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# In planta novel starch synthesis

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## 1. Introduction

Starch is the most abundant reserve carbohydrate present in higher plants, where it is predominantly found in the amyloplasts of storage organs such as roots, tubers and seeds. The green leaves have the unique ability to harvest light energy in the presence of carbon dioxide and water to synthesize carbohydrates, which are converted into starch. Starch present in the leaves is known as transitory starch, because it is broken down into simple sugars, which are transported to storage organs such as roots, tubers or seeds, where storage starch is synthesized for utilization by the plants at a later stage. Approximately 2050 and 679 million tonnes of storage starch is produced annually by the cereal and tuber crops, respectively, (Tester and Karkalas 2002) and harvested by humans for food, feed and industrial applications.

Starch is a staple in the diet of the world's population. Almost fifty percent of the calories in human diet are supplied by starch based products. In the tropics, the indigenous starch crops of the tropics, cassava, sorghum, millet or yam, are considered wonders of nature, because with the sun and rain, and little or no artificial inputs, they are able to grow in great abundance. For centuries, tropical starches have served as staple food for millions of people, throughout the hot and humid regions of the world. These starch crops are so proficient to supplying essential calories to even the



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very poorest people of the world that they are considered to be the quintessential subsistence crop.

In the developed countries, starch is also used in the food and beverage industries as a thickener and a sweetener, in a multitude of processed food products. Starch is one of the most preferred industrial raw materials and it is being used to produce hundreds of products for use in several industries such as paper and pulp, textiles, cosmetics, pharmaceutical, biodegradable plastic film, construction and mining. However, for industrial applications, native starches have to be chemically modified to confer the desired physical and chemical attributes (Ellis et al. 1998). The use of starch for industrial purposes will only become economically viable when its use as a raw material can compete with petroleum-based products. The renewable and biodegradable nature of starch makes it increasingly attractive in response to the environmental concerns about the industrial wastes generated from petroleum products and the growing awareness of the potential deleterious consequences of greenhouse gas emissions from these activities. However, if starch can be modified *in planta* to confer some of the desirable traits, which will reduce post-harvest processing and modification, the utility of starch as an industrial raw material will be significantly enhanced. Therefore, in the late twentieth century it was emphasized that the focus of starch production will shift from low value bulk starch to high value specialty starches (Stroh 1997). The non-food uses of starch are also a prime indicator of a country's economic condition. During periods of reduced economic growth, the volume of starch going into non-food use also drops considerably. On the other hand, during periods of rapid growth, the demand for construction materials for building industrial plants and housing, paper for packaging and wrapping various products, place a high demand on industrial raw materials (Jobling 2004).

The demand for industrial starches is increasing, but four major crops, potatoes, maize, wheat and tapioca supply most of the requirements for non-food industrial applications. Starch properties differ between the plant sources, but for specialized applications, the native starches are chemically modified. Therefore, it is advantageous to produce novel or tailor-made specialty starches *in planta* as it would decrease the currently imperative post-harvest modification, some of which are environmentally damaging (Slattery et al. 2000). Recent progress in the understanding of starch structure and biosynthesis and the development of molecular biology strategies to alter cellular metabolism has provided an opportunity to change starch structure *in planta*. In this chapter, we will discuss the storage starch structure, biosynthesis and strategies for its genetic modification for food, feed and industrial uses.

## 2. Starch structure

Starch is stored as crystalline or semi-crystalline water-insoluble granules with an internal lamellar structure, which consists of two types of glucan polymers, amylose and amylopectin, trace amounts of lipids and proteins (Buléon et al. 1998). Amylose is a predominantly linear glucan polymer, composed of α-1,4-linked glucose residues, that is sparsely branched with α-1,6-linkages (approximately one branch per 1,000 glucose residues) (Takeda et al. 1986; Takeda and Hizukuri 1987). The degree of polymerization (dp) of amylose molecules is species dependent, ranging from around 800 in maize and wheat to approximately 4500 in potato (Morrison and Karlakas 1990; Alexander 1995). Amylopectin on the other hand is a much larger (dp $10^5$ to $10^7$) and more complex glucan polymer. It is composed of hundreds of short α-1,4 glucan chains joined together by α-1,6-linkages, with approximately 5 % of the residues having both α-1,4 and α-1,6-linkages (Myers et al. 2000). Studies have shown that both the length of the glucan branch chains and their pattern of branching are non-random. The ordered nature of the α-1,6-linkages indicates that the branches in the amylopectin molecule are clustered. The branch length distributions of amylopectins from a variety of different plants were shown to be polymodal, with peaks at approximately dp 15, and dp 45 (Hizukuri 1985). The unique and highly-ordered structure of the amylopectin molecule, in terms of both branch location and branch length, is essential to the formation of the starch granule (Myers et al. 2000; Buléon et al. 1998). A normal starch granule is made up of one-quarter amylose and three-quarters amylopectin. The ratio of amylose to amylopectin is an important determinant of starch physical properties and its end-use and its alteration will be discussed in subsequent sections. Other components within all starch granules are proteins (0.5% in cereal endosperm and 0.05% in potato tuber), which include the enzymes of starch biosynthesis and may contribute to the flavor of starch, and lipids (1% in cereal endosperm and 0.1% in potato tuber).

The granular structure is relevant when considering the mechanism of starch degradation, as many glucan metabolizing enzymes appear to be unable to act upon intact granules as substrate (Buléon et al. 1998). Starch is accumulated in a diurnal fashion in chloroplast of leaves, and in photosynthetic competent cells (transitory starch) to store sugar produced from photosynthesis. Starch stored in this way is degraded at night (dark period) for energy source and growth. Starch is stored in tuberous tissues (roots) and seeds are also used for an energy source for the formation of the next plant generation. Transitory starch has small granules, while reserve starch granules have species specific size and shapes (Davis et al. 2003).

## 3. Starch biosynthesis

Studies on starch biosynthesis in plants have mainly been focused on pea, maize, rice, potato, wheat and barley where starch properties are important factors for crop quality. These studies have been aided by the availability of a large number of starch mutants primarily in pea, maize and barley, but also mutants created in the non-crop plant *Arabidopsis* and the unicellular green algae *Chlamydomonas*. Several recent reviews have summarized the extensive data related to starch biosynthesis (Ball and Morell 2003; Morell and Myers 2005; Smith 2001). Studies of starch mutants have so far produced a general consensus that ADP-glucose pyrophosphorylase (AGPase), soluble starch synthases (SS), starch branching enzymes (SBE), starch debranching enzymes (DBE; pullulanase; isoamylase) and possibly also disproportionating enzyme (D-enzyme) catalyze the final steps leading to amylopectin synthesis (Figure 7.1). Granule bound starch synthase 1 (GBSS 1) is the only enzyme committed to amylose synthesis. Several of the starch biosynthetic enzymes exist in different isoforms, some of which vary in their sub-cellular distribution, enzyme specificity, temporal activity and interaction with other enzymes, thus causing variation of the starch biosynthetic pathway in different plant species and tissues. A minimal subset of 14 conserved starch biosynthetic enzymes (two AGPases, five SS, three SBE and four DBE) is homologous in all the plant species studied to-date (Morell and Myers 2005). Besides the core enzymes, additional enzymes such as starch phosphorylases, disproportionating enzymes and glucan water kinases also play important roles in starch biosynthesis.

AGPase is the first enzyme committed to starch biosynthesis and catalyzes synthesis of ADP-Glu and pyrophosphate from ATP and Glu-1-P (Fig. 7.1). ADP-Glu is then used as glucose donor for synthesis of linear chains on amylose and amylopectin, the two glucan polymers accumulated in the water-insoluble starch granules. AGPase has long been considered a key regulatory enzyme in the starch pathway and is composed of two large and two small subunits, both affecting allosteric and catalytic properties of the enzyme (Cross et al. 2004; Hwang et al. 2005). AGPase is activated by 3-phosphoglyceric acid (3-PGA), a product of photosynthesis, and inhibited

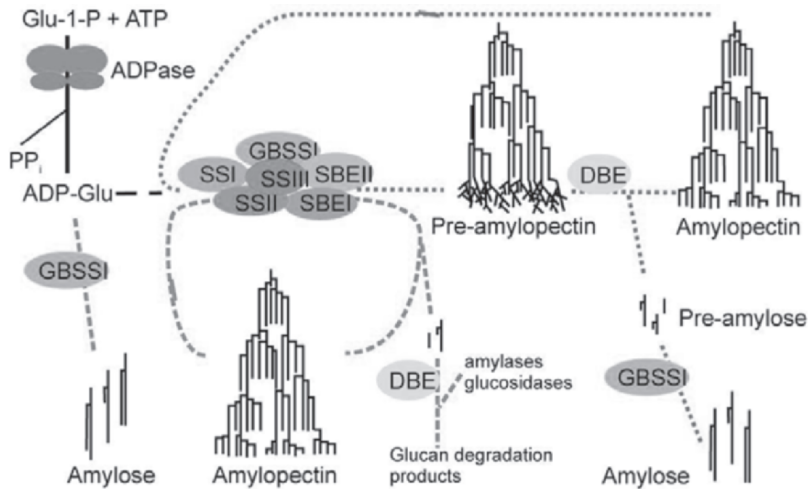


**Fig. 7.1.** Schematic illustration of two models for starch biosynthesis in plants. The "preamylopectin trimming model" is illustrated by the dark grey pathway and the "soluble glucan recycling model" is shown by the light grey pathway.

by inorganic phosphate ($P_i$) in many plant tissues (Preiss and Sivak 1988). In addition, some cereal AGPases are sensitive to heat stress, which affects crop yields (Singletary et al. 1994). AGPase is also under tight redox control in leaves, where the reducing conditions during the light cycle causes a thioredoxin-mediated reduction of a regulatory disulphide bond present between the two small subunits (Hendriks et al. 2003). The reduction of AGPase results in increased activity and higher activation by 3-PGA than obtained from the oxidized form present during the dark cycle. Due to redox and allosteric regulation of AGPase, the ADP-Glu production in green tissues is tightly coordinated with the day/night cycles and carbon status of the source tissue (Gibon et al. 2004).

In addition to ADP-Glu synthesized by the plastidial AGPase, an alternative source of ADP-Glu for starch biosynthesis in leaves has been proposed (Munoz et al. 2005). According to this study, a substantial amount of ADP-Glu is produced in the cytosol by the action of sucrose synthase (SuSy) and imported into the chloroplast for starch synthesis. In this alternative model, the role of plastidial ADPase is merely as a scavenger of breakdown products from starch degradation occurring during the dark cycle.

In developing grains of cereals, most of the AGPase activity is localized to the cytosol of the endosperm (Denyer et al. 1996b; Thorbjørnsen 1996; Beckles et al. 2001). This contrasts the location of AGPase in other cereal tissues and sink tissues of non-cereal plants where the AGPase activity is

exclusively found in plastids (Beckles et al. 2001). The presence of ADP-glucose transporter channels in the plastid membrane allows ADP-Glu formed in the cytosol of cereal endosperm to be imported into plastids and utilized for starch biosynthesis (Shannon et al. 1998; Patron et al. 2004). It has been speculated that the presence of a cytosolic, as well as, a plastidial AGPase in cereals will allow preferential channeling of available sucrose to starch biosynthesis at the expense of competing pathways utilizing sucrose-derived hexose phosphates as substrates (Beckles et al. 2001). In contrast to the leaf AGPases, the AGPases in sink tissues of different plant species show variable levels of allosteric regulation. In barley, wheat and maize, the endosperm AGPases are far less dependent on 3-PGA for activation than the leaf enzyme (Burger et al. 2003; Gómez-Casati and Iglesias 2002).

The main enzymes catalyzing synthesis of amylose and amylopectin from ADP-Glu are all located within plastids and include SS, SBE and DBE (Figure 7.1). It has long been believed that SS requires a primer to initiate glucan synthesis and it has been poorly understood how the polymerization reaction is primed. One hypothesis is that a protein with similar function as the self-glycosylating glycogenin, the initiator of glycogen biosynthesis in yeast and animal cells (Cheng et al. 1995), is involved in the priming reaction. The latter hypothesis is supported by RNAi experiments in *Arabidopsis* where down-regulation of a gene encoding a glycogenin-like protein in plastids resulted in reduced starch production (Chatterjee et al. 2004). A model that excludes the need of a primer to initiate glucan polymerization has also been put forward (Mukerjea and Robyt 2004). This model is based on growth of the glucan chain from the reducing end, as opposed to the long held belief that glucose units are added to the non-reducing end of the glucan.

The glucan chain elongation is catalyzed by four main SS: SSI, SSII, SSIII and granule bound SS (GBSSI). These enzymes catalyze formation of α-1,4-linkages between the glucose units resulting in linear glucan chains. SSI, SSII and SSIII are primarily involved in amylopectin biosynthesis, whereas GBSSI is the only enzyme associated with amylose production. The shortest chains on the amylopectin molecule are mainly synthesized by SSI and the longer chains are generally the result of SSII, SSIII and GBSSI actions. The relative contribution of each SS differs between plant species/tissues (Smith et al. 1997), and alterations to SS activities will affect the amylopectin fine-structure and/or starch granule morphology (Craig et al. 1998; Edwards et al. 1999; Morell et al. 2003).

The branches on the amylose and amylopectin molecules are introduced by SBE, which catalyzes cleavage of α-1,4 linkages and attaches the released chain through an α-1,6 bond to a new site on the glucan molecule. Two classes of SBE, SBEI and SBEII, with different substrate specificities

have been identified in plants. Long chain branches are introduced by SBEI activity, whereas SBEII prefer transfer of shorter chains (Preiss and Sivak 1998). A larger form of SBEI, SBEIc, has been identified in plants of the *Triticeae* tribe and is preferentially associated with the large A-type granules (Båga et al. 2000; Peng et al. 2000).

Debranching activities, exerted by isoamylase and pullulanase enzymes, have been postulated to have an active role in starch biosynthesis (Ball et al. 1996; Myers et al. 2000). The exact role of DBE in starch biosynthesis is not clear and two models for amylose and amylopectin biogenesis have emerged that includes different roles for DBE (Fig. 7.1). The "preamylopectin-trimming model" suggests that the outer branches of preamylopectin molecules are being trimmed by DBE to facilitate chain elongations by SS (Mouille et al. 1996). This will form amylopectin with an ordered branch structure and allow packaging of the molecule into starch granules. In addition, the glucan chains released by the DBE action on amylopectin can be elongated by GBSSI to form the amylose fraction. The role of DBE in the "soluble glucan recycling model" is to participate in degradation of short chain glucan molecules that may be produced by SS or SBE action to prevent accumulation of highly branched soluble polymers at the expense of amylopectin formation (Zeeman et al. 1998). Several studies have suggested that amylose is synthesized within the starch granules (Kuipers et al. 1994; Denyer et al. 1996a), whereas the much larger and highly branched amylopectin biosynthesis occurs at the surface of the granules (Smith et al. 1997). The spatial separation of amylose and amylopectin synthesis may explain why the two polymers with very different structures and sizes, can be produced simultaneously in plastids.

## 4. In planta starch modification strategies

The complexity of the starch biosynthetic pathway has been a challenge for the targeted *in planta* modification of starch to cater to novelty end uses for the food, health and industrial applications. Nonetheless, significant progress made towards better understanding of starch biosynthesis and altering biochemical pathways in plants has made possible to *in planta* alter the physico-chemical properties of starch. The availability of mutagenized populations, gene knockouts, over-expression and silencing approaches can all aid in gene function assessment and alteration. Therefore extensive efforts have been made to achieve similar end results within the plant itself. From screening for naturally occurring starch mutants to mutation breeding strategies to the use of newer technologies, including genetic transformation and genomics tools, the ability to alter starch

functionality has been demonstrated. A few strategies that have been successfully employed for *in planta* starch modification are described in the following sections.

### 4.1 Mutation breeding

To incorporate new desirable traits into their breeding programs, plant breeders screen for naturally occurring mutants from wild species of cultivated crops and land races. However, such screening procedures are sometimes time-consuming and labor-intensive and identification of the desired traits may often be challenging. Screening for starch mutants has, in the past, been restricted to the phenotypic aberrances exhibited by the seeds and characterized as the commonly used terms such as waxy, sugary, shrunken, brittle and so on (Kossmann and Lloyd 2000). Among the naturally occurring starch biosynthetic variants, over a century ago maize mutants, *sugary1* (*su1*) were identified (Correns 1901; cited in Jones 1924), which have been associated with sweet corn. It is now known that the *su1* mutation in maize is due to a deficiency in the debranching enzyme (DBE) of the isoamylase-type (James et al. 1995). The food industry has benefited tremendously from such naturally occurring mutants and subsequently others such as *sugary enhancer* and *shrunken2* (Young et al. 1997), which has further improved the sweetness of corn. However, most of the available cereal mutants are in diploid species like maize, rice and barley. Maize mutants for the *Waxy* locus have also been identified and grown for commercial purposes. The *waxy* maize starch has found uses as food stabilizers and thickeners, and emulsifiers for salad dressings (Jobling 2004). *Waxy* maize starch is also used in the manufacture of gummed tapes as remoistening adhesives, in adhesives and in the paper industry (Fergason 2001). As opposed to high amylopectin maize, the *sugary2* mutants have increased amylose up to 40% (Takeda and Preiss 1993) due to a lack of SSIIa activity (Zhang et al. 2004). In recent years high amylose starches have come to be recognized for their health benefits (Jobling 2004) as well as other industrial applications. In cereal species like wheat the identification of mutants is more difficult due to its hexaploid nature. Screening starch granule bound polypeptides useful natural starch mutants of wheat have been identified (Båga et al. 1999; Chibbar and Båga 2003). Naturally occurring starch mutants have not been found in potato (*Solanum tuberosum* L.) and other means of generating mutants by induced mutation, tissue culture and genetic transformation have been attempted. In the tropically grown cassava plant (*Manihot esculenta* Crantz) natural mutants for starch

have been identified with high free sugar content and lacking expression of the gene for starch branching enzyme (Carvalho et al. 2004).

Although screening for naturally occurring mutants has been and is still practiced, alternative ways to create variability are being explored. Even though induced mutations may eventually result in the release of varieties with altered traits, the resource-demanding nature of the process can preclude the benefits. This is because induced mutations, whether physical or chemical, are non-targeted leading to a large number of wasteful mutants. Induced mutants for starch biosynthesis have been generated in many plants. The model plant, *Arabidopsis thaliana*, is probably one of the best examples for induced mutation studies for starch biosynthesis, although it is not a starch storing plant. It has been suggested that study of transitory starch synthesis in leaves of mutant *Arabidopsis* may help to better understand the role of starch debranching enzymes in cereals (Zeeman et al. 1998). Due to the ease of inducing mutations and ease of genetically transforming *Arabidopsis*, it has been useful for studying starch biosynthesis and other metabolic pathways. Hovenkamp-Hermelink et al. (1987) induced mutations by X-ray irradiation to produce amylose-free starch in potato. In barley chemical mutagenesis was used to produce starch synthase IIa deficient lines with amylose content of 65-70% (Morrell et al. 2003) and one of the lines was shown to improve large bowel health in rats (Bird et al. 2004). In rice, retrotransposon-mediated insertion led to the production of mutants deficient for starch synthase I (SSI), wherein there was a reduction in amylopectin chain length (Fujita et al. 2006).

## 4.2 Genetic transformation

Among the starch storing plants, dicotyledonous species, such as potato, have been more amenable to genetic transformation as opposed to the monocotyledonous species such as the cereals. Potato has, therefore, served as a model system for genetic engineering of starch biosynthesis *in planta* using both the particle bombardment as well as *Agrobcaterium* species. In recent years, however, significant progress has also been made in the genetic transformation of cereals, primarily due to improved regeneration from cultured tissues and the ability to use *Agrobacterium tumefaciens*, which was earlier thought to be non-competent for the transformation of cereals. Since the first reports on the transformation of a Japonica rice (Hiei et al. 1994) and an Indica rice (Rashid et al. 1996) using *Agrobacterium*, there have been numerous reports on the *Agrobacterium*-mediated transformation of all the major cereals. Genetic transformation for *in planta* modification of starch was first successfully achieved in

potato, wherein an antisense construct for *GBSS1* gene was introduced using *Agrobacterium rhizogenes* to down-regulate expression of the endogenous *GBSSI* (Visser et al. 1991). Transformed potato lines exhibiting 70-100% reduction in GBSSI activity were produced, with reduced to amylose-free starch. The availability of efficient physical and biological DNA delivery systems for the transformation of dicotyledonous species has resulted in a number of reports of *in planta* starch alteration; albeit with varying success as to the extent the desired change occurred. For dicotyledonous species like potato, *Agrobacterium* is still the preferred method due to its efficiency. However, in spite of the perceived ease for *in vitro* culture and transformation of potato, not all varieties have been amenable to transformation and those that have been transformed have not always shown consistent expression levels of transgenes (Dale et al. 1995; Heeres et al. 2002). This genotype-dependent effect implied that tissue culture and transformation strategies had to be re-visited for poorly responsive genotypes and different regeneration and/or transformation protocols needed to be used for different genotypes. Heeres et al. (2002) attempted the transformation of 16 varieties of potato with an antisense construct for *GBSSI* and found variable degree of suppression of *GBSSI*, reduced yield and reduced starch content in some of the transformants compared to wild-type plants. The antisense inhibition of ADP-glucose pyrophosphorylase (AGPase) in potato resulted in reduced starch in tubers and accumulation of sucrose and glucose (Muller-Rober et al. 1992), while over-expression of an *E. coli* AGPase resulted in increase in sucrose to starch conversion (Stark et al. 1992). The transformation of other starch root crops has also been attempted. Starch crops such as cassava (*Manihot esculanta* Crantz) and sweet potato (*Ipomoea batatas*) are tropical habitat plants and consumed in many parts of the world. Raemakers et al. (2003) produced transgenic cassava plants with amylose free starch as a result of the integration of an antisense *GBSS* construct. Silencing of *GBSSI* gene in a transgenic sweet potato line led to the production of amylose free starch in tubers, in spite of the gene being introduced in sense orientation (Kimura et al. 2001).

For cereal transformation, the particle bombardment approach has been most widely used, prior to *Agrobacteirum* being shown to be transformation competent. There have been a number of obstacles for cereal transformation to become routine, particularly for the temperate cereals. First, the lack of efficient regeneration systems for cereals prevented production of high transformation frequencies of transformants. Furthermore, the tissue culture responses were found to be highly genotype-dependent and only a few model genotypes were consistently used for transformation experiments. The high yielding commercial varieties were generally less

responsive to tissue culture and were not targeted for transformation. Generally, immature embryos, immature scutella and inflorescences have been found to be responsive in tissue culture and competent for transformation. However, growth and maintenance of donor plants for isolation of immature explant tissues is very critical for high efficiency tissue culture response. Therefore the plants have to be grown under carefully controlled conditions and adequately fertilized to maintain their optimum physiological state and thereby improve tissue culture response. Consequently, growth of donor plants under such conditions adds to the cost, besides being time-consuming. Isolation of the immature tissues is also laborious and has to be done with minimal damage to the explants in order not to affect regeneration response. Furthermore the callus phase prior to plant regeneration has been known to be susceptible to somaclonal variation (Larkin and Scowcroft 1981). This occurs in monocotyledonous as well as dicotyledonous plants. This variation has been exploited for the regeneration and identification of useful variants and perceived, especially in the eighties as a novel source of *in vitro*-induced mutation. In combination with selection pressure in culture, regeneration of a number of plants with improved traits such as disease resistance and herbicide resistance have been obtained and released as varieties.

With the development of more specialized media, commercial varieties of the major temperate cereals are now amenable to high frequency regeneration in a fairly genotype independent manner using mature embryos (Ganeshan et al. 2006). The mature embryo has an added advantage since there is no requirement for the growth and maintenance of donor plants for explant isolation for *in vitro* culture. Mature embryos can be readily isolated from available mature seeds even from field-grown plants. The need for vernalization of winter cereals for obtaining immature explants for culture is also eliminated. The mature embryo culture is thus a simple, efficient and expedited approach for regeneration from cereals. The mature embryos have also been shown to be transformation competent using particle bombardment.

With the transformation methodology now being a routine process in most laboratories involved in transgenics, the focus has shifted from single gene transfer to studying and assessing multiple transgene functions. This becomes more relevant for the manipulation of the complex starch biosynthetic pathway, wherein multiple control points and interactions occur, including pleiotropic effects. To this end transfer of large inserts carrying genes involved in starch biosynthesis would be valuable for *in planta* starch modification. Recent advances in plant transformation technology using *Agrobacterium* and specialized vectors have led to the possibility of inserting large fragments of DNA into the plant genomes. In order to

achieve this, binary bacterial artificial chromosomes (BIBAC) vectors were developed for plant transformation (Hamilton et al. 1996; Hamilton 1997). More recently transformation–competent artificial chromosome (TAC) vectors (Liu et al. 1999) containing large-insert genomic DNA of hexaploid wheat has been developed (Liu et al. 2000). Using TAC vectors in *Agrobacterium*, transgenic rice carrying an 80 kb insert was recovered (Liu et al. 2002). Even though the stability of these large inserts has been questioned, progress has been made in determining factors influencing such instability and efficiency for transformation of tomato (Frary et al. 2001), potato (Song et al. 2003) and rice (Nakano et al. 2005). With the availability of such vectors for large inserts, it would be possible to use a multi-gene linking approach to place a number of the cloned starch biosynthetic genes in tandem and study their effects on *in planta* changes in starch. Using this approach, Lin et al. (2003) were able to stack multiple genes (including 10 genes and functional DNA elements) into a TAC vector and successfully transformed rice plants. This approach also offers the possibility of studying potential gene interactions by pyramiding only those starch biosynthetic genes suspected to be involved in the concerted metabolism of starch.

The available loss-of-function production systems such as induced mutations, insertional knock-out mutagenesis and antisense down-regulation have been effective to varying degrees in plants, and as mentioned earlier, *Arabidopsis thaliana* being the most versatile. But generally these approaches are time-consuming, unpredictable and desired alterations may not always occur in the more complex plant genomes such as wheat. The hexaploid wheat genome is made up of three different genomes - A, B and D genomes. Due to the occurrence of mostly three homoeologues of a particular gene in the wheat genome, there is a compensation effect from loss-of-function of one homoeologue. Therefore, studying the effect of a null allele on starch biosynthesis for example becomes complicated and confounded. More recently there has been interest in RNA interference (RNAi) as an approach at gene silencing. RNAi is a post-transcriptionally occurring gene silencing mechanism induced by double-stranded RNA and was first reported in the nematode worm, *Caenorhabditis elegans* (Fire et al. 1998). RNAi-mediated silencing in plants has also been shown (Waterhouse et al. 1998). Although RNAi is not a knock-out strategy per se, its down-regulatory potential can be specifically targeted to each gene of interest and would be valuable for polyploid species like wheat for both starch biosynthetic gene function analysis as well as for targeted *in planta* starch modification.

The application of the RNAi technology to plants is straightforward and relies on the availability of a transformation system and carefully designed transgene construct such that the transgenes produce hairpin RNA (hpRNA) with double-stranded RNA (dsRNA) regions (Waterhouse and Helliwell 2002). Several studies have explored the possibility of using the RNAi technology for reverse genetics or for *in planta* starch alterations using model plants. Using an RNAi approach in tobacco plants Chen et al. (2005) targeted the gene that codes for sucrose-6-phosphate phosphatase, which catalyzes the last step in the sucrose biosynthetic pathway converting sucrose-6-phosphate to sucrose. Transgenic tobacco lines were found to accumulate 3-5 times more starch than non-transformed plants. Silencing of β-amylase8 by RNAi in transgenic *Arabidopsis* plants led to starch accumulation in the leaves, with or without a six hour cold treatment at 4°C (Kaplan and Guy, 2005). Induction of β-amylase occurs during temperature stresses and leads to increase in maltose content to protect membrane proteins and the electron transport chain (Kaplan and Guy 2004). A similar observation has been reported in potato tubers stored at low temperatures, wherein maltose accumulation occurred (Nielsen et al. 1997). Potato could therefore be an ideal candidate for RNAi-mediated silencing of β-amylase to increase starch content. More recently, Regina et al. (2006) produced high-amylose wheat by RNAi silencing of two starch branching enzyme II isoforms (SBEIIa and SBEIIb), leading to more than 70% amylose content. This resistant starch wheat was shown to improve large bowel indices in rats and offers potential for designing healthy starches for humans (Regina et al. 2006).

## 4.3 Tilling

As a retort to induced mutations, a targeted mutation strategy termed TILLING (Targeting Induced Local Lesions IN Genomes) was developed (McCallum et al. 2000). TILLING combines classical chemical mutation approach with high-throughput molecular biology techniques for the identification of desired mutants. It offers the possibility of identifying single base pair changes in a gene of interest (Till et al. 2003). The TILLING strategy involves mutagenizing seeds with ethlylmethane sulfonate (EMS), growing plants (M1), allowing selfing, planting of individual M2 seeds for DNA extraction from leaves and cataloguing M3 seeds. EMS primarily causes G/C to A/T transitions and in *Arabidopsis thaliana* up to 99.5% of mutations were shown to be randomly distributed G/C to A/T transitions (Greene et al. 2003). DNA extracted from the M2 plants is pooled for PCR of target gene. The PCR products are denatured and allowed to re-anneal

for the formation of heteroduplexes. In the original protocol, the heteroduplexes were analyzed for mutations on denaturing HPLC (McCallum et al. 2000). This was subsequently modified wherein the heteroduplexes were treated with an endonulcease, CELI, which cleaves mismatches in the heteroduplexes between mutated and non-mutated variants (Colbert et al. 2001). CELI, identified in celery, reconizes single base mismatches and cleaves on the 3'-side of the mismatch (Oleykowski et al. 1998). The cleaved heteroduplexes are separated by electrophoresis and mutations are identified. DNA from individual samples of the pool are then screened to identify the mutant plant. TILLING populations have been established for plant species such as *Arabidopsis thaliana* (Till et al. 2003), *Lotus japonica* (Perry et al. 2003), maize (Till et al. 2004), barley (Caldwell et al. 2004) and wheat (Slade et al. 2005). The wheat TILLING population is of significance since it has enabled the identification of a range of *waxy* phenotypes from close to normal to near-null (Slade et al. 2005).

## 5. Targets for in planta modification of starch synthesis

### 5.1 Increased starch quantity

Increased starch quantity in storage organs has several advantages therefore it was one of the first targets to successfully apply molecular techniques to alter starch biosynthesis. In the first instance, AGPase, the rate limiting enzyme in starch biosynthesis, was genetically manipulated to increase starch production and/or content in potato and maize. A 30% increase in starch content was observed in transgenic potato expressing an *E. coli* AGPase that is insensitive to $P_i$, a feed-back regulator of AGPase (Stark et al. 1992). Similarly, site-specific mutagenesis was used in maize to produce a mutant AGPase large subunit that was also insensitive to Pi. In this mutant, seed weight was increased 11-18% without increasing or decreasing the percentage of starch (Giroux et al. 1996). The mutant AGPase gene when introduced into wheat resulted in altered large AGP subunit which reduces $P_i$ insensitivity and stable large and small subunit interactions (Smidansky et al. 2002). The transgenic wheat showed enhanced AGPase activity and 38% more seed yield and an increase in total plant biomass by 31% (Smidansky et al. 2002). These results are similar to those reported in maize (Giroux et al. 1996) and rice (Sakulsingharoj et al. 2004), where $P_i$ insensitive AGPase large subunit gene did not increase grain starch concentration, but the seed weight was increased by 11-18%. Increased starch content in potato was also achieved through the manipulation

of a different enzyme, inorganic pyrophosphatase (PPi). Expression of an *E. coli* PPase in transgenic potato increased starch content by 20-30% (Geigenberger et al. 1998). Another approach to increase starch content in cereals is the manipulation of Bt-1, the plastidial adenylate transporter (Shannon et al. 1998). Bt-1 mutants in maize have markedly reduced starch contents (Creech 1965), and thus over-expression of Bt-1 may lead to increased starch content.

The increase of total starch concentration is not an economically viable alteration in starch metabolism. However, in some cases augmentation of starch synthesis may be needed to make the production of unique starches or other traits economically viable. Naturally occurring mutants and/or transgenic plants with altered starch biosynthesis often have significantly reduced starch contents associated with qualitative changes to the starch (Marshall et al. 1996; Creech 1965; Nishi et al. 2001). Therefore, increasing the starch content in mutants/transgenics may be necessary to increase the yields of the starch with altered structure.

## 5.2 Phosphorylated cereal starches

Phosphorous is present in minor quantities, but the relatively higher quantity and its chemical nature in tuber starch contributes to their superior functional qualities over cereal starch for several applications (Alexander 1995). Using the *in planta* modification strategies described in this chapter, it may be possible to produce phosphorylated starches in wheat, barley and maize with endosperm-specific expression of the R1 protein (Lorberth et al. 1998). Down regulating the R1 protein gene expression in potato and the concomitant reduction in starch phosphate content resulted in starch with reduced peak viscosity and elevated setback viscosity (Albertsson and Karlsson 1995). Therefore, transgenic expression of R1 protein in cereal grains will likely increase peak viscosity and reduce setback, in phosphorylated cereal starches. Such changes could enhance the utility of cereal starches for industrial purposes. However, the precise changes due to the phosphorylated cereal starch cannot be predicted as there are other differences between cereal and potato starch, such as the structure and content of amylose molecules, phospholipids content and granule size.

## 5.3 High phytoglycogen starches

The *sugary-1* mutants accumulate phytoglycogen, a highly-branched, water-soluble polysaccharide, in addition to starch in maize (Creech 1965) and rice (Nakamura et al. 1997), and the *Sta7* mutant in *Chlamydomonas* (Mouille et al. 1996). In both the maize and rice mutants, the phytoglycogen content is approximately 25-30% of total carbohydrates. Increasing the phytoglycogen content of maize to more than 30% could result in a storage polysaccharide with reduced viscosity, gel formation and retrogradation rate with increased water holding capacity (Johnson et al. 2001a). In addition, a grain with high phytoglycogen-starch combination would have increased digestibility as a livestock feed, which could potentially have an enormous economic impact (Johnson et al. 2001a,b). Attempts have been made to increase phytoglycogen concentration by stacking different mutant alleles in maize but have been unable to markedly increase phytoglycogen concentration.

The naturally occurring phytoglycogen accumulating sugary-1 mutants are often associated with a reduction in dry seed weight (Creech 1965). Increasing starch quantity through genetic engineering, as discussed in the previous section, may be necessary for the development of plants with a high phytoglycogen phenotype. In addition, the physiological role of the starch granule as a non-hygroscopic, non-osmotic energy store must also be considered. Higher phytoglycogen content may result in reduced starch concentration, and this could cause problems with germination and therefore agronomic viability. Thus, there may be a limit to which phytoglycogen content may be increased, without interfering with the physiological characters of the grain.

## 5.4 Starch with reduced amylose concentration

Amylose free (waxy) starch has unique properties and it is used in various industries such as textiles, corrugating and adhesive industries. These result from their clear film forming properties. Moreover, chemical modification of waxy starch further extends its industrial applications and an enormous array of applications in the food industry (Fergason 2001). Natural mutants lacking GBSSI activity produce starch granules containing only the amylopectin glucan polymer. The *waxy* mutants have been shown to be present in maize, rice and barley, but till recently had not been identified in crops with multiple genomes, such as wheat. During the last decade, through breeding it has been possible to recombine the three null GBSSI present in different wheat germplasms to develop lines with <1%

amylose (Nakamura et al. 1995; see recent reviews Seib 2000; Chibbar and Chakraborty 2004). "Partially *waxy*" starch, i.e. starch with amylose levels between normal and *waxy* starch, was identified as a potentially valuable quality trait in maize (Johnson et al. 2001a) and in wheat it is used for Asian noodle production (Hoshino et al. 2000; Yamaguchi et al. 2000). In wheat, the GBSS1 produced by the three genomes A, B and D, show activity levels in the order B>D>A (Miura and Sugiwara 1996) and therefore different combinations result in lines with varying amylopectin concentrations, known as 'partially waxy' starch. Partially waxy starch, i.e. starch with amylose levels between normal (25%) and *waxy* starch is desired for production of certain foods, e.g. Japanese noodles (Seib 2000). Partially waxy wheat starch has been obtained through breeding of germplasm carrying one or two GBSSI null alleles (Demeke et al. 1999). The partially waxy wheat starches contain about 12% amylose and have higher swelling power and show crystallization patterns and gelatinization properties distinct from both normal and waxy starch (Seib 2000; Chibbar and Chakraborty 2004).

## 5.5 Starches with high amylose concentration

Starch with elevated amylose concentration (≥ 40%) is preferred for gums and candies and in the paper and pulp industry. Recently, an increased demand for high amylose wheat starch within the food industry has emerged as high-amylose starch can be converted to resistant starch (RS) upon heating and subsequent cooling. RS is not digested in the small intestine, but is broken down by the bacteria in the colon. As a result, RS acts as dietary fiber (DF), reduces the calories from food, has low glycemic index and is considered beneficial for colon health. An increase in amylose/amylopectin ratio and higher average amylopectin branch length in starch is found in certain maize, rice and pea mutants, which lack expression of one of the two SBEII isoforms (Chibbar and Baga 2003). The high amylose mutants in maize and pea have amylose concentrations of 50% or greater as compared to 25-30% for the normal endosperm and embryo starches. In mutant indica rice, the amylose content is 39-41% compared to 24-25% for the wild type (Kaushik and Khush 1991). High amylose barley lines with grain endosperm amylose concentration up to 40% have been developed (B.G. Rossnagel, personal communication). The amylose fraction in some mutants has a lower molecular weight and wider size distribution than normal amylose. The amylopectin fractions of these starches are also more linear with longer branch length than normal starch. In high amylose barley (Himalaya) the mutation has been attributed to SSIIa deficiency (Morell et al. 2003). Similarly, elimination of SSII polypeptides in

wheat increased apparent amylose (colorimetric assays) concentrations to 30-37% (Yamamori et al. 2000). In a similar approach, wheat lines deficient in SSII A and B genome polypeptides, resulted in lines showing up to 35% amylose as determined by HP-SEC analyses (Chibbar et al. 2005). However, in rice, barley and wheat amylose concentrations could not be increased beyond 40%, although a recent report (Regina et al 2006) suggest that RNAi silencing of SBEIIb genes in primary wheat transgenics amylose concentrations can be increased to 70%.

## 5.6 Large or small granule starches

Starch granule size is an important factor in several industrial processes including the production of thin films (Lim et al. 1992), paper coatings, cosmetic products (Ellis et al. 1998), and carbonless copy paper (Nachtergaele and Van Nuffel 1989). Furthermore, starch granule size is important in the brewing process. A significant portion of the small B-type granules from barley is not completely gelatinized in the mash and the undegraded residue causes mechanical problems during subsequent processing, e.g. filtration (Tillett and Bryce 1993). There are significant additional processing costs required for the isolation of large or small granules. Thus, the *in planta* production of starches with predominantly large or small granules would be very desirable.

Very little is known about the processes determining starch granule size. A few studies have indicated that SBEI may be involved. Studies using antisense SBEI constructs have reported increased granule size in transgenic potato (Flipse et al. 1996). It has also been suggested from studies in wheat that an SBEI isoform, SBEIc, may be involved in determining bimodal starch granule size distribution in this crop. Production of the two size fractions of starch granules is only found in plants belonging to the Triticeae tribe, including wheat, barley rye and triticale. Analysis of wheat starch granule proteins has revealed a difference in the abundance of SBEIc, an isoform of starch branching enzyme, in small and large granules (Båga et al. 2000; Peng et al. 2000). SBEIc is a large (~150 kD) protein that, in contrast to the 87 kD SBEI, is preferentially incorporated into the large starch granules. SBEIc is only found in wheat, barley and rye starch granules, and thus, is associated with plants showing bimodal starch granule size distribution in the endosperm. Down-regulation or a mutation in either SBEI or SBEIc can alter the proportion of A- and B-type granules. Both transgenic-antisense SBEI and/or SBEIc approach or traditional plant breeding techniques with mutagenesis could be taken to develop starches with altered starch granule size.

## 5.7 Starches with altered chain lengths and branching patterns

The molecular structures of amylose, amylopectin and the starch granule are important determinants of the functional properties of a starch. There is potential to produce a wide range of new starches through the alteration of the glucan chain lengths, branching patterns and granule crystallinity (Johnson et al. 2001a). Mutations in several different starch synthetic enzymes have been shown to affect the branch length and degree of branching of amylopectin (Craig et al. 1998; Yamamori et al. 2000; Edwards et al. 1999) resulting in altered starch physicochemical properties (Yamamori et al. 2000; Edwards et al. 1999). Recently, transgenic expression of a bacterial glycogen branching enzyme gene in rice resulted in a marked increase in the degree of branching of amylopectin with high number of short branches (Kim et al. 2005). Given the many different enzymes involved in amylopectin biosynthesis, various modifications to amylopectin structure and a diverse range of concomitant functional changes are possible. Some functional changes in maize, including improved gel formation and stabilized viscosity have been postulated to have a significant economic value in maize (Johnson et al. 2001a).

# 6. Concluding remarks

Starch is one of the most abundant and versatile storage carbohydrates, which has a multitude of applications. Several modifications of starch structure have been made which have further enhanced its utility. Recent advances in our understanding of plant processes and cellular metabolism present numerous opportunities to modify starch structure for the benefit of humans. For example, the recent expression of the cellulose synthase-like (*CslF*) genes from rice in *Arabidopsis* resulted in the synthesis of (1,3:1,4)-β-D-glucan like polymers (Burton et al. 2006), which demonstrates that novel glucan polymers can be synthesized in plants. Maize starch with beta glucan linkages has been suggested as a potentially valuable trait in maize (Johnson et al. 2001a). Similarly, introduction of 1,3 and 1,6 linked glucosyl residues catalyzed by the transgenic expression of bacterial glucansucrases is another approach to modify glucan biosynthesis in plants (Kok-Jacon et al. 2003). The use of starch granules as carriers of valuable therapeutic drugs or other useful compounds has not been fully explored. Some of the starch biosynthetic enzymes are completely granule bound and this unique feature may be used to deposit compounds of interest in the starch granules. The hydrophobic nature of starch granules will facilitate purification of the compound of interest. New approaches to

study grain development and starch biosynthesis (Jansson et al. 2006) will further improve our understanding of starch biosynthesis which can be used to produce genetically modified starches *in planta*.

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