AMYLOSE BIOSYNTHESIS IN POTATO: INTERACTION BETWEEN SUBSTRATE AVAILABILITY AND GBSSI ACTIVITY, REGULATED AT THE ALLELIC LEVEL

AMYLOSE BIOSYNTHESE IN AARDAPPEL: INTERACTIE TUSSEN BESCHIKBAAR SUBSTRAAT EN KGZ ACTIVITEIT, GEREGULEERD OP ALLEL NIVEAU

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Amylose biosynthesis in potato:

interaction between substrate availability and GBSSI activity, regulated at the allelic level

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Stellingen:

1 In tegenstelling tot het model van Denyer *et al.* (1996) kan amylose gevormd worden in afwezigheid van malto-oligosacchariden (dit proefschrift).

2 De produktie van amylopectine-vrij zetmeel is een utopie (Denyer *et al*, 1999; dit proefschrift).

3 Een toename in de korrel-gebonden zetmeelsynthase activiteit door multi allelie leidt alleen in aanwezigheid van voldoende hoge concentraties glucose-donor tot hogere amylose gehalten. (Flipse *et al.*, 1996; Lloyd *et al.*, 1999; dit proefschrift).

4 De aanwezigheid van amylose in opslagzetmeel heeft een duidelijk selectief voordeel.

5 Verschillen in promoter expressie kunnen beter met het eigen gen (dit proefschrift) dan met een reporter gen (Rohde *et al.*, 1990) zichtbaar gemaakt worden.

6 Het feit dat homologie tussen de promoter van het endogene allel en de transgen promoter bepalend is voor de mate van antisense remming duidt erop dat dit proces complexer is dan hybridisatie tussen mRNA en antisense RNA (dit proefschrift).

7 Het Nederlandse beleid m.b.t. de biotechnologie was de afgelopen jaren in overeenstemming met het dubieuze drugs "gedoogbeleid".

8 Het feit dat veel Nederlanders de Fransen arrogant vinden zegt meer over de Nederlanders dan over de Fransen.

9 In tegenstelling tot voetballers worden politici ten onrechte niet afgerekend op hun kwaliteiten, maar op hun imago.

10 Milieu organisaties zijn vaak te veel bezig met het leveren van kritiek en te weinig met het kritisch medebegeleiden van zaken.

11 Er zijn meer mensen die het leuk vinden oranje pionnen op de weg te zetten, dan dat er mensen zijn die ook daadwerkelijk de moeite nemen om aan de weg te werken (Lebbis en Jansen, 1999).

Stellingen behorende bij het proefschrift getiteld:
Amylose biosynthesis in potato:
interaction between substrate availability and GBSSI activity, regulated at the allelic level

door Marion van de Wal

Wageningen, 23 mei 2000

Contents

GENERAL INTRODUCTION

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Starch is a reserve carbohydrate, which is present in most green plants and in practically every type of tissue. In the plant, starch is deposited as starch granules in chloroplasts of photosynthetic tissues (transient starch) or in the amyloplasts of storage tissues (storage starch). These granules vary in size, shape, composition and properties between species, organs and stage of development. In leaves, starch is deposited during active carbon dioxide fixation by photosynthesis during the day and degraded by respiration during darkness (Sachs, 1887). In sink tissues such as cereal grain endosperm, potato tubers, pea seed cotyledons and tuberous roots of cassava, sucrose is imported from the photosynthetic tissues and converted before it is taken up by the amyloplast and stored in the form of starch granules for a long period of time. The ultimate role of the storage of starch is to provide energy for plant metabolism, for example for the sprouting of potato tubers or the germination of seeds.

Besides the role of starch as the major primary source of calories in both human and animal diet, it is used as raw material for industrial applications, both for food and non-food applications, such as the paper industry, textile industry, chemical industry, and pharmaceutical industry. Annually 20 - 30 x 10^6 tons of starch is isolated for a wide range of industrial applications, while EC starch production is approaching 10 x 10^6 tons. Starch is processed into over 600 commercial products and the market for starch is still increasing especially as other resources such as crude oil are shrinking. Moreover, new markets for starch are arising, for example, the use of starch for the manufacture of biodegradable polymers such as "bio-plastics". Starch isolated for industrial applications, is mainly derived from corn. However, in Europe a significant proportion of starch is isolated from potato (Hyer *et al.*, 1999). The advantage of potato starch over starches isolated from other sources is the low protein and fat content and the high molecular weight amylopectin.

STARCH STRUCTURE AND PROPERTIES

Industrially important properties which are responsible for the functional quality of starch, such as gelatinization, retrogradation, starch swelling, viscosity, and behaviour as granules, are highly affected by the starch structure. Starch consists only of glucose residues, which are linked by α-1,4 bonds and branched in α-1,6. However, the starch

granule structure (for review see Manners, 1989) is much more complex than expected from its composition. Starch is constituted of two glucose polymers, amylose, which is essentially linear and amylopectin, which is highly branched. The ratio of amylose to amylopectin is one of the key factors determining industrial important properties. In most plants, starch consists of 20 – 30 % amylose and 70 – 80 % amylopectin. In amylose, which has a molecular weight of 10^4 – 10^6 Da, the glucose residues are linked by α-1,4 bonds. Some amylose molecules are branched to a small extent (less than 1 %). Amylopectin is much more highly branched with about 4 % to 5 % of α-1,6 linkages. It has a molecular weight of 10^7 – 10^8 Da and is one of the largest biological molecules in nature (Buléon *et al.*, 1998). The branch-points in amylopectin do not occur randomly, but are arranged in clusters thereby allowing the formation of double helices, which can pack together in organised crystalline and amorphous lamellae, that are the basis of the semi-crystalline structure of the starch granule (Fig. 1).

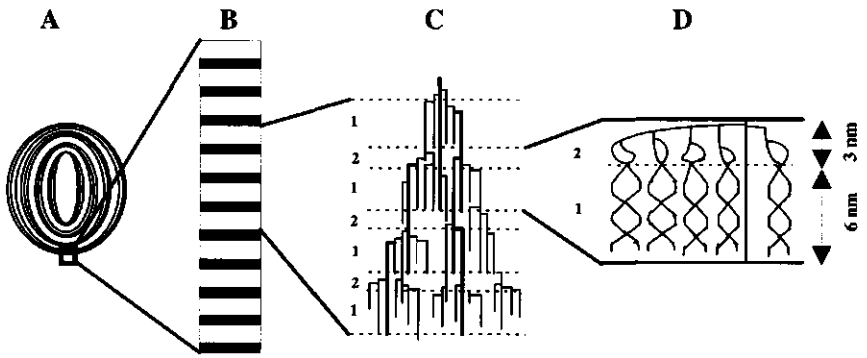

Fig. 1 Schematic overview of the structure organisation of amylopectin within the starch granule (copied from Ball *et al.*, 1998b). (A) Schematic view of a 1,5 μm thick starch granule showing a succession of amorphous and crystalline growth rings. (B) Section of a 0.1 μm thick crystalline growth ring showing the molecular organisation of amylopectin. Each black and each white section represents an amorphous and a crystalline lamella, respectively. Thus, the crystalline growth ring enlarged in (B) consists of regular succession of 11 amorphous and crystalline lamellae. (C) Succession of seven lamellae in relation to the primary structure of a portion of an amylopectin molecule. Each line represents an α-1,4 linked glucan chain. The chains are inter-linked by α-1,6 branches. The broken line delimits the sections appearing in the crystalline (1) and amorphous (2) lamellae. Note that most of the α-1,6 branches are included in the amorphous lamellae at the root of the chain clusters and that the glucans are pointing towards the granule's surface. (D) Part of the primary structure depicted in (C) is shown in relation to the secondary structure proposed for amylopectin. Each line represents an α-1,4 linked glucan chain. A single cluster is displayed. The parallel α-1,4 linked glucan double helices define the crystalline lamellae. The base of the cluster contains most of the α-1,6 branches. The 6 nm size of the crystalline lamella corresponds to a length of 18 glucose residues.

Besides the amylose to amylopectin ratio and the degree of branching of both glucan polymers affecting the starch structure, other critical parameters affecting starch quality are the granule size, the presence or absence of particular starch modifying enzymes and other storage tissue constituents such as proteins and lipids. An important property of starch for the characterisation and selection of mutants and transgenics with altered starch composition is its interaction with iodine. Iodine interacts with α-1,4 linked glucan chains by inserting in the hydrophobic cavity of the linear glucan helices, which are generated if the chains are sufficiently long, over 12 glucose residues at 20 °C. The iodine binding capacity of amylose is approximately 20 % (weight/weight), while the binding capacity of amylopectin is less than 1 %. The colour of the iodine glucan complex is dependent on length (Banks *et al.*, 1971). Short chains from glycogen will stain brown with a λ_{max} (wave-length of the maximal absorbency) between 450 – 490 nm. Medium size chains from amylopectin will stain purple red, with a λ_{max} between 530 - 560 nm and long chains from amylose will stain green blue with a λ_{max} of 600 - 650 nm. Starch containing a mixture of purple red amylopectin and greenish blue amylose will stain dark blue.

STARCH BIOSYNTHESIS

The complexity of starch, as was shown by the extremely ordered structure of the amylopectin chains, is also indicated by the multiplicity of enzymes involved in starch biosynthesis. The major reactions involved in the final steps of starch biosynthesis are:

1. The synthesis of ADP-Glc

Glc-1-P + ATP ↔ ADP-Glc + PPi

2. The transfer of a glucose residue from ADP-Glc to the growing α-1,4 linked polysaccharide

ADP-Glc + (glucosyl)n → ADP + (glucosyl)n+1

3. The formation of α-1,6 branch-points

α-1,4 linked glucan → glucan with α-1,6 branch-points

Formation of ADP-Glc,

ADP-Glc pyrophosphorylase (AGPase) catalyses the conversion of Glc-1-P into ADP-Glc, which is the preferred substrate for starch formation. The formation of ADP-Glc is generally accepted to be the rate-limiting step of starch biosynthesis. A reduction in

ADP-Glc supply by a decrease in AGPase activity reduces the amount of starch produced (Preiss, 1969; Smith *et al.*, 1989; Müller-Röber *et al.*, 1992). Moreover, a reduction in amylose content was observed when the synthesis of starch was restricted through limited supply of ADP-Glc. The rb and rug3 mutants from pea, which are affected in their AGPase and plastidial phosphoglucomutase activity respectively, accumulate less starch and have a reduced amylose content (Lloyd *et al.*, 1996; Harrison *et al.*, 1998). Phosphoglucomutase catalyses the formation of Glc-1-P from Glc-6-P in a reversible reaction, thereby affecting the substrate for the formation of ADP-Glc. Lowered expression of AGPase in transgenic potato reduced the total amount of starch to 4 % of the *wild type* amount and the amylose content from 19 % down to 13 % (Lloyd *et al.*, 1999b). A *Chlamydomonas* mutation in AGPase resulted in a reduction of total starch content to 5 – 10 % of the *wild type* amounts while this starch contained no amylose (Ball *et al.*, 1991). By increasing the supply of ADP-Glc through the over-expression of a plastidial ATP/ADP transporter it was possible to increase amylose and starch contents of potato tubers (Tjaden *et al.*, 1998). The ATP/ADP transporter is responsible for the import of ATP into amyloplasts, which is necessary for the conversion of G-1-P to ADP-Glc by AGPase.

Chain elongation,

Starch synthases catalyse the transfer of a glucose residue from ADP-Glc to the non-reducing end of a growing α-1,4 linked glucan. Multiple isoforms of starch synthases have been characterised. Comparing the starch synthase sequences revealed four distinct classes. The starch synthase I (SSI), starch synthase II (SSII) and starch synthase III (SSIII) exist as soluble forms or as both soluble and minor granule-bound isoforms. The major granule-bound isoform, referred to as granule-bound starch synthase I (GBSSI) appears to be exclusively granule-bound. The homology between and within the starch synthase classes and with bacterial glycogen synthases is shown in Fig. 2.

Starch synthases SSI, SSII and SSIII,

The starch synthase isoforms SSI, SSII and SSIII, are found in a diverse range of species, including monocots and dicots. These isoforms are found mainly in the soluble phase and are supposed to be involved in amylopectin biosynthesis. The relative contribution of each of the starch synthases, SSI, SSII and SSIII, to starch biosynthesis is highly variable between different species and in different organs within a species. Therefore it is not possible to extrapolate results obtained with one species to other plant species. SSIII for example appears to be the major starch synthase in potato tubers, while SSII seems to be the major enzyme in pea embryo amylopectin synthesis (Marshall *et al.*, 1996; Denyer and Smith, 1992). The location of the SSI, SSII and SSIII is not completely

restricted to the soluble phase. Again the association of these starch synthases with the starch granule is variable between plant species. However, even though some isoforms are to some extent bound to the starch granule, they do not contribute to amylose biosynthesis.

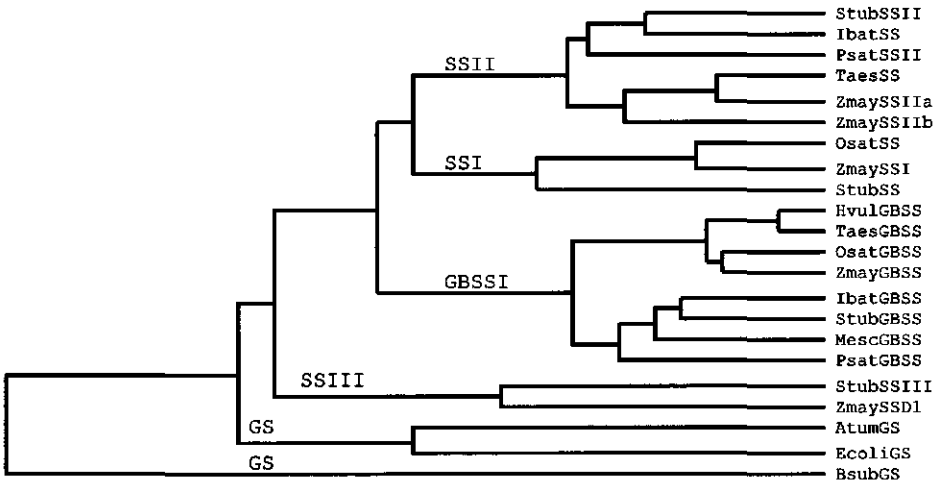

Fig. 2. Dendrogram showing the relationships between the different starch synthases and bacterial glycogen synthases (copied from Ball *et al.*, 1998b). The amino-acid sequences were aligned using the clustal method with PAM250 residue weight table. The identities of the proteins are: StubSSII, (*Solanum tuberosum* SSII; Denyer and Smith, 1992); IbatSS (*Impomoea batatas* SS; Harn *et al.*, unpublished); PsatSSII (*Pisum sativum* SSII; Dry *et al.*, 1992); TaesSS (*Triticum Aestivum* SS; Walter *et al.*, unpublished); ZmaySSIIa (*Zea mays* SSIIa; Harn *et al.*, 1998); ZmaySSIIb (*Zea mays* SSIIb; Harn *et al.*, 1998); OsatSS (*Oryza sativa* SS; Baba *et al.*, 1993); ZmaySSI (*Zea mays* SSI; Knight *et al.*, 1998); StubSS (*Solanum tuberosum* SS; Abel *et al.*, unpublished); HvulGBSS (*Hordeum vulgare* GBSSI; Rohde *et al.*, 1988); TaesGBSS (*Triticum aestivum* GBSSI; Clark *et al.*, 1991); OsatGBSS (*Oryza sativa* GBSSI; Hirano and Sano, 1991); ZmayGBSS (Zea mays GBSSI; Kloesgen *et al.*, 1986); IbatGBSS (*Ipomoea batatas* GBSSI; Wang *et al.*, unpublished); StubGBSS (*Solanum tuberosum* GBSSI; van der Leij *et al.*, 1991b); MescGBSS (*Manihot esculenta* GBSSI; Salehuzzaman *et al.*, 1993); PsatGBSS (*Pisum sativum* GBSSI; Dry *et al.*, 1992); StubSSIII (*Solanum tuberosum* SSIII; Abel *et al.*, 1996); ZmaySSD1 (*Zea mays* SS dull1; Gao *et al.*, 1998); AtumGS (*Agrobacterium tumefaciens* glycogen synthase; Uttaro and Ugalde, 1994); EcoliGS, (*Escherichia coli* glycogen synthase; Kumar *et al.*, 1986); BsubGS, (*Bacillus subtilis* glycogen synthase; Kiel *et al.*, 1994).

Granule-bound starch synthase I (GBSSI),

The major granule-bound starch synthase (GBSSI), the most extensively studied isoform both *in vivo* and *in vitro*, is involved in amylose biosynthesis. GBSSI from higher plants ranges in molecular weight from 59 - 61 kDa, while GBSSI from *Chlamydomonas* has a molecular weight of 76 kDa (Delrue *et al.*, 1992), which is due to an extension at the C-terminus (D'Hulst, 1997). In the first *in vitro* starch synthesis experiments purified starch granules were incubated with radio-labelled UDP-Glc (Leloir *et al.*, 1961). In these

experiments the label was incorporated in both amylose and amylopectin. Subsequent *in vitro* experiments performed by Recondo and Leloir (1961) demonstrated that ADP-Glc was the preferred donor substrate. At that time however, it was not yet known that they were assaying GBSSI, the enzyme responsible for amylose biosynthesis. In 1987 *in vitro* experiments performed with sweet potato starch granules also showed incorporation of label in both amylopectin and amylose by GBSSI (Baba *et al.*, 1987). Since they were then aware that GBSSI activity was studied under these conditions, they questioned the genetic evidence that GBSSI was solely responsible for amylose synthesis as label was also incorporated in amylopectin. However, mutants lacking GBSSI in corn (Nelson and Rines. 1962), barley (Rohde *et al.*, 1988), wheat (Nakamura *et al.*, 1995), potato (Hovenkamp-Hermelink *et al.*, 1987), sorghum (Hseih, 1988), amaranth (Okuno and Sakaguchi, 1982), pea (Denyer *et al.*, 1995) and the unicellular green algae *Chlamydomonas reinhardtii* (Delrue *et al.*, 1992) all synthesise amylose-free starch while normal amounts of starch granules were obtained containing *wild type* levels of amylopectin. The absence of amylose in these mutants clearly indicates that GBSSI is responsible for amylose biosynthesis. The presence of *wild type* levels of amylopectin shows that GBSSI makes little or no contribution to amylopectin synthesis.

Although it is clear that GBSSI determines whether or not amylose is formed the genetics behind amylose content still needs to be unravelled. The effect of allelic composition on GBSSI activity and amylose content could be proven for rice, a homozygous diploid species (Cai *et al.,* 1998) and hexaploid wheat, an allopolyploid species (Miura and Sugawara, 1996). Also in foxtail millet different GBSSI alleles, regulating quantitative levels of GBSSI protein and amylose content, have been identified (Nakayama *et al.*, 1998). The inheritance of amylose content in non-mutant cultivars of potato is complicated due to the heterozygosity and the tetraploid level of potato. In addition to the potato mutant *amf* allele (Jacobsen *et al.*, 1989) three complete *wild type* sequences have been reported, while five sequences of the promoter region were published (Dai *et al.*, 1996; Hofvander *et al.*, 1992; van der Leij *et al.*, 1991b; Rohde *et al.*, 1990). Comparing both the GBSSI gene product and the amylose content within a group of simplex plants, containing only one *wild type* GBSSI allele, it was suggested that the allelic composition had an influence on amylose content (Flipse *et al.*, 1996a). However, no differential effect of the *wild type* alleles on amylose content could be demonstrated.

As amylose content is one of the major factors affecting starch quality different strategies have been applied to obtain starch with altered amylose content. For example amylose-free potato starch was obtained by the suppression of GBSSI using antisense technology (Visser *et al.*, 1991; Kuipers *et al.*, 1994a). As an antisense gene can act as a

dominant suppressor of the endogenous genes, amylose-free cultivars can be obtained directly by *Agrobacterium* mediated transformation of existing varieties. In comparison, in conventional breeding programs, four copies of the mutant GBSSI allele are required to obtain amylose-free starch in tetraploid potato (Jacobsen *et al.*, 1991). Introduction of additional copies of the *wild type* potato GBSSI gene did not increase the amylose content above that of *wild type* potato (Flipse *et al.*, 1994) although, full restoration of amylose content could be obtained after complementation of the potato *amf* mutant with the cloned *wild type* GBSSI gene (Van der Leij *et al.*, 1991a). Experiments in which partially complemented transformants were crossed with both the *amf* and *wild type* potato showed that the GBSSI transgene was capable of inhibiting the expression of the introduced as well as the endogenous *wild* type GBSSI alleles (Flipse *et al.*, 1996b). This indicated that co-suppression influenced the level of complementation and thereby might have an impact on the final amylose content. Another reason why over-expression of GBSSI in potato did not result in an increase in amylose content (Flipse *et al.*, 1994; Flipse *et al.*, 1996a) could be that not the amount of active GBSSI but the ADP-Glc concentration is the rate-limiting factor in *wild type in vivo* amylose biosynthesis.

The Michaelis Menten constant (Km) of GBSSI is five to ten-fold higher than that of the soluble starch synthases indicating that GBSSI has a lower affinity for ADP-Glc than the other starch synthases. Amylose synthesis by GBSSI is therefore more sensitive to variations in the ADP-Glc supply than the amylopectin biosynthesis catalysed by the other starch synthase isoforms. This was also indicated by the alterations in amylose content when ADP-Glc concentrations are either increased or decreased (as described under the formation of ADP-Glc).

Apart from the higher Km for ADP-Glc GBSSI has unique properties, which allow it to catalyse the synthesis of amylose. One obvious property is its binding to the starch granule as amylose is formed within the amylopectin matrix and GBSSI is the major protein bound to the starch granule. However, other isoforms of starch synthase, which are to some extent bound to the starch granule do make little or no contribution to amylose synthesis. For example mutants of maize and pea, which lack GBSSI, contain 10 –15 % granule-bound starch synthase activity due to other starch synthase isoforms bound to the starch granule. Despite this granule-bound starch synthase activity no amylose is formed (Denyer *et al.*, 1995; Hylton *et al.*, 1996). These results indicated that binding to the starch granule of the synthases was not sufficient for amylose biosynthesis.

Another difference between GBSSI and the other starch synthase isoforms is the N-terminal extension, which GBSSI is lacking as compared to SSI, SSII and SSIII. The function of this N-terminal domain also referred to as a 'flexible arm' (Martin and Smith,

1995; Knight *et al.*, 1998) is unknown and comparison of the amino-acid sequences of the N-terminal domain revealed very little homology. Comparing the catalytic properties of GBSSI and SSII revealed two other major differences. The first is the processive rather than distributive manner in which GBSSI elongates glucans (Denyer *et al.*, 1996). The second is the affinity of GBSSI for amylopectin as an effector (Denyer *et al.*, 1999). The specific interaction of GBSSI with amylopectin is a unique property of GBSSI. This interaction influences besides the rate also the mode of elongation. Denyer *et al.* (1999) suggested that the ability of GBSSI to interact with amylopectin and to processively elongate glucans are the primary features of GBSSI distinguishing it from other starch synthases.

Formation of branch-points,

The formation of α-1,6 branch-points is catalysed by different isoforms of starch branching enzyme (SBE). This reaction involves the hydrolysis of an α-1,4 bond and the subsequent reattachment of the severed chain to form an α-1,6 branch. Although, more than two isoforms of starch branching enzyme often occur, all isoforms isolated so far can be grouped into two classes (A and B) based on their predicted primary amino-acid sequences deduced from the respective genes coding for them (Burton *et al.*, 1995). Both isoforms are found mainly in the soluble fraction and are presumably involved in amylopectin biosynthesis. However, the exact roles played by the two classes remains unclear. As branching enzymes are also found among the minor proteins associated with the starch granule it is likely that those are responsible for the low level of α-1,6 branches found in amylose.

In vitro studies with the starch branching enzymes A and B from maize showed that SBEB transferred longer chains than SBEA. Moreover, SBEB had a lower affinity for amylopectin and a higher affinity to amylose in comparison with SBEA (Guan *et al.*, 1994). These differences indicated that the SBE isoforms may have differential functions in determining the amylopectin structure. This was also indicated by the results obtained by analysing SBE mutants. Comparing the amylopectin isolated from pea leaves of the rugosus (r) mutant, containing a point mutation in the gene encoding SBEA, with *wild type* amylopectin, more chains of longer length were found (Tomlinson *et al.*, 1997). In the amylose extender (ae) mutants of maize (Boyer and Preiss, 1978) and rice (Mizuno *et al.*, 1993) reduction of the SBEA activity also led to a reduction of starch accumulation, an increase in the amylose to amylopectin ratio and an alteration in the chain-length distributions within amylopectin. The function of the SBEB isoforms is less well understood. Reduction of the activity of the SBEB isoform of potato by antisense or co-

suppression techniques (Flipse *et al.*, 1996c) showed no increase of the amylose content of the starch despite the fact that the expression of total SBE was largely inhibited. Over-expression of plastid-targeted bacterial glycogen branching enzymes (glgB) from *Anacystis nidulans* or *Escherichia coli* led to the synthesis of starch in which the amylose fraction was completely replaced by a branched material that was an intermediate between amylose and amylopectin (Kortstee *et al.*, 1998).

The role of starch debranching enzymes in starch synthesis,

Results obtained from the studies of four mutants with a similar phenotype in maize, rice, *Arabidopsis* and the green algae *Chlamydomonas reinhardtii* have provided evidence that starch debranching enzymes, capable of hydrolysing the α-1,6 branch-points, also have a role in starch synthesis. Two different types of debranching enzymes have been classified based on their substrate affinity. Isoamylases (also referred to as R-enzyme) have a higher affinity for glycogen, whilst pullulanases (also known as limit dextrinases) have a higher affinity for pullulan (Doehlert and Knutson, 1991). The evidence that the debranching enzymes have a role in starch synthesis came from a study of the sta-7 mutant of *Chlamydomonas rheinhardtii*. In this mutant only 0.1 % of the granular material was accumulated. The production of water-soluble polysaccharides (WSP) amounted to 5 % of what would have been the amount of starch in a *wild type* strain. These WSPs were more highly branched than amylopectin and not crystalline. Due to its similarity in structure to glycogen, which accumulates in bacteria and mammals it is referred to as phytoglycogen. The enzyme activity lacking in this mutant was that from a 88 kDa debranching enzyme (Mouille *et al.*, 1996). A model explaining the biosynthesis of amylopectin was proposed based on the results from the characterisation of the sta-7 mutant (Ball *et al.*, 1996). In this model debranching enzymes are involved in ordering the branches produced by trimming the pre-amylopectin to produce a more ordered amylopectin molecule and to prevent phytoglycogen synthesis.

Similar mutants accumulating phytoglycogen have also been found in maize, rice and *Arabidopsis*. The sugary-1 mutant of maize (Pan and Nelson, 1984), is deficient in an isoamylase type starch debranching enzyme (James *et al.*, 1995). In an *Arabidopsis* mutant lacking the activity of an isoamylase, phytoglycogen was also accumulated at the expense of starch (Zeeman *et al.*, 1998). As both starch and phytoglycogen accumulate at the same time, in the same plastids Zeeman *et al.*, (1998) proposed a model in which amylopectin was made by a combination of starch branching enzymes and synthases only. In this model debranching enzymes were involved in the degradation of phytoglycogen, which was a by-product of the starch synthesis.

Interaction between enzymes,

The existence of multiple distinct isoforms of the enzymes involved in starch biosynthesis and the interaction between these isoforms probably form the basis for the specific chain-length profiles of starch formed in different species and organs. The biochemistry of the starch synthesis is further complicated as variation in expression of specific enzymes can affect the activities of other enzymes in the pathway. This could be due to a direct interaction between enzymes, the lack of substrate for enzymes active further downstream of the pathway, competition between enzymes for the same substrate or because novel structures are formed that cannot act as substrate for other enzymes. It is therefore important to establish how enzymes interact in the starch biosynthesis pathway. A few examples are given to emphasis the importance of interactions between different isoforms.

Comparing the data obtained from potato lines in which both SSII and SSIII are repressed to those obtained from lines containing mutations in one of the two isoforms alone revealed a synergistic effect on amylopectin synthesis. This indicated that the different SS isoforms interact with each other (Edwards *et al.*, 1999; Lloyd *et al.*, 1999a). Moreover, an additive effect on the starch structure is observed by the simultaneous mutation of GBSSI and other isoforms of starch synthase. In homozygous maize lines containing both the *waxy* and *dull1* mutant alleles (Yuan *et al.*, 1993) the relative proportion of shorter chains from amylopectin was further increased as compared to the single dull1 mutant. This can be explained by the decrease in intermediate size and long glucans, which are synthesised by GBSSI. A combination of the *Chlamydomonas* GBSSI mutant *sta2* with the *sta3* mutant, containing a mutation in the major starch synthase, showed analogous effects on the starch structure. However, the reduction in starch synthesis and the increase in water-soluble polysaccharide was much higher (Maddelein *et al.*, 1994). Similar results were also obtained when double mutants of *waxy* maize and *sugary2* were produced (Fuwa *et al.*, 1987).

MODIFICATION OF STARCH

To adapt starch to its applications it is often chemically or physically modified after purification. These modifications are expensive, time consuming and sometimes environmentally hazardous. Therefore altering starch composition within the plant will have both environmental and economical advantages. Modification of starch inside the plant can be achieved by reduction of the activity of existing starch synthesising enzymes either by introducing mutations or through antisense or co-suppression or by the introduction of novel activities. Especially the combined reduction of existing activities and

the introduction of novel activities will contribute to the number of variable starch structures that can be generated.

Altering the production of starch inside the plant requires an understanding of the function and regulation of the enzymes involved in starch biosynthesis. The suppression of existing or introduction of novel enzyme activities requires the identification and isolation of genes involved in starch biosynthesis or alternative metabolic pathways such as the glycogen synthesis. And a prerequisite for the use of genetic manipulation to specifically alter starch synthesis is a successful transformation and regeneration system. As potato was one of the first crop plants amenable to genetic transformation it is very well suited for the production of modified starch polymers. Genetic modification of potato starch combines the advantageous potato starch characteristics with the improved processing techniques for starch production.

OUTLINE OF THIS THESIS

The amylose to amylopectin ratio highly affects industrially important properties of starch. Subtle changes in amylose content may significantly improve quality as was shown by the effect of GBSSI on amylose content and noodle quality in wheat (Zhao *et al.*, 1998). The goal of this thesis research was to unravel the amylose biosynthesis in potato and to determine the role of GBSSI in this process. Therefore amylose biosynthesis was studied *in vitro* and *in vivo*.

To study the *in vitro* amylose synthesis starch was isolated from *Chlamydomonas* (Chapter 2) and higher plants (Chapter 3) and incubated with radio-labelled ADP-Glc. *Chlamydomonas reinhardtii*, a green algae, is the only starch storing unicellular organism that is intensively studied by geneticists as starch polysaccharides are not found in bacteria and fungi. *Chlamydomonas* can be used as a model system as starch produced in nitrogen-starved *wild type Chlamydomonas reinhardtii* cultures, is virtually identical to storage starches of higher plants (Ball, 1998a). Moreover, all enzymes, reported to be involved in higher plant starch synthesis, are also found in *Chlamydomonas* with similar biochemical properties. A *Chlamydomonas* mutant strain defective for the large subunit of AGPase was used, as starch produced in this mutant contained no amylose despite the presence of *wild type* GBSSI. Therefore *in vitro* synthesised amylose could be compared to that produced *in vivo*. Based on the results obtained with both mutant and *wild type Chlamydomonas* starch a model explaining the amylose synthesis within the starch granule is presented (Chapter 2). The value of this model for the amylose synthesis in higher plants is described in Chapter 3.

Small changes in amylose content, which can affect the starch quality, can be obtained by classical breeding as was shown for rice (Pooni *et al.*, 1993) and wheat (Miura *et al.*, 1994). In Chapter 4 the differential effect of *wild type* GBSSI alleles on GBSSI activity and amylose content in potato is proven significant. This effect was determined in a population of *Solanum tuberosum* cultivars and *Solanum* breeding lines. A marker test based on Southern and PCR analysis was developed to readily enable the distinction between the different potato GBSSI sequences. Differentiation between the alleles will be useful in breeding programs aimed at altering amylose content. To achieve a more drastic effect on amylose content antisense technology can be used to obtain amylose-free starch (Visser *et al.*, 1991). A major drawback of the use of antisense technology is the variation in inhibition of the target gene after introduction of the antisense gene. This variation can be partly explained by copy-number and position effects of the T-DNA inserts. The effect of allelic composition of the variety used for transformation on antisense inhibition was studied in Chapter 5. It is shown that the allelic composition can be used as a pre-selection criterion in breeding programs in which antisense technology is used to obtain amylose-free potato varieties. In Chapter 6 a general discussion is given, connecting the different chapters and placing the results in the context of the intended goal: the unravelling of the amylose biosynthesis in potato.

AMYLOSE IS SYNTHESIZED *IN VITRO* BY EXTENSION OF AND CLEAVAGE FROM AMYLOPECTIN.

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ABSTRACT

Amylose synthesis was obtained *in vitro* from purified *Chlamydomonas reinhardtii* starch granules. Labeling experiments clearly indicate that initially the major granule-bound starch synthase extends glucans available on amylopectin. Amylose synthesis occurs thereafter at rates approaching or exceeding those of net polysaccharide synthesis. While these results suggested that amylose originates from cleavage of a pre-existing external amylopectin chain, such transfer of chains from amylopectin to amylose was directly evidenced from pulse-chase experiments. The structure of the *in vitro* synthesized amylose could not be distinguished from *in vivo* synthesized amylose by a variety of methods. Moreover, high molecular mass branched amylose synthesis preceded that of the low molecular mass suggesting that chain termination occurs consequently to glucan cleavage. Short pulses of synthesis followed by incubation in buffer with or without ADP-Glc prove that transfer requires the presence of the glucosyl-nucleotide. Taken together these observations make a compelling case for amylopectin acting as the *in vivo* primer for amylose synthesis. They further prove that extension is followed by cleavage. A model is presented which can explain the major features of amylose synthesis in plants. The consequences of intensive amylose synthesis on the crystal organization of amylopectin are reported through wide-angle X-ray analysis of the *in vitro* synthesized polysaccharides.

INTRODUCTION

Starch is usually defined as a mix of 2 distinct polymer fractions: amylopectin and amylose. Amylopectin the major compound is composed of intermediate size α-1,4 linked glucans that are organized in clusters of parallel chains by a dense packing of α-1,6 linkages. Amylose which accounts for 20 to 30 % in weight of the starch granule is often referred to as a smaller linear molecule with very few α-1,6 branches (for review see Preiss and Sivak, 1996). It was apparent ever since the pioneering work of Leloir and Recondo (Leloir *et al.*, 1961; Recondo and Leloir 1961) that glucose was transferred from ADP-Glc to the non-reducing end of a growing α-1,4 linked glucan thus coupling an extra glucose residue to this chain with the simultaneous release of ADP.

The enzyme was identified by Fekete *et al.* as associated with starch granules (de Fekete *et al.*, 1960) and was subsequently called granule-bound starch synthase. Due to the position of GBSSI inside the granule, diffusion of both donor and acceptor substrate might be a limiting factor for activity. GBSSI was first reported to use non-physiological concentrations of UDP-Glc (Leloir *et al.*, 1961) while ADP-Glc was shortly discovered thereafter as the preferred donor substrate (Recondo and Leloir 1961). These observations opened an altogether new area of research for both glycogen and starch synthesis in bacteria and plants respectively. It is known ever since the foundation work laid by Nelson and Rines (1962), that GBSSI is responsible for the biosynthesis of the amylose fraction. Mutations leading to defects for GBSSI have been isolated in an ever increasing number of species including *waxy* (*wx*) maize (Weatherwas, 1922), *wx* rice (Murata *et al.*, 1965), *wx* barley (Erikson, 1969), *wx* wheat (Nakamura *et al.*, 1995), amylose-free (*amf*) potato (Hovenkamp-Hermelink *et al.*, 1987), low amylose (*lam*) pea (Denyer *et al.*, 1996a), *wx* amaranth (Konishi *et al.*, 1985) and *sta2 Chlamydomonas reinhardtii* (Delrue *et al.*, 1992). All mutants accumulate during storage normal amounts of starch granules containing amylopectin with *wild type* crystalline organization (Buléon *et al.*, 1997). These important results establish that amylose is not required for the biogenesis of normal granules. A number of studies approaching the synthesis of amylose *in vitro* (Leloir *et al.*, 1961; Postein *et al.*, 1991; Baba *et al.*, 1987; Denyer *et al.*, 1996b), establish that GBSSI incorporates glucose both in amylopectin and amylose according to the conditions used. Leloir *et al.* (1961) originally noted a stimulation of GBSSI by high concentrations of malto-oligosaccharides and found incorporation of radioactive glucose into both starch fractions. In a very recent study Denyer *et al.* (1996b) showed that, in the absence of these oligosaccharides, the labeled product synthesized *in vitro* by GBSSI was confined to the amylopectin fraction. However, in the presence of high malto-oligosaccharide concentrations, GBSSI incorporated massively glucose into amylose-like glucans. Denyer

et al. (1996b) thus speculate that malto-oligosaccharides could trigger amylose synthesis in storage starch when their concentration becomes high enough to be physiologically active. It is thus tempting to suppose that the oligosaccharides are used as primers for the synthesis of amylose within starch granules. *In vivo* evidence supporting the involvement of GBSSI in amylopectin synthesis was produced by Maddelein *et al.* (1994). In this study, genetic interaction experiments clearly showed that defects in GBSSI strongly reduced amylopectin synthesis in particular mutant backgrounds.

We have shown that growth arrested (nitrogen starved) *Chlamydomonas reinhardtii* cells accumulate a polysaccharide that bares strong structural resemblance to maize endosperm storage starch (Delrue *et al.*, 1992; Maddeleine *et al.*, 1994; Fontaine *et al.*, 1993). Moreover, we have demonstrated that similar enzymes synthesize it and that it responds in an identical fashion to mutations affecting these activities (Ball *et al.*, 1991). We have reported that the starch accumulated during log-phase growth differs markedly from storage starch (Libessart *et al.*, 1995). The polysaccharide, which is similar to vascular plant transient starch, harbors little or no amylose while the amylopectin displays an altered chain-length distribution. Van den Koornhuyse *et al.* (1996) showed that in *Chlamydomonas reinhardtii,* mutants defective either for phosphoglucomutase or for the large subunit of AGPase accumulate, under storage conditions, polysaccharides whose structure were identical to those of transient starch. Transient starch structures are those naturally found in plant storage organs prior to storage starch and consequently amylose biosynthesis. Storage starch extracted from mutants defective for ADP-Glc supply together with transient starches extracted from *wild type* algae offer a unique opportunity to study the biosynthesis of amylose *in vitro*. Indeed prior to their incubation with ADP-Glc, these starches contain virtually no amylose (Libessart *et al.,* 1995; van den Koornhuyse *et al.*, 1996). In addition, the reported levels of GBSSI activity and protein increased four to five-fold with respect to those found for storage starches. Following the *in vitro* synthesis experiments reported in this paper, we propose an entirely new mechanism for amylose synthesis in plants.

MATERIALS AND METHODS

Materials,

ADP-[U-^{14}C]Glc was purchased from Amersham (U.K.). ADP-Glc, maize amylopectin, potato amylose, potato starch, sweet potato α-amylase, Pronase and Proteinase K were obtained from Sigma. Amyloglucosidase maltose and maltotriose were obtained from Boehringer (Germany). *Pseudomonas amyloderamosa* isoamylase was purchased from Hayashibara Biochemical Laboratories (Japan).

***Chlamydomonas* strains, growth conditions and media,**

The reference strain of *C. reinhardtii* used in this study is 137C (*mt⁻ nit1 nit2*). I7, defective for the large subunit of AGPase (*sta1-1*), was generated by X-ray mutagenesis from 137C and was previously described (Ball *et al.*, 1991). The GBSSI defective strain BAFR1 (*mt⁺ nit1 nit2 sta2-29:ARG7*) contains a disruption of the *STA2* gene that was generated through random integration of the pARG7 plasmid in the nuclear DNA of *Chlamydomonas reinhardtii* (Maddelein *et al.*, 1994). Standard media are fully detailed by Harris (1989) while growth conditions and nitrogen-starved media are described by Delrue *et al.* (1992), Ball *et al.* (1991), van den Koornhuyse *et al.* (1996) and Ball *et al.* (1990).

Determination of amylopectin/amylose content, starch purification and spectral properties of the iodine-starch complex,

A full account of amyloglucosidase assays, starch purification on Percoll gradients and λ_{max} determinations can be found in Delrue *et al.* (1992).

***In vitro* synthesis of amylose, protease protection experiments,**

500 µg starch was incubated with 3.2 mM ADP-Glc in the presence of 50 mM glycine (pH 9.0), 100 mM $(NH_4)_2SO_4$, 0.4 % β - mercaptoethanol, 5 mM $MgCl_2$, 0.05 % BSA and 2.2 µM ADP-[U-^{14}C]Glc at 10.5 GBq/mmol in a total volume of 2 ml at 30 °C for different periods of time. The reaction was terminated by adding 3 volumes of 96 % ethanol. After centrifugation at 3000 x g for 10 min the supernatant was discarded and the starch was suspended in 100 % DMSO and boiled for 20 min. The polysaccharide was precipitated over night at –20 °C by adding 3 volumes of 96 % ethanol. After centrifugation at 15,000 x g for 20 min at 4 °C, the pellet was dried in air for at least 10 min, dissolved in 500 µl 10 mM NaOH and subjected to gel permeation chromatography. To investigate the sensitivity of GBSSI to proteases, concentrations of 0.1 and 0.2 mg x ml^{-1} were used, respectively, for proteinase K and Pronase using the standard GBSSI assay at 30 min and 1 h after a 30 min preincubation.

Separation of starch polysaccharides by gel permeation chromatography,

0.5 - 1.0 mg starch dissolved in 500 µl 10 mM NaOH was applied to a column (0.5 cm i.d. x 65 cm) of Sepharose CL2B or CL4B which was equilibrated and eluted with 10 mM NaOH. Fractions of 300 - 320 µl were collected at a rate of one fraction per 1.5 min. Radioactivity was determined by liquid scintillation counting. Glucans in the fractions were

detected by their reaction with iodine and the levels of amylopectin and amylose were quantified by determining the amount of glucose after amyloglucosidase treatment.

Debranching analysis,

Isoamylase-mediated debranching of GPC-purified fractions of amylopectin and amylose was achieved as previously described (Libessart *et al.,* 1995). After completion of the debranching reaction, samples were kept at 80 °C in 10 % DMSO to avoid retrogradation of the long glucans into insoluble material. The debranched polysaccharides were subjected to TSK HW-50(F) chromatography as detailed in Libessart *et al.,* 1995. Debranching of amylopectin for CL4B gel permeation chromatography was performed in 50 mM NaAc (pH 4.0) containing 59 units of isoamylase.

Determination of the β-amylolysis limit,

Amylose and amylopectin were dissolved in 25 µl 0.8 M NaOH, diluted with 25 µl distilled water and incubated for 24 h at 30°C in 200 µl 50 mM NaAc (pH 3.5). The latter contained either no β-amylase, 22 units of β-amylase or both 22 units of β-amylase and 59 units of isoamylase. After adding 150 µl of 1 % 3,5- dinitrosalicylic acid to 50 µl sample, the mixture was boiled for 10 min and the number of reducing ends was determined spectrophotometricaly at 540 nm. Maltose was used as a standard. As a control the β-amylolysis limit was determined for maize amylopectin, potato amylose and potato starch.

X-Ray diffraction measurements,

Samples (10 mg) were sealed between two aluminium foils, to prevent any significant change in water content during the measurement. Diffraction diagrams were recorded using Inel (Orléans - France) X-ray equipment operating at 40 kV and 30mA. $CuK_{\alpha 1}$ radiation (l=0.15405 nm) was selected using a quartz monochromator. A curved position sensitive detector (Inel CPS120) was used to monitor the diffracted intensities using 2 h exposure periods. Relative crystallinity was determined, after bringing all recorded diagrams at the same scale using normalization of the total scattering between 3 and 30 °(2θ). Dry extruded starch and spherolitic crystals of amylose were used as amorphous and crystalline standards respectively.

RESULTS

Synthesis of amylose occurs *in vitro* from transient starches in the absence of added malto-oligosaccharides,

Starch was purified both from nitrogen starved cultures of mutants defective for the large subunit of AGPase or from nitrogen supplied *wild type* algae. The granules extracted from these strains displayed GBSSI activities ranging between 15 to 20 nmoles of ADP-Glc incorporated into insoluble polysaccharide per min and per mg starch. This activity displayed an apparent Km of 3.5 mM for ADP-Glc and was entirely protected from pronase and (or) proteinase K action. When using 3.2 mM ADP-Glc, incorporation was linear with time for periods ranging from 10 min to 2 h at 30 °C. Although all experiments reported in this paper were performed from freshly purified material, no loss of GBSSI activity could be evidenced after one week storage at 4 °C. Starch purified from strain BAFR1 (containing a gene-disrupted GBSSI structural gene) displayed less than 0.5 % of the *wild type* activity which fell below background when fractionated on Sepharose CL2B columns. This proves that only GBSSI was monitored under our experimental conditions. Fig. 1A and 1B show the separation of amylopectin and amylose by CL2B chromatography before and after *in vitro* synthesis respectively.

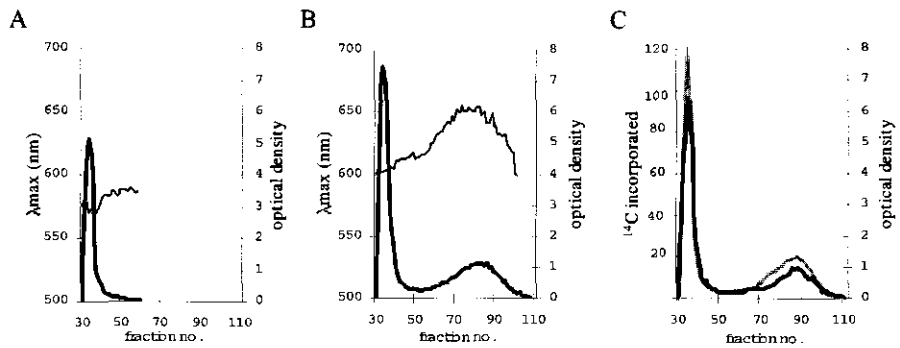

Fig. 1. Separation of amylopectin and amylose by CL2B-Sepharose chromatography. The optical density (bold black line) of the iodine-polysaccharide complex was measured at λ_{max} (thin black line) before (Panel A) and after (Panel B) 24 h *in vitro* synthesis. The starch extracted from the mutant defective for the large subunit of AGPase (17) displays no detectable amounts of amylose (Panel A). After 24 h of *in vitro* synthesis the amylose content raises to over 24 % of the total starch (Panel B). Panel C displays the optical density (bold black line) and the incorporation of ^{14}C from ADP-[^{14}C]Glc (bold gray line) after mixing 1 mg *wild type* storage starch and 0.05 mg of 17 starch subjected to 24 h *in vitro* synthesis. The radioactivity was determined by liquid scintillation counting. Because of the relative abundance of cold *wild type* reference polysaccharides, the OD reflects exclusively the *wild type* amylopectin and amylose while the radioactive material represents the *in vitro* synthesized glucans.

After 24 h we were able to raise the amylose content from less than 2 % to over 24 % of the total starch. The polysaccharide synthesized under these conditions could not be distinguished from standard *Chlamydomonas reinhardtii* amylose. Fig. 1C displays an experiment where 0.05 mg of starch subjected to *in vitro* synthesis for 24 h in the presence of labeled ADP-Glc was mixed and chromatographed with 1 mg of storage starch (20 % amylose) extracted from *wild type* cultures. We can conclude from these experiments that the molecular mass distribution of the *in vitro* synthesized material is identical to that of native amylose.

Moreover, the fine structure of the *in vitro* synthesized product was investigated using debranching analysis followed by gel permeation (Fig. 2A). By all these criteria, the *in vitro* synthesized polysaccharide proved to be identical to native *Chlamydomonas reinhardtii* amylose. As with algal native amylose, we detected up to 1 % branches within the *in vitro* synthesized product. We confirmed the presence of a similar branching pattern by measuring the β-amylolysis limit. β-amylase is known to be an exo-type of enzyme digesting external chains up to 2 to 3 residues from a branch-point. The β-amylolysis limit (the percentage of digested material) for both the *in vitro* and *in vivo* synthesized amyloses ranged between 70 to 75 %. Together with the identical chain-length distributions revealed by our debranching analyses (see above), these results prove that both polysaccharides contain an identical distribution of α-1,6 linkages with a strong bias towards the reducing end of the molecules (for review see Buléon *et al.*, 1998). From all these experiments we conclude that authentic amylose biosynthesis has been achieved. It must be stressed that this synthesis occurred at the expense of major changes in the structure of amylopectin. The λ_{max} of the iodine-polysaccharide complex of amylopectin increased from 570 to 600 nm (Fig. 1) while debranching analysis (Fig. 2B) clearly show that the label is incorporated in the fraction excluded from TSK-HW50 chromatography. The OD of the intermediate and small chain-length amylopectin fractions (26 - 39) before and after *in vitro* synthesis were identical while the OD of the long glucan fractions increased dramatically after *in vitro* synthesis (data not shown). We believe these chains to be external since the β-amylolysis limit of amylopectin increased before and after synthesis from 50-55 % to 58-63 % respectively. That these chains are covalently bound to amylopectin is confirmed by the fact that all attempts to dissociate them from amylopectin were unsuccessful. Indeed the long glucan fraction of amylopectin could only be recovered by enzymatic debranching as was reported both by Baba *et al.* (1987) and by Denyer *et al.* (1996b)

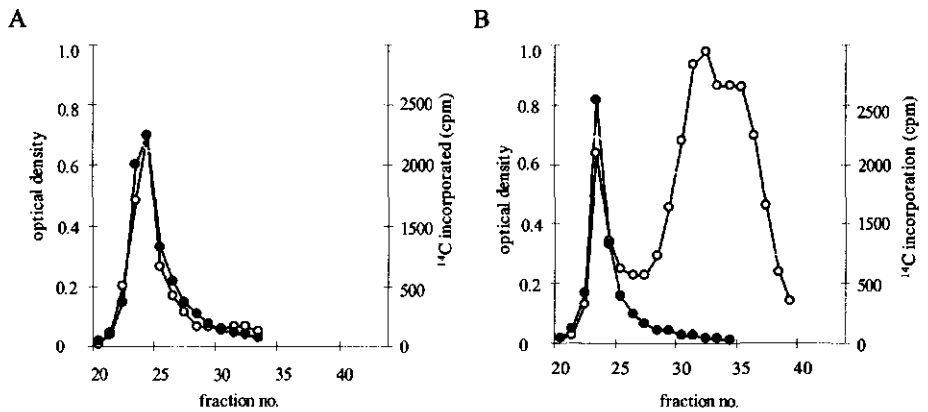

Fig. 2. Separation of isoamylase debranched glucans by TSKHW-50 chromatography. Three milligrams of *wild type* amylose and amylopectin separated by CL2B-Sepharose chromatography were mixed with 0.05 mg of amylopectin and amylose from the I7 mutant subjected to 24 h *in vitro* synthesis. After debranching with isoamylase the amylose (Panel A) and amylopectin (Panel B) were subjected to TSKHW-50 chromatography. The optical density (o) of the iodine-polysaccharide complex was measured at λ_{max}. The incorporation of ^{14}C from ADP-[U-^{14}C]Glc (•) was determined by liquid scintillation counting. Because of the relative abundance of cold *wild type* reference polysaccharides, the OD reflects exclusively the *wild type* amylopectin and amylose chain-length distribution while the radioactive material represents the *in vitro* synthesized glucans.

Kinetics of amylose deposition,

Denyer *et al.* (1996b) have reported that, in the absence of malto-oligosaccharides, after one hour incubation, amylopectin was the predominant if not only labeled species. While these results could be confirmed by us it was furthermore evident that this situation changed dramatically when longer incubations were analyzed. Our results reported above show that after 24 h of incubation more than 40 % of the label is incorporated in the amylose fraction. To study amylose deposition, we followed the kinetics of *in vitro* synthesis in the presence of labeled ADP-Glc. For this purpose, we used amylose-less starch granules purified from a nitrogen starved mutant defective for the large subunit of AGPase (I7). For each time point, a complete analysis involving Sepharose CL2B chromatography of dissolved starch granules was performed. The OD at λ_{max} and amount of labeled material were thus recorded in each fraction and are displayed in Fig. 3A (from 10 min to 2 h) and Fig. 3B (from 2 to 24 h). We were thus able to monitor total incorporation of label, as well as the amount selectively synthesized within amylose and amylopectin (Fig. 4). It is clear that *Chlamydomonas* starch behaves very much like that of peas in the initial steps. Indeed very little if any amylose synthesis occurred during the first hour (Fig. 3A) while active amylopectin elongation is witnessed.

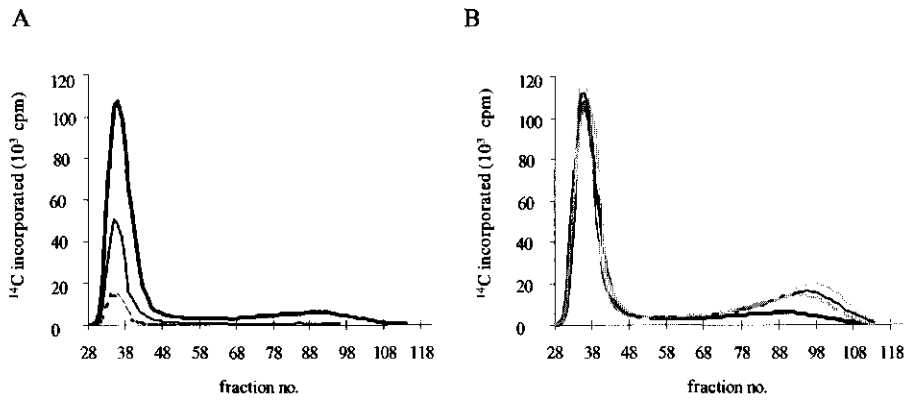

Fig. 3. Kinetics of *in vitro* synthesis. Starch from the mutant defective for the large subunit of AGPase (17) was subjected to *in vitro* synthesis in the presence of ^{14}C- labeled ADP-Glc. After *in vitro* synthesis the amylopectin and amylose were separated by CL2B-Sepharose chromatography. Panel A shows the incorporation of ^{14}C from ADP-[U-^{14}C]Glc after 10 min (thin dotted line), 30 min (thin black line) and 2 h (bold black line) *in vitro* synthesis. Panel B shows the incorporation of ^{14}C after 2 h (bold black line), 6 h (bold gray line), 12 h (thin black line) and 24 h (thin gray line). After 2 h the amount of label incorporated in the amylose fractions increases, while there is no further increase in the amount of label incorporated in the amylopectin fractions.

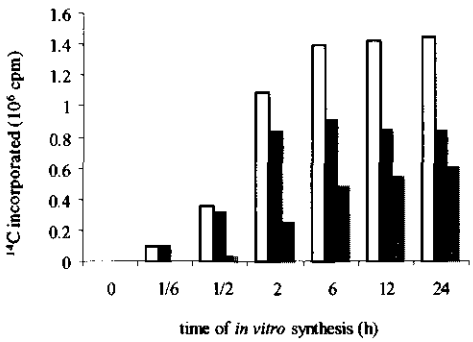

Fig. 4. Incorporation of ^{14}C from ADP-[U-^{14}C]Glc in amylopectin and amylose. After *in vitro* synthesis in the presence of ^{14}C labeled ADP-Glc the amylopectin and amylose were separated by CL2B-Sepharose chromatography. The total incorporation of label after different times of *in vitro* synthesis is shown for starch (white column), amylopectin (black column) and amylose (gray column). After 2 h incubation, amylopectin synthesis becomes progressively substituted by that of amylose.

That external amylopectin chains are getting longer is proven by the increase in λ_{max} of the iodine-polysaccharide complex from 570 to 600 nm that is completed within the very first 30 min (Table I).

Table I. λ_{max} of the amylopectin-complex after different times of *in vitro* synthesis.

	Time of *in vitro* synthesis (h)						
	0	1/6	½	2	6	12	24
λ_{max}	570	580	600	615	615	610	605

To ascertain that single chains are indeed getting longer, we debranched amylopectin and compared the length of these chains to those characterizing mature debranched *Chlamydomonas reinhardtii* amylopectin.

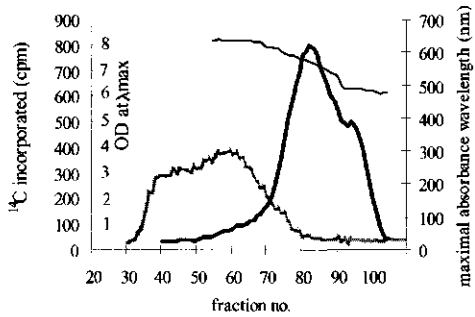

Fig. 5. Separation of isoamylase debranched amylopectin by CL4B-Sepharose chromatography. One milligram of *wild type* amylopectin separated by CL2B-Sepharose chromatography was mixed with 0.05 mg of amylopectin from the I7 mutant subjected to 1 h *in vitro* synthesis. After debranching with isoamylase the amylopectin was subjected to CL4B-Sepharose chromatography. The optical density (bold black line) of the iodine-polysaccharide complex was measured at λ_{max} (thin black line). The incorporation of ^{14}C from ADP-[U-^{14}C]Glc (gray line) was determined by liquid scintillation counting. Because of the relative abundance of cold *wild type* reference polysaccharides, the OD reflects exclusively the *wild type* amylopectin chain-length distribution while the radioactive material represents the *in vitro* synthesized glucans The chains elongated by GBSSI are considerably longer than the average chains of *wild type* amylopectin. A bimodal distribution reflecting the existence of two types of external amylopectin chains elongated by GBSSI can be already evidenced after 1 h *in vitro* synthesis.

Results displayed in Fig. 5 together with our β-amylolysis (see above) studies confirm that external amylopectin chains are getting elongated by GBSSI. Moreover, a bimodal distribution of the long glucans of amylopectin is detected. After 2 h incubation, amylopectin synthesis becomes progressively substituted by that of amylose (Fig. 3B and Fig. 4). After 12 h amylose synthesis rates exceeded those of incorporation by GBSSI. In addition and at the same time the λ_{max} of the amylopectin-iodine complex is decreasing from 615 to 605 nm (Table I). This result indicates that incorporation in amylose is accomplished at the expense of amylopectin. It is striking to note that high molecular mass amylose

biosynthesis occurs before that of the low molecular mass species mimicking thus the bimodal distribution of the amylopectin external long glucan fractions. At this point of our analysis we already suspected that external amylopectin chains were used to generate mature amylose by a single endo-type of cleavage event. We thus undertook experiments specifically designed to test this hypothesis.

Pulse-chase experiments,

The best way to prove the function of amylopectin as a primer for amylose biosynthesis would be to pulse-label the former and check if we can subsequently chase the label into the latter. We chose to pulse-label amylopectin for 30 min at t_0 from starch extracted from the same strain as that was used in the time-course experiments described above. As predicted from our previous experiments, amylopectin was found as the sole labeled species immediately after the radioactive pulse. As synthesis proceeded with unlabeled substrate the label was slowly but clearly chased into amylose (Fig. 6A). Again the label appeared first in the high molecular mass amylose fraction (Fig. 6B). We double-checked that pulse-labeled amylopectin external chains could also be chased into amylose in the case of both transient and storage starches from *wild type* algae. An example of such an experiment can be found in Fig. 6C, which displays a pulse-chase experiment with storage starch from *wild type* algae. Similar pulses gave similar results yielding a net chase from amylopectin into amylose. In this case, a low but substantial amount of radioactivity in the amylose fraction was immediately detected following the pulse. This result can be simply explained by assuming that a percentage of amylopectin outer chains are already physically ready to be transferred into amylose at t_0. It is worth noting that there again, high molecular mass amylose appears first. Pulse-chase experiments performed on transient starches from *wild type* strains behaved in a fashion virtually identical to that which we reported for the low ADP-Glc synthesizing mutants. In addition during time-course experiments extension of amylopectin chains also preceded the appearance of amylose. Moreover, we double-checked that time-course and pulse-label results obtained from transient starch using 0.5 mM ADP-Glc and pH 7.8 were identical after 4 days incubation to those obtained after 24 h at 3.2 mM ADP-Glc and pH 8.2. At this stage, the only reasonable interpretation of our results would be to assume that GBSSI extends amylopectin chains. When these chains become "long enough" they are released into mature amylose by a single cleavage event. The systematic appearance of high before low molecular mass amylose argues against significant elongation occurring after cleavage. Because we were not entirely satisfied with the meaning of chains "long enough" to be

cleaved we proceeded to search for experimental conditions uncoupling synthesis by GBSSI from that of the postulated cleavage reaction.

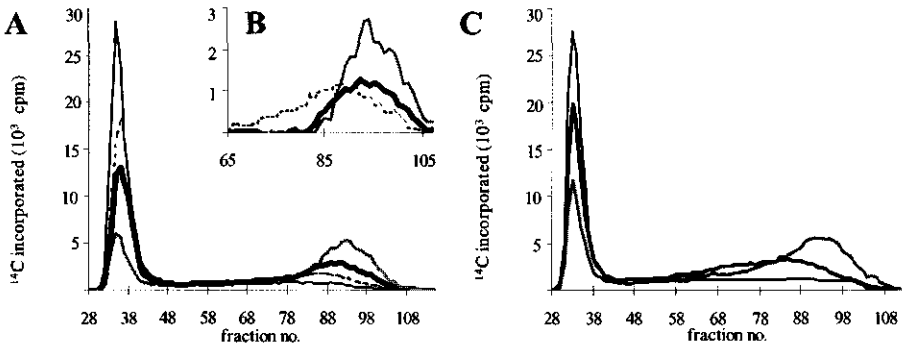

Fig. 6. Pulse-chase experiment of I7 and *wild type* storage starch. Panel A shows the incorporation of ^{14}C from ADP-[U-^{14}C]Glc in the mutant defective for the large subunit of AGPase (I7), analyzed with CL2B. After a 30 min pulse-label (thin black line), amylopectin was the sole labeled species. After a chase of respectively 2 h (dotted line), 6 h (bold black line) and 24 h (gray line) the amount of label incorporated in the amylopectin decreased and the amount of label incorporated in the amylose fraction increased. The label appeared first in the high molecular mass amylose fraction and later in the low molecular mass fraction. This is shown in Panel B. Panel B's y axis represents the increase in label between the different times and does not represent the total amount of radioactivity. As shown in panels A and C there is no net chase of the high molecular mass material into the low mass material. Panel C shows the incorporation of ^{14}C from ADP-[U-^{14}C]Glc in *wild type* storage starch. After a 30 min pulse-label (thin black line) amylopectin is the predominantly labeled species, however, a substantial amount of radioactivity is detected in the amylose fraction. After 6 h (bold black line) and 24 h (gray line) a net chase from amylopectin to amylose is detected. Again high molecular mass amylose appears first.

Cleavage of amylose requires continuous chain elongation and is not stimulated by maltotriose,

In order to uncouple cleavage from synthesis we used transient starch or storage starch from low ADP-Glc containing mutants. As predicted from our time course experiments pulse-labeling for 30 min at t_0 gave incorporation confined to amylopectin. The labeled starch granules were, as usual washed twice, but this time in the absence of any traces of ADP-Glc and in the presence or absence of 50 mM maltotriose. Absence of the substrate blocked cleavage and release into amylose. Because this reaction was not triggered by the presence of 50 mM maltotriose we reasoned that the previously reported malto-oligosaccharide stimulation of amylose synthesis (Leloir *et al.*, 1961; Denyer *et al.*, 1996b) did not seem to involve a selective function of the dextrins on chain termination at least not on its own. We therefore conclude that chain cleavage is tightly coupled to synthesis through GBSSI.

The influence of maltotriose on ^{14}C incorporation by GBSSI,

A number of reports in the literature suggest that amylose biosynthesis is stimulated by addition of maltodextrins such as maltotriose. Therefore we repeated the experiments performed by Denyer *et al.* (1996b) using 50 mM maltotriose and 3.2 mM ADP-Glc concentrations. Synthesis of amylose-like material was indeed massive within one hour incubation (Fig. 7) whereas relatively little glucose was incorporated in amylopectin. GBSSI incorporated 2-3 times more glucose in the starch granules under these conditions. Two important conclusions can be drawn from this experiment (i) The approximately 3-fold reduction of label in amylopectin in the presence of maltotriose suggests that the non-reducing ends of maltotriose compete with amylopectin molecules as acceptor substrates. Apparently a large number of maltotriose molecules have been elongated to such an extent that they became too large too escape from the granule. (ii) The increase in GBSSI activity upon maltotriose supply indicates that the amount of available acceptor substrate in the granule is limiting. This suggests that the immobilized GBSSI can not use the full potential of non-reducing termini within the starch granule. However, in the presence of a diffusable acceptor substrate such as maltotriose a larger number of GBSSI molecules can participate in the synthesis reaction. To illustrate the number of non-reducing ends the following estimation was made. Assuming an amylopectin content of 30 %, a volume for one glucose residue of 0.125 nm^3 and an average chain-length of 20 glucose units, we have calculated a non-reducing end concentration of 450 mM within the granule.

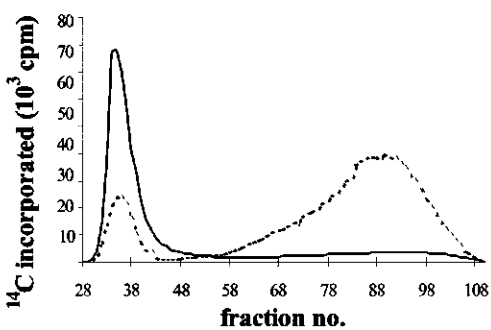

Fig. 7. Effect of malto-oligosaccharides on incorporation of ^{14}C in amylopectin and amylose. After one hour *in vitro* synthesis of I7 starch in the presence of 3.2 mM cold ADP-Glc and 2 μM ADP-[U-^{14}C]Glc the samples were run on a CL2B-Sepharose column. In the absence of 50 mM maltotriose (black line) amylopectin was the predominantly labeled species. In the presence of 50 mM maltotriose (dotted line) the total amount of radioactivity incorporated doubled, and amylose was the predominantly labeled species.

The above observations prompted us to investigate ^{14}C incorporation in starch granules with varying ADP-Glc and maltotriose concentrations (Table II). In the absence of maltotriose, ^{14}C incorporation increases proportionally with the ADP-Glc concentration until a concentration of 1 mM is reached. At this stage the donor substrate concentration is limiting. After this point the ^{14}C incorporation *vs.* ADP-Glc concentration curve starts to level off. At an ADP-Glc concentration of 3.2 mM, the incorporation of label increased with the maltotriose concentration, until a maltotriose concentration of 50 mM was reached. At 200 mM maltotriose a reduced ^{14}C incorporation is observed. Because of the abundance of maltotriose, most of the label will be transferred to this acceptor molecule instead of to amylopectin. Assuming that small maltodextrins as maltotetraose and maltopentaose dissociate readily from GBSSI, the consequence of a very high maltotriose concentration is that many of these molecules are extended with only a few glucose units. After 1 h of incubation these molecules have not grown long enough to be retained in the granules upon washing and consequently ^{14}C incorporation is reduced. In fact, this means that the actual GBSSI activity (at least) with 3.2 mM ADP-Glc and 200 mM maltotriose is underestimated. With lower ADP-Glc concentration also an "optimal" maltotriose concentration for label incorporation was observed. However, the optimum shifted to lower maltotriose concentrations as expected.

Table II. GBSSI activity at different ADP-Glc and maltotriose concentrations. GBSSI activities are expressed as nmoles ADP-Glc incorporated into insoluble polysaccharide per min.

ADP-Glc conc.	Maltotriose conc. (mM)					
(mM)	0	2	5	10	50	200
0.01	0.12	0.10	0.09	0.08	0.07	0.06
0.04	0.44	0.49	0.48	0.46	0.39	0.29
0.2	2.0	2.6	2.7	2.7	2.5	2.1
1.0	8.8	10	12	14	14	12
3.2	18	26	33	42	47	43

The consequences of massive amylose biosynthesis on granule crystallinity,

Our *in vitro* synthesis experiments gave us a unique opportunity to test the impact of massive amylose synthesis in complete absence of concomitant crystalline amylopectin biosynthesis. Crystalline diffraction patterns of plant starches fall into two distinct types, namely the so-called A and B-type. A-type diffraction patterns are found in cereal endosperm and *Chlamydomonas reinhardtii* starches while the B-type is found in potato tuber and high amylose mutant starches. GBSSI defective mutants from algae and cereals

display the very same A-type patterns. However, high amylose mutants from maize and *Chlamydomonas reinhardtii* switch to the B-type of crystalline organization. Because these high-amylose mutants are affected in the amylopectin biosynthesis pathway, it is not known if the switch is due a modification in amylopectin structure or to the increase in amylose which by itself could influence the amylopectin crystalline organization. We therefore compared the wide-angle X-Ray diffraction analysis of a sample of t_0 transient starch with less than 1 % in weight amylose to that of the very same starch that was subjected to intensive *in vitro* synthesis for over 48 h with 3.2 mM ADP-Glc leading to 45 % final amylose content.

The X-ray diffraction diagrams are displayed in Fig. 8. The crystallinities measured for the t_0 and t_{48} samples amounted to 27 and 16 % respectively. Transient starch displays an A-type diffraction diagram with a crystallinity of about 27 % very close to those described previously for *Chlamydomonas* storage starch (Buléon *et al.*, 1998). After prolonged synthesis the diagram clearly switched to the B-type with a lower crystallinity (16 %). Nevertheless, the degree of crystallinity of B-type starches is well known to depend strongly on the water content. Therefore the calculated value is only relative, since it was not possible to manage the hydration level on a so small amount of substrate. Moreover, some A-type can be still present in t_{48} starch. Indeed it is impossible in B-type starch diffraction diagrams to detect less than 15 % A-type (Planchot *et al.*, 1997), because of the high similarity of spectra and the low crystallinity of native starches.

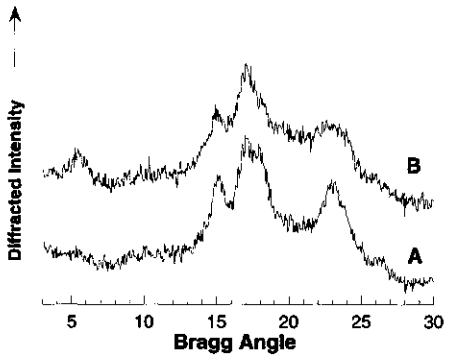

Fig. 8. Wide angle X-Ray diffractograms of starches before and after *in vitro* synthesis of amylose. The starch X-Ray diffractogram before and after 48 h *in vitro* synthesis are displayed respectively in panels A and B. Diffraction peaks at 2θ (Bragg angle) values of 9.9°; 11.2°; 15°; 17°; 18.1° and 23.3° characterize the A-type starches while diffraction peaks at 2θ values of 5.6°; 15°; 17°; 22° and 24° typify B-type starches. It is clear that the t_{48} (panel B) starch sample has switched from the A (panel A) to the B-type.

DISCUSSION

In the present study we have used both mutant and *wild type Chlamydomonas* starch granules to elucidate the process of amylose biosynthesis. Comparison of amylose synthesis in these backgrounds with that of starch purified from a strain containing a gene-disrupted GBSSI structural gene showed that only GBSSI enzyme activity was measured under our experimental conditions. We have proven among other by pulse-chase experiments that extension of amylopectin external-chains by GBSSI occurs *in vitro* (Table I) with subsequent cleavage into amylose (Fig. 6A, 6C). We have not found evidence for chain elongation within the amylose fraction itself. In addition, we have repeatedly observed a progressive switch from high to low molecular mass amylose synthesis (Fig. 6B). Comparison of *wild type* and *in vitro* synthesized amylose by CL2B chromatography (Fig. 1C), debranching analysis followed by gel permeation chromatography (Fig. 2A) and determination of the β-limit dextrin have shown that *in vitro* synthesis yields a branched polymer which is indistinguishable from native amylose. Based on the results described in this paper we propose an entirely new route for amylose biosynthesis.

Active GBSSI can be localized bound at the surface, bound within the granule, or both. The physical location of active enzyme is of paramount importance because it is only at the surface that the enzyme can eventually be considered as moving with the growing amylose molecules. Within the polysaccharide matrix itself, there is very little room if any for the enzyme movement. We have chosen to discuss only the latter possibility. Indeed, evidence for the presence of actively moving and readily dissociating surface enzyme is presently lacking. Three possible models accounting for amylose synthesis within the starch granule are displayed in Fig. 9. The three models share a number of major assumptions, which will be discussed first. We assume that each GBSSI enzyme is tightly bound to the amylopectin matrix and is in fact an immobilized enzyme. While this assumption remains to be formally proven the resistance of GBSSI activity to proteases, the pronounced decrease of the apparent Km following solubilization of the enzyme (Macdonald *et al.*, 1985) and the immunolocalization of this enzyme within the granule (Denyer *et al.*, 1993) are all in agreement with it. Moreover, we have to take into account the inability of large proteins to diffuse into the polysaccharide matrix, the likelihood of amylose deposition within the amorphous cavities of the granule, and the availability of GBSSI for multiple rounds of amylose chain synthesis. Assuming an amylose content of 30 %, an average molecular weight of 500,000 (Buléon *et al.*, 1998) and an amount of approximately 2.5 µg of 76 kDa GBSSI protein per mg starch (estimated by SDS gel electrophoresis) we have calculated a 1:18 ratio of GBSSI to amylose molecules. The major difference between model A on the one hand and models B and C on the other is that the former uses malto-

oligosaccharides as an acceptor substrate, whereas the latter two use amylopectin. When ADP-Glc is supplied, extension of the polysaccharide chain occurs at the non-reducing end of the molecule pushing the nascent glucan into the amorphous cavities of the granule (Fig. 9). The part of the glucan situated the furthest away from the non-reducing end of the glucan is more likely to encounter the less abundant branching enzyme (BE) trapped in the granule (Fig. 9). Amylose branching is thus a stochastic event requiring a close encounter with BE within the path of the growing glucan.

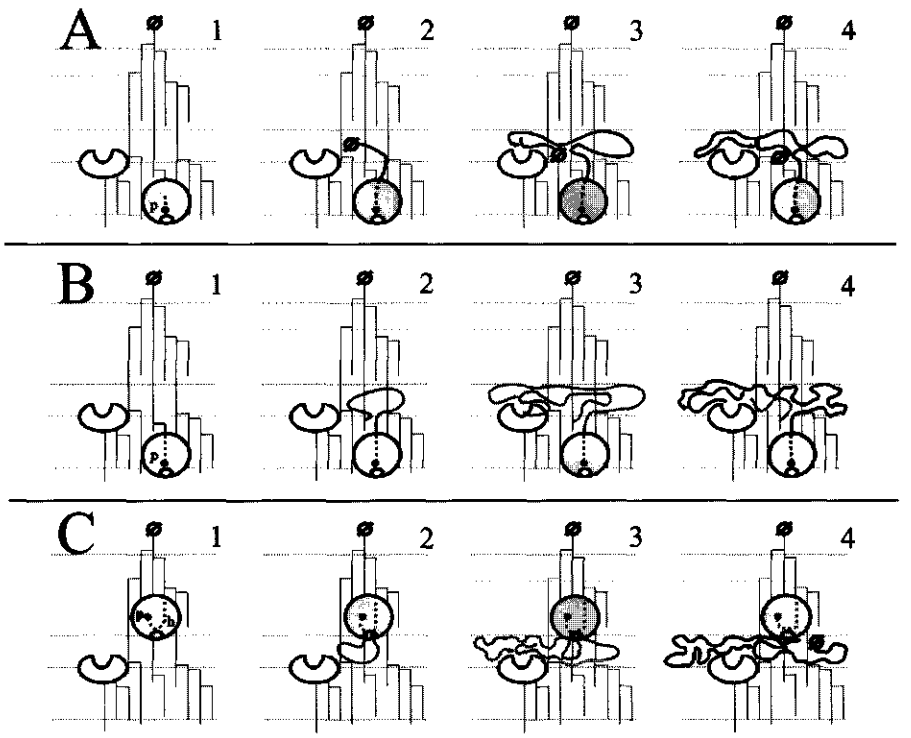

Fig. 9. Possible models explaining amylose synthesis within the starch granule by immobilized GBSSI. All reaction steps occur within the very dense polysaccharide matrix inside the granule. The GBSSI is displayed as a tunnel-shaped enzyme, while branching enzyme is drawn as a moon-shaped structure. (•) symbolizes a glucan non-reducing end while Ø symbolizes a reducing end. The catalytic sites of GBSSI are given as "p" and "h", indicating a polymerase and a postulated hydrolase activity respectively. Amylopectin is shown as a light gray line while amylose is shown as a dark line. Panel A shows the malto-oligosaccharide-primed amylose synthesis, whereas B and C show the amylopectin-primed amylose biosynthesis. In Panel B we show a BE-mediated cleavage through intra-molecular transglycosylation. Cleavage by a hydrolytic enzyme is also possible but not shown in this figure. In contrast to B, panel C shows that hydrolysis (h) can occur very near the site of polymerization (p) within GBSSI, ensuring that a non-reducing end is close to the site of synthesis for reinitiating the next round of amylose biosynthesis.

We have been able to reproduce the effect of malto-oligosaccharides on *in vitro* amylose synthesis previously reported by Denyer *et al.* (1996b) in our system (Fig. 7). The reduction of label in amylopectin in the presence of maltotriose indicated that high concentrations of malto-oligosaccharides compete with amylopectin as acceptor substrate for GBSSI. Incorporation of ^{14}C in the insoluble polysaccharide at different concentrations of ADP-Glc and maltotriose (Table II) suggests that at high ADP-Glc concentrations the length of the glucans produced is long enough to be retained within the granule while at lower concentrations the relatively short oligosaccharides will escape from the granule. Such small labeled oligosaccharides were indeed reported by Leloir and his colleagues (1961) in experiments involving UDP-Glc concentrations well below the apparent Km of the enzyme. Our present evidence points to normal amylose synthesis occurring in the absence of malto-oligosaccharides. After prolonged incubation in the absence of malto-oligosaccharides, massive amylose synthesis was achieved while our incubation media contained less than 1 μM malto-oligosaccharides (data not shown). Whether or not malto-oligosaccharide-primed amylose biosynthesis occurs *in vivo* is a matter of available acceptor substrate concentrations. The cluster-like structure of amylopectin provides a formidable potential of non-reducing ends in the starch granule (see results section). The increase of ^{14}C incorporation upon addition of diffusable malto-oligosaccharides (Table II) strongly indicates that GBSSI cannot use this full potential. However, the large number of possible priming sites within the starch granule together with the absence of detectable amounts of malto-oligosaccharides in *wild type Chlamydomonas* undergoing amylose biosynthesis, make it very unlikely that malto-oligosaccharide-priming is important *in vivo*.

Model B and C account for the amylopectin-primed amylose synthesis that we have observed (Fig. 6). In these models the external amylopectin chain is secured in the active site of GBSSI and we assume that enzyme and substrate do not easily dissociate, due to the immobilized character and processivity of the enzyme and the organized structure of amylopectin. Amylose is formed when the side-chain is detached from the amylopectin molecule. Model B suggests that cleavage occurs far from GBSSI, either by BE through an intra-molecular transglycosylation (Fig. 9B) or by a hydrolytic enzyme trapped within the granule e.g. α-amylase (not indicated in the figure). Takata *et al.* (1996a; 1996b) have demonstrated that BE from *B. stearothermophilus* can catalyze inter and intra-molecular branching of both amylose and amylopectin and they suggest that these reactions are common to BEs from various sources. As a result of the intra-molecular transfer by BE the newly formed amylose will not have a reducing end and is cyclic at the point of cleavage. However, because the number of amylose molecules more or less agrees with the number of reducing ends documented for amylose, we believe that downstream cleavage through

hydrolases is presently a more likely hypothesis. Cleavage far from GBSSI implies that the non-reducing end of the amylopectin chain will not be easily available for a next round of synthesis. In this case multiple rounds of amylose synthesis will depend on the accessibility of new amylopectin non-reducing ends to GBSSI and the possibility of dissociation of the GBSSI-substrate complex. In model C the assumption is made of an hydrolytic event occurring very near the site of synthesis within GBSSI. We postulate that steric hindrance of the glucan's progress will trigger hydrolysis by the GBSSI enzyme itself, assuming that GBSSI has a dual activity, i.e. synthase (or polymerase) and hydrolase. Such a dual activity has been observed before in the Klenow fragment of DNA polymerase of *Escherichia coli*. The N terminus contains a 3' - 5' exonuclease (hydrolase) activity, whereas the C terminal part contains a polymerase function (Blanco *et al.*, 1992). After release of the amylose chain, the old external amylopectin chain, which has never left the acceptor binding sites, can reinitiate the next round of amylose biosynthesis. In this model growth of the glucan will be finally stopped by the lack of space within the amylopectin matrix. This will be a late event at the beginning of amylose synthesis and will happen progressively sooner as the starch granule fills with amylose. This can explain why long-chain amylose precedes that of the low molecular mass material. Also the position of GBSSI within the granule can be of influence on the synthesis of low and high molecular mass amylose. Jane and Shen (1993) have shown that both concentration and size of amylose is dependent on its position within the starch granule. A detailed biochemical characterization of GBSSI is required to determine whether amylose biosynthesis proceeds via mechanism B or C.

Our results also prove that filling the starch granule with amylose *in vitro* in the absence of concomitant crystalline amylopectin synthesis is sufficient to change the crystalline organization within the granule. There are presently two possibilities to explain these results. First because of the large amount of amylose present after synthesis it is possible that the newly synthesized material crystallizes in the B-type under *in vitro* conditions. Another intriguing and perhaps more likely hypothesis would be that the massive amylose synthesis within the amylopectin matrix would push the preexisting A-type into B-type crystals. Indeed the structure proposed for the B-type crystals displays a central cavity that could be easily filled by one or two amorphous amylose chains (Imberty *et al.*, 1991) while the denser A-type packing does not allow for amylose infiltration within the crystal. Our present estimates of crystallinity levels before and after *in vitro* synthesis of amylose do not allow us to discriminate between these two possibilities. However, our results do establish *in vitro* synthesis of amylose from transient *Chlamydomonas* starches as an extremely powerful system to investigate the selective impact of amylose on the structure of amylopectin within the granule.

We believe that the model we propose is useful in that it makes a number of experimentally testable predictions. One of them is that a radioactive pulse given at the time of amylose synthesis should lead to a net chase of radioactive material from amylopectin to amylose. We are thus proceeding to confirm our *in vitro* approach by similar experiments performed *in vivo* in the presence of normal amylopectin synthesis.

FOOTNOTES

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AMYLOPECTIN-PRIMED AMYLOSE BIOSYNTHESIS IN HIGHER PLANTS.

ABSTRACT

Amylose is synthesized by granule-bound starch synthase I (GBSSI), which catalyzes the transfer of a glucose residue from ADP-Glc to the non-reducing end of a growing α-1,4 linked glucan. Two acceptor substrates for amylose biosynthesis have been put forward, amylopectin and malto-oligosaccharides. The elongation of glucans available on amylopectin and the transfer of chains from amylopectin to amylose were evidenced from pulse-chase experiments performed with *Chlamydomonas* starch. The aim of this study was to determine whether amylopectin-primed amylose synthesis also occurs in higher plants. Despite the fact that GBSSI activities in plant starches were 15 to 100-fold lower than the GBSSI activity in *wild type Chlamydomonas* starch, chase experiments performed with plant starches showed small but consistent decreases in label previously incorporated in amylopectin during the pulse. These decreases led to increases in label incorporated in amylose, which were proportional to the GBSSI activities of these starches. Based on these results we postulate that the results on amylopectin-primed amylose synthesis, obtained with *Chlamydomonas* starch, can be extrapolated to higher plants. Pulse-chase experiments in the presence of varying concentrations of malto-oligoaccharides showed that amylopectin-primed amylose synthesis can occur side by side with malto-oligosaccharide-primed amylose biosynthesis, thus offering the plant the opportunity to adapt to different conditions during biosynthesis.

INTRODUCTION

Starch, the major reserve polysaccharide in higher plants, is stored in the form of granules that, dependent on their source, vary in size and shape. Starch consists primarily of two glucose polymers: amylose and amylopectin. Amylopectin, one of the largest biomolecules, contains a complex organization of α-1,4 and α-1,6 linked glucose residues. Amylose, the minor fraction of starch, consists of a smaller glucan polymer with few α-1,6 branches (for review see Buleon *et al.*, 1998).

Amylose is synthesized by granule-bound starch synthase I (GBSSI), the major protein bound to the starch granule. GBSSI, like other starch synthases, catalyzes the transfer of a glucose residue from ADP-Glc (donor substrate) to the non-reducing end of a growing α-1,4 linked glucan (acceptor substrate). In the first *in vitro* synthesis experiments, performed by Leloir *et al.* (1961), in which purified starch granules were incubated with radio-labelled UDP-Glc, it was found that label was incorporated in both the amylopectin and amylose fractions. The incorporation of label by GBSSI in both amylose and amylopectin was also shown in *in vitro* experiments with starch granules of sweet potato (Baba *et al.*, 1987) and pea (Denyer *et al.*, 1996b) and in semi *in vivo* experiments with potato tuber slices (Ponstein, 1990), in which ADP-Glc was used as donor substrate. Whether label is incorporated mainly into amylopectin, amylose or both is dependent on the experimental conditions used for *in vitro* synthesis. Adding maltotriose to an *in vitro* synthesis experiment with starch granules isolated from pea embryos and potato tubers resulted in an increase of total label incorporated, which was mainly present in the amylose fractions (Denyer *et al.*, 1996b). It was suggested that these malto-oligosaccharides were used as acceptor substrate for amylose biosynthesis. In a time course experiment performed with *Chlamydomonas* starch it was shown that label was first incorporated mainly in amylopectin, while thereafter the label incorporated in the amylose fractions increased. Moreover, transfer of label from amylopectin to amylose was evidenced from pulse-chase experiments (van de Wal *et al.*, 1998). In the model which was put forward, amylopectin was used as acceptor substrate and amylose was formed by extension of and cleavage from amylopectin (van de Wal *et al.*, 1998; Ball *et al.*, 1998b).

In the present study, the *in vitro* synthesis with starch isolated from higher plants was compared to that with *Chlamydomonas* starch granules to determine whether the results on amylopectin-primed amylose biosynthesis can be extrapolated to higher plants.

MATERIALS AND METHODS

Materials,

ADP-[U-^{14}C]Glc was purchased from Amersham (United Kingdom). ADP-Glc was obtained from Sigma. Amyloglucosidase and maltotriose were obtained from Boehringer Mannheim (Mannheim, Germany).

The *Chlamydomonas reinhardtii* strains used in this study were WT strain 137C and the mutant strains I7 (generated from 137C by X-ray mutagenesis, defective for the large subunit of AGPase, Ball *et al.*, 1991) and BAFR1 (containing a gene-disrupted GBSSI structural gene, Maddelein *et al.*, 1994). Starch isolated from I7 contained very little to no amylose despite the presence of *wild type* GBSSI. The higher plants used in this study were cassava, potato, taro and wheat. Moreover, starch was isolated from the amylose-free (*amf*) mutant from potato (Hovenkamp-Hermelink *et al.*, 1987).

Starch purification and granule size measurements,

Chlamydomonas strains were grown in nitrogen starved media as described by Ball *et al.* (1990) and starch was isolated according to Delrue *et al.* (1992). Growth arrested (nitrogen-starved) *Chlamydomonas reinhardtii* cells accumulate a polysaccharide that bears structural resemblance to storage starch from higher plants. Starch from higher plants was isolated by homogenizing either cassava roots, potato tubers or taro roots in extraction buffer (10 mM EDTA, 50 mM Tris pH 7.5, 1 mM DTT, 0.1 % $Na_2S_2O_5$). The suspension was filtered through a cheese cloth and the filtrate was allowed to settle at 4 °C or centrifuged at 3000 x g for 10 min. The supernatant was discarded and the starch granules were washed twice with extraction buffer and three times with water. Wheat starch was obtained from Niko TNO food Sciences (Groningen). Average granule size analyses were performed with a Coulter multisizer II as described by Visser *et al.* (1997). The average granule diameter of the *Chlamydomonas* starch granules was determined using microscopy.

GBSSI activity *in vitro* and separation of starch polysaccharides by gel permeation chromatography,

The GBSSI activity was measured as the incorporation of labeled [U-^{14}C]Glc from ADP-[U-^{14}C]Glc in the insoluble fraction per min per mg starch (Vos-Scheperkeuter, 1986). For the *in vitro* synthesis, starch was incubated with 3.2 mM ADP-Glc in the presence of 50 mM glycine (pH 9.0), 100 mM $(NH_4)_2SO_4$, 0.4 % β-mercaptoethanol, 5 mM $MgCl_2$ and 0.05 % bovine serum albumin in a total volume of 2 ml at 30 °C. Depending on the GBSSI activities of the different starches, variable amounts of starch and different concentrations of ADP-[U-^{14}C]Glc were added to the reaction mixtures (Table I). After incubation the

reaction mixture was centrifuged at 13.000 rpm for 10 min. The supernatant was removed and the starch was suspended in 100 % me2SO and boiled for 20 min. The polysaccharide was precipitated overnight at –20 °C by adding three volumes of 96 % ethanol. After centrifugation at 13.000 rpm for 20 min at 4 °C the pellet was air dried for at least 10 min, dissolved in 500 µl 10 mM NaOH and subsequently applied to a CL2B Sepharose column (0.5 cm x 65 cm, equilibrated and eluted with 10 mM NaOH) to separate the amylopectin and amylose fractions. Fractions of 300-320 µl were collected at a rate of one fraction per 1.5 min. The glucans were detected by their reaction with iodine and the radioactivity was determined by liquid scintillation counting. 100 µl of the fractions were used for iodine staining and 100 µl of the I7 and 137C fractions and 200 µl of the potato and taro fractions were used for liquid scintillation counting (Table I). Quantification of polysaccharides was done by determining the amount of glucose after amyloglucosidase treatment as described by Delrue *et al.* (1992).

Table I. Differential conditions for pulse chase experiments with starch isolated from *Chlamydomonas* strains I7 and 137C and WT potato and taro. Due to large differences in GBSSI activity of the starches variable amounts of starch and different concentrations of ADP-[U-^{14}C]Glc were added to the *in vitro* synthesis mixtures.

	Starch (µg)	ADP-[U-^{14}C]Glc (µM)	CL2B sample (µl), Used for liquid scintillation counting
I7	500	2.2	100
137C	500	2.4	100
Potato	2000	8.8	200
Taro	2000	8.8	200

RESULTS

Starch characteristics.

The starch isolated from *Chlamydomonas* mutant strain I7, defective for the large subunit of AGPase, contained no detectable amounts of amylose, while starch isolated from WT *Chlamydomonas* strain 137C and WT potato contained around 20 % amylose. The granule-bound starch synthase activity of starch isolated from WT strain 137C was approximately 10-fold lower than that of starch isolated from I7, while starch isolated from potato tubers displayed a starch synthase activity which was approximately 200-fold lower than that of I7 starch (Table II). Starch isolated from *Chlamydomonas* strain BAFR1 (containing a gene-disrupted GBSSI structural gene, Maddelein *et al.*, 1994) and from the

amylose-free (*amf*) mutant from potato (Hovenkamp-Hermelink *et al.*, 1987) displayed less than 2 % of granule-bound starch synthase activity compared to the activity in starch granules containing active GBSSI (data not shown). This implied that mainly GBSSI was monitored under our experimental conditions. The GBSSI activity was in addition determined for starch isolated from cassava, taro and wheat (Table II). It was shown that the GBSSI activity in the higher plants studied was much lower than that in *Chlamydomonas*. Of the plants studied potato displayed the highest GBSSI activity and was, therefore, selected to perform a pulse-chase experiment.

Table II. GBSSI activity and average granule size of starch isolated from *Chlamydomonas* strains I7 and 137C and various higher plants.

	Starch					
	I7	137C	potato	cassava	taro	wheat*
GBSSI activity nmol/min/mg	12.000 - 18.000	1.000 – 2.000	60 - 90	10 – 30	10 - 30	20 – 40
Average granule diameter (μm)	< 0.5	0.8	30	10	5	5-50

* Wheat starch contains a bimodal distribution of starch granules.

Another major difference between starch granules isolated from *Chlamydomonas* and that from higher plants was the granule size (Table II). The ratio of granule surface to granule volume is more favorable with respect to diffusion of substrate for smaller granules as an increase in granule size results in a surface increase to the power of two, while the volume increases to the third power. It was shown earlier that the ADP-Glc concentration influences the amylose to amylopectin ratio in both *Chlamydomonas* and higher plants (Ball *et al.*, 1991; Lloyd *et al.*, 1999b). If the diffusion of ADP-Glc was the limiting factor in amylose biosynthesis, than the granule size would influence GBSSI activity. The granule size of starch isolated from *Chlamydomonas* was much smaller than that of higher plant starch granules. The average diameter of starch granules from *Chlamydomonas* mutant strain I7 was somewhat smaller than that of WT *Chlamydomonas* strain 137C. Of the higher plants studied, taro contained the smallest starch granules and was, therefore, in addition to potato selected to perform a pulse-chase experiment. The starch granules from taro were still more than ten-fold larger than the granules isolated from I7. In *Chlamydomonas* the decrease in granule size coincided with an increase in GBSSI activity.

However, this increase in GBSSI activity was probably caused by an increase in the amount of GBSSI protein bound per µg starch. Comparison of the higher plants showed no increase in GBSSI activity with decreasing size of starch granules, therefore, the diffusion of ADP-Glc does not seem to be the limiting factor for donor substrate availability. As it was impossible to determine the amount of active GBSSI per µg starch, these results are only indicative of the effect of ADP-Glc diffusion on GBSSI activity.

Kinetics of *in vitro* synthesis,

To study the kinetics of *in vitro* synthesis a time course experiment was performed (Fig. 1). The *in vitro* synthesis proceeded even after 12 h. For the higher plants studied there was a linear relation between the time of *in vitro* synthesis and the total label incorporated in starch, indicating that the GBSSI activity was stable over a period of at least 24 hours. Incorporation of label in starch isolated from *Chlamydomonas* strain 137C stagnated after 12 h *in vitro* synthesis. Until 12 h the GBSSI activity was stable and comparable to that in higher plants although the activity in *Chlamydomonas* strain 137C was much higher. Already after 0,5 h the incorporation of label in I7 starch stagnated. This stagnating incorporation of label in *Chlamydomonas* starch was probably due to the fact that the ADP-Glc concentration became limiting. Radioactive ADP-[U-^{14}C]Glc in the supernatant was measured by liquid scintillation counting. The label counted in the supernatant before the *in vitro* synthesis was 260.000 cpm. For I7 the amount of label in the supernatant dropped below 90.000 and 60.000 cpm after 0,5 and 1 h respectively. For 137C the label in the supernatant dropped below 100.000 cpm after 12 h of *in vitro* synthesis. For the higher plants the label in the supernatant after 24 h *in vitro* synthesis was in between 235.000 and 255.000 cpm. This indicated that the ADP-Glc concentration became limiting during the *in vitro* synthesis time course experiments with *Chlamydomonas* starch, but not for the experiments performed with higher plant starches.

After 12 h *in vitro* synthesis the label incorporated in the I7 starch slightly decreased (Fig. 1), while there was a slight increase from 20.000 to 40.000 cpm in label incorporated in the supernatant. This might indicate that malto-oligosaccharides were released. The oligosaccharides in the supernatant were quantified after amyloglucosidase treatment. After 4 h *in vitro* synthesis indeed small amounts (20 µg / ml) of glucans were detected in the supernatant of I7. The exact structure of these glucans is not known. If these were all maltotriose than the concentration after 12 h (50 µg / ml) would be 100 µM. The absence of these glucans in the supernatant after shorter periods of time for I7 and in the time course experiments with 137C and potato starch is probably caused by the fact that these glucans are elongated by GBSSI and end up in the amylose fraction. When a higher concentration

of ADP-Glc was added to the I7 *in vitro* synthesis mix (40 mM in stead of 3.2 mM) no glucans were detected in the supernatant after 12 h. This indicated that elongation of small glucans and subsequent precipitation was dependent on the ADP-Glc concentration.

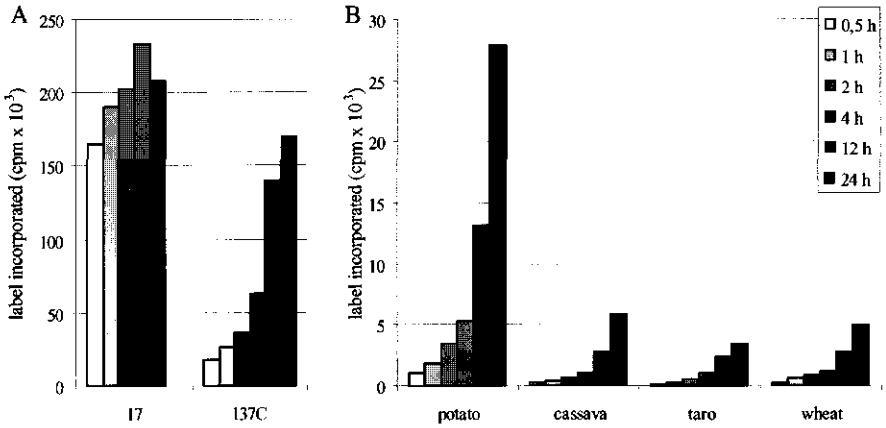

Fig. 1 In vitro synthesis time course experiment with starch isolated from the Chlamydomonas strains I7 and 137C (A) and the higher plants potato, cassava, taro and wheat (B).

Pulse-chase experiments,

To study amylopectin-primed amylose biosynthesis in higher plants similar pulse-chase experiments were performed as described previously for *Chlamydomonas* (van de Wal *et al.*, 1998). Since potato starch showed the highest GBSSI activity of the higher plants, starch isolated from WT potato was used. Moreover, starch isolated from taro was selected for the pulse-chase experiments as taro starch contained the smallest starch granules of the higher plants studied (Table II). The pulse-chase experiments performed with starch isolated from potato and taro were compared to those performed with starch isolated from I7 and 137C. The experimental conditions used were adjusted to the starch characteristics as described under Material and Methods.

Comparing the absorbency and the radioactive profile after CL2B separation of amylopectin and amylose from 137C and potato starch it is clear that WT *Chlamydomonas* and WT potato show comparable profiles (Fig. 2). Both starches contain amylose at the start of the *in vitro* experiment and after 30 min pulse-labeling a substantial amount of label was detected in the amylose fractions. As it was not clear whether the fractions 45 to 55 contained long linear amylopectin or high molecular weight amylose chains these fractions were assigned intermediate.

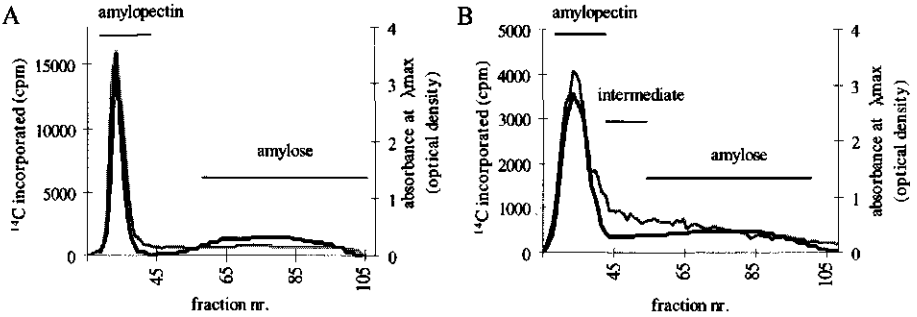

Fig. 2 Separation of amylopectin and amylose by CL2B-Sepharose chromatography after 30 min *in vitro* synthesis with starch isolated from 137C (A) and WT potato(B) in the presence of radiolabeled ADP-[U-^{14}C]Glc. The optical density (black line) of the iodine-polysaccharide complex was measured at λmax. The incorporation of 14C from ADP-[U-^{14}C]Glc (gray line) was determined by liquid scintillation counting.

After 30 min pulse-labeling I7 starch almost all label was incorporated in amylopectin (Fig. 3A). Starch isolated from *Chlamydomonas* strain 137C, potato and taro contained substantial amounts of label incorporated in the intermediate and amylose fractions (Fig. 3B, 3C and 3D). As synthesis proceeded with unlabeled substrate, the label incorporated in amylopectin of I7 was chased into amylose (Fig. 3A). After 1 h chase around 45.000 cpm were transferred, while after 4 h only 90.000 cpm were transferred from amylopectin into amylose. A decrease in GBSSI activity due to a limited ADP-Glc concentration, as was shown in the *in vitro* synthesis time course experiment, might account for the decline in transfer after 1 h chase.

In agreement with the results obtained from the pulse-chase experiment with I7 starch chases of label from amylopectin to amylose, 6.000 cpm after 1 h and 19.000 cpm after 4 h, were observed for 137C (Fig. 3B). These chases were slower and less obvious than those obtained with I7. The transfer of label from amylopectin to amylose after 1 h for 137C was approximately 7.5-fold less than that observed for I7. This was in agreement with the 7.5-fold decrease in GBSSI activity of the starches used for these experiments. The total label incorporated in I7 and 137C were 450.000 and 120.000 cpm respectively, while the concentrations of ADP-[U-^{14}C]Glc added to the *in vitro* synthesis mixes were respectively 2.2 μM and 4.4 μM.

The 1 h chase with potato starch showed a decrease of label incorporated in amylopectin and an increase of label in amylose of approximately 2000 cpm (Fig. 3C), which was also in line with the GBSSI activity. Based on the 200-fold lower GBSSI activity of potato starch as compared to I7 a transfer of 1800 cpm (45.000 / 200 x 4 x 2)

was expected for potato. In this calculation we accounted for the fact that 2 mg potato starch were incubated instead of 500 µg I7 (x 4) and that 200 µl of the CL2B fractions were used for liquid scintillation counting instead of 100 µl (x 2).

Based on the 1000-fold lower GBSSI activity of taro starch as compared to I7 a transfer of no more than 360 cpm (45.000 / 1000 x 4 x 2) was expected for taro. Again a slight decrease of label in the amylopectin fractions and a slight increase of label in the amylose fractions could be observed after the 1 h (approximately 300 cpm) and 4 h (approximately 600 cpm) chases with taro starch (Fig. 3D), which were in line with the GBSSI activity. Although the activities were low, the results on the transfer of label were consistent and in accordance with the predicted values.

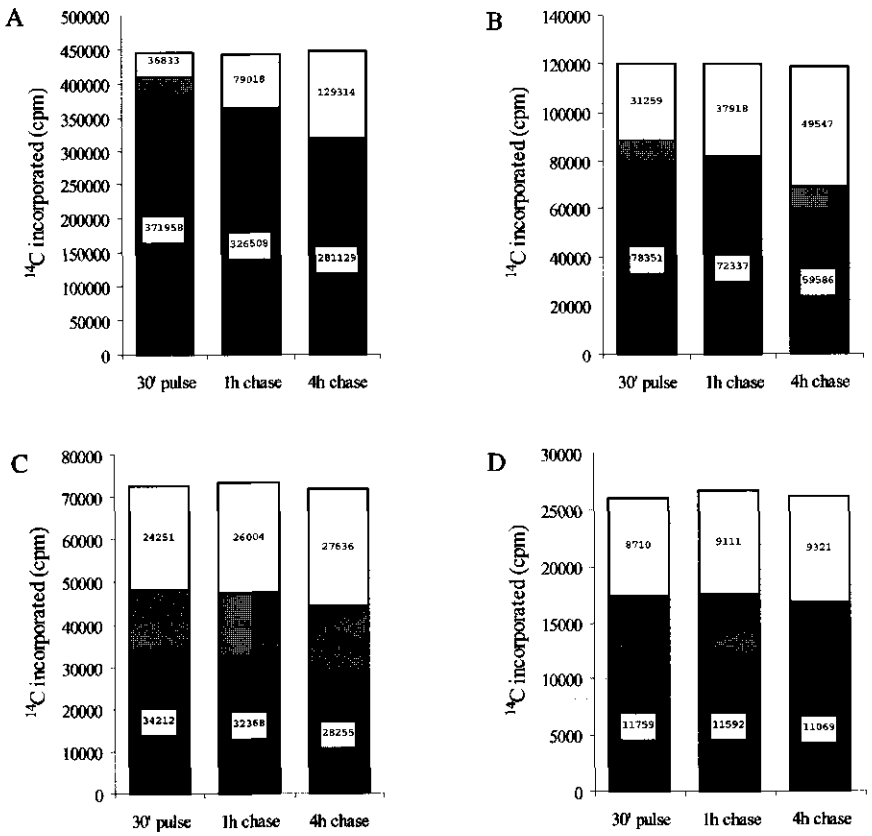

Fig. 3 Incorporation of ^{14}C from ADP-[U-^{14}C]Glc after pulse chase experiments with starch isolated from Chlamydomonas strains I7 (A) and 137C (B) and the higher plants potato (C) and taro (D). The total incorporation after the 30 min pulse and the 1 h and 4 h chase is shown for the amylopectin (black), intermediate (gray) and amylose (white) fractions.

A comparison between *Chlamydomonas* and higher plants for the label incorporated in amylose as a percentage of the total label incorporated visualized the increases found for higher plants more clearly (Fig 4). From these values it was also clear that the relatively large amounts of label incorporated in amylose directly after the pulse for 137C, potato and taro had a severe effect on the distinctiveness of the pulse-chase experiments. The total values of label incorporated after the 30 min pulse and the 1 h and 4 h chases were very consistent for all pulse-chase experiments performed (Fig. 3), which indicated the reproducibility of the experiments.

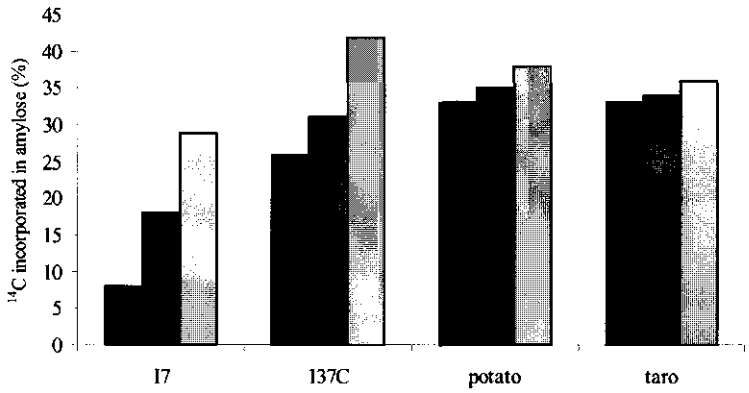

Fig. 4 Label incorporated into amylose as a percentage of the total label incorporated after 30 min pulse (black), 1 h chase (dark gray) and 4 h chase (light gray). The pulse-chase experiments were performed with starch isolated from the *Chlamydomonas* strains I7 and 137C and the higher plants potato and taro.

As it was shown that small amounts of malto-oligosaccharides were formed during a 12 h *in vitro* synthesis experiment with I7 starch, the influence of malto-oligosaccharides on pulse-chase experiments was determined. To study the effect of malto-oligosaccharides on the transfer of label from amylopectin to amylose during the pulse-chase experiment, maltotriose was added to the chase (Fig. 5). I7 starch was pulse-labeled for 30 min in the absence of malto-oligosaccharides. After the pulse the starch was washed and the *in vitro* synthesis was proceeded with unlabeled ADP-Glc in the presence of no, 200 μM or 50 mM maltotriose. The presence of 200 μM maltotriose showed very little effect on the transfer of label from amylopectin to amylose. In the presence of 50 mM maltotriose less label was transferred from amylopectin to amylose. This was probably due to a reduction in the elongation of amylopectin. When the elongation of amylopectin is slowed down by the

presence of high concentrations maltotriose presumably fewer chains will be cleaved and released into amylose, resulting in a lower transfer of label.

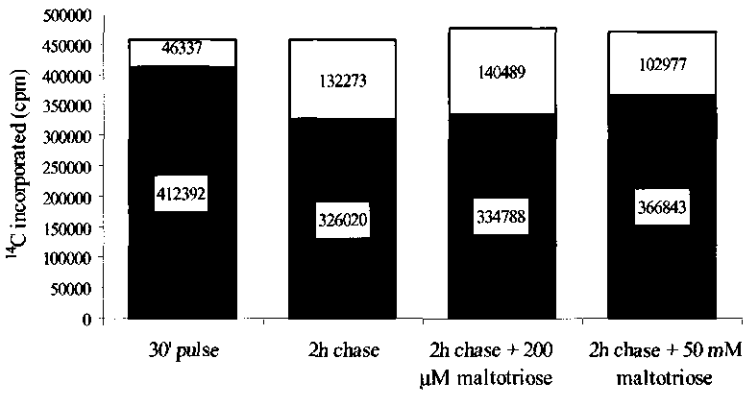

Fig. 5 Incorporation of ^{14}C from ADP-[U-^{14}C]Glc in amylopectin (black) and amylose (white). The total incorporation of label is shown after 30 min pulse labeling I7 starch and after a 2 h chase in the presence of no, 200 µM and 50 mM maltotriose.

DISCUSSION

Two models accounting for amylose biosynthesis have been put forward (Ball *et al.*, 1998b). One model shows the mechanism of malto-oligosaccharide-primed amylose biosynthesis as was suggested by Denyer *et al.* (1996b) based on the results obtained with pea and potato starch. In the other model amylopectin is used as acceptor substrate, which was concluded from results obtained with *Chlamydomonas* starch (van de Wal *et al.*, 1998). To determine whether amylopectin-primed amylose synthesis also occurred in higher plants, pulse-chase experiments with starch isolated from potato and taro were compared to those performed with starch isolated from *Chlamydomonas* WT strain 137C and *Chlamydomonas* mutant strain I7.

The GBSSI activity of starch isolated from *Chlamydomonas* is much higher than that of the higher plants investigated (Table II). Of the higher plants studied, potato starch contained the highest GBSSI activity and was therefore selected to perform a pulse-chase experiment. In addition starch isolated from taro was selected as taro contained the smallest granules of the higher plants studied. Starch isolated from I7 contained no detectable amounts of amylose which facilitated the characterization of *in vitro* synthesized amylose, while starch isolated from strain 137C and potato contained around 20 % amylose.

Besides these differences in starch characteristics the GBSSI protein from *Chlamydomonas* differed from that from higher plants in that it contained a C terminal extension of 119 amino-acids. This extension might account for an additional function of GBSSI from *Chlamydomonas*, lacking in higher plants. Studying GBSSI from cassava in a potato background, Salehuzzaman *et al.* (1999) showed that GBSSI from potato and cassava, two more related species, already exhibited different intrinsic properties. In addition to the differences in GBSSI characteristics, hydrolytic enzymes bound to or entrapped in the starch granules from *Chlamydomonas* might occur in different abundance in higher plants.

Comparing the pulse-chase experiments performed with starch isolated from potato, taro, *Chlamydomonas* WT strain 137C and mutant strain I7, we showed that directly after the pulse all label was incorporated in the amylopectin fractions of I7 starch, while for starch isolated from 137C, potato and taro substantial amounts of label were already incorporated in the intermediate and amylose fractions. After 1 h and 4 h chase the label was transferred from amylopectin to amylose (Fig. 3). The chase of label for 137C (3B) was less obvious than that of I7 (3A). Lower GBSSI activities and label incorporated in the amylose fractions directly after the pulse (Fig. 4), reduced the distinctiveness of the pulse-chase experiments. Nevertheless for both higher plant systems a decrease in label incorporated into amylopectin and an increase in label incorporated in amylose could be observed (Fig. 3C and 3D). These increases in label incorporated into amylose were in line with the differences in GBSSI activities. The size of the starch granules appeared to show no significant effect on the transfer of label, as the chases of label from amylopectin to amylose for I7, 137C, potato and taro were in line with the GBSSI activities, despite the differences in granule size.

Pulse-chase experiments performed with I7 starch in the presence of maltotriose showed that low concentrations of malto-oligosaccharides, which were detected after 12 h *in vitro* synthesis, hardly affect the transfer of label from amylopectin to amylose (Fig. 5). In the absence of ADP-Glc no transfer of label could be observed indicating that hydrolytic cleavage was linked to chain elongation. Therefore it can be concluded that in the presence of low concentrations of malto-oligosaccharides elongation of amylopectin is not reduced. The elongation of malto-oligosaccharides is probably catalyzed by latent GBSSI, which has no accessibility to the non-reducing ends of amylopectin. An increase in the total label incorporated with no subsequent decrease of label incorporated in amylopectin was also shown by Denyer *et al.* (1996) in *in vitro* experiments in which soluble extracts of pea embryos and potato tubers were added to the *in vitro* mixture. These results indicate that

amylopectin-primed and malto-oligossaccharide-primed amylose synthesis can occur side by side.

In the presence of high concentrations of maltotriose less label was transferred from amylopectin to amylose, which can be explained by competition between maltotriose and amylopectin for elongation by GBSSI. Competition between maltotriose and amylopectin was also indicated by the approximately 3-fold reduction of label incorporated in amylopectin in the presence of 50 mM maltotriose (van de Wal *et al.*, 1998). This competition could be due to competition as acceptor substrate for the active site of GBSSI or due to competition for ADP-Glc, the limited donor substrate. The transition from amylopectin elongation to malto-oligosaccharide elongation by GBSSI did not result in a release of elongated chains from amylopectin into amylose as otherwise an increase in the transferred label should be observed.

In conclusion, decreases of label incorporated in amylopectin and increases of label incorporated in amylose, which were in line with the GBSSI activity, could be observed in pulse-chase experiments performed with potato and taro. Based on these results it can be postulated that the results on amylopectin-primed amylose biosynthesis obtained with starch isolated from *Chlamydomonas* strain I7 and 137C starch also holds true for starch of higher plants.

MULTIPLE ALLELISM AS A CONTROL MECHANISM IN METABOLIC PATHWAYS: GBSSI ALLELIC COMPOSITION AFFECTS ENZYME ACTIVITY AND STARCH COMPOSITION IN POTATO.

ABSTRACT

Multiple allelism in heterozygous autopolyploid species like potato not only occurs for morphological characteristics but also for genes involved in metabolic pathways. Based on a combination of Southern and PCR analysis at least eight alleles encoding granule-bound starch synthase I (GBSSI), responsible for amylose biosynthesis, could be identified. These alleles were grouped into four classes, distinguishable by Southern analysis, and subdivided based on the results from PCR. Despite the heterozygous and polyploid character of potato it was possible to assign variation in GBSSI activity to the GBSSI allelic composition within a large population of *Solanum tuberosum* cultivars and *Solanum* breeding lines. Moreover, the availability of an *amf* allele enabled a reduction in heterogeneity and made it possible to demonstrate an effect of GBSSI allelic composition on amylose content. The major difference between the alleles identified was the absence or presence of a 140 bp fragment, 0.5 kb upstream of the ATG start codon of the GBSSI gene. The absence of this 140 bp fragment had a major effect on GBSSI activity and amylose content, while the presence of small bp deletions and simple sequence repeats led to no apparent effects.

INTRODUCTION

Starch, the major storage carbohydrate in higher plants, comprises two different glucose polymers, amylose and amylopectin. Amylose is composed of linear chains of α-1,4 linked glucose residues with very few α-1,6 branches. Amylopectin on the other hand is a highly branched glucan with a specific "clustered" distribution of α-1,4 and α-1,6 linkages (Hizikuru, 1986; Manners, 1989). The ratio of amylose to amylopectin is one of the key factors in determining industrially important properties such as gelatinisation, retrogradation and viscosity. Subtle changes in amylose content and starch structure may significantly improve quality as was shown by the effect of granule-bound starch synthase I (GBSSI) on noodle quality in wheat (Zhao *et al.*, 1998).

Although there is a body of evidence showing that GBSSI determines whether or not amylose is produced (Denyer *et al.*, 1995; Hovenkamp-Hermelink *et al.*, 1987; Hseih, 1988; Kuipers *et al.*, 1994a; Nelson and Rhines, 1962; Rohde *et al.*, 1988; Visser *et al.*, 1991) the genetics behind amylose content still needs to be unravelled. Understanding the genetic control of GBSSI activity and amylose production will be important in designing breeding strategies aimed at controlling starch composition.

In hexaploid wheat, an allopolyploid species, the three GBSSI loci (referred to as *Wx* loci) are organised as a triplicate set of single-copy homoeoloci. For wheat it was shown that three *Wx* genes (*Wx*-A1, *Wx*-B1 and *Wx*-D1), present on homoeologous chromosomes, have differential effects on amylose content (Miura and Sugawara, 1996). Absence of the *Wx*-B1 gene reduced the amylose content by more than 3 % while absence of the *Wx*-A1 or the *Wx*-D1 gene reduces the amylose content by less than 2 %. Moreover, comparison of double null lines has shown that the amylose synthesis capacity of the *Wx*-B1 is higher than the amylose synthesis capacity of *Wx*-D1, which is higher than that of *Wx*-A1 (Miura *et al.*, 1999). The differences in amylose content in wheat were partly correlated with the variation in the amounts of *Wx* proteins produced by the different *Wx* genes.

In rice, a homozygous diploid species, the ratio of amylose to amylopectin is also heritable and varies from cultivar to cultivar. The apparent amylose content in rice has been found to be controlled by a series of alleles at one locus with major effects (*Wx* locus) and one or more genes with minor effects (Pooni *et al.*, 1993). It is shown that inefficient and aberrant splicing of intron 1 from the 5' UTR results in a decreased expression of the waxy gene and a reduced amylose content (Cai *et al.*, 1988). Also in foxtail millet different *Wx* alleles have been characterised, which regulate quantitative levels of *Wx* protein and amylose content (Nakayama *et al.*, 1998).

Potato in comparison to wheat and rice is a tetraploid heterozygous vegetatively propagated species. Multiple allelism is common in heterozygous autopolyploid species. However, the effect of allelic composition on various properties due to the heterogeneity is less obvious than the effect in a diploid homozygous species like rice. Van Eck *et al.* (1994) showed the effect of multiple allelism in potato on tuber shape.

Amylose production in potato has been shown to be completely dependent on the presence of GBSSI, which is encoded by a single copy gene. An amylose-free (*amf*) potato mutant was obtained after irradiation of a monohaploid with X-rays (Jacobsen *et al.*, 1989). The *amf* allele produces mRNA equivalent to that of *wild type* level, however, no protein is tightly linked to the starch granule due to a point mutation in the transit peptide. As a consequence the *amf* allele does not contribute to amylose biosynthesis. In addition to this mutant allele three independently isolated complete GBSSI sequences have been reported while five sequences of the GBSSI promoter region were published (Dai *et al.*, 1996; Hofvander *et al.*, 1992; van der Leij *et al.*, 1991b; Rohde *et al.*, 1990). The homology between the alleles in the coding region is very high, over 99 % at amino-acid level between the three sequences published. The presence of multiple deletions and simple sequence repeats (SSR) in the untranslated regions enabled the use of Southern hybridisation and PCR-based techniques to differentiate between alleles.

To study the allelic composition of GBSSI and the effect on GBSSI activity and amylose content in potato a population of *Solanum* breeding lines and *Solanum tuberosum* cultivars was analysed. In addition to the five promoter sequences described in literature another four new alleles have been described, which could be distinguished by a combination of Southern and PCR analysis. An effect of allelic composition on GBSSI activity was proven within the studied population. The availability of an *amf* allele enabled a reduction in heterogeneity and made it possible to study the effect of single alleles. After reduction of the variation in genetic background a significant effect of allelic composition on amylose content could be demonstrated.

MATERIALS AND METHODS

Materials,

Fourteen *Solanum* breeding lines, twelve diploid and two tetraploid, were obtained by crossing various *Solanum tuberosum* cultivars with different wild *Solanum* species and selection for high starch content (Hutten, R., Lab of Plant Breeding, unpublished clones). In addition to these *Solanum* breeding lines, fourteen *Solanum tuberosum* cultivars also containing a high starch content and the tetraploid amylose-free (*amf*) clone 93-6704-18 were grown for DNA isolation and tuber analysis. Per clone 10 plants were used for a field

trial and 3 plants were grown in the greenhouse. For the field trial the plot was set up in two replicates surrounded with the cultivar (cv) Bintje. To obtain full sib offspring clones which share on average the same genetic background crosses were made between selected *Solanum* clones, both diploid and tetraploid, the diploid *amf* clone 5002-18 and the tetraploid *amf* clones 93-6706-2 and 93-6707-6. The amylose-free clones used were derived from the *amf* mutant described by Jacobsen *et al.* (Jacobsen *et al.*, 1989). Twenty progeny plants of the tetraploid crosses and twelve progeny plants of the diploid crosses were grown in the greenhouse.

Southern analysis,

Genomic DNA was isolated from 0.5 - 2.0 g of leaves as described by Rogers and Bendich (1988). DNA (4 µg) was digested with *Hin*dIII, electrophoresed on a 0.8 % agarose gel for 16 h at 40 V and subsequently vacuum blotted (Pharmacia) onto Hybond N^+ membranes (Amersham) in 10 x SSC. A 1.3 kb *Eco*RI fragment containing the 3' part of GBSSI cDNA and a 1.1 kb *Eco*RI fragment containing the 5' part of the GBSSI cDNA were used as probes (Visser *et al.*, 1989) (Fig. 1). Probes were radioactively labelled with the Megaprime DNA labelling system (Amersham). Hybridizations were performed in glass bottles in a Hybaid hybridisation oven, at 65 °C for 16 – 18 h. The blots were rinsed to a stringency of 0.2 x SSC, 0.5 % SDS at 65 °C.

PCR analysis,

Genomic DNA isolated according to Rogers and Bendich (1988) was used as template for PCR amplification. The primer pairs used to distinguish between the five GBSSI promoter regions were combinations of the forward primer cdf1 (gaaccatgcatctcaatctt) and the reverse primers cdf2 (gtactggtccctcc), mwpr1 (ggcaactgtggacgctgt), mwpr2 (gagtgagtgagtgtgag), mwpr3 (cagtagggtcaaattcag), mwpr4 (gaagaagaagaagaagagg) and mwpr5 (ctaccagagaataagc) (Fig. 1).

The PCR reactions with the primer combinations cdf1/cdf2, cdf1/mwpr1, cdf1/mwpr2 or cdf1/mwpr3 contained 50 ng DNA, 0.1 mM deoxynucleotides, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1 % Triton X-100, 0.2 µM oligo cdf1, 0.2 µM oligo cdf2, mwpr1, mwpr2 or mwpr3 and 0.25 units Taq polymerase (Perkin & Elmer). The PCR reactions were denaturated at 95 °C for 4 min, followed by 35 cycles of 95 °C for 45 s, 55 °C for 30 s and 72 °C for 60 s. The final extension was at 72 °C for 5 min. After PCR 1 µl loading buffer (98 % formamide, 10 mM EDTA, 0.025 % bromophenol blue, 0.025 % xylene cyanol) was added to 10 µl sample. The samples were loaded on a 1.0 % agarose gel and electrophoresed for 6 h at 60 V.

For the PCR reactions containing the primer combinations cdf1/mwpr4 and cdf1/mwpr5 the cdf1 primer was radioactively labelled using [γ-^{33}P]ATP and T4 polynucleotide kinase according to Vos *et al.* (1995). A 20 µl PCR reaction was performed containing 0.5 µl labelled cdf1 primer (5 ng), 30 ng of the unlabeled reverse primer, 5 µl template DNA (5 ng), 0.4 unit Taq polymerase, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 0.2 mM of all four dNTPs. The PCR amplification reactions were performed for 36 cycles with the following cycle profile: denaturation at 95 °C for 30 s, followed by an annealing step at 65 °C for 30 s and an extension step at 72 °C for 60 s. After the first cycle the annealing temperature was repeatedly reduced by 0.7 °C for each of the next 12 cycles, and was maintained at 56 °C for the remaining 23 cycles. The PCR product was run on a 5 % denaturing polyacrylamide gel as described by Vos *et al.* (1995). After vacuum drying the gel was exposed to standard X-ray film at room temperature for 48 hours.

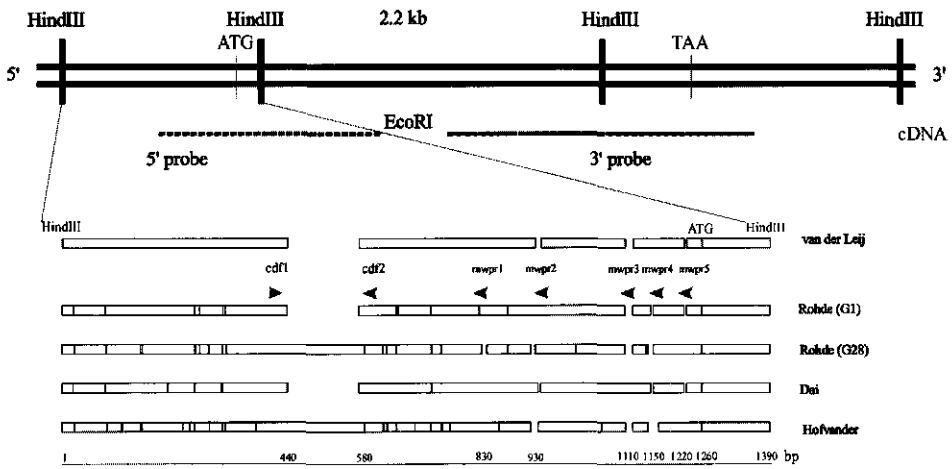

Fig. 1 Schematic overview of the GBSSI gene. *Hin*dIII restriction sites and 5' and 3' *Eco*RI cDNA probes were used for Southern analysis. For the PCR analysis seven primers (cdf1, cdf2, mwpr1, mwpr2, mwpr3, mwpr4, mwpr5) were designed based on the five GBSSI promoter sequences published (Dai *et al.*, 1996; Hofvander *et al.*, 1992; van der Leij *et al.*, 1991; Rohde *et al.*, 1990). The thin vertical lines in the promoter sequences indicate single basepair changes.

Northern analysis,

RNA was isolated according to Kuipers *et al.* (1994b) blotted and hybridised against the cDNA of potato GBSSI as described by Sambrook *et al.* (1989). A 24S ribosomal probe was used as a standard (Landsmann *et al.*, 1985).

Western analysis,

The granule-bound protein fraction was isolated and electrophoresed on a 12,5 % polyacrylamide-SDS phastsystem gel as described by Kortstee *et al.* (1996). Immunoblotting was carried out as described by the manufacturer, using anti-serum raised against potato GBSSI (Vos-Scheperkeuter *et al.*, 1986).

Determination of amylose content and GBSSI activity,

The amylose content and GBSSI activity of the *Solanum* breeding lines and cultivars were determined separately in tubers from three greenhouse grown plants, while the GBSSI activity and amylose content of the progeny from the crosses were determined in three individual tubers. Potato tubers were homogenised in extraction buffer (10 mM EDTA, 50 mM Tris pH 7.5, 1 mM DTT, 0.1% $Na_2S_2O_5$) and filtered through a cheesecloth. The filtrate was allowed to set at 4 °C and the supernatant was discarded. The starch granules were subsequently washed twice with extraction buffer and three times with water. The starch granules were analysed in triplicate for amylose content and GBSSI activity. The apparent amylose content was determined spectrophotometrically in 1 to 2 mg isolated starch (Hovenkamp-Hermelink *et al.*, 1988). GBSSI activity was determined as described by Vos-Scheperkeuter *et al.* (1986) and was expressed as pmol ADP-Glc incorporated per min per mg starch.

RESULTS

GBSSI allele classification by Southern analysis,

The allelic composition of a population of *Solanum tuberosum* cultivars and *Solanum* breeding lines was first determined by Southern analysis. The 3' *Eco*RI GBSSI cDNA probe hybridised with a 2.2 kb internal fragment and a 3' fragment of the GBSSI gene as shown in Fig. 1. The length of this 3' fragment was 2.5 kb, 1.9 kb or 1.7 kb (Fig. 2a). The 2.2 kb internal fragment and the 1.9 kb 3' fragment were found in all clones indicating that all clones contained at least one allele with a 1.9 kb 3' fragment. The 5' *Eco*RI GBSSI cDNA probe hybridised with a 5' fragment of 1.2 kb, 1.30 kb or 1.34 kb in addition to the 2.2 kb internal fragment which was also recognised by the 3' probe (Fig. 2a). The 1.2 kb fragment was present in all clones except breeding line 90-027-6. This diploid clone contained only a 1.34 kb 5' fragment in addition to the 2.2 kb internal fragment. The intensity of the bands was indicative of the copynumber of alleles with those particular fragments. For example cv Elkana contained three alleles with a 1.2 kb and one allele with a 1.34 kb 5' fragment. In comparison, cv Ponto contained one allele with a 1.2 kb, one allele with a 1.30 kb and two alleles with a 1.34 kb 5' fragment.

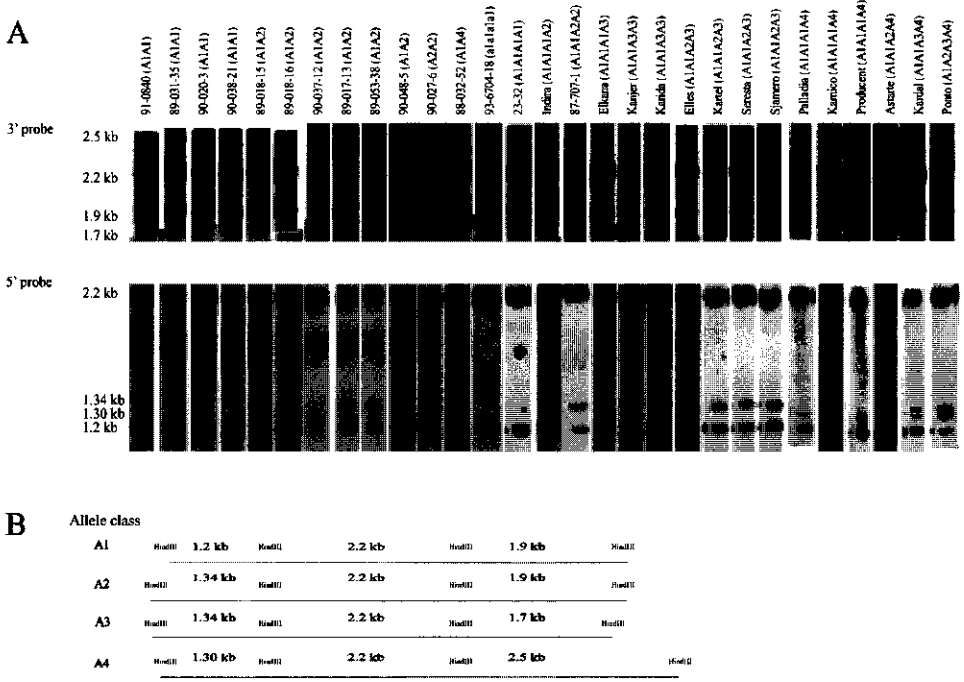

Fig. 2 Allele classification after Southern analysis. Genomic DNA was digested with *Hin*dIII, blotted and hybridised with a 3' and 5' *Eco*RI GBSSI cDNA probe (A). The alleles were classified according to the hybridisation pattern (B) and the allelic composition is shown in brackets. The composition of *amf* mutant 93-6704-18 is indicated as a1a1a1a1, since this clone contains four mutant alleles belonging to the A1 class.

Comparison of the results obtained with the 3' probe with those of the 5' probe it was noticed that a 2.5 kb 3' fragment always coincided with a 1.30 kb 5' fragment and vice versa (Ponto, Palladia, Astarte, Kardal, Karnico, Producent, 88-032-52). Clones with a 1.7 kb 3' fragment always showed a 1.34 kb 5' fragment (Elkana, Ponto, Elles, Kardal, Kartel, Seresta, Sjamero, Kanjer, Karida). Note, that sometimes a 1.34 kb 5' fragment was found without the 1.7 kb 3' fragment (Astarte, Indira, 89-018-15, 89-018-16, 87-707-1, 90-027-6, 90-037-12, 89-017-13, 89-053-38, 90-048-5). Moreover, cultivars Ponto, Elles and Kartel contained twice a 1.34 kb 5' fragment and only once a 1.7 kb 3' fragment. Based on these results obtained by Southern analysis the alleles could be grouped into the four classes A1, A2, A3 and A4 (Fig. 2b). Alleles belonging to the A1 class contained a 1.2 kb 5' and a 1.9 kb 3' fragment. All clones except breeding line 90-027-6 possessed at least one allele belonging to the A1 class. The A2 class had a 1.9 kb 3' and a 1.34 kb 5' fragment. This class was recognised by the presence of a 1.34 kb 5' fragment in the absence of a 1.7 kb 3'

fragment. Allele class A3 contained in combination a 1.34 kb 5' and a 1.7 kb 3' fragment. In allele class A4 a 1.30 kb 5' fragment was found in combination with a 2.5 kb 3' fragment. The allelic composition of the investigated clones is shown in brackets (Fig. 2a). The tetraploid *amf* clone 93-6704-18 showed the same fragment composition as breeding line 23-32 indicating the presence of four alleles belonging to the A1 class. The mutant allele however, does not contribute to amylose biosynthesis it is therefore noted as a1. The occurrence of the different alleles was not evenly distributed over the allele classes. Alleles belonging to the allele class A1 (62 %) were much more frequent than those belonging to the A2 (18 %), A3 (12 %) or A4 (8 %) classes.

GBSSI allele subclassification by PCR analysis,

To assign earlier published alleles to the four GBSSI allele classes and to further characterise the allelic composition of the *Solanum* breeding lines and *Solanum tuberosum* cultivars a PCR analysis was performed. PCR primers were designed based on the five GBSSI promoter sequences published (Dai *et al.*, 1996; Hofvander *et al.*, 1992; van der Leij *et al.*, 1991b; Rohde *et al.*, 1990) (Fig. 1). Since it was impossible to recognise polymorphism obtained by primer pair cdf1/mwpr4 and cdf1/mwpr5 on a 1.0 % agarose gel, the cdf1 primer was radioactively labelled and the PCR fragments were separated by polyacrylamide gel electrophoresis.

Table I. Allele subclassification. PCR fragment lengths in bp after amplification with various primer combinations annealing to the 5' ends of the alleles classified by Southern analysis.

	Primer combinations						
Allele	Cdf1/ Cdf2	Cdf1/ Mwpr1	Cdf1/ Mwpr2	Cdf1/ Mwpr3	Cdf1/ Mwpr4	Cdf1/ Mwpr5	Homologous to sequence:
A1[a]	60	300	400	np	590	np	van der LEIJ
A1[b]	60	300	400	np	610	np	ROHDE G1
A1[c]	60	300	400	570	610	np	DAI
A2[a]	200	np	np	np	720	770	---
A2[b]	200	np	np	np	730	780	ROHDE G28
A3[a]	200	430	np	690	np	780	---
A3[b]	np	np	520	Np	720	770	---
A4	np	430	np	690	np	770	---

np indicates that no product is formed with this primer combination
--- indicates that this sequence has not been published before

Based on the PCR results at least eight in stead of four alleles could be identified. Comparison of the results from Southern analysis with those of PCR allowed subdivision of the alleles as shown in Table I. Allele A1 could be subdivided into three and the alleles A2 and A3 into two subclasses respectively. The difference between $A1^c$ on one and $A1^a$ and $A1^b$ on the other hand and the difference between $A3^a$ and $A3^b$ were clearly visible after agarose gel electrophoresis (Fig. 3a, 3b). The differences between $A1^a$ and $A1^b$ and $A2^a$ and $A2^b$ could be observed after polyacrylamide gel electrophoresis (Fig. 3c). The promoter regions of the different alleles were sequenced and compared to the published sequences (Fig. 4). The clones from which these sequences were obtained are given in brackets. The promoter sequences of $A1^a$ (Ponto) $A1^b$ (91-0840) and $A1^c$ (89-018-16) showed complete homology to the sequences published van der Leij *et al.* (1991b), Rohde *et al.* (1990, G1) and Dai *et al.* (1996) respectively. Promoter $A2^b$ (89-037-12) was identical with the G28 sequence published by Rohde *et al.* (1990). $A2^a$ (89-017-13), $A3^a$ (Kanjer), $A3^b$ (Kanjer) and A4 (88-032-52) were new alleles. In our population the allele published by Hofvander *et al.* (1992) (indicated as HvD) was not present. This allele does not contain the 140 bp fragment and does therefore not belong to the A1 class. However, from these results it is not clear whether the allele published by Hofvander *et al.* (1992) can be placed in the A2, A3, A4 class or not.

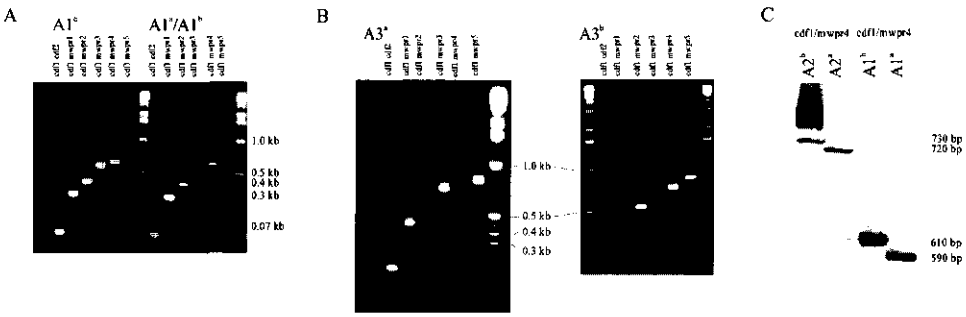

Fig. 3 PCR amplification pattern of the different alleles using the primer combinations cdf1/cdf2, cdf1/mwpr1, cdf1/mwpr2, cdf1/mwpr3, cdf1/mwpr4 and cdf1/mwpr5. Differentiation between $A1^c$ on the one hand and $A1^a$ and $A1^b$ on the other hand (A) and between $A3^a$ and $A3^b$ (B) was possible by agarose gel electrophoresis. Polyacrylamide gel electrophoresis was used to differentiate between $A1^a$ and $A1^b$ and $A2^a$ and $A2^b$ (C).

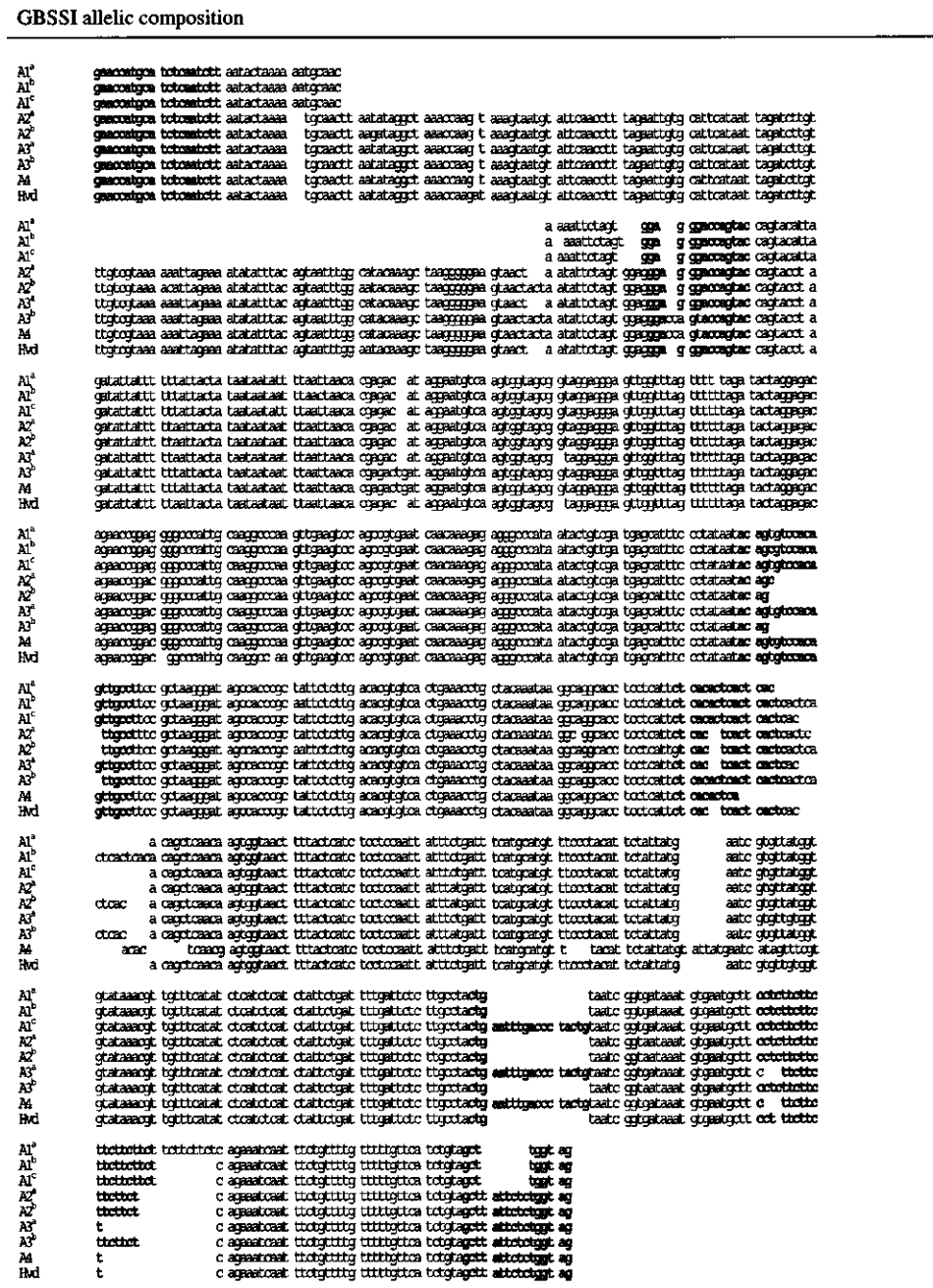

Fig. 4 Sequences of the GBSSI promoter region of the nine alleles identified. Annealing sites of the primers used for identification are given in bold. The allele published by Hofvander *et al.*, (1992) is indicated as HvD.

GBSSI allele classification, GBSSI activity and amylose content within a large population of *Solanum tuberosum* cultivars and *Solanum* breeding lines,

The GBSSI activity and amylose content were determined in three individual tubers per clone. The clones were subdivided into two groups containing tetraploids and diploids respectively. Statistical evaluation of the group of tetraploid clones with an analysis of variance showed a dosage effect for alleles belonging to the A1 class, which was significant at the 0.05 level. Using a t-test analysis it was shown that the GBSSI activity of starch isolated from clones containing A1A1A1A1 (34.1 pmol/min/mg starch) or A1A1A1A* (35.4 pmol/min/mg starch) was significantly higher than that of starch isolated from clones with A1A1A*A* (22.8 pmol/min/mg starch) or A1A*A*A* (22.7 pmol/min/mg starch) (Fig. 5a). The average GBSSI activity of the diploid clones containing A1A1, A1A* and A*A* was 30.2, 28.5 and 20.9 pmol/min/mg starch respectively. However, using a t-test analysis these differences were not significant at the 0.05 level. No significant correlation between GBSSI activity and amylose content was found (Pearson correlation test) either in the group of diploids (not shown) or in the group of tetraploids (Fig. 5b). The amount of GBSSI-protein in the starch granule fraction and the amount of GBSSI-RNA from potato tubers were analysed. Differences in GBSSI activity could, however, not be deduced to the GBSSI protein or RNA level (Fig. 6) which is in agreement with the results of Flipse *et al.* (1996a).

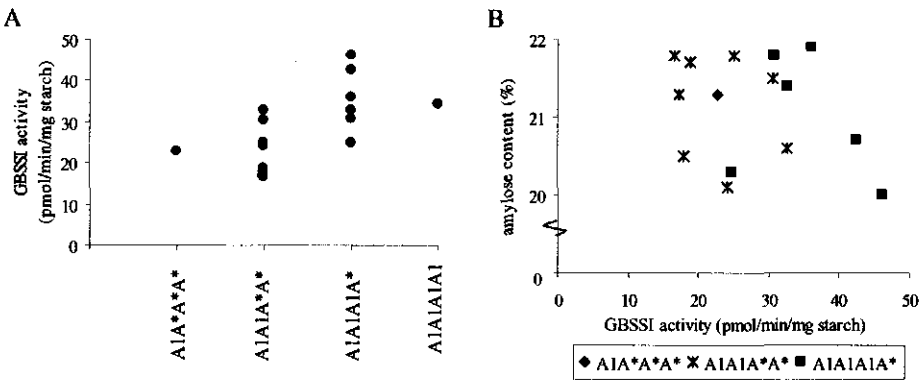

Fig. 5 Allele classification, GBSSI activity and amylose content of the tetraploid genotypes. Each point represents the mean value of three individual tubers from three greenhouse grown plants. The GBSSI allele A1 dosage effect on GBSSI activity is shown under A. Amylose content versus GBSSI activity is shown under B.

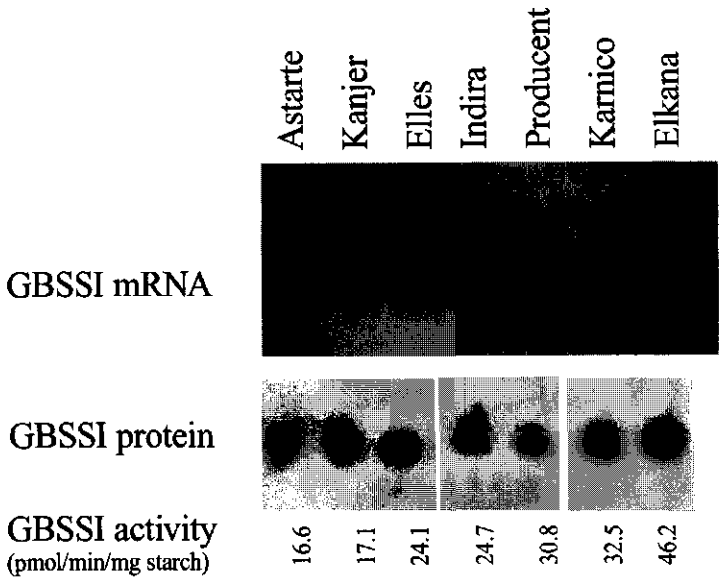

Fig. 6 GBSSI mRNA and protein level and GBSSI activity of some tetraploid *Solanum tuberosum* cultivars.

Subclassification of the GBSSI alleles did not result in a more detailed characterisation of the effect of allelic composition on GBSSI activity and amylose content. Besides the additive effect of alleles belonging to the A1 class, no significant effects for other alleles could be observed. This could be due to the very heterogeneous character of the population studied and the interaction between alleles. All clones except breeding line 90-027-6 contained at least one time allele A1. To study the effect of single alleles in an *amf* background, as well as to obtain clones with a more comparable genetic background, crosses were made between selected clones and the *amf* mutant.

GBSSI allele classification, GBSSI activity and amylose content within full sib families,

Single alleles in an *amf* background were obtained by crosses between the diploid *amf* clone 5002-18 ($a1^a a1^a$) and the diploid breeding lines 88-032-52 ($A1^c A4$), 89-053-38 ($A1^b A2^a$) and 90-027-6 ($A2^a A2^b$). Since there were no diploid clones containing an allele belonging to the A3 class crosses were made between the tetraploid *amf* clones 93-6706-2 ($a1^a a1^a a1^a a1^a$) and 93-6706-6 ($a1^a a1^a a1^a a1^a$) and cv Kanjer ($A1^c A1^c A3^a A3^b$) (Table II). For the tetraploid crosses twenty progeny plants and for the diploid crosses twelve progeny

plants were grown in the greenhouse. However, due to disease and poor tuber formation less progeny plants were analysed.

Table II. Crosses between selected Solanum genotypes with different GBSSI wild type alleles and the amf mutant containing only the a1 allele.

Parents	Allelic composition	Number of progeny plants analysed
88-032-52 x 5002-18	$A1^cA4$ x $a1^aa1^a$	10
89-053-38 x 5002-18	$A1^bA2^a$ x $a1^aa1^a$	7
90-027-6 x 5002-18	$A2^aA2^b$ x $a1^aa1^a$	7
Kanjer x 93-6706-2	$A1^cA1^cA3^aA3^b$ x $a1^aa1^aa1^aa1^a$	12
Kanjer x 93-6707-6	$A1^aA1^cA3^aA3^b$ x $a1^aa1^aa1^aa1^a$	11

The GBSSI allelic composition of the progeny was determined by a combination of Southern and PCR analyses. Statistical evaluation with an analysis of variance of the F1 of the crosses Kanjer x 93-6706-2 and Kanjer x 93-6706-6 showed that there was a significant effect of the allelic composition on GBSSI activity and amylose content (Fig. 7).

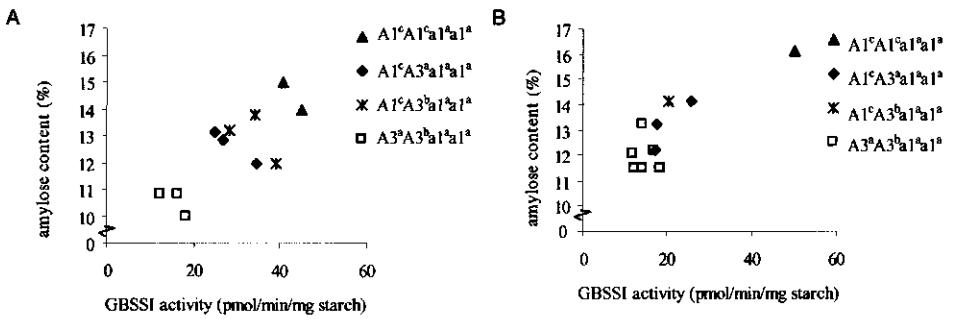

Fig. 7 Allelic composition, GBSSI activity and amylose content of the progeny of the crosses cv Kanjer x 93-6706-2 (A) and cv Kanjer x 93-6707-6 (B). Each point represents the mean value of three individual tubers per progeny plant.

By using the t-test it was shown that the GBSSI activity of the $A1^cA1^ca1^aa1^a$ containing plants was significantly higher than that of plants containing $A1^cA3^aa1^aa1^a$ or $A1^cA3^ba1^aa1^a$. Plants containing $A1^cA3^a$ $a1^aa1^a$ or $A1^cA3^ba1^aa1^a$ had a higher GBSSI activity than those with $A3^aA3^ba1^aa1^a$. No significant differences were found between the $A1^cA3^aa1^aa1^a$ and

$A1^cA3^ba1^aa1^a$ plants. By using the t-test it was also shown that the amylose content of the $A1^cA1^ca1^aa1^a$ plants was significantly higher than that of the $A1^cA3^aa1^aa1^a$ or $A1^cA3^ba1^aa1^a$ plants, which was in turn higher than that of $A3^aA3^ba1^aa1^a$ plants. By using the Pearson correlation test correlations of 0.675 and 0.856 were found between the GBSSI activity and the amylose content within the crosses Kanjer x 93-6706-2 and Kanjer x 93-6707-6 respectively which were significant at the 0.01 level (1-tailed).

A t-test analysis of the crosses 88-032-52 ($A1^cA4$) x 5002-18 ($a1^aa1^a$), 89-053-38 ($A1^bA2^a$) x 5002-18 ($a1^aa1^a$) and 89-027-6 ($A2^aA2^b$) x 5002-18 ($a1^aa1^a$) showed that the amylose content of the progeny containing an allele belonging to the A1 class was significantly higher than that of progeny containing no A1 allele. No significant differences in amylose content were found between progeny containing allele $A2^a$ or $A2^b$ (Fig. 8).

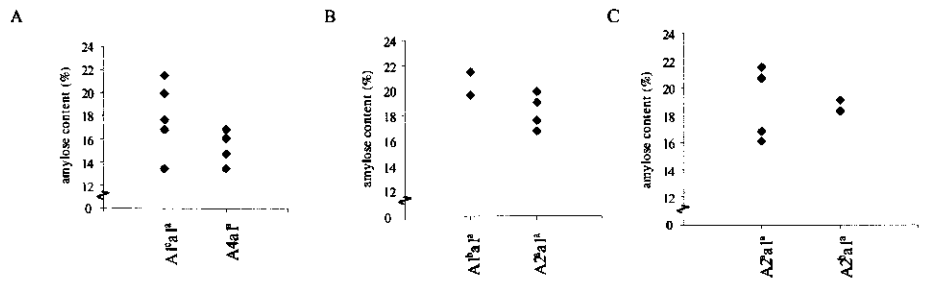

Fig. 8 Relation between allelic composition and amylose content within the progeny of the crosses 88-032-52 x 5002-18 (A), 89-053-38 x 5002-18 (B) and 90-027-6 x 5002-18 (C). 5002-18 is a diploid *amf* clone.

DISCUSSION

It was shown that multiple allelism in a heterozygous autopolyploid species like potato not only occurs for morphological characteristics like tuber size and shape, but also for genes involved in metabolic pathways like starch synthesis. A combination of Southern and PCR analysis allowed the identification of at least eight alleles coding for GBSSI, which is involved in amylose production. Based on the results obtained with Southern analysis the alleles were divided into four classes: A1, A2, A3 and A4 (Fig. 1), which could be subdivided by PCR (Table I).

In allele class A1 the three subclasses $A1^a$, $A1^b$ and $A1^c$ were found, which showed complete homology in the GBSSI promoter sequence to the sequences published by van der Leij *et al.* (1991b), Rohde *et al.* (1990, G1) and Dai *et al.* (1996) respectively. In A2 two subclasses, $A2^a$ and $A2^b$, were determined. The $A2^b$ sequence was identical to that of G28 (Rohde *et al.*, 1990). A3 was also subdivided into two subclasses, $A3^a$ and $A3^b$. The

promoter sequences of $A2^a$, $A3^a$, $A3^b$ and A4 are described for the first time in this paper (Fig. 4).

The major difference between the alleles was the absence or presence of a 140 bp fragment, 0.5 kb upstream of the ATG start codon. Alleles $A1^a$, $A1^b$ and $A1^c$ in contrast with $A2^a$, $A2^b$, $A3^a$, $A3^b$ and A4 lack this fragment. The homology at DNA level for the promoter sequences within the A1, A2 and A3 classes was 92 %, 98 % and 94 % respectively. The homology between the A2, A3 and A4 classes was over 90 %, while A1 only showed 80 % homology to the other classes. In comparison, the homology in the coding region both within and between allele classes is over 98 %.

In a population of *Solanum tuberosum* cultivars and *Solanum* breeding lines the alleles were not evenly distributed over the different classes. It appeared that allele class A1 was present more frequently than the other classes. The cultivars and breeding lines were all selected for their high starch content. However, it is not clear whether this selection process influenced the GBSSI allelic composition by means of a selective advantage of allele A1. Based on the clustal multiple allignment of the promoter sequences using a standard algorithm (Altschul *et al.*, 1990) a phylogenetic tree was created, which shows that it is likely that A1 is derived from the other sequences by deletion of the 140 bp fragment (Fig. 9).

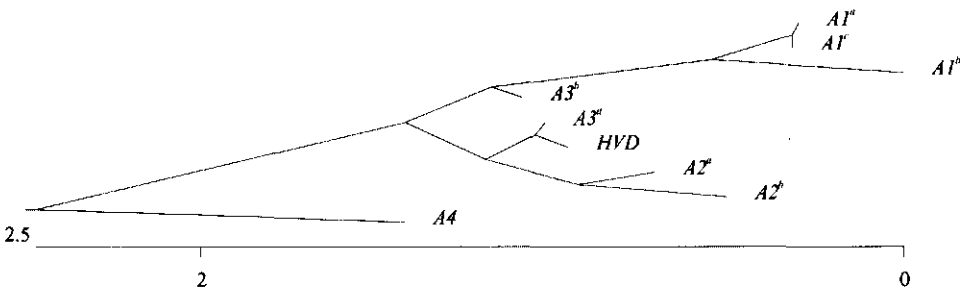

Fig. 9 Phylogenetic tree of GBSSI promoter sequences. The sequences were alligned using a standard algorithm according to Altschul *et al.* (1990).

In wheat the three *Wx* genes (*Wx*-A1, *Wx*-B1 and *Wx*-D1), present on homoeologous chromosomes have differential effects on amylose content (Miura and Sugawara, 1996; Miura *et al.*, 1999). The three *Wx* genes can be seen as a fixed intergenomic multiple allelism. In rice, a homozygous diploid species, an intragenomic multiple allelic effect on GBSSI activity and amylose content was proven (Cai *et al.*, 1988; Pooni *et al.*, 1993). It is known that intragenomic multiple allelism is common in heterozygous polyploid species

like potato. However, due to the heterogeneous character, the effects of allelic compositions are less obvious.

Despite the heterozygous autopolyploid nature of cultivated potato it was possible to show a significant effect of allelic composition on GBSSI activity. In a population of *Solanum tuberosum* cultivars and Solanum breeding lines a dosage effect of allele A1 on GBSSI activity was shown indicating an additive effect of the allele A1 (Fig. 5). Moreover, in a more homogeneous background obtained by crossing selected diploid and tetraploid clones to the *amf* mutant an effect of the allelic composition on amylose content was also proven (Fig. 7). In the progeny of the crosses a positive correlation between GBSSI activity and amylose content was determined. No such correlation was found in the studied population. A large variation in other factors influencing amylose content, such as the activity of other enzymes involved in starch biosynthesis, the structure of the starch granule and the interactions between these characters and the GBSSI activity, probably caused this absence of correlation.

Statistical analysis showed that there were no significant differences between alleles A2, A3 and A4 within the population of *Solanum tuberosum* cultivars and *Solanum* breeding lines. Moreover, no significant differences were found between allele $A2^a$ and $A2^b$, and $A3^a$ and $A3^b$ within the progeny of the crosses. This indicated that the presence or absence of the 140 bp fragment has a major effect on GBSSI activity. It turned out that the presence of small bp deletions and simple sequence repeats led to no apparent effects on GBSSI activity. A BLAST (Altschul *et al.*, 1990) search of the NCBI (National Center of Biotechnology Information, Bethesda, MD) promoter databank using the 140 bp sequence showed no significant alignments with other identified regulatory promoter elements.

Due to the high degree of homology at amino-acid level between the different alleles it is likely that the specific activities of the gene products are similar. Based on this and the fact that variation in GBSSI activity and amylose content could be explained mainly by the absence or presence of a 140 bp fragment in the GBSSI promoter region it was expected that the variation in GBSSI activity and amylose content could be explained by differences at protein and/or RNA level. In wheat it was shown that the amylose content was indeed correlated to the amount of *Wx* protein formed. However, in potato the variation in GBSSI activity and amylose content could not be assigned to large differences at GBSSI RNA and protein level (Fig. 6).

In summary, significant effects of allelic composition on GBSSI activity and amylose content were demonstrated, which could be mainly explained by the absence or presence of a 140 bp fragment, 0.5 kb upstream of the ATG start codon. The absence or presence of this fragment can be easily monitored with either Southern analysis or PCR

techniques. Further characterisation of GBSSI allelic composition by Southern analysis, using other restriction enzymes and/or PCR analysis, using other primer combinations will reveal more alleles with small bp changes. However, the effect on GBSSI activity and amylose content will probably be minor in comparison to the effect of the absence or presence of the 140 bp fragment. Thus in order to obtain cultivars with a higher amylose content screening for clones lacking this fragment would be a simple and worthwhile activity.

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GBSSI allele A1 displaying the highest GBSSI activity is most frequently inhibited by a homologous antisense construct containing both the GBSS1 A1 promoter and cDNA.

ABSTRACT

Amylose-free potato varieties can be obtained after the suppression of granule-bound starch synthase I (GBSSI) by the introduction of an antisense GBSSI cDNA. However, a large variation in amylose content of the transformants was observed both within and between genotypes. The variation in GBSSI activity and amylose suppression within genotypes is caused by the variability in expression of the transgene, which is influenced by positional effects, copy number and repetitiveness of the T-DNA insert. These factors influencing transgene expression are supposed to be similar for all genotypes. By analysing a large number of transformants per genotype, the values used to represent the level of suppression could be averaged. The additional variation found between genotypes was expected to be due to differences in genetic composition. An effect of allelic GBSSI composition on antisense inhibition was proven significant. Although it was previously shown in non-transformed clones that a positive correlation existed between the number of A1 alleles and the amylose content, a negative correlation between A1 copy-number and amylose content was observed after transformation, indicating a more severe antisense effect. As both the GBSSI A1 promoter and the A1 GBSSI cDNA were used for the antisense construct, homology between the transgene and the endogenous allele appear to be major factors influencing the level of suppression. The allelic composition of a variety can therefore be used as a pre-selection criterion in breeding programs in which antisense technology is used to obtain amylose-free potato varieties. Moreover, knowledge on the allelic composition may provide information to come to an educated guess on the number of transformants necessary for the selection of a sufficient number of transformants with complete suppression.

INTRODUCTION

Starch consists mainly of two components: amylopectin, a branched glucose polymer and amylose, an essentially linear glucose polymer. The synthesis of amylose is catalysed by granule-bound starch synthase I (GBSSI), which in potato is encoded by a single copy gene containing multiple alleles. The recessive amylose-free (*amf*) allele results from a point mutation in the GBSSI transit peptide (van der Leij *et al.*, 1991b). Although *wild type* levels of GBSSI mRNA are produced in the *amf* mutant no protein is present tightly linked to the starch granule and, therefore, no amylose is synthesised (Hovekamp-Hermelink *et al.*, 1987). In addition to the mutated *amf* allele, nine active *wild type* alleles have been identified which were characterised by a combination of Southern and PCR analysis (Chapter 4). The differentiation between these alleles was enabled by the presence of multiple deletions and simple sequence repeats in the promoter and downstream of the TAA stop codon.

Industrially important properties of starch such as gelatinization, retrogradation and viscosity are influenced by the ratio of amylose to amylopectin and, therefore, starches with different ratios are preferred for various applications. This can be achieved by conventional breeding methods (Jacobsen *et al.*, 1991) using mutant alleles like *amf* or by the use of antisense technology (Visser *et al.*, 1991). Due to the recessive character of the *amf* allele four copies are required to obtain amylose-free starch in tetraploid potato, and they have to be combined with many other important agricultural traits. In contrast an antisense gene can act as a dominant suppressor gene, and hence can be directly applied to develop amylose-free starch potato cultivars with *Agrobacterium* mediated transformation of existing varieties. Moreover, amylose-free transgenic clones can be used as progenitors in an established breeding program to develop amylose-free potato varieties (Heeres *et al.*, 1997).

One major drawback of the use of antisense technology is the variation in inhibition of the target gene after introduction of the antisense gene. This variability could be the result of position effects of the T-DNA inserts that are assumed to be caused by influences of adjacent plant genomic DNA sequences. Other factors that might influence transgene expression and, thereby, the level of antisense inhibition are the copy number of T-DNA insertions, repetitiveness of the transgene insert, DNA methylation, promoter characteristics of the antisense constructs and the level of homology between the transgene and the endogenous gene (Bourque, 1995; Hobbs, Kpodar and DeLong, 1990; Kuipers *et al.*, 1995; Mol *et al.*, 1994; Stam *et al.*, 1997).

The amylose content among individual transformants of potato obtained by introduction of an antisense GBSSI gene varied from 0 to 20 %. A positive correlation between T-DNA copy number and inhibition of the GBSSI gene expression was shown by

Kuipers *et al.* (1995). Moreover, an effect of the construct promoter on antisense inhibition was proven. GBSSI antisense constructs driven by the GBSSI promoter resulted in a higher number of transformants with complete inhibition as compared to constructs containing a 35S CaMV promoter. Crosses between *wild type* and transgenic amylose-free potato clones showed a strong correlation between the genetic composition of the *wild type* parent and the level of antisense inhibition (Heeres *et al.*, 1997). Wolters *et al.* (1998) found that the composition of endogenous GBSSI alleles influences the degree of antisense effect of some T-DNA-containing linkage groups. This effect could be explained by variation in the strength of expression of the endogenous GBSSI-genes, homology between each of the endogenous alleles and the transgene, and susceptibility to inhibition of the endogenous alleles.

As potato is vegetatively propagated, tetraploid and highly heterozygous multiple allelism is common also for GBSSI. Recently a quantitative effect of allelic composition on GBSSI activity and amylose content was shown for potato (Chapter 4). The major difference between the GBSSI alleles identified was the absence or presence of a 140 bp fragment in the GBSSI promoter, 0.5 kb upstream of the ATG start codon. The absence of this fragment, which can be easily monitored by Southern analysis, correlated with increased GBSSI activity and amylose content.

In this study the allelic composition of 11 potato varieties, used for transformation with antisense GBSSI, was analysed by Southern analysis and the effect of allelic composition on antisense inhibition was determined. Data on the allelic classification of the transformants provided an indication how varieties can be selected for transformation or as progenitor in a breeding program to enhance the level of antisense RNA mediated suppression and the chance of obtaining complete suppression of the target gene.

MATERIALS AND METHODS

Materials,

The starch potato varieties Atillo, Calgary, Elkana, Florijn, Indira, Kanjer, Karida, Karnico, Kurola, Nika, and Producent, used for transformation with antisense GBSSI and classified by iodine staining of starch (Heeres *et al.*, unpublished results), were analysed for allelic composition by Southern blotting. The varieties were transformed with the binary vector pKGBA50 (Kuipers *et al.*, 1995) containing the GBSSI cDNA in antisense orientation between the GBSSI promoter and the Nopaline synthase (Nos) terminator (Fig. 1). Both the GBSSI cDNA and the GBSSI promoter were derived from the genomic sequence as published by van der Leij *et al.* (1991b). According to our classification this allele belongs to the A1 class (Chapter 4).

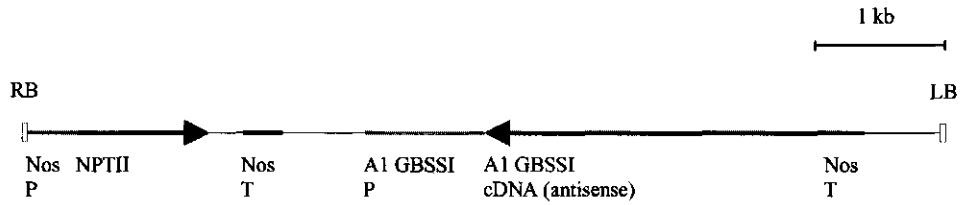


Fig. 1 Schematic representatation of the T-DNA of plasmid pKGBA50 used for transformation. RB, right border; LB, left border; Nos P, promoter of the nopaline synthase gene; Nos T, polyadenylation sequence of the nopaline synthase gene; NPTII, kanamycine resistance gene; A1 GBSSI P, GBSSI promoter belonging to the allele class A1; A1 GBSSI cDNA, GBSSI cDNA belonging to allele class A1.

Southern analysis,

Genomic DNA (4 µg), isolated from 0.5 - 2.0 g of leaves as described by Rogers and Bendich (1988), was digested with *Hin*dIII and electrophoresed on a 0.8 % agarose gel for 16 h at 40 V. The DNA was subsequently vacuum blotted (Pharmacia) onto Hybond N^+ membranes (Amersham) in 10 x SSC. A 1.3 kb *Eco*RI fragment containing the 3' part of GBSSI cDNA and a 1.1 kb *Eco*RI fragment containing the 5' part of the GBSSI cDNA were used as probe (Visser *et al.*, 1989). Probes were radioactively labelled with the Megaprime DNA labelling system (Amersham). Hybridizations were performed in glass bottles in a Hybaid hybridization oven, at 65 °C for 16 - 18 h. The blots were rinsed to a stringency of 0.2 x SSC, 0.5 % SDS at 65 °C. Alleles were classified as described in Chapter 4 (Fig. 2).

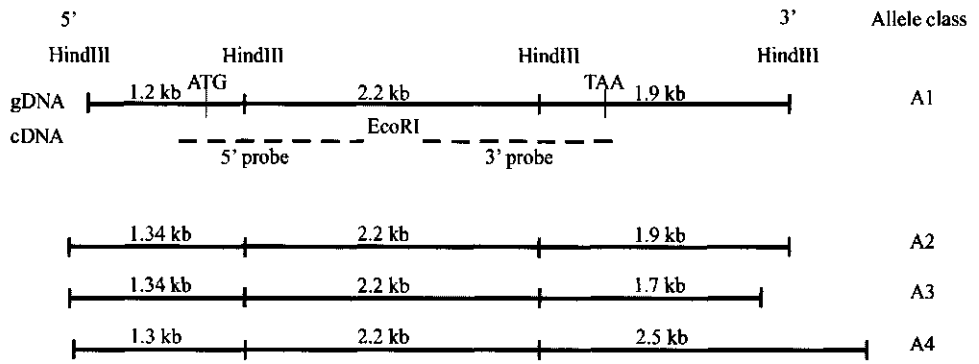


Fig. 2 GBSSI allele classification. The 5' and 3' *Eco*RI cDNA probes were used for Southern analysis of *Hin*dIII digested gDNA.

Classification of transformants and determination of the average amylose content by iodine staining of starch,

The inhibition of amylose production by antisense GBSSI in the transformants was determined by staining potato tuber starch granules with lugol/H_2O (1:1) (Merck). Starch containing both amylose and amylopectin stains blue while starch containing only amylopectin stains red. In the presence of reduced amounts of amylose the starch granule stains red with a blue core of varying size (Kuipers *et al.*, 1994a). Based on the size of the blue core the transformants were classified into four groups: rk, rm, rg and b.

The size of this blue core was found to be closely related to the amylose content (Kuipers *et al.*, 1994a). In rk transformants the blue core represents less than 10 % of the granule diameter (< 1 % amylose). Rm transformants contain starch granules with a blue core of 10 – 50 % of the granule diameter (~ 5 % amylose). Rg transformants contain blue starch granules with a red outer layer (~13 % amylose) and b transformants show no decrease in amylose content (~ 20 %). The average amylose content per group of transformants per genotype was determined by the formula:

$$\frac{(1 \times n_{rk}) + (5 \times n_{rm}) + (13 \times n_{rg}) + (20 \times n_{b})}{n}$$

n_{rk}: number of transformants classified in the rk group
n_{rm}: number of transformants classified in the rm group
n_{rg}: number of transformants classified in the rg group
n_{b}: number of transformants classified in the b group
n: total number of transformants derived after transformation of a genotype

RESULTS

GBSSI allele classification,

The allelic composition of the *Solanum tuberosum* varieties was determined by Southern analysis. Results of Fig. 3a and 3b were obtained after hybridisation of *Hin*dIII digested genomic DNA of 11 varieties with a 5' and 3' GBSSI *Eco*RI cDNA probe respectively. The alleles were grouped into four classes (Fig. 2) according to Chapter 4. The 2.2 kb internal fragment was recognised by both the 5' and the 3' probe. variety Calgary contained only a 1.2 kb 5' fragment and a 1.9 kb 3' fragment, indicating that all four alleles belong to the A1 class. In addition to these two fragments the varieties Indira, Florijn and Kurola showed a 1.34 kb 5' fragment, indicating the presence of an allele belonging to the A2 class. The varieties Elkana, Kanjer and Karida contained besides the

1.34 kb 5' fragment an additional 1.7 kb 3' fragment indicative for allele A3. The varieties Karnico, Producent, Atillo and Nika contained alleles belonging to the A4 class, which were indicated by the presence of a 1.3 kb 5' fragment and a 2.5 kb 3' fragment. The intensity of the bands was used as indication for the copy number of alleles with those particular fragments. For example Karnico and Producent contained only one allele belonging to the A4 class, while Atillo and Nika contained two alleles of the A4 class (Fig. 3). The varieties were grouped into three genotype classes containing four (Calgary), three (Indira, Florijn, Elkana, Karnico, and Producent) and two (Kurola, Janjer, Karida, Atillo and Nika) A1 alleles.

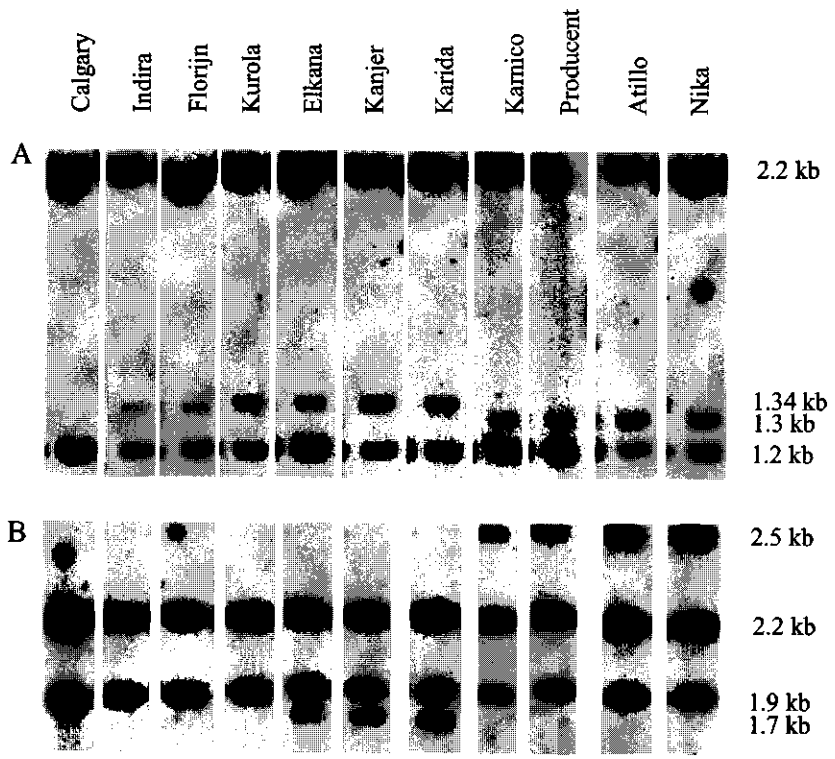


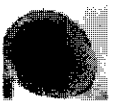
Fig. 3 Genotyping of varieties for GBSSI alleles by Southern analysis of *Hin*dIII digested genomic DNA, hybridised with a 5' (A) and 3' (B) *Eco*RI GBSSI cDNA probe.

Effect of allelic composition on antisense inhibition,

After transformation of the varieties with the pKGBA50 construct (Fig. 1) containing a GBSSI cDNA in antisense orientation behind a GBSSI promoter belonging to

the allele class A1, the transformants (2 - 145 independent transformants per variety) were classified for starch composition by iodine staining of the starch (Table I).

Table I Allelic composition of varieties transformed with the pKGBA50 construct and classification of transformants after iodine staining of starch granules from cut tuber surfaces.

Variety	Allelic composition	# clones				
		Total	rk	rm	rg	b
						
Calgary	A1A1A1A1	78	8	18	19	33
Indira	A1A1A1A2	64	6	8	20	30
Florijn	A1A1A1A2	76	8	14	9	45
Kurola	A1A1A2A2	5	0	1	1	3
Elkana	A1A1A1A3	131	10	21	40	60
Kanjer	A1A1A3A3	139	5	17	21	96
Karida	A1A1A3A3	78	5	11	9	53
karnico	A1A1A1A4	145	20	20	22	83
Producent	A1A1A1A4	100	9	12	23	56
Atillo	A1A1A4A4	29	0	3	4	22
Nika	A1A1A4A4	2	0	0	1	1

The varieties Kurola and Nika had a very low transformation affinity using the pKGBA50 construct and the number of transformants obtained after transformation of variety Atillo with this construct was relatively low. The amylose in the different transformants was confined to a core of varying size at the hilum of each granule. The transformants could be grouped into four classes depending on the size of this blue core. Rk transformants contained no or very little amylose while in transformants classified as b the amylose percentage was around *wild type* level (~ 20 %). Rm and rg are two intermediate classes with a blue core of 10 – 50 % and 50 – 90 % of the granule diameter respectively.

A large variation in the size of the blue core could be observed both within and between varieties. Transformation of all varieties, except for Kurola, Atillo and Nika, resulted in the formation of transformants belonging to all four different classes. After transformation of the varieties Kurola, Atillo and Nika no transformants were classified as rk, which was partly caused by the low number of transformants that could be analysed for these varieties.

The varieties were grouped into three classes of genotypes, containing four, three and two A1 alleles respectively. The variation within genotypes was supposed to be caused by position and copy number effects of the inserted T-DNA. Suppression of GBSSI by highly expressed T-DNA loci, containing multiple copies of the transgene, may lead to complete inhibition of amylose content independent of the allelic GBSSI composition as was shown by Wolters *et al.* (1998). On the other hand a T-DNA copy inserted in an unfavourable position will presumably never lead to strong suppression of the endogenous alleles even if the endogenous allelic composition is favourable for inhibition. Therefore transformants classified as rk, rm, rg as well as b were found among the transformants derived from one genotype. The variability in T-DNA copy number and due to position effects is supposed to be similar for all genotypes. By classifying a large numbers of transformants per genotype the variation within genotypes can be set as a standard.

Besides the variation within genotypes an additional variation between genotypes was observed, which was expected to be due to differences in GBSSI allelic composition. Two values were used to determine the effect of allelic composition on antisense inhibition. The first value was the percentage of transformants from a genotype classified as rk, indicating complete or nearly complete inhibition of amylose biosynthesis. Statistical analysis of the transformants with an analysis of variance showed a positive dosage effect of allele A1 on the % rk transformants, which was significant at the 0.05 level (Fig. 4A). No significant differences in the percentage of transformants belonging to the rk class were observed between the genotype A1A1A1A1 (10.3 %, Calgary) and A1A1A1A* (10.3 %, Indira, Florijn, Elkana, Karnico and Producent). However, this percentage was significantly higher than that of the genotype A1A1A*A* (4.0 %, Kurola, Kanjer, Karida, Atillo and Nika). A* represents a GBSSI allele not belonging to the A1 class.

The second value used was the average amylose content of the transformants, derived per genotype (as described in the Material and Methods). A negative dosage effect of allele A1 on the average amylose content was proven significant (Fig. 4B). The average amylose content of the transformants obtained from genotype A1A1A1A1 was 12.9 % (Calgary). The transformants derived from genotype A1A1A1A* showed an average amylose content of 14.3 % (Indira, Florijn, Elkana Karnico and Producent) while those obtained from genotype A1A1A*A* had an average content of 16.4 % (Kurola, Kanjer, Karida, Atillo and Nika).

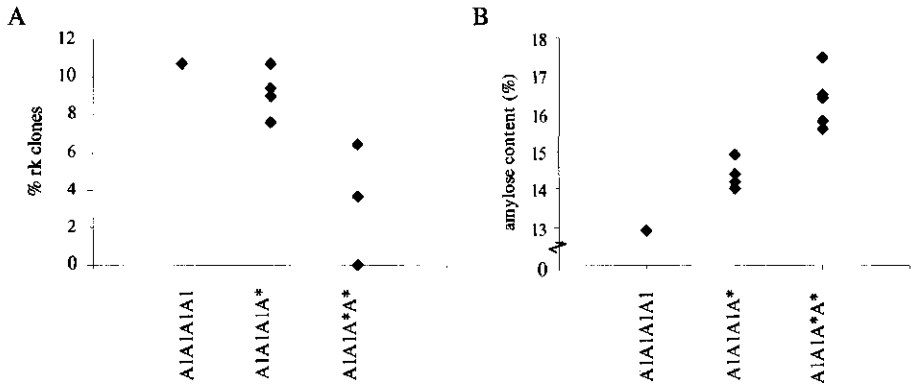


Fig. 4 Effect of allele A1 copynumber on the two parameters representing antisense inhibition: percentage of transformants belonging to the rk class (A) and average amylose content after transformation (B).

These data imply a positive correlation between the number of A1 alleles and the success rate of the antisense inhibition. As the construct contained the GBSSI A1 cDNA in antisense orientation driven by the GBSSI A1 promoter, homology between the endogenous alleles and the transgene appear to affect this correlation. Despite the strong expression of GBSSI alleles belonging to the A1 class (Chapter 4), a relatively high percentage of rk phenotypes was obtained in plants with four A1 alleles. The effect of allelic composition on antisense inhibition could therefore not be explained by the strength of the endogenous alleles.

DISCUSSION

For the development of amylose-free potato varieties the expression of GBSSI can be suppressed by the introduction of an antisense GBSSI cDNA. In a tetraploid crop like potato this is less time consuming than the introduction of a recessive allele followed by homozygotisation of it combined with selection for many agricultural traits in a normal breeding program. A complicating factor of the use of antisense technology is the large variation of suppression in the transformants obtained after transformation.

One factor that is expected to influence the level of antisense inhibition is the endogenous allelic composition of the variety that has to be altered in starch composition. Based on the results obtained with Southern analysis the GBSSI allelic composition of varieties used for transformation was determined. The alleles were divided into four classes: A1, A2, A3 and A4 (Fig. 2) according to chapter 4 and the varieties were grouped into three classes depending on the number of A1 alleles.

The differences between the published GBSSI alleles in the coding region are very small, only a few bp between the alleles (Dai *et al.*, 1996; Hofvander *et al.*, 1992; van der Leij *et al.*, 1991b). The major difference between the alleles has been found in the promoter region. Alleles belonging to the A1 class lack a 140 bp promoter fragment, 0.5 kb upstream of the ATG start codon which is present in alleles belonging to the A2, A3 and A4 classes.

To study the effect of allelic composition on antisense inhibition the transformants were classified after iodine staining of the starch granules. Two values were used to express the level of antisense inhibition. The first value was the percentage of transformants from a genotype classified as rk, indicating complete or nearly complete inhibition of amylose biosynthesis. This value can be used to predict the number of transformants necessary to obtain sufficient rk phenotypes for selection of an amylose-free cultivar. The second value is the average amylose content, which was derived from the size of the blue core as it was earlier found that these two values were closely related (Kuipers *et al.*, 1994a).

A large variation in amylose content was found both between and within genotypes. The copy number and the position of the inserted T-DNA (not investigated), which are known to affect the expression of inserted genes in transgenic plants, are generally supposed to cause the variation within genotypes. Kuipers *et al.* (1995) has shown a correlation between the T-DNA copy number and the level of antisense inhibition. Moreover, Wolters *et al.* (1998) have shown that the antisense inhibition of some T-DNA loci was effected by the allelic composition while that of others was not. The variation in T-DNA copy number and position effects is supposed to be similar for all genotypes. If relatively large numbers of transformants per genotype are analysed then the observed variation can be set as a standard. The additional variation found between genotypes was, therefore, expected to be due to differences in genetic composition.

An effect of allelic composition on antisense inhibition was indeed found. Both a positive effect of allele A1 on the percentage of rk-transformants and a negative effect on the average amylose content in the transformants were proven significant. In comparison, in non-transformed clones a positive dosage effect of allele A1 on GBSSI activity and amylose content was proven (Chapter 4). If strength of the endogenous alleles was one of the major factors influencing the amylose content in the transformants a positive correlation between the number of A1 alleles and the amylose content would be expected. However, a negative correlation was observed indicating that the effect of allelic composition on antisense inhibition could not be explained by the strength of the endogenous alleles.

Homology between each of the endogenous alleles and the transgene and susceptibility of the endogenous alleles to antisense inhibition are more important. As both the GBSSI A1 promoter and the A1 GBSSI cDNA were used for the antisense construct,

homology between transgene and the endogenous alleles is likely to influence the interaction. As the differences in the coding region between the alleles are very small, it is postulated that the promoter sequences of the endogenous GBSSI alleles influence the antisense inhibition.

An effect of the construct promoter on antisense inhibition was already proven by Kuipers *et al.* (1995). The introduction of the GBSSI promoter containing antisense constructs coding for GBSSI resulted in a higher percentage of transformants with complete inhibition as compared to those driven by the 35S CaMV promoter. The percentage of transgenic clones with low or moderate inhibited GBSSI gene expression was highest for constructs driven by the 35S CaMV promoter. This clearly indicates that the effectiveness of inhibition is influenced by the promoter used. Moreover, expression of the antisense GBSSI gene from the GBSSI promoter resulted in a higher stability of inhibition in tubers of field grown plants as compared to expression from the 35S CaMV promoter (Kuipers *et al.* 1994b).

Homology between the construct promoter and each of the endogenous GBSSI promoters can influence the allelic inhibition in that the transgene expression level is regulated in a similar way as the endogenous gene. No reports on regulation at the allelic level have been reported so far. Methylation of the target gene and other factors influencing the susceptibility to inhibition may also attribute to the effect of allelic variation in the promoter sequences on GBSSI gene suppression.

In conclusion allelic composition of the endogenous GBSSI gene affects antisense inhibition. Determining the allelic composition by Southern analysis might be worthwhile as it can be used as an additional selection criterion for breeding programs in which antisense technology is used to obtain sufficient transformants with altered starch composition for selection of a new variety without negative side-effects. Moreover, an educated guess can be made on the number of transformants necessary for the selection of a clone with complete suppression of the activity.

GENERAL DISCUSSION

The experiments described in this thesis were performed to unravel the amylose biosynthesis in potato and to study the factors influencing the amylose content. As amylose content is one of the critical parameters affecting starch quality, understanding the amylose synthesis pathway can contribute towards a directed alteration of the amylose content and an increase in the application possibilities of the starch produced. In Chapter 2 and 3 the results obtained after studying the amylose biosynthesis *in vitro* are described. In the Chapters 4 and 5 the factors determining amylose content *in vivo* were studied both in *wild type* potato clones (Chapter 4) and after transformation (Chapter 5). A general discussion of the results will be given in this chapter.

AMYLOSE BIOSYNTHESIS *IN VITRO* VERSUS *IN VIVO*

The reaction,

Amylose is synthesised by the elongation of a growing α-1,4 linked glucan (Fig. 1), which is catalysed by GBSSI as was shown by the synthesis of amylose-free starch in mutants lacking GBSSI. The low level of α-1,6 branches, present in amylose are probably formed by branching enzymes, which are found among the minor proteins associated with the starch granule.

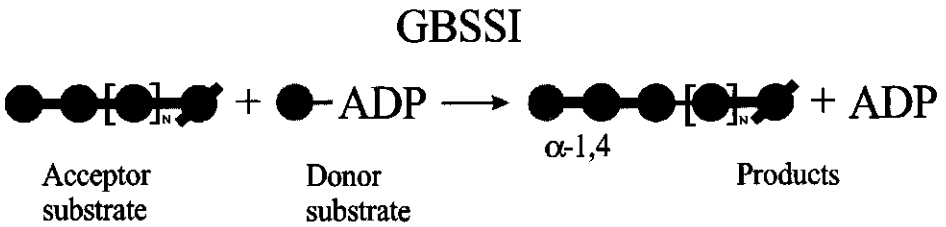


Fig. 1 Transfer of a glucose residue from ADP-Glc to the non-reducing end of a growing α-1,4 linked glucan catalysed by GBSSI.

To study the reaction catalysed by GBSSI *in vitro* (Chapter 2 and 3), starch granules were purified and incubated with a radio-labelled Glc-donor. By isolating starch granules only enzymes bound to or entrapped in the starch granule can contribute to the *in vitro* synthesis. As GBSSI is the major protein bound to the starch granule, GBSSI activity is mainly monitored under these conditions. Comparing the results from *in vitro* experiments with *wild type* starch granules to those obtained with granules isolated from GBSSI mutants

enables the correction for any possible contribution of other starch synthases (Chapter 2 and 3).

In vitro experiments performed by Leloir *et al.* (1961), Recondo and Leloir (1961) and Baba *et al.* (1987) showed that label was incorporated in both amylopectin and amylose. The incorporation of label in amylopectin was remarkable as genetic evidence had shown that GBSSI was mainly responsible for amylose synthesis and that GBSSI makes no or very little contribution to amylopectin synthesis.

The donor substrate,

In the first *in vitro* experiment UDP-Glc was used as Glc-donor (Leloir *et al.*, 1961), while in later experiments starch granules were incubated with radio-labelled ADP-Glc (Recondo and Leloir., 1961), which is now generally accepted to be the preferred donor substrate. In most of the *in vitro* experiments performed a concentration of 3.2 mM ADP-Glc was supplied. In time-course experiments performed with 500 µg starch isolated from higher plants it was shown that even after 24 h the ADP-Glc concentration did not become limiting (Chapter 3). However, already after 1 hour incubation of 500 µg *Chlamydomonas* starch the Glc-donor became limited, which was due to the higher GBSSI activity of *Chlamydomonas* starch (Chapter 3).

In vivo the ADP-Glc concentration is also the rate limiting factor in amylose biosynthesis as was shown by data obtained from both mutant and transgenic plants. A reduction in ADP-Glc supply lowered the amylose content as was shown in the AGPase mutant from *Chlamydomonas* and the rb and rug3 mutants from pea, which are affected in their AGPase and plastidial phosphoglucomutase activities (Ball *et al.*, 1991; Lloyd *et al.*, 1996; Harrison *et al.*, 1998). Lowered expression of AGPase in transgenic potato also reduced the amylose content (Lloyd *et al.*, 1999b). By increasing the supply of ADP-Glc through the over-expression of a plastidial ATP/ADP transporter it was possible to increase amylose and starch contents of potato tubers (Tjaden *et al.*, 1998).

The acceptor substrates,

Two acceptor substrates for GBSSI have been put forward, amylopectin and malto-oligosaccharides. In a time course *in vitro* experiment performed with starch isolated from *Chlamydomonas* (Chapter 2) it was shown that initially label was incorporated in amylopectin while thereafter significant amounts of label were found in the amylose fraction. These results suggested that amylose was formed by cleavage of external chains from amylopectin. Transfer of chains from amylopectin to amylose was indeed evidenced from pulse-chase experiments performed with *Chlamydomonas* starch (Chapter 2), which

proves that amylopectin can act as acceptor substrate for amylose biosynthesis by GBSSI. Amylopectin-primed amylose synthesis in higher plants was shown in Chapter 3, in which *in vitro* synthesis experiments performed with starch isolated from higher plants were compared to those performed with *Chlamydomonas* starch. Pulse-chase experiments with starch isolated from potato and taro showed small decreases in label incorporated in amylopectin and small increases in label incorporated in amylose, which were in proportion to the GBSSI activities of these starches. Both the much lower GBSSI activity of starch isolated from higher plants and the label incorporated in amylose at the start of the chase complicated the detection of a transfer of label from amylopectin to amylose. However, the transfers observed, although small, were continuous over time and the amounts as expected by comparison of the GBSSI activities. Therefore it was concluded that amylopectin-primed amylose synthesis also takes place in higher plants. The incorporation of label in amylopectin as was shown already during the first *in vitro* experiments performed can be explained by the fact that amylopectin is the acceptor substrate for GBSSI.

The other acceptor substrates for GBSSI are malto-oligosaccharides. *In vitro* elongation of malto-oligosaccharides was already shown by Leloir *et al.* in 1961. In those experiments it was shown that adding malto-oligosaccharides to the *in vitro* synthesis mixture yielded soluble products with small extensions. These results were interpreted as competition between the added malto-oligosaccharides and the native primer present within the granules. Incubation of isolated pea leaf starch granules with ADP-Glc and maltotriose showed an increase in the total label incorporated, which was found mainly in the amylose fraction (Denyer *et al.* 1996). This effect of malto-oligosaccharides on the incorporation of label in amylopectin and amylose was also shown with *in vitro* experiments performed with *Chlamydomonas* starch (Chapter 2). Denyer *et al.* (1996) suggested that these malto-oligosaccharides, which are lost during starch isolation, are required for amylose synthesis as they are used as acceptor substrate by GBSSI. However, in the earlier performed *in vitro* experiments it was shown that significant amounts of label were incorporated in amylose even if no malto-oligosaccharides were added, indicating that malto-oligosaccharides can be elongated to form amylose, but are not required for amylose synthesis.

Three models explaining amylose biosynthesis,

Based on the results obtained from the *in vitro* experiments three models explaining the amylose synthesis within the starch granule by GBSSI were presented (Chapter 2). Model A showed amylose synthesis with malto-oligosaccharides as acceptor substrate for GBSSI, while models B and C showed the amylopectin-primed amylose biosynthesis. The difference between model B and C is the enzyme catalysing the cleavage of the elongated

chains from amylopectin. In model B cleavage is mediated by branching enzyme or a hydrolytic enzyme present within the starch granule. In model C hydrolysis occurs within GBSSI. In this model the assumption was made that GBSSI had a dual, both a polymerase and a hydrolase, activity.

Malto-oligosaccharide- versus amylopectin-primed amylose synthesis

When soluble extracts from pea embryos or potato tubers were added to the *in vitro* mixture the label incorporated in amylose increased while there was no decrease in label incorporated in the amylopectin fraction (Denyer *et al.*, 1996). The factors influencing the incorporation in these extracts were believed to be malto-oligosaccharides as they were heat-stable and α-glucosidase degradable. The fact that the label incorporated in amylopectin did not decrease indicated that there was no competition between the added malto-oligosaccharides and the amylopectin. The total label incorporated increased by adding an extra soluble acceptor substrate as the elongation of malto-oligosaccharides and amylopectin occurred side by side. Pulse-chase experiments performed with *Chlamydomonas* starch in the presence of maltotriose showed that low concentrations of malto-oligosaccharides did not affect the transfer of label (Chapter 3) from amylopectin to amylose. This also indicated that malto-oligosaccharide- and amylopectin-primed amylose synthesis could take place at the same time and that there was no competition between amylopectin- and malto-oligosaccharide-primed amylose synthesis.

If GBSSI molecules, which are present in the starch granule but which do not participate in the amylopectin-primed amylose synthesis, start synthesising amylose only after adding an extra acceptor substrate like malto-oligosaccharides then there will be no competition between the amylopectin and malto-oligosaccharide-primed amylose synthesis. A prerequisite is that the ADP-Glc concentration is sufficient for both amylopectin- and malto-oligosaccharide-primed amylose biosynthesis. GBSSI molecules, which have no access to the non-reducing ends of amylopectin and can therefore be referred to as latently present GBSSI molecules, could be responsible for the malto-oligosaccharide-primed amylose synthesis (Fig. 2).

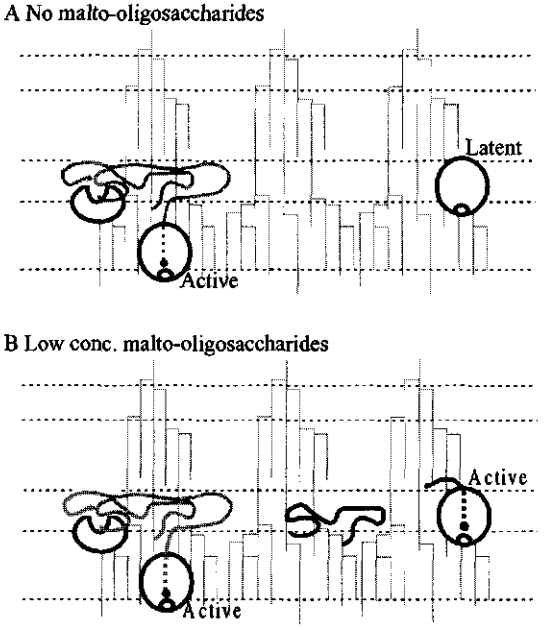


Fig. 2 Schematic representation of the GBSSI activity in the absence (A) and presence (B) of low concentrations of malto-oligosaccharides. GBSSI is displayed as a tunnel-shaped enzyme, whereas branching enzyme is drawn as a moon-shaped structure. Amylopectin is shown as a light grey line, whereas amylose and malto-oligosaccharides are shown as black lines. In the absence of malto-oligosaccharides (A) only GBSSI molecules, which have access to the non-reducing ends of amylopectin will participate in amylose synthesis. If low concentrations of malto-oligosaccharides are present (B) there is no competition between the amylopectin-primed amylose synthesis and the malto-oligosaccharide-primed amylose synthesis. GBSSI molecules, which were inactive in the absence of malto-oligosaccharides become active in the presence.

However, competition between amylopectin and malto-oligosaccharides as acceptor substrate for amylose biosynthesis has been shown in several *in vitro* experiments. Leloir *et al.* (1961) already suggested that there was competition between the added malto-oligosaccharides and the native primer present within the starch granules. Incubation of 500 µg *Chlamydomonas* starch with 3.2 mM ADP-Glc and 50 mM maltotriose resulted in an 2 – 3 times increase of the total label incorporated, while the label incorporated in amylopectin showed a 3-fold reduction as compared to *in vitro* experiments where no maltotriose was added (Chapter 2). Competition was also shown by incubating starch granules with varying concentrations of ADP-Glc and maltotriose (Chapter 2). At low concentrations of ADP-Glc the label incorporated in starch even decreased with increasing maltotriose concentrations, as the short oligosaccharides, which are elongated with only a

few glucose molecules, escape from the starch granule and are not precipitated with the starch. Moreover, in the presence of high concentrations of maltotriose during the chase, less label was transferred from amylopectin to amylose (Chapter 3).

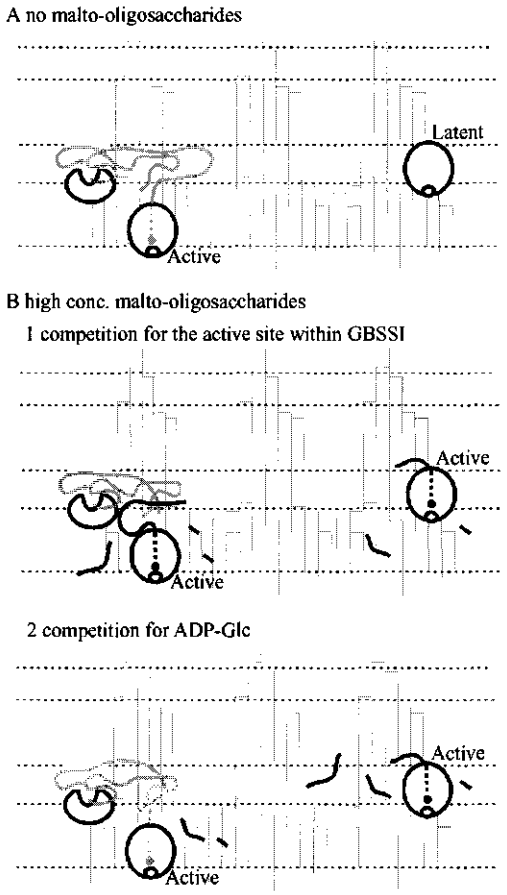


Fig. 3 Schematic representation of the GBSSI activity in the absence (A) and presence (B) of high concentrations of malto-oligosaccharides. GBSSI is displayed as a tunnel-shaped enzyme, whereas branching enzyme is drawn as a moon-shaped structure. Amylopectin is shown as a light grey line, whereas amylose and malto-oligosaccharides are shown as black lines. In the absence of malto-oligosaccharides (A) only GBSSI molecules, which have access to the non-reducing ends of amylopectin will participate in amylose synthesis. At high concentrations of malto-oligosaccharides (B) competition between amylopectin- and malto-oligosaccharide-primed amylose synthesis will occur. This competition can be either due to competition for the active site within GBSSI (B-1) or due to competition for ADP-Glc (B-2). If malto-oligoasaccharides compete for the active site within GBSSI (B-1) then the amylopectin chain will not be further elongated and therefore not released as amylose. If there is competition between the amylopectin- and malto-oligosaccharide-primed amylose synthesis then the amylopectin elongation will be slowed down.

From these results it can be concluded that if the GBSSI activity or the ADP-Glc concentration is the limiting factor in amylose synthesis than there will be a competition between amylopectin and malto-oligosaccharides as acceptor substrate (Fig. 3). This could either be due to competition for the active site within GBSSI (Fig. 3B-1) or due to competition for ADP-Glc, the donor substrate for amylose biosynthesis (Fig. 3B-2).

The ADP-Glc concentration is the rate-limiting factor for *in vivo* amylose biosynthesis, which was shown by the analysis of different mutants and transgenic plants. Therefore competition between amylopectin- and malto-oligosaccharides-primed amylose synthesis *in vivo* is likely and dependent on the concentrations of the two acceptor substrates.

In Chapter 2 we have estimated the concentration of non-reducing ends of amylopectin within the starch granule at 450 mM. Although GBSSI is immobilised within the granule and can therefore not use the full potential of non-reducing termini this concentration probably exceeds the concentration of malto-oligosaccharide non-reducing termini. Moreover, label was found incorporated in amylopectin even if maltotriose was added to 50 mM (Chapter 2) or 100 mM (Denyer *et al.*, 1996). Therefore amylopectin-primed amylose synthesis probably forms the basis for amylose biosynthesis *in vivo*.

As amylopectin- and malto-oligosaccharide-primed amylose synthesis can occur side by side, malto-oligosaccharide-primed amylose synthesis may occur *in vivo* if high concentrations of malto-oligosaccharides are formed. At an ADP-Glc concentration of 3.2 mM we showed that the available amount of acceptor substrate inside the starch granules was limiting as the GBSSI activity increased by adding a diffusable acceptor substrate such as maltotriose (Chapter 2). When no maltotriose was added to the *in vitro* mixture containing 500 µg starch, ADP-Glc was the limiting factor at a concentration lower than 0.5 mM. In comparison, at a 10 mM maltotriose concentration the ADP-Glc was the limiting factor at a concentration lower than approximately 2.0 mM. So determining the ADP-Glc and malto-oligosaccharide concentrations will give insight in the impact of amylopectin and malto-oligosaccharides-primed amylose synthesis *in vivo*.

Cleavage of the elongated chain: GBSSI versus branching enzyme or hydrolytic enzymes entrapped in the starch granule

Amylose formation by elongation of and cleavage from amylopectin requires a hydrolytic activity inside the starch granule, where amylose is formed. As branching enzymes are found among the minor proteins associated with the starch granule, these might mediate this cleavage through an intra-molecular transglycosylation (amylose synthesis model B, Chapter 2). Amylose formed by an intra-molecular transfer catalysed by

branching enzyme does not contain a reducing end and is cyclic at the point of cleavage. As the number of amylose molecules is more or less in agreement with the number of reducing ends documented for amylose it is not very likely that branching enzyme is the main enzyme responsible for the release of the elongated chain. Other hydrolytic enzymes, which are bound to or entrapped in starch granules such as α-amylases, might also account for the cleavage of the elongated glucan chain from amylopectin.

In the amylose synthesis model C (Chapter 2) the assumption was made that GBSSI had a dual activity, both a synthase (or polymerase) and a hydrolase activity. Such a dual activity has been observed before for example in the Klenow fragment of DNA polymerase of *Escherichia coli* (Blanco *et al.*, 1991). The advantage of this model is the occurrence of a hydrolytic cleavage very near the site of synthesis within GBSSI, thereby providing the enzyme with an acceptor substrate for the next round of amylose synthesis. Cleavage by another hydrolytic enzyme far away from GBSSI implies that the released non-reducing end of amylopectin is not easily available and that the next round of synthesis will depend on the availability of another non-reducing end. Multiple rounds of amylose synthesis are likely, as we have calculated a 1:18 ratio of GBSSI to amylose molecules (Chapter 2).

Whether amylose biosynthesis proceeds via mechanism B or C requires a more detailed characterisation of GBSSI and the reaction catalysed. As all mutants, that have been described so far, containing amylose-free starch have been identified as containing a disruption in GBSSI we might speculate that model C is the most likely. If other enzymes were also involved in amylose synthesis we would have expected to find amylose-free mutants affected in another gene than GBSSI. However, it is possible that the hydrolytic activity is not restricted to one enzyme and therefore no other mutants are found. The results obtained from the different pulse-chase experiments on the other hand indicate that model B is more likely. The relative large amounts of label still incorporated in amylopectin after 6 h chase (Chapter 2 and 3) point out to cleavage of the elongated chain away from the site of synthesis. If the chain is cleaved near the site of synthesis according to the amylose synthesis model C we would have expected a total transfer of label from amylopectin to amylose after 6 h chase. However, it is possible that some chains never reach the critical length for chain cleavage and are thereby responsible for the label in amylopectin after the chase. A more thorough understanding of the GBSSI activity is required to give a definite explanation on the mechanism of amylose synthesis.

CONTROLLING AMYLOSE CONTENT IN POTATO

Effect of allelic composition on GBSSI activity and amylose content,

Using conventional breeding methods with the aim to control starch content and composition in potato requires the understanding of the genetic control of amylose synthesis. In a heterozygous autopolyploid species like potato multiple allelism is common, not only for morphological characteristics but also for genes involved in metabolic pathways like starch synthesis. It had been established for some time that GBSSI was required for amylose synthesis; however, the genetics behind amylose content in potato was still unclear. Although multiple alleles of GBSSI had been characterised no differential effect on amylose content was demonstrated except for the mutant *amf* allele (Jacobsen *et al.*, 1989). The mutant *amf* allele contains a point mutation in the GBSSI transit peptide coding sequence, and does therefore not contribute to amylose biosynthesis. In addition to this mutant allele three complete *wild type* sequences have been reported, while five sequences of the promoter region were published (Dai *et al.*, 1996; Hofvander *et al.*, 1992; van der Leij *et al.*, 1991b; Rohde *et al.*, 1990). Based on these sequences a PCR and Southern based marker test was developed to readily identify the different alleles (Chapter 4). At least eight alleles could be identified, which were grouped into four classes, based on Southern analysis and subclassified by the results from PCR (Chapter 4).

A large population of *Solanum tuberosum* cultivars and *Solanum* breeding lines was tested for their GBSSI allelic composition, their GBSSI activity and amylose content (Chapter 4). Within this population it was possible to assign variation in GBSSI activity to the GBSSI allelic composition. The major difference between allele class A1 on one hand and allele classes A2, A3 and A4 on the other hand is the absence of a 140 bp fragment, 0.5 kb upstream of the ATG start codon, which was absent in alleles belonging to the A1 class. The absence of this fragment had a major effect on GBSSI activity, while the presence of small bp deletions and simple sequence repeats in both the 5' and 3' ends led to no apparent effects.

The effect of this 140 bp fragment, located within the GBSSI promoter, could not be observed by studying the promoter activities in a promoter-GUS assay. Expression studies of the GBSSI promoter sequences G1 (belonging to the A1 class) and G28 (belonging to the A2 class) fused to the beta-glucuronidase (GUS) reporter gene revealed no significant variation in GUS activity and expression pattern (Rohde *et al.*, 1990). This indicated that promoter-reporter gene studies are only limited informative in showing fluctuations in gene expression.

As the 140 bp fragment, which showed a major effect on GBSSI activity is located within the promoter region of GBSSI it is likely that regulation of expression is involved in

the effect of allelic composition on GBSSI activity. However, no effect of GBSSI allelic composition on the amount of GBSSI RNA and protein could be demonstrated. Either the detection methods for GBSSI RNA and protein are not sensitive enough or GBSSI proteins encoded by one allele are more active at the time of amylose synthesis then proteins encoded by another allele. As the variation in GBSSI activity ranged from 16 to 46 pmol/min/mg starch we would have expected to detect such a variation both at the RNA and protein level. This indicated that only part of the GBSSI RNA was translated into an active protein.

No effect of GBSSI allelic composition on amylose content could be demonstrated within the population of *Solanum tuberosum* cultivars and *Solanum* breeding lines. By crossing the *amf* mutant to selected diploid and tetraploid clones an effect of allelic composition on amylose content could be shown. Moreover, a positive correlation between GBSSI activity and amylose content was determined. Three factors affecting the amylose content are the concentrations of acceptor (amylopectin or malto-oligosaccharides) and donor substrate (ADP-Glc) and the GBSSI activity. If the acceptor or donor substrate is the limiting factor for amylose biosynthesis the effect of GBSSI activity and therefore the effect of allelic composition on amylose content will be less clear.

Crossing of selected diploid and tetraploid clones to the *amf* mutant resulted in a reduction of the variability in the genetic background. If the genetic background is more homogenous then the effect of GBSSI allelic composition on amylose content will be better visible. Moreover, by crossing *wild type* diploid and tetraploid clones to the *amf* mutant only half of the alleles are *wild type* and do therefore contribute to amylose synthesis. A reduction in the number of *wild type* alleles might result in a clearer effect of allelic composition on amylose content as GBSSI activity becomes the rate limiting factor in amylose synthesis.

In a gene dosage population obtained by crossing two duplex plants (Flipse *et al.*, 1996a) it was shown that there was a dosage effect for the *wild type* GBSSI genes on amylose content, which was not linear. The amylose content increased with an increasing number of *wild type* GBSSI genes from the nulliplex, simplex and duplex plants. However, the amylose content was similar for the duplex and the triplex/quadruplex group. Increasing the number of *wild type* GBSSI genes above two within the progeny of this cross did not further increase the amylose content, indicating that the number of *wild type* GBSSI genes was no longer the limiting factor for amylose content. Transformation of the potato *amf* mutant with cassava or potato / cassava hybrid constructs containing the GBSSI gene never resulted in full restoration of the amylose content, although full phenotypic complementation could be obtained with the hybrid construct (Salehuzzaman *et al.*, 1999).

Moreover, *wild type* levels of GBSSI activity were measured in these transformants, indicating that GBSSI from potato and cassava have different intrinsic properties. As GBSSI from potato and cassava have different intrinsic properties, GBSSI becomes the rate limiting factor in amylose biosynthesis causing the incomplete restoration.

Crossing tetraploid *wild type* clones to the *amf* mutant, two out of four alleles are *wild type*, which causes the reduction of GBSSI to a level where it becomes limiting. Within the progeny of the crosses it was possible to find a linear correlation between GBSSI activity and amylose content, while no such correlation could be found within the population of *Solanum tuberosum* cultivars and *Solanum* breeding lines. If GBSSI activity is the rate-limiting factor in amylose biosynthesis then there will be a positive correlation between GBSSI activity and amylose content and a visible effect of allelic composition on amylose content can be observed.

Within the population of *Solanum tuberosum* cultivars and *Solanum* breeding lines all clones analysed contained at least one copy of an allele belonging to the A1 class (Chapter 4), which showed the highest GBSSI activity. Therefore it is not clear whether clones containing four copies of *wild type* alleles belonging to either the A2, A3 or A4 class might still be limited in their GBSSI activity and thereby showing an effect of allelic composition on amylose content. The production of homozygous plants for one allele (A1A1A1A1, A2A2A2A2, A3A3A3A3 or A4A4A4A4) could directly give answers to this question. To come so far is in potato however time consuming.

Effect of allelic composition on antisense inhibition,

Amylose-free potato cultivars can be obtained by inhibition of GBSSI expression after introduction of an antisense GBSSI cDNA. A major drawback of the use of antisense technology is the variation in inhibition of the target gene after introduction of the antisense gene. Factors concerning the variety (ploidy level, strength of the endogenous alleles, homology with the antisense construct and susceptibility to antisense inhibition), the transgene construct (construct composition and promoter characteristics) and the expression of the transgene (T-DNA copy-number, T-DNA position effects, repetitiveness of the insert and DNA methylation) can cause large variation in amylose content both within and between varieties after transformation (Fig. 4). Moreover, factors influencing antisense inhibition may interact with each other. For example construct composition, promoter characteristics of the construct and repetitiveness of the insert may influence DNA methylation, while DNA methylation may influence the susceptibility of endogenous alleles to antisense inhibition.

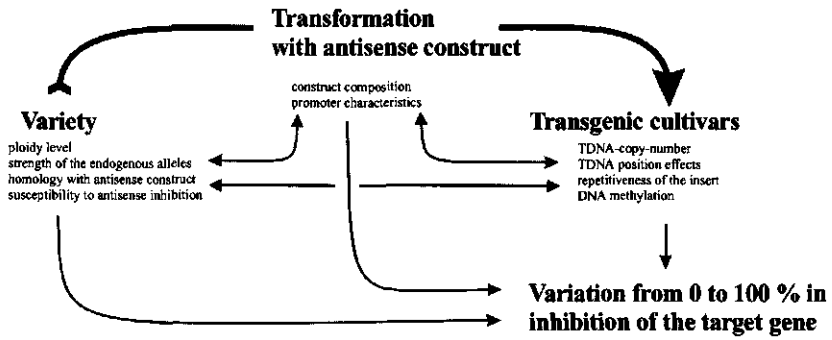


Fig. 4 Factors influencing antisense inhibition in transgenic plants.

The influence of T-DNA copy-number, repetitiveness of the transgene insert, DNA methylation and promoter characteristics of the antisense construct have been described by Kuipers *et al.* (1995), Stam *et al.* (1997) and others. The effect of the variety on antisense inhibition is described in Chapter 5. To study the effect of allelic composition on antisense inhibition a large number of transgenics was isolated per variety and the allelic composition of these varieties was determined by Southern analysis. The transformed clones were classified for starch composition by iodine staining of the starch granules. In the presence of reduced amounts of amylose the starch granule stained red with a blue core of varying size (Kuipers *et al.*, 1994a). Based on the size of this blue core the transformed clones were classified as rk (blue core < 10 %), rm (blue core 10 – 50 %), rg (blue granules with red outer layer) or b (completely blue granules). As the size of the blue core was found to be closely related to the amylose content (Kuipers *et al.*, 1994a) the size was used to determine the average amylose content. Both the percentage of rk-clones, indicating complete or nearly complete inhibition of GBSSI and the average amylose content were used to express the level of antisense inhibition. A positive effect of allele A1 on the percentage of rk clones and a negative effect on the average amylose content were proven significant (Chapter 5).

Different factors concerning the variety and more specifically the effect of allelic composition on antisense inhibition are ploidy level, strength of the endogenous alleles, homology of the endogenous alleles with the antisense construct and level of susceptibility of the endogenous alleles to DNA methylation and antisense inhibition. A positive dosage effect of allele A1 on GBSSI activity and amylose content was proven for non-transformed clones (Chapter 4). The amylose content in A1A1A1A1 varieties transformed with an antisense GBSSI construct would be relatively high as compared to varieties with less A1

alleles if the strength of the endogenous alleles determined the effect of allelic composition on antisense inhibition. However, these varieties showed a low amylose content after transformation indicating that the effect of allelic composition on antisense inhibition can not be explained by the strength of the endogenous alleles.

Homology between the endogenous alleles and the antisense construct and more specifically between the endogenous GBSSI promoter and the promoter used to express the transgene is more likely to be responsible for the specific effect of allelic composition on antisense inhibition. Comparing the effectiveness of inhibition between GBSSI antisense constructs driven by the GBSSI promoter and those driven by the 35S CaMV promoter it was shown that there was an effect of the construct promoter on antisense inhibition (Kuipers *et al.*, 1995). The level of susceptibility to DNA methylation and antisense inhibition of the endogenous alleles can however not be ruled out as a factor influencing the effect of allelic composition on antisense inhibition. Susceptibility to DNA methylation and antisense inhibition might very well be regulated at the 5' promoter or 3' end, where most of the differences between the alleles were found. By transformation of different varieties with GBSSI antisense constructs driven by the A2, A3 or A4 GBSSI promoter it would be possible to discriminate more directly between the effect of homology to the construct promoter and susceptibility of the endogenous alleles to antisense inhibition.

AMYLOSE BIOSYNTHESIS IN CONCLUSION

From the *in vitro* experiments it was concluded that amylopectin was used as acceptor substrate for amylose biosynthesis. This implies that amylose is formed downstream of amylopectin. Indications on the formation of amylose downstream of amylopectin synthesis were also found by Kuipers *et al.* (1994b) and Tatge *et al.* (1999) who showed that the synthesis of amylose occurs within the matrix of the starch granule. The results obtained from the *in vitro* experiments performed with varying concentrations of ADP-Glc and maltotriose and the calculations made to estimate the concentration of non-reducing ends from amylopectin indicated that amylopectin-primed amylose biosynthesis formed the basis for amylose biosynthesis *in vivo*. Based on these results a new scheme displaying the final steps of amylopectin and amylose synthesis can be drawn up. (Fig. 5).

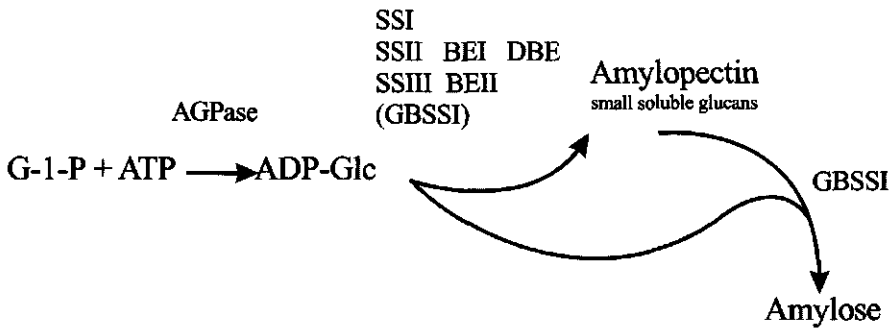


Fig. 5 Amylose and amylopectin biosynthesis. Amylopectin is formed by the combined action of starch synthases, starch branching enzymes and debranching enzyme. GBSSI plays no or only a minor role in amylopectin synthesis. GBSSI uses amylopectin as the acceptor substrate to form amylose. Amylose is formed downstream of amylopectin. Small soluble glucans can also be elongated by GBSSI to form amylose. ADP-Glc is the donor substrate for both amylopectin and amylose biosynthesis.

If malto-oligosaccharides are formed these can be elongated by GBSSI, thereby reducing the concentration of small oligosaccharides in the cell. In this way GBSSI uses the products from amylopectin synthesis, resulting in a reduction of the osmotic value and a clearance of the cell. In tetraploid potato the GBSSI activity was present in abundance. Only after reduction of the number of *wild type* alleles an effect of allelic composition and GBSSI activity on amylose content could be proven significant. Amylose biosynthesis is therefore an efficient system using the products from amylopectin synthesis, thereby filling the available spaces within the amylopectin matrix. As the Km of GBSSI for ADP-Glc is much higher than that of the soluble starch synthases only small effects of the presence or absence of amylose biosynthesis on amylopectin synthesis should be expected, as was shown by the low reduction in yield in mutants containing no amylose.

SUMMARY

Besides the role of starch as the primary source of calories in both human and animal diet, it is used as raw material for industrial application such as the paper industry, textile industry, chemical industry, and pharmaceutical industry. Starch isolated for industrial applications, is mainly derived from corn. However in Europe a significant proportion of starch is isolated from potato tubers. Starch consists of two glucose polymers, amylose, which is essentially linear and amylopectin, which is highly branched. Industrially important properties, which are responsible for the functional quality of starch, are highly affected by the ratio of amylose to amylopectin. Therefore, different strategies have been applied to obtain starch with altered amylose content. Mutations leading to the selective loss of amylose have been described in many species, including potato. From the synthesis of amylose-free starch in mutants lacking granule-bound starch synthase I (GBSSI) it is clear that GBSSI is responsible for amylose biosynthesis.

The goal of this thesis research was to unravel the amylose biosynthesis in potato and to determine the role and regulation of GBSSI in this process. Therefore amylose biosynthesis was studied *in vitro* and *in vivo*. GBSSI, like other starch synthases, catalyses the transfer of a glucose residue from ADP-Glc (donor substrate) to the growing α-1,4 linked glucan (acceptor substrate). This reaction can be studied *in vitro* (Chapter 2 and 3) by the incubation of purified starch granules, containing the active GBSSI, with radio-labelled ADP-Glc. In this thesis we show a new mechanism for amylose biosynthesis, using amylopectin as acceptor substrate. Transfer of chains from amylopectin to amylose was evidenced from pulse-chase experiments performed with starch isolated from *Chlamydomonas wild type* strain 137C and mutant strain I7 (defective for the large subunit of AGPase) (Chapter 2). Starch isolated from I7 contained very little to no amylose, despite the presence of *wild type* GBSSI.

As amylopectin-primed amylose synthesis was proven for *Chlamydomonas, in vitro* experiments were performed to determine whether it also occurred in higher plants. *In vitro* experiments performed with starch isolated from higher plants were compared to those performed with *Chlamydomonas* starch. Despite the fact that GBSSI activities in plant starches were 15 to 100-fold lower than the GBSSI activity in *wild type Chlamydomonas* starch, chase experiments performed with plant starches showed small but consistent decreases in label previously incorporated in amylopectin during the pulse. These decreases led to increases in label incorporated in amylose, which were proportional to the GBSSI activities of these starches. Based on these results we postulate that the results on

amylopectin-primed amylose synthesis, obtained with *Chlamydomonas* starch, can be extrapolated to higher plants.

Other acceptor substrates of GBSSI for amylose biosynthesis are malto-oligosaccharides. The effect of malto-oligosaccharides on the incorporation of label in amylopectin and amylose was shown with *in vitro* experiments performed with *Chlamydomonas* starch (Chapter 2 and 3). Based on these results obtained from the *in vitro* experiments performed with varying concentrations of ADP-Glc and maltotriose it is shown that amylopectin- and malto-oligosaccharide-primed amylose synthesis can occur side by side. However, as ADP-Glc concentration is the rate-limiting factor for *in vivo* amylose biosynthesis, competition between amylopectin- and malto-oligosaccharide-primed amylose synthesis *in vivo* is likely. Malto-oligosaccharide-primed amylose synthesis may occur *in vivo* if high concentrations of malto-oligosaccharides are formed. However, calculations made to estimate the concentration of non-reducing ends from amylopectin indicated that amylopectin-primed amylose biosynthesis formed the basis for amylose biosynthesis *in vivo*.

From the *in vitro* experiments, it can be concluded that amylose is formed downstream of amylopectin. Amylose biosynthesis is an efficient system using amylopectin itself, or side-products from amylopectin synthesis, thereby reducing the osmotic pressure and filling the available spaces within the amylopectin matrix. The availability of acceptor substrate (amylopectin and malto-oligosaccharides) and donor substrate (ADP-Glc) influence the mode of amylose biosynthesis.

To alter the amylose content within the plant, different strategies can be applied. Subtle changes in amylose content, can be obtained by classical breeding. These relatively small changes may significantly improve quality as was shown by the effect of GBSSI on amylose content and noodle quality in wheat. Although it is clear that GBSSI determines whether or not amylose is formed, the genetics behind amylose content in potato still needed to be unravelled. The effect of allelic composition on GBSSI activity and amylose content was demonstrated for rice, wheat and foxtail millet. The inheritance of amylose content in potato is, however, complicated due to the heterozygosity and the tetraploid level of potato.

In potato three complete *wild type* sequences have been reported, while five sequences of the promoter region were published. Based on these sequences a PCR and Southern-based marker test were developed to readily identify the different alleles (Chapter 4). Within a population of *Solanum tuberosum* cultivars and *Solanum* breeding lines it was possible to assign variation in GBSSI activity to the GBSSI allelic composition. By crossing the amylose-free potato mutant (*amf*) with selected diploid and tetraploid clones an

effect of GBSSI allelic composition on amylose content (10 –15 %, versus 20 % in *wild type* potato) could be shown. The major difference between the alleles identified was the absence or presence of a 140 bp fragment, 0.5 kb upstream of the ATG start codon. The absence of this fragment, in alleles classified as A1, had a positive effect on GBSSI activity and amylose content. As this fragment was located within the promoter region of GBSSI it is likely that regulation of expression is involved in the effect of allelic composition on GBSSI activity.

The effect of allelic composition on antisense inhibition was tested in Chapter 5. Amylose-free potato starch can be obtained by suppression of GBSSI using antisense technology. As an antisense gene can act as a dominant suppressor of the endogenous genes, amylose-free cultivars can be obtained directly by *Agrobacterium*-mediated transformation of existing varieties. For the transformation constructs were used containing a GBSSI A1 cDNA in antisense orientation behind a A1 GBSSI promoter. A significant effect of allelic composition on the level of antisense inhibition was shown after analysing a large number of transgenics per variety. The allelic composition was determined by Southern analysis and the transformants were classified for starch composition by iodine staining of the starch granules. Starch containing both amylose and amylopectin stains blue while starch containing only amylopectin stains red. Both the percentage of red colouring (rk) clones, indicating complete or nearly complete inhibition of GBSSI and the average amylose content were used to express the level of antisense inhibition. As varieties containing multiple alleles belonging to the A1 class showed the highest antisense effect, it was postulated that homology between the endogenous alleles and the antisense construct, and more specifically between the endogenous GBSSI promoter and the promoter used to express the transgene, is more important for the specific effect of allelic composition on antisense inhibition than strength of the endogenous alleles.

SAMENVATTING

Zetmeel is de belangrijkste bron van calorieën voor zowel mensen als dieren. Bovendien wordt zetmeel veelvuldig gebruikt als grondstof voor o.a. de papier- textiel- olie- en farmaceutische-industrie. Zetmeel wordt voornamelijk geïsoleerd uit maïs, maar in Europa is aardappel ook een belangrijke bron voor de isolatie van zetmeel. Zetmeel bestaat uit twee hoofdbestanddelen, amylose en amylopectine. De eigenschappen van zetmeel, die voor een groot deel haar toepassingmogelijkheden bepalen, worden sterk beïnvloed door de amylose:amylopectine verhouding. Verschillende strategieën worden daarom toegepast om zetmeel te produceren met een gewijzigde samenstelling. Mutaties die leiden tot de produktie van amylose-vrij zetmeel zijn in een groot aantal gewassen, waaronder aardappel, beschreven. Deze mutanten hebben een defect in het korrel gebonden zetmeel synthase (kgz), wat er op duidt dat kgz verantwoordelijk is voor de produktie van amylose.

Het doel van het onderzoek beschreven in dit proefschrift is de opheldering van de amylose biosynthese in aardappel en meer specifiek de rol en regulatie van kgz in dit proces. Kgz is verantwoordelijk voor de overdracht van een glucose eenheid van ADP-Glc (donor substraat) naar de groeiende glucaan keten (acceptor substraat). Door zetmeel korrels met radioactief gelabeld ADP-Glc te incuberen kan deze reactie *in vitro* bestudeerd worden (Hoofdstuk 2 en 3). Het acceptor substraat voor amylose biosynthese was niet bekend. In dit proefschrift is aangetoond dat amylose gevormd wordt na afsplitsing van een verlengde amylopectine keten. Met de resultaten van *pulse-chase* experimenten uitgevoerd met zetmeel geïsoleerd uit een wild type en mutante groenalg (*Chlamydomonas*) is aangetoond dat ketens van amylopectine overgedragen worden naar amylose (Hoofdstuk 2). Uit deze resultaten kan geconcludeerd worden dat amylopectine als acceptor substraat voor amylose synthese kan dienen.

Amylopectine als acceptor substraat voor amylose synthese in hogere planten is aangetoond in hoofdstuk 3. *In vitro* experimenten met zetmeel uit hogere planten zijn vergeleken met die uitgevoerd met *Chlamydomonas* zetmeel. Ondanks het feit dat de kgz-activiteit van zetmeel geïsoleerd uit hogere planten veel lager is dan die van *Chlamydomonas,* kon ook in hogere planten een overdracht van ketens van amylopectine naar amylose aangetoond worden.

Een ander acceptor substraat voor kgz zijn malto-oligosacchariden. Het effect van malto-oligosaccharides op de incorporatie van label in amylopectine en amylose is aangetoond met *in vitro* experimenten uitgevoerd met *Chlamydomonas* zetmeel. (Hoofdstuk 2). Gebaseerd op de resultaten uit *in vitro* experimenten met variërende

concentraties ADP-Glc en maltotriose kan geconcludeerd worden dat synthese van amylose met amylopectine en malto-oligosaccharides als acceptor substraat naast elkaar kan voorkomen. De ADP-Glc concentratie is echter de limiterende factor bij amylose biosynthese *in vivo*, waardoor er een competitie ontstaat tussen de twee acceptor substraten. Malto-oligosaccharides worden gebruikt als acceptor substraat wanneer deze in hoge concentraties in de cel aanwezig zijn. Berekeningen, uitgevoerd om de concentratie van amylopectine uiteinden te bepalen, laten zien dat amylopectine het belangrijkste acceptor substraat voor amylose biosynthese is.

Uit de *in vitro* experimenten kan geconcludeerd worden dat amylose synthese plaats vindt na amylopectine synthese. Amylose biosynthese is een efficiënt systeem dat de produkten van amylopectine synthese gebruikt waardoor de osmotische druk afneemt en de beschikbare ruimtes in het amylopectine opgevuld worden. De beschikbaarheid van acceptor (amylopectine en malto-oligosaccharides) en donor substraat (ADP-Glc) beïnvloeden de manier waarop het amylose gevormd wordt.

Om het amylosegehalte in de plant te wijzigen kunnen verschillende technieken toegepast worden. Relatief kleine veranderingen kunnen verkregen worden door gebruik te maken van klassieke veredelingstechnieken. Deze kleine veranderingen in het amylosegehalte kunnen een grote invloed hebben op de kwaliteit van het zetmeel. Dit is onder andere aangetoond bij tarwe. De genetica van het amylosegehalte in aardappel was echter nog niet opgehelderd. Een effect van allelcompositie op kgz-activiteit en amylosegehalte is eerder beschreven voor rijst, tarwe en gierst. De overerving van het amylosegehalte in aardappel is echter gecompliceerd door de heterozygotie en het tetraploïde karakter van aardappel.

Drie volledige sequenties van het aardappel kgz-gen zijn gepubliceerd. Bovendien zijn vijf sequenties van kgz-promoter beschreven. Gebaseerd op deze sequenties is met behulp van PCR en restrictie-analyse een merkertest ontwikkeld waarmee op een snelle en eenvoudige wijze de verschillende allelen (sequenties) onderscheiden konden worden. Binnen een populatie van aardappel (*Solanum tuberosum*) cultivars en veredelingslijnen was het mogelijk om de variatie in kgz-activiteit (23 – 35 pmol/min/mg zetmeel) te relateren aan de kgz-allelcompositie. Door het maken van gerichte kruisingen tussen een amylose-vrije (*amf*) aardappel en wild type aardappel was het bovendien mogelijk om een significant effect van kgz-allelcompositie op het amylosegehalte (10 – 15 %; in verhouding tot 20 % in *wild type* aardappel) aan te tonen. Het belangrijkste verschil tussen de allelen was de aan- of afwezigheid van een 140 bp fragment, 0.5 kb voor het ATG start codon. De afwezigheid van dit fragment, in allelen geclassificeerd als A1, had een positief effect op de kgz-activiteit en het amylosegehalte. Aangezien dit fragment ligt in de kgz-promoter is het

waarschijnlijk dat regulatie van expressie van het kgz-gen verantwoordelijk is voor de gevonden effecten.

Amylose-vrij aardappel zetmeel kan verkregen worden door de toepassing van antisense technieken. Aangezien een antisense gen functioneert als een dominante onderdrukker van het endogene gen, kunnen amylose-vrije cultivars direct verkregen worden door transformatie van bestaande variëteiten met behulp van *Agrobacterium*. Ter vergelijking, vier kopieën van het recessieve mutante kgz-allel zijn nodig om een amylose-vrije aardappel te verkrijgen met behulp van de klassieke veredeling. Het verkrijgen van amylose-vrije variëteiten met deze laatste methode vergt daardoor veel meer tijd. De mate van antisense remming is onder andere afhankelijk van de variëteit die gebruikt wordt voor de transformatie. Analyse van een groot aantal transformanten per variëteit heeft aangetoond dat er een significant effect van allelcompositie op antisense remming bestaat. De allelcompositie van de transformanten werd bepaald met behulp van restrictie analyses en de zetmeel korrels zijn gekleurd met jodium om de zetmeel samenstelling te bepalen. Zetmeel dat zowel amylose als amylopectine bevat kleurt blauw, terwijl amylose-vrij zetmeel rood kleurt. Het percentage rood kleurende transformanten per variëteit en het gemiddelde amylosegehalte zijn gebruikt als standaarden om de antisense remming weer te geven. Beide werden significant beïnvloed door de allelcompositie.

Dit effect van allelcompositie op antisense remming kan verklaard worden door meerdere factoren. Ten eerste de sterkte van het endogene allel dat onderdrukt moet worden. Ten tweede de mate van homologie tussen antisense allel en het endogene allel. En ten derde de gevoeligheid van verschillende endogene allelen voor antisense remming. Gebaseerd op de resultaten verkregen uit de verschillende experimenten kan geconcludeerd worden dat homologie tussen het endogene allel, en in het bijzonder de endogene promoter en het antisense allel waarschijnlijk het meest bepalend is voor het effect van allelcompositie op antisense remming.

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CURRICULUM VITAE

Marion van de Wal werd geboren op 17 juli 1971 te Oirschot. In 1990 behaalde zij haar VWO diploma aan het Jacob Roelands Lyceum te Boxtel, waarna zij Plantenveredeling ging studeren aan de Landbouwuniversiteit Wageningen. Tijdens deze studie werden twee afstudeervakken en een stage voltooid gecoördineerd via de vakgroepen Plantenveredeling (Prof R. Visser) en Fytopathologie (Prof. P. de Wit). De stage vond plaats aan de Universiteit van Californië in het laboratorium van Prof. B. Staskawicz. In mei 1995 begon zij als AIO bij de vakgroep Plantenveredeling (onderzoeksschool Experimentele Plantenwetenschappen) aan een door ABON gefinancierd project. Dit onderzoek heeft geleid tot dit proefschrift. Sinds mei 1999 is ze als toegevoegd onderzoeker werkzaam bij het Laboratorium voor Virologie (Wageningen Universiteit) en verricht ze onderzoek naar de replicatie van TSWV. Vanaf 1 oktober 2000 zal Marion als moleculair bioloog werkzaam zijn bij het veredelingsbedrijf Nunhems.