate contains a clearly defined equatorial groove [Fig. 1 panels (d) and (e)]. The equatorial plate then expands further at the rim, with some deposition on the faces of the plate, to a diameter approaching the maximum granule diameter [Fig. 1 panel (g)]. A period of active deposition on the faces of the equatorial plate then occurs, producing the characteristic lenticular shape of the mature wheat A-granule [Fig. 1 panel (h)]. The deposition on the faces of the equatorial plate of the A-granule appears to proceed in a diurnal or circadian manner, producing the characteristic alternating layers of starch, differing in their susceptibility to amylase digestion [Fig. 1 panel (i)]. The initiation of B granules has been most intensively investigated by Parker[8] who described the appearance of B granules in amyloplasts containing a single A-granule, mid way through endosperm development. The B-granules were seen in lateral evaginations of the amyloplast membrane, and multiple small B-granules were seen in a single evagination. B granules remain spherical or orthorhombic and do not proceed through the equatorial plate formation pathway described above for the A-granule. There are marked differences in the patterns of starch deposition in different cereals that result in different starch granule size distributions and morphologies. The size and properties of various cereal starch granules are summarised in Table I.

In addition to starch, the starch granule contains two other important components. Firstly, the interior of the starch granule contains a range of starch biosynthetic enzymes that account for about 0·5% of the mass of the granule[9,10]. The nature of these proteins is discussed in the next section. Secondly, the granule contains lipids, complexed within the amylose fraction[11]. These lipids are thought to exert important effects on the interactions of the granule with water during gelatinisation and swelling[12] and will be explored in greater detail in a later section.

The properties and functionality of wheat starch are controlled not only by the nature and composition of the starch granule, but are also strongly influenced by the nature of the endosperm material in which the granule is embedded in the desiccated grain. The hardness of the grain controls the manner in which the endosperm and starch granule is fractured during the milling process, leading to important effects on processing performance.

In summary the key features of the deposition of starch in the wheat endosperm that control functionality are starch content, grain hardness, granule size distribution and shape, the presence of endogenous lipids in the granule, amylopectin structure, and the ratio of amylose to amylopectin (Fig. 2). These differences in starch deposition define the ways in which starch responds to heat and water during the utilisation of starch in the complex foods prepared from cereal flours. Each of these features may be amenable to modification by molecular/genetic changes in genomic DNA.

GENETIC CONTROL OF STARCH SYNTHESIS IN PLANTS

The delivery of sucrose to the developing endosperm and the transformation of that sucrose to

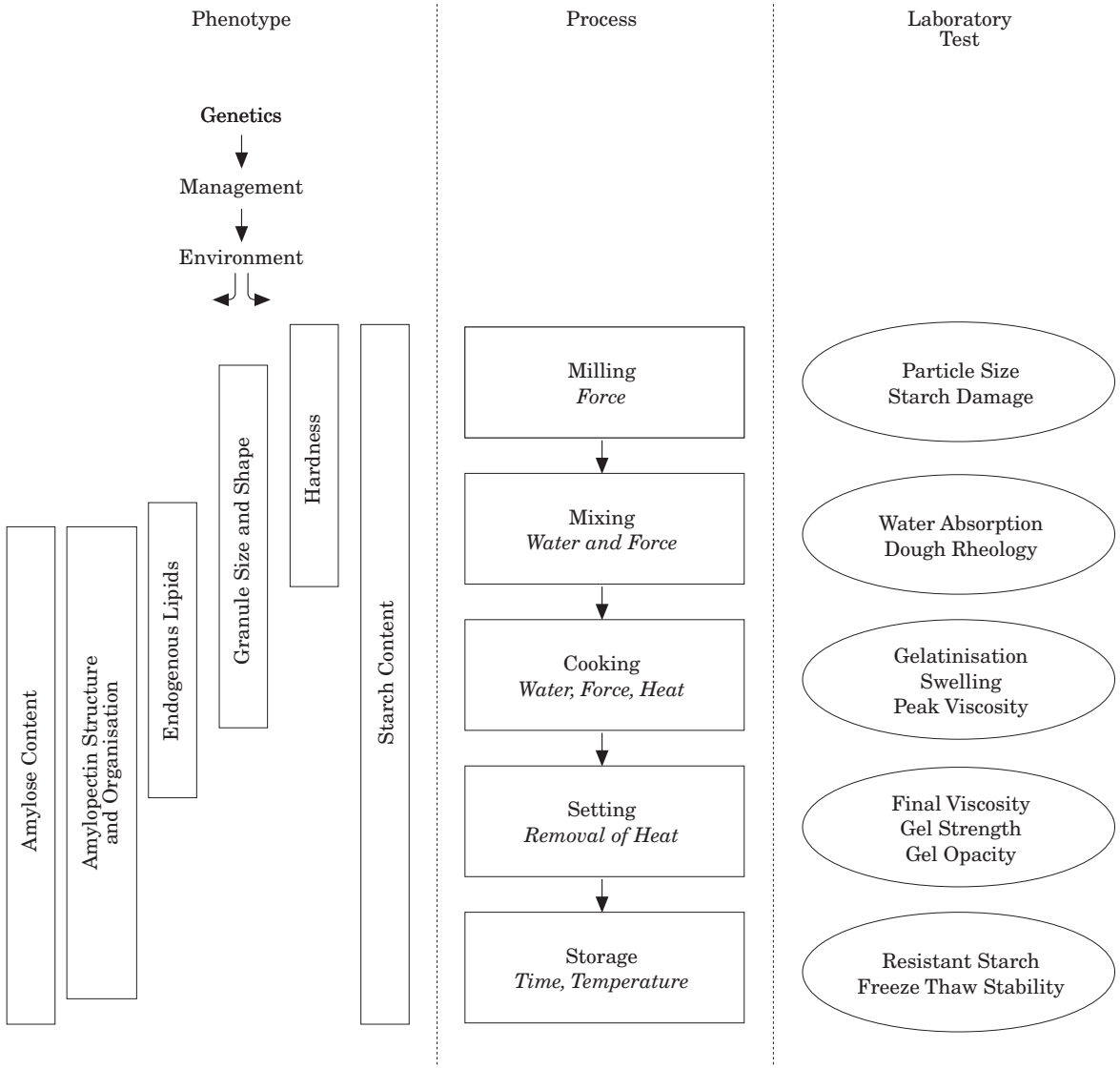

Figure 2 Flow diagram describing typical transformations involved in the production of wheat based foods, starch diagnostic tests used at each stage of the transformation, and the properties of starch that have the greatest impact on each stage of the transformation.

glucose-1-phosphate, the precursor of starch biosynthesis, is integral to the overall process of starch deposition. However, these processes are beyond the scope of this review and will not be considered in depth here. For references concerning aspects of the transformation of sucrose to glucose-1-phosphate in wheat endosperm, see[13–16].

A scheme for starch synthesis in wheat endosperm is shown in Figure 3. The first committed step of the starch biosynthetic pathway is the formation of ADP glucose (ADPG) from glucose-1-phosphate and ATP by ADPG pyrophosphorylase (ADPGPP). ADPG is a common substrate for the various starch synthases responsible for the synthesis of amylose and amylopectin through addition of the glucosyl moiety of ADPG to the non-reducing end of a pre-existing starch molecule. The extended starch polymer is then branched through the action of starch branching enzymes and there is strong evidence for the involvement of starch debranching enzyme in forming the final structure of amylopectin[17–20].

In cereals there appear to be at least four classes of starch synthases important to starch synthesis

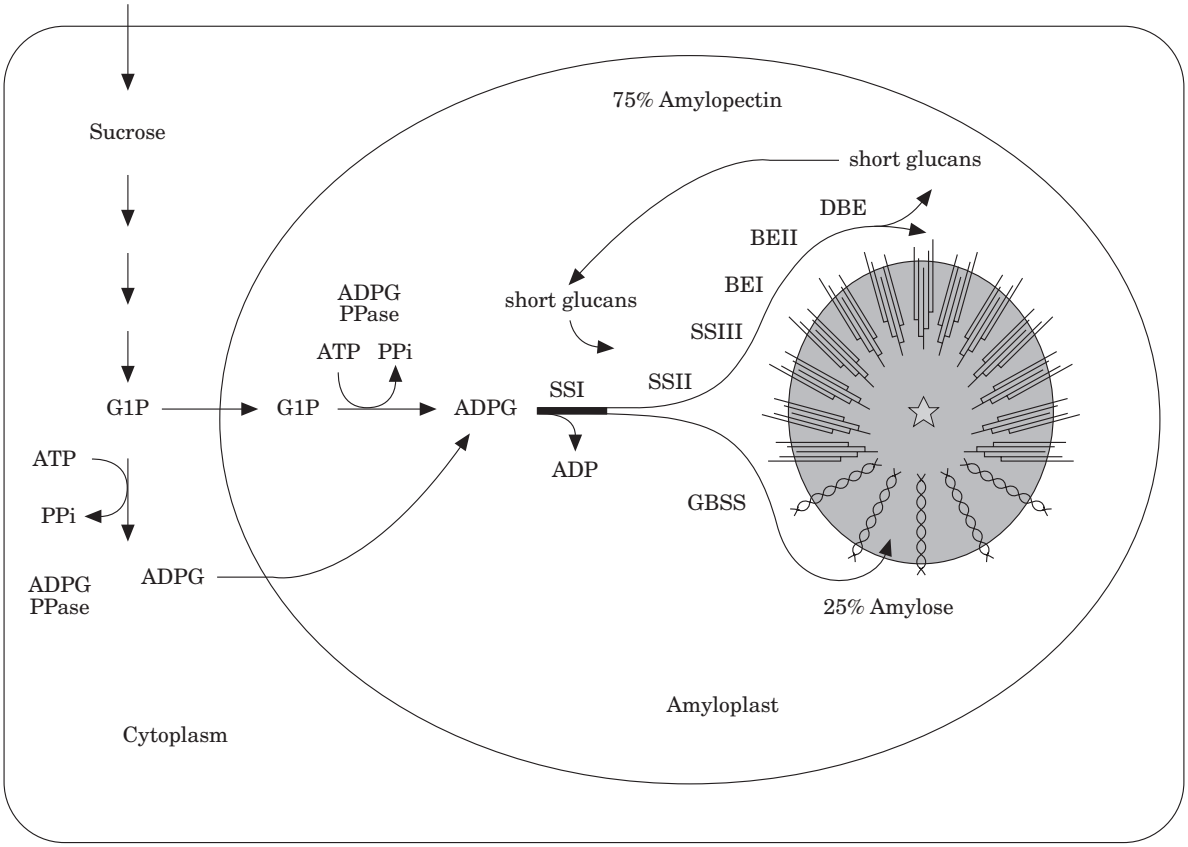

Figure 3 General scheme for starch biosynthesis in cereals. Sucrose is transformed to glucose-1-phosphate through the action of invertase, sucrose synthase, UDPglucose pyrophosphorylase, hexokinases, and phosphoglucomutase (not shown).

in the endosperm: granule bound starch synthase (GBSS: *wx1* in maize[21]), starch synthase I (SSI[22]), starch synthase II (SSII[23]) and starch synthase III (originally designated as SSII, *du1* in maize[24]). GBSS is essential for amylose synthesis, and may be a contributor to amylopectin synthesis. The roles of SSI, SSII and SSIII are thought to be predominantly in amylopectin synthesis[22,24,25] although they may also be non-essential contributors to the synthesis of amylose.

Two classes of branching enzymes (BE) are known in cereals, designated BEI and BEII. The BEII class in maize contains two members, BEIIa and BEIIb[26]. Natural mutants lacking BEI have not been reported in any species and the characteristics of potato tubers starches lacking BEI activity following antisense suppression of BEI synthesis[27,28] suggests that this enzyme does not have a major role in defining the branching frequency in amylopectin in the presence of BEII. In contrast, the absence of BEIIb leads to the high amylose mutants known in maize[26,29] pea[30] and rice[31].

The role of debranching enzymes (DBE) in starch synthesis is the subject of considerable ongoing debate. It has been suggested that they trim excess branches in the amylopectin[32,33]. Although there may be speculation about the precise function of debranching enzymes the genetic evidence for a key role of isoamylase-type debranching enzymes in starch biosynthesis in the cereal endosperm is very powerful[32]. The scheme outlined in Figure 3 is based on studies of starch biosynthesis in species such as maize, pea, potato and *Chlamydomonas* (for reviews see[32,34–37]). While the principles of synthesis are expected to be similar in wheat, there may be significant differences in detail in the wheat endosperm. The relationships between specific enzymes and starch functionality are discussed further in a later section.

FUNCTIONALITY OF WHEAT STARCH

The functionality of wheat starch must be considered with reference to the end-uses of wheat starch. The range of end-uses of wheat starch in the food industry are very wide and varied, from use in leavened breads, flat breads, steamed breads, biscuits, cakes, pastas, noodles to a vast array of regional specialties. In non-food industries, wheat starch finds application as a sizing agent in the paper and textile industries, and as a substrate for the production of glucose syrups and adhesives. Further applications require chemical modification and such applications will not be considered here. While the range of applications of wheat starch is wide, some common processing steps are employed in the utilisation of wheat starch as a component of flours or semolinas for food use (Fig. 2). As a wheat starch moves through these classes of processing steps to a final product, different aspects of wheat starch structure and functionality assume prominence in defining the suitability of the starch for the process. Figure 2 summarises relationships between aspects of starch structure and functionality and processing steps. In order to predict how the structure and properties of starch will perform in a given process, a range of tests have been devised to mimic process steps and quantify the response of a starch to that process. These tests are also indicated in Figure 2. General principles concerning the relationships between the composition and properties of starch and functionality can be drawn which can be used to guide the genetic manipulation of starch functionality in wheat; no attempt is made to be comprehensive or specific because of the vast range of end-uses.

GENES CONTROLLING WHEAT STARCH FUNCTIONALITY

Starch content

There is evidence from enzymological studies that ADPGPP is the rate-limiting step for starch synthesis in leaves[38] (Fig. 3). In leaf ADPGPP is a tetrameric enzyme consisting of two large and two small subunits of approximately 60 kDa and 55 kDa respectively[39]. Genes for large and small subunits of ADPGPP have been described from rice and maize[40–42] and there are also cDNA sequences from wheat[43].

It has been reported that potatoes with increased levels of starch have been produced by introducing bacterial ADPGPP genes[44]. The particular advantage of the bacterial gene is that it is not subject to feedback inhibition by 3-phosphoglycerate. Whether such modification would have an effect in cereals is debatable, as it is not clear if ADPGPP is a major rate-limiting step in the cereal grain. Early work[45,46] using developing grains led to the conclusion that in the endosperm the flux through the pathway was mainly controlled by starch synthase activity. In contrast, increases of 15% in seed weight of maize have been obtained by site-specific mutagenesis of ADPGPP[47] and it has been suggested that manipulation of this enzyme may lead to heat-stable grain filling in maize[48].

ADPGPP may occur in the amyloplast or in the cytoplasm. The situation in wheat endosperm is not yet resolved but in maize and barley, there is strong evidence for cytosolic ADPGPP being the major contributor to starch biosynthesis[49,50]. Thus it may be possible to alter the amount and structure of the starch produced by altering the ratios of ADPGPP in the two cellular locations by targeting the relative amounts of the two isoforms in genetic engineering experiments. It is also possible that increasing starch synthase or branching enzyme activity in the endosperm could increase the amount of starch formed during endosperm development.

Grain hardness

Grain hardness of wheat is an important criterion for starch quality and wheat-end use because grain hardness is a major determinant of the level of starch damage during milling. The level of starch damage in turn influences the level of absorption of water by the flour. In soft grains the adhesion between the starch granules and the protein matrix is weaker than in hard wheats. Consequently during milling the fracture planes run between the starch granules and the protein matrix in soft wheats but within the starch granules in hard wheats. Thus starch from hard grains fracture more during milling and this leads to greater water absorption when water is added to the flour. Hard grains are used for baking breads and noodle production whereas soft wheats are used for biscuit flour.

The major factor controlling grain hardness is a single locus, *Ha*, on chromosome 5D of wheat[51–53] but so far no definitive biochemical explanation of the difference between soft and hard wheats

has been provided. In 1986 a correlation was noted between the presence of a 15 kDa protein associated with the starch and grain softness[54]. This 15 kDa protein has been purified and found to be a mixture of at least three polypeptides[55–58]. The proteins, puroindoline a, puroindoline b and GSP-1, are encoded on chromosome 5 and there is tight linkage between these genes and grain hardness[59–61]. The three polypeptides so far defined are closely related and it has been shown that the puroindolines can interact with lipids[62]. It has been proposed that the major component of the mixture, puroindoline b, is the candidate product of the *Ha* gene[63]. The purodindoline b gene from the soft cultivar Chinese Spring and the hard cultivar Cheyenne was sequenced and only a glycine to serine change found at position 75 of the deduced amino acid sequence in the hard cultivar compared to the soft cultivar. This change occurs in a position that has been speculated to interact with lipids. Recently it has been suggested that a combination of puroindoline a and purodindoline b polypeptides affect grain hardness[64]. Other results suggest that grain hardness can occur independently of alterations in puroindoline a or puroindoline b and further genes may be involved in determining this trait (Turnbull *et al.*, pers. comm.). The definition of the specific molecular genetic cause(s) of hardness in our view remains an open question.

In barley the major locus controlling milling energy (which is analogous to grain hardness) has been mapped to a quantitative trait loci (QTL) on chromosome 5H spanning 13 cM[65]. Clearly, the ability to modulate the hardness of the grain for specific end purposes will have significant practical applications.

Granule size distribution and shape

There has been little work on the impact of granule size distribution and shape on the processing or performance of starch containing foods because of a lack of diversity available through wheat germplasm. Starch granule size is important in the starch processing industry, where there is separation of A granules from B granules during starch washing, with B granules typically being lost into effluent streams, incurring additional processing and disposal costs. Changes in rheological properties of dough based on granule size distribution might also be expected because an increase in the proportion of the small B granules provides a much higher surface area for the binding of proteins (including amylases), lipids and water. The impact of granule size on dough rheology has been examined in a reconstitution system in which wheat starch was replaced by starches from various species, or by glass beads, with differing size distribution[66]. It was concluded that it was difficult to separate the direct rheological effect of granule size from the impact of substituting wheat starch by starches from other botanical sources. Kulp[67] noted the increased water binding of small granule starches and concluded that the baking quality of resconstituted flour containing small-granule starch was inferior to unfractionated wheat starch. In a study of the effect of granule size on dough extension it was found that small starch granules increase the extensibility of the dough, whereas large granules increase resistance to extension[68]. More recently, preliminary studies indicated that flours with starch containing only purified B granules show markedly longer mixing times and higher water absorbtion compared to a reconstituted flour containing only A granules (P. Gras, E. Asp, pers. comm.). The importance of the starch granule surface properties in influencing the rheological properties of wheat flour dough has been highlighted by recent work[69].

For many years there has been speculation regarding the requirement of an initiator protein for starch biosynthesis. The search for the initiator of glycogen in mammalian systems led to the discovery of glycogenin, a self-glucosylating protein required for glycogen biosynthesis[70]. Recently a number of glycogenin-like sequences have been isolated from plants including rice (GenBank: accession number: D26537) and wheat[71]. It may be that a sub-class of these proteins is involved in starch granule initiation but this has yet to be demonstrated. Clearly identification of such starch-initiating proteins would provide an answer to a persistent question and be very useful in terms of manipulation of starch properties.

It is not known what factors specifically control the shape of the granule. It is clear that if the composition of the granule is grossly affected then the shape of the granule is also affected. For example, in the high amylose maize starches, the granules have irregular and elongated shapes compared with the near-spherical shapes in the normal maize. The dramatically higher amylose in the starch presumably prevents the normal packing mechanisms from operating efficiently in these cultivars. Similarly in the embryos of peas bearing

the *rug5* mutation in the starch synthase II gene, the starch is deposited in the form of compound granules[25]. Highly distorted starch granules have been reported[72] in wheat lines that are missing sgp-1 which has been shown to correspond to SSII[73]. It seems that alterations in amylopectin structure will change the shape of starch granules.

Endogenous lipids

The lipid content of cereal starches is low, about 1%, but lipids have been shown to affect the viscosity characteristics and quality of the starches[74]. The lipids in a sample of starch granules can be assigned to one of three operational classes: non-starch, surface and internal. The internal lipids of wheat starch granules consist entirely of lysophospholipids. There is little work to date on defining the biochemical steps or the molecular genetics involved in starch lysophospholipid synthesis but there is evidence that cultivars of wheat differ in the percentage of lysophospholipid in their starches[11,74] and hence of the percentage of lipid-complex amylose in their starches[75]. A gene that has been shown to influence the amount of extractable free polar lipid in the grain, *fpl-1*, has been shown to be tightly genetically linked to the grain hardness locus, *Ha*, on the short arm of chromsome 5D[76]. A second gene, *fpl-2*, that also controls starch lipid content, has been mapped to the long arm of chromosome 5D[76] but the biochemical basis of the action of these genes is not known. It has been shown that bound polar lipids are probably involved in the interaction between puroindolines (see grain hardness) and the starch granule surface[77].

Starch structure in relation to gelatinisation, swelling and hot paste viscosity

Starch granule gelatinisation involves the heat driven transformation of granules in excess water from an ordered state to a disordered state. The initial stages of gelatinisation involve the uptake of water lowering the glass transition temperature in the amorphous regions and causing the crystalline regions to begin to melt. Further heating and water uptake results in the mobilisation of amylose and the initiation of its leaching from the granule and the complete melting of the crystalline regions, marked by the loss of birefringence. Further swelling of the granule occurs, typically producing greatly enhanced viscosity in the starch-water paste. The overall process of gelatinisation is similar in both normal and waxy starch granules, although by definition, amylose is not present in a waxy starch and cannot be lost by leaching. These observations suggest that the properties of the amylopectin fraction of starch are most critical in controlling gelatinisation and granule swelling in cereal starches. Examples of manipulations that alter amylopectin structure by reducing the external chain length distibution, and reduce granule gelatinisation temperature, can be found in the *su-2* mutation in maize[78] and in transgenic potato with reduced levels of SSII and SSIII[79,80]. The packing of amylopectin side chains in the amylopectin fraction is basic to the formation of crystalline regions, known as crystallites, in the granule. Three types of packing have been thought to occur, based on x-ray diffraction studies. Densely packed A-type crystallites are found in cereals with waxy or normal amylose contents, while B-type crystallites are found in potato and a range of other tuber starches. C-type starches are typically found in the grain legumes, and contain both A- and B-type crystallites in the same granule. It has been shown that these differing x-ray structures are related to differences in chain length and the arrangement of branch points in the amylopectin fraction[80]. It has been demonstrated that the temperatures differ at which disordering of A- and B-type crystallites occurs in pea starch during gelatinisation[81]. For reviews of the importance of granule crystallinity in controlling gelatinisation, see[82–84].

Amylose content is thought to be a major influence on starch granule swelling, mediated through the reduced mobility of amylose/lipid complexes in the granule that restrict water movement and swelling[12]. In wheat, a pioneering study[85] discovered a link between a null allele at the GBSS locus on chromosome 4A and the eating quality of Japanese noodles. Initially this seemed to correlate, as expected, with reduction in the proportion of amylose in the starch. More detailed analyses, however, have revealed that flour swelling volume rather than amylose content correlated with noodle quality[86–89]. The study of wheat lines that are missing the GBSS gene and the GBSS protein on chromosome 4A[89] indicates that starch viscosity, as well as swelling volume, are increased without a significant change in the relative amylose content. Other lines of wheat missing the GBSS protein encoded by chromosome 4A as a result of

point mutation have not been characterised in detail with respect to starch properties[174]. Although the presence or absence of GBSS loci have major effects on wheat flour suitability for noodles it is also clear that minor effects are due to other genetic factors and these remain to be determined. The presence of a GBSS protein characteristic of pericarp tissue (GBSS2) has been reported[167,168].

From the pathway of starch biosynthesis illustrated in Figure 3, one could expect that mutations in the genes encoding BEI, BE II, SSI, SSII, SSIII and DBEs to profoundly affect amylopectin structure. In this section we will consider the role of each of these enzymes (with the exception of BE II which will be discussed in the next section together with GBSS as major contributors to the control of the amylose/amylopectin ratio).

BEI is an enzyme of approximately 88 kDa in wheat[90]. The gene consists of 14 exons in rice, wheat and maize[91–93,169] and a variant with only 10 exons has also been described in wheat[94] but despite this detailed structural knowledge no clear definition of its role in cereals is yet available. Three properties of BEI differentiate this gene from BEII. Firstly, BEI transfers longer chains than BEII during catalysis[95]. Secondly, BEI is expressed later in endosperm development in wheat and maize than BEII[90,96]. Thirdly, unlike BEII, BEI is not found within the starch granule in wheat[10] or maize[97].

SSI is a 75 kDa starch synthase that is found in wheat endosperm, partitioned between the soluble fraction and the granule[9,10,98]. Clones of cDNA coding for SSI in cereals are available in rice[99], maize[22] and wheat[98]. The gene has been described from rice[100] and recently in wheat[98]; like other starch biosynthetic genes described so far the gene is complex with 15 exons over 10 kb in wheat. The gene is genetically linked to the GBSS gene in rice on chromosome 6, at a distance of 5 cM[100]. In wheat a locus controlling the accumulation of SS is on chromosome 7[101] and it appears that this is also the structural gene[98]. Natural mutants lacking all SSI activity have not been identified although Yamamori and Endo[101] have identified lines with mutations affecting SSI genes in the A genome and in the B genome.

A group of three polypeptides of 100, 108 and 115 kDa apparent molecular weight are associated with wheat starch granules[101] and have been clearly shown to be starch synthase[9,10,73]. Sequence comparisons indicate that these proteins are most highly homologous to maize SSIIa[23] and in wheat have been referred to as SSII[73]. Yamamori has recently reported the isolation of wheat lines lacking each of the isoforms encoded by chromosomes 7A, 7B and 7D[72]. These lines have unusual starch properties but the proportion of amylose was not drastically different from that in wild-type starches. The properties of these wheats will be of great interest to the elucidation of the function of these proteins. Similar reports in peas also indicate that loss of soluble starch synthase II in the *rug5* mutant leads to abnormal granule morphology[25].

The genetic lesion responsible for the dull phenotype has been deduced from maize by transposon tagging and the disrupted gene encodes a 180 kDa starch synthase, designated SSII[24]. This gene is the homologue of the SSIII gene of potato[102,103]. Lack of this enzyme in maize results in kernels with a tarnished appearance, slightly lower total carbohydrate content and slightly or greatly increased amylose content, depending on genetic background[104]. Although the gene for the 180 kDa starch synthase is directly affected in the *dull1* mutation[24], the amount of SBE IIa is also reduced, probably as a secondary effect. The characterisation of the homeologous gene has not yet been reported from other cereals. The simultaneous antisense repression of both SSII and SSIII in potato has been reported and the changes in structure are dramatic with an increase in both short and very long chains in amylopectin[79,80].

Debranching enzymes, which remove the α-1,6 branch from branched glucose polymers, have been shown in mutation studies to be involved in the biosynthesis of amylopectin in the endosperm of maize[17], rice[19] and *Chlamydomonas*[18]. Loss of debranching activity leads to the accumulation of sugars in the endosperm, presumably because starch synthesis is inhibited, and in addition there is the production of a highly branched polymer of glucose, phytoglycogen. The role of debranching enzyme in starch biosynthesis has been suggested to involve the removal of branch points, introduced by branching enzymes, which are inappropriately positioned for amylopectin crystallisation to occur[18,32]. The *sugary 1* mutation in maize has been demonstrated by transposon tagging to be caused by inactivation of a gene encoding an isoamylase. The gene is over 11 kb in length and the mRNA codes for an enzyme of about 80 kDa with 32% amino acid identity overall to bacterial isoamylase[17]. In maize there appears to be a multigene family of about

10–15 members encoding isoamylase like sequences, however, it seems that only one of these is expressed in the developing endosperm[17]. In both maize and rice, mutation of the *sugary-1* type isoamylase gene also has the concomitant effect of reducing the expression of a pullulanase-type debranching enzyme[17,19]. In rice, a cDNA[19] and gene[105] for a pullulanase-type enzyme have been described. The encoded protein is 102 kDa and located on chromosome 4 of rice and it has been suggested that there is only a single copy of the gene in rice. A pullulanase cDNA from maize[106] and from barley[107] have also recently been described and both genes are expressed in the endosperm. It has not yet been established whether the loss of pullulanase activity is also important to the impact of the *sugary-1* mutation on starch biosynthesis, or is a secondary effect.

Starch structure in relation to final viscosity and gelation

Polyethylene of different densities have been studied extensively in order to determine the relationship between weight/branching at a molecular level, and rheological properties such as viscosity[157–160]. Although a detailed review of the rheological studies on synthetic polymers is beyond the scope of this review it is evident that these studies suggest increases in either molecular weight or branching can lead to increases in maximum viscosity (elongation). Furthermore the studies suggest that small, defined, alterations to a polymer such as amylopectin could lead to significant changes in physical characteristics. A study by Dintzis and Bagley[161], for example, has suggested that the amylopectin in waxy maize starch had shear-thickening properties that were not present in the amylopectin present in normal starch. Chemical analyses of these two amylopectins have not, to date, had the resolution to determine the structural change underpinning the physical differences.

The smaller size of the unbranched glucan polymer of amylose ($0{\cdot}2–8 \times 10^5$ daltons) provides opportunities for entanglement and the formation of double helices in domains where regions of molecules are in close contact[162,163]. This behaviour leads to precipitation of amylose and the gels thus formed when suspensions of amylose are cooled are opaque because of light-scattering by the precipitated polysaccharide, but strong because of the intermolecular entanglements. This phenomenon is also referred to as retrodegration when it occurs in foods. Amylopectin (up to 590×10^6 daltons)[164] tends not to form the domains of intimate interaction between molecules as easily as amylose (Fig. 4) and does not precipitate as readily. As a result, it forms clearer gels, of lower strength. Studies on low concentration amylopectin gels have suggested that the molecules aggregate side-by-side with intimate interactions between outer branches (see Fig. 4) allowing the formation of a network[165].

The pasting of starch is typically analysed in an instrument such as the Viscoamylograph or Rapid Visco Analyser (RVA), which measures the resistance of starches to shearing forces under defined hydration and temperature regimes[108]. Following complete gelatinisation at high temperatures (for example 95 °C), peak viscosity is reached, the starch is then cooled (typically to 50 °C) while continuing to be stirred. A final viscosity is measured and while this viscosity reflects the interactions between amylose and amylopectin in the formation of a gel structure, there is a general trend towards increasing strength with increasing amylose. Further cooling of a starch paste to ambient or sub-ambient temperatures results in the formation of either a gel, in the case of starches containing amylose, or a very weak gel or viscous solution in the case of waxy starches. Depending on composition, the gel or starch solution may show the classic signs of retrogradation, in which aggregation of amylose molecules occurs, leading to an opaque appearance and the presence of a form of resistant starch[109]. Amylose content is of central importance to the strength and visual appearance of gels formed from gelatinised dispersed starches[84]. High amylose starches are utilised to produce firm opaque gels that tend to retrograde over time. In contrast, amylose-free starches form viscous solutions rather than gels, which are typically translucent. Synergistic effects on paste viscosity and gel strength have been observed when amyloses and amylopectins are mixed[110] and the blending of starches from different sources produced novel paste properties[111]. It also needs to be remembered that during baking the gelatinisation of the starch granule occurs in a limited water system where competition from other components, such as gluten, changes as the baking process progresses and thus products vary in the gelatinisation of the starch depending on both the initial components of the system and the heating regime used[166].

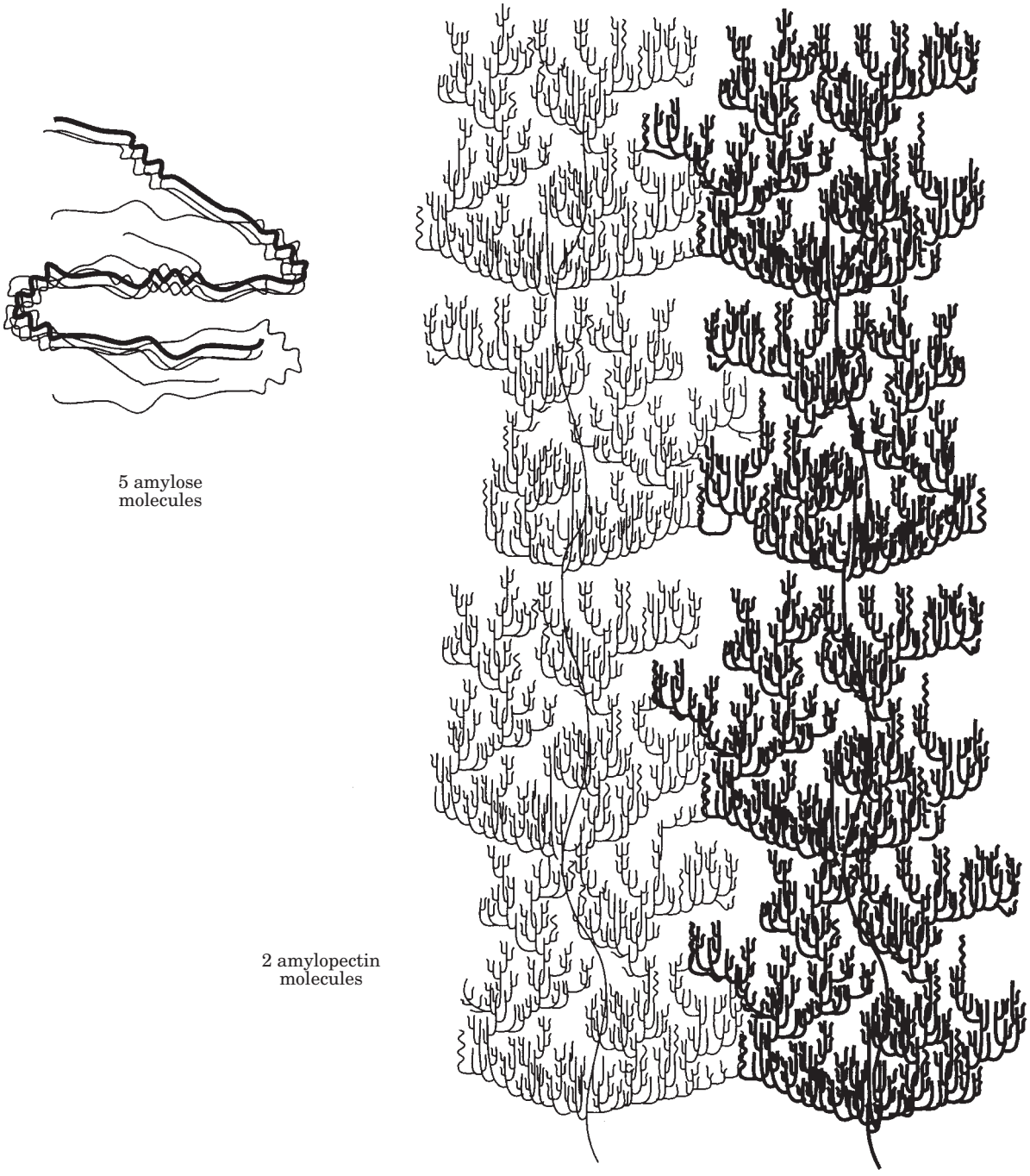

Figure 4 Schematic diagram that illustrates the scale of the molecular size differences between amylose and amylopectin, and indicates the types of interactions involved in amylose-amylose intermolecular interactions, and amylopectin–amylopectin interactions. Amylose, with its low molecular mass produces low viscosity solutions but its ability to align and form double helices, is the primary determinant of gel strength and the primary cause of retrogradation in starches. Pure amylopectin forms clearer gels of lowered strength as only the outer branches of two molecules can interact.

The 60 kDa GBSS (also known as the waxy protein) is of critical importance in the synthesis of amylose, but not for amylopectin synthesis. The enzyme is thus a primary target if the goal is to reduce the amylose/amylopectin ratio. The waxy gene has been described from a number of cereals

including rice[112], barley[113], maize[114] and wheat[115]. The gene structure in barley shows that there are 11 introns[113]. In wheat the genes are on chromosomes 7A, 4A and 7D[116]; the 4A location arises because of a translocation of a chromosome segment from chromosome 7B to chromosome 4A[117]. The efficiency of removal of intron 1 to produce the mRNA for an active GBSS varies between cultivars in rice; the efficiency is high in most indica varieties and low in japonica varieties and this is correlated to the difference in amylose content between these groups of varieties[118].

In barley, rice and maize there are natural mutations which produce starches that are essentially amylose-free. In wheat lines were identified which are lacking one or other of the isoforms[119,170,171] and by conventional breeding waxy wheat has been produced[120]. Antisense technology based on a portion of the rice waxy gene has been used to produce rice grains with reduced amylose content[121]. Waxy wheats have also been produced by mutagenesis[122,123] and the properties of waxy wheat starches are currently under investigation[124,125].

Amylose content is also affected by mutations in BEII. In maize two forms of BEII were distinguished, II a (the predominant form in the leaf) and II b (the predominant form in the endosperm). In the *ae* (amylose extender) mutation BEII b was entirely missing and the total branching enzyme activity was only 20% of the normal[26]. High amylose maize lines have been produced by crossing *ae* mutants with normal cultivars, in order to maintain the high amylose phenotype but remove other less desirable properties. Similarly in rice, mutants have been identified where the loss of BE II has been correlated with the alteration of starch structure[31]. Clearly then BE II b is an attractive target for gene manipulation for increasing the amylose content of starch. A BE II gene was mapped to rice chromosome 2 and it was suggested that it occurs on chromosome 5 of maize and wheat chromosome 6[126]. In wheat, there is strong evidence for the existence of a BEII gene on chromosome 2 in wheat[127] (and authors' unpublished observations) and the corresponding cDNA has also been reported[128]. Recently the isolation of BE IIb cDNA and genes from barley have been reported[129]. While high amylose wheats have not been reported, a well-characterised barley line, Glacier Ac38, has an amylose content of 45 to 50%[130].

High amylose content is not only important for the properties of dispersed starches. In the well known *amylose extender (ae)* mutation in maize, the granule is distorted in shape and starch digestibility declines markedly. This provides a source of 'resistant starch'[131], delivering unfermented starch to the colon of humans and other monogastric animals, which has been implicated in improved bowel health[132–134].

GENETIC MANIPULATION OF STARCH FUNCTIONALITY

Two broad approaches for genetic alteration are genetic engineering and conventional breeding. During the last decade methods for the production of transgenic cereal plants have been rapidly developed[135–138]. Although transgenic cereal plants have been obtained by direct gene transfer to protoplasts[135,139–141], the majority of transgenic plants are currently produced by either particle bombardment-mediated transformation or *Agrobacterium*-mediated transformation[142].

At the present, particle bombardment is still a popular and repeatable technique for the transformation of wheat as well as other cereal crops including rice, maize and barley. Using this technique, transgenic cereal plants have been obtained by many laboratories. The list of species includes rice[143], maize[144,145], wheat[136,146–148,172,173] and barley[137]. Generally speaking, this method produces a low frequency of transgenic cereal plants and high percentage of transgenic plants contain multiple insertions. The frequency of transformation ranges between 0·9–3·5% for rice, and 0·1–1% for barley, maize and wheat.

Rice was the first cereal crop successfully transformed using *Agrobacterium*-mediated transformation[149,150]. Since then this technique has been rapidly adapted to other crops which include maize[151], barley[138] and wheat[152]. Although *Agrobacterium*-mediated transformation of rice, maize and barley has been successfully used in many laboratories, transformation of wheat using this techniques has been reported only by one group so far[152].

Compared to particle bombardment methods, *Agrobacterium*-mediated transformation has an advantage in reducing the copy number of transgenes, reducing the frequency of transgene silencing and increasing the transformation frequency. Under the ideal conditions, the efficiency of recovery of transgenic plants can reach 12 to

29% in rice[150], 5 to 30% in maize[151], 1·7% in barley[138] and 1% to 4·3% in wheat[152]. The single insertion of transgenes in wheat can be increased from 17% (particle bombardment) to 35% (*Agrobacterium*-mediated transformation)[152]. These results are very encouraging and have led to a concentrated effort at transforming these cereal crops using *Agrobacterium*-mediated transformation.

Conventional breeding is still an attractive option when the characteristics desired can be identified in a natural population. Parent cultivars are then crossed. Progeny (generally at the F2 generation) can be selected which contain the desired phenotype and then backcrossed to elite cultivars. After at least six backcrosses to the elite cultivar, progeny plants that are satisfactory for agronomic use can be obtained. Clearly selection is much easier if the selected phenotype is dominant.

Breeding waxy wheat by conventional means has been achieved by combining null alleles of granule bound starch synthase[3,123]. Using a similar strategy, Yamamori[72] has produced wheat lines containing null alleles of SSII. The properties of starch from these lines are novel, based on preliminary studies, and are being further analysed. Exploiting wild germplasm and making wide crosses in breeding programs can be an attractive proposition to obtain greater variation in desired characteristics.

Another strategy to obtain variation in properties that are not available in breeding lines is to use mutagenesis. Using this approach waxy wheats have been produced by ethyl methane sulphonate treatment of cv. Kanto[122]. However, it is important to note that in this example of mutagenesis, Kanto is deficient in both Wx-A1 and Wx-B1 proteins and the mutagenesis was targeted to developing a line also deficient in the Wx-D1 protein

CONCLUSION

We can anticipate the development of wheat starches with a wide range of properties in the future. This will be due to a number of factors: further elucidation of the genes involved in the starch biosynthesis pathway and their roles, development of improved transformation technologies, improvements in marker assisted selection and improved analytical tools to define changes in starch structure and properties. Development of the required germplasm will come from two routes. One route is selective crossing using molecular markers to follow progeny, such as has been achieved with waxy wheats. In addition transformation technologies will be used to manipulate the expression of endogenous wheat genes, and to introduce novel genes form diverse sources. Although particle bombardment is still the most widely used and reliable method for transformation of wheat, *Agrobacterium*-mediated transformation will play a far more important role in wheat transformation as well as other cereal crops, maize, rice and barley in the future. The commercial use of transgenic technologies in food crops remains in the balance because of regulatory and consumer acceptance issues.

Some aspects of starch synthesis and structure that are important to functionality are still poorly understood at the biochemical or molecular genetic level. Grain hardness has, for example, been linked to markers on the tip of chromosome 5D and yet the causal lesion is still to be definitively identified. Little is known about genes controlling the synthesis of the suite of lipids present in the wheat starch granule. Similarly, while it is clear that the ratio of A granules to B granules has a genetic determinant[153], the causal gene(s) directing B granule initiation have not yet been elucidated. The process of starch granule development in wheat is clearly complex (see Fig. 1) and there is little known about how the three-dimensional growth of the granule in wheat (or other plants) is programmed. Other genes not identified through mutagenesis approaches in cereals could also be involved in starch biosynthesis and useful for the alteration of starch properties. Two examples are the R-protein[154] and disproportionating enzyme[155].

In addition to genetics it is clear that starch structure can be manipulated by the environment[175,176]. An understanding of the complex interactions between specific isoforms of the various enzymes involved in starch biosynthesis and the environment is in its infancy.

The coming decade holds much promise as a period when linkages between the roles of specific genes and the functionality of the wheat starch will be further defined. It is reasonable to anticipate that at least some of these advances in understanding and in the identification of genes and germplasm will translate into economically important new opportunities for the wheat industry and for the consumers of wheat-based products.

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