Modification of potato starch composition by introduction and expression of bacterial branching enzyme genes.

Promoter:	Dr. Ir. E. Jacobsen Hoogleraar in de Plantenveredeling, in het bijzonder in de genetische variatie en reproductie
Co-promoter:	Dr. R.G.F. Visser Universitair Hoofddocent, vakgroep Plantenveredeling

Anne Kortstee

Modification of potato starch composition by introduction and expression of bacterial branching enzyme genes

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen
Dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 6 mei 1997
des namiddags te vier uur in de Aula

Het onderzoek beschreven in dit proefschrift werd uitgevoerd by de vakgroep Plantenveredeling van de Landbouwuniversiteit Wageningen. Het onderzoek werd gefinancierd door AVEBE BA, en Hettema Zonen Kweekbedrijf B.V., Emmeloord in het kader van de subsidieregeling programmatische bedrijfgerichte technologiestimulering (PBTS) van het ministerie van Economische zaken.

CIP-Data Koninklijke Bibliotheek, Den Haag.

Kortstee, A.J.

Modification of potato starch composition by introduction and expression of bacterial branching enzyme genes / Anne J. Kortstee - [S.l.:s.n.]

Thesis Wageningen Agricultural University - With summaries in Dutch and English. Department of Plantbreeding, P.O. Box 386, 6700 AJ, Wageningen, NL.

ISBN 90-5485-675-0

Subject headings: branching enzyme/glgB, genetic modification, starch composition, physico-chemical properties.

Abstract

The thesis describes the introduction and expression of bacterial branching enzyme genes (glgB) in potato. The glgB genes of *E.coli* and *A.nidulans* were transformed via *A.tumefaciens* to diploid potato clones with or without amylose containing starch. Expression of the introduced genes led to a 25-35% increase in the starch branching degree. In the amylose containing background sometimes the increased branching degree was accompanied by a lowered amylose content. The essentially linear amylose was believed to be further branched, resulting in a so-called intermediate fraction. Physico-chemical properties of the genetically modified starches were determined. The presence of amylose was found to be the most important characteristic in determining the swelling and gelatinization properties of the starch. The influence of an increased branching degree of the starch was seen as a lowering of the peakviscosity during heating of starch-water suspensions.

Contents

General introduction

1

Kortstee A.J., Vermeesch A.M.G., Suurs L.C.J.M., Jacobsen E. and Visser R.G.F.

Part of this chapter will be published in: Proceedings of the conference on Engineering Crops for Industrial End Uses. (Editors P.R. Shewry, J.A. Napier and P. Davis).16-18 september, 1996, IACR-Long Ashton Research Station, Bristol, England.

General introduction

Starch biosynthesis and structure

In higher plants the major reserve polysaccharide is starch. Starch is stored in the form of granules in special starch storing plastids, the amyloplasts. The last steps in the synthesis of starch in the amyloplast involve the combined action of a number of enzymes starting with ADP-glucose pyrophosphorylase (AGPase), which synthesizes ADP-glucose. In the next step the glucosyl part of ADP-glucose is linked to the non-reducing end of a pre-existing glucan chain by a starch synthase (SSS), forming an α-1,4 linkage while releasing ADP. Branching of linear α-1,4 chains is catalyzed by branching enzymes (BE), which hydrolyze an α-1,4 linkage within a chain and then form an α-1,6 linkage between the reducing end of the chain which was cut, and another glucose residue probably from the hydrolyzed chain. One isoform of starch synthases, which is tightly bound to the granule (GBSS), synthesizes an α-1,4 linked glucan which is called amylose and usually makes up for 15-30% of the total starch. The glucan formed by the starch synthases and subsequently branched by the branching enzymes is called amylopectin and forms the major part of starch (reviewed by Shannon and Garwood, 1984; Martin and Smith, 1995).

Branching enzyme

Multiple isoforms of starch branching enzyme, starch synthase and AGPase, were found in different botanical sources like maize, pea and rice (Boyer and Preiss, 1981; Smith, 1988; Nakamura, 1992). For potato and cassave so far only one BE isoform has been found (Kossmann *et al.*, 1991; Salehuzzaman *et al.*, 1992).

Based on nucleotide sequence and amino acid homology, branching enzymes can be divided into two groups; Type-I and Type-II corresponding to either maize BE-I or maize BE-II (Boyer and Preiss, 1981;for different nomenclature see Burton *et al.*, 1995). The group of Type-I branching enzymes contains maize BE-I, pea BE-II, rice BE-I, cassava BE and potato BE. Type-II branching enzymes form a group to which e.g. maize BE-II, rice BE-II, pea BE-I and *E.coli* glgB belong.

Determination of substrate preference of the isoforms of maize branching enzyme showed that BE-I preferred amylose over amylopectin and BE-II preferred branching amylopectin. BE-I was also shown to transfer longer chains and BE-II shorter chains (Guan and Preiss, 1993). From these results it was concluded that in maize amylopectin biosynthesis, BE-I may be responsible for the formation of the long chains, while BE-II forms the short outer chains within the amylopectin cluster. Supporting this view are the results from studies involving the amylose extender mutant of maize, in which the BE-II isoform is missing (Hedman and Boyer, 1982). The starch of this mutant contains a higher amount of amylose and amylopectin with longer chains compared to normal maize starch (Takeda *et al.*, 1989). Branching enzyme is known to introduce α-1,6 glucosidic linkages in amylose or amylopectin *in vitro*. Recently however, it was described how the branching enzyme of *Bacillus stearothermophilus* was able to catalyze a cyclization reaction with amylose as a substrate. The formation of a cyclic glucan composed of α-1,4 and α-1,6 linkages was suggested to be possible for plant branching enzymes as well (Takata *et al.*, 1996). Whether this reaction also takes place during starch biosynthesis or not is not known.

Amylopectin cluster model

Amylopectin consists of short linear α-1,4-linked chains with an average length of about 25 glucose residues. The current model of amylopectin structure as depicted in Fig.1 was first proposed by Hizukuri (1986). In this model of amylopectin structure three types of chains can be distinguished: the A chains

which are connected to the amylopectin by their potential reducing endgroups, the B chains which are similar to A chains, but carry one or more A and/or B-chains, and the C chain, which carries the sole reducing endgroup of the molecule (Manners, 1989).

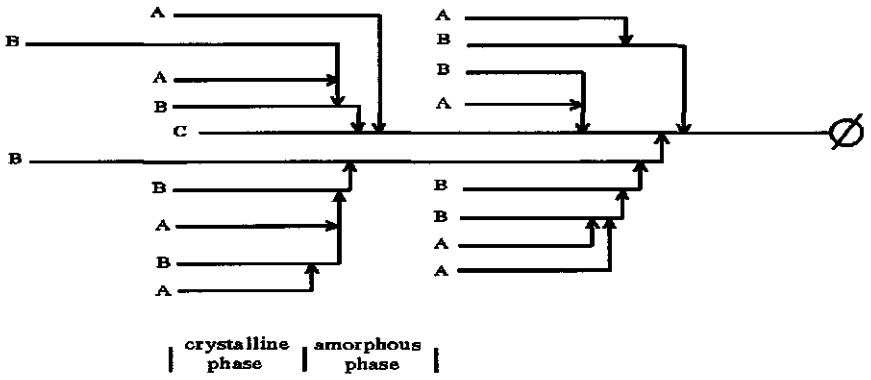


Figure 1. Amylopectin fine structure according to Hizukuri (1986). A, B, and C are the designated chains. ϕ, reducing endgroup; ——, α-1,4 linked chain; →, α-1,6 linkage.

Amylopectin fine structure

Despite the highly conserved ultra-structure of the starch granule there is, within certain limits, some variation. The degree of branching of the amylopectin and the distribution of side chains are specific for each species (Hizukuri, 1986). About the crucial factor in determination of the branching characteristics of amylopectin, different views exist.

According to studies of Tolmasky and Krisman (1987) the branching degree of glucans is an intrinsic property of the branching enzyme. In an *in vitro*

experiment with branching enzymes of different sources a branched glucan was produced similar to that found in the tissue from which the enzyme originated. Work by Takeda *et al.* (1993) and Guan and Preiss (1993) on branching enzyme isoforms of maize supported this view. Based on the differential enzymatic activity found for the maize BE isoforms, Guan and Preiss (1993) postulated that Type-I branching enzymes form a less branched amylopectin than Type-II branching enzymes.

Another idea about the determining factor for amylopectin structure is that the branching degree of starch depends on the ratio branching : debranching enzymes and is supported by work of Pan and Nelson (1984) on the *Sugary-1* (*Su1*) mutant of maize which has a decreased debranching enzyme activity (relative to branching enzyme activity) and an abnormally highly branched starch (phytoglycogen). The debranching enzymes are thought to be required to balance the excess branching activity present at the surface of the granule. Recently mutants similar to the maize *sugary* mutant were found in rice (Nakamura *et al.*, 1996) and in the monocellular alga *Chlamydonomas reinhardtii* (Mouille *et al.*, 1996).

Amylopectin has a polymodal distribution of chain lengths, meaning the linear side chains can be divided in two or three populations of chains with distinct lengths (Hizukuri, 1986) or degree of polymerization (dp). The polymodal chain length distribution is characteristic for amylopectin isolated from each botanical source. The formation of a chain of a given length is probably not determined by the branching enzyme but by the starch synthase, acting in concert with branching enzyme. The idea of an important role for starch synthases in the determination of amylopectin fine structure resulted mainly from work on mutants of *Chlamydonomas reinhardtii* (Fontaine *et al.*, 1993; Maddelein *et al.*, 1994). SSS-II defective mutants accumulated less starch and the amylopectin showed a decrease in the number of relative short chains with a dp 8-50 and an increase in

very short chains with a dp 2-7. This suggested that SSS-II is responsible for the formation of intermediately sized chains. A double mutant for SSS-II and GBSS-I was found to contain a small amount of highly branched glucan which resembled phytoglycogen. These results indicated that GBSS-I is involved in the formation of long chains of amylopectin (and amylose), SSS-II for the formation of intermediately sized chains and SSS-I can only make short chains.

The maize SSS isoforms appeared to have different substrate preferences as well. SSS-I was shown to have a high affinity for a more branched glucan (glycogen) whereas SSS-II preferred a lesser branched substrate and probably longer chains (MacDonald and Preiss, 1985). So, like the branching enzymes, the starch synthase isoforms have different substrate specificity *in vitro*.

In a preliminary model for amylopectin synthesis by Kram (1995) considering all the above mentioned results, it was stated that while the branching enzymes are responsible for the formation of branchpoints, the distribution of chain lengths is dependent on the ratio of starch synthases and their interaction with the amylopectin. Longer chains are formed by a SSS with stronger interaction with the amylopectin. According to this model, at least two different starch synthases and possibly two different branching enzymes are needed, closely working together, for the formation of short and medium sized chains in the amylopectin cluster.

Starch composition

Apart from amylose and amylopectin, the starch granule contains protein (of which GBSS is the most abundant), phosphorus (both starch-bound and free), lipid and some ash (Swinkels, 1985a). In addition to the two major starch fractions, amylose and amylopectin, some researchers have reported the existence of a third starch fraction, the intermediate fraction (Whistler and Duoane, 1961, Wang *et al.*, 1993a). This fraction is evident from comparison of gel permeation elution profiles of native and debranched starch and is believed to

contain highly branched amylose and/or long chained amylopectin. However, the existence of the intermediate fraction is still under discussion (Tester and Karkalas, 1996), as other researchers argue that it does not give a distinct peak on gel permeation analysis of isoamylase debranched starch, as can be seen for amylose and amylopectin.

The starch composition is determined by the amylose content, the molecular size of amylose and amylopectin, the average side chain length, the chain length distribution and the non-carbohydrate components and is considered to be unique for each botanical source (Swinkels, 1985). Genetic variation within species can contribute considerably to the wide variation found in structural characteristics for starch of different origins. This is also the reason for the distinctive properties of native starches from different botanical origins and even different genotypes within a species (Howling, 1980; Sanders *et al.*, 1990; Campbell *et al.*, 1995).

Relationship between structural and functional properties of starch

The influence of structural characteristics on the physico-chemical properties of the starch is evident (Swinkels, 1985b). In attempts to define the relationship between the structural and functional features of starch, many studies were undertaken involving native starches of different botanical origin (Howling, 1980; Swinkels, 1985a/b; Jacobs *et al.*, 1995) and mutants with altered starch compositions (Sanders *et al.*, 1990; Wang *et al.*, 1992, 1993b; Bogracheva *et al.*, 1995; Campbell *et al.*, 1995b; Cheng-Yi Lii *et al.*, 1995). Most mutants are found in maize where they exist for almost every isoform of the enzymes involved in starch biosynthesis, resulting in starches with different compositions and characteristics (Wang *et al.*, 1992, 1993a/b). The best studied example of mutant

starch is that of the *waxy* mutant of maize (Echt and Schwartz, 1981). This mutation has also been identified and studied in other crops including potato, rice, barley and wheat (Shannon and Garwood, 1984; Sano, 1984;. Hovenkamp-Hermelink *et al.*, 1987; Nakamura *et al.*, 1995). These mutants contain amylose-free starch as a result of a defect in the gene encoding Granule-Bound Starch Synthase (GBSS) and a subsequent absence of GBSS activity (Echt and Schwartz, 1981). Other mutants contain a relatively high amount of amylose in the starch as a result of a mutation in one of the isoforms of starch branching enzyme and are found in maize (*amylose-extender)* (Boyer and Preiss, 1981; Hedman and Boyer, 1982) and pea (*wrinkled)* (Edwards *et al.*, 1988; Smith, 1988). Analysis of starches of mutant genotypes in four inbred maize lines (Sanders *et al.*, 1990) showed the importance of the ratio of short to longer chains for the thermal behavior of starch. Starch from the double mutant *aewx* contained more chains of high molecular weight, the population of lower molecular weight chains consisted of longer chains and a higher T-max compared to starch from the single mutant *wx*. This effect was thought to be caused by an increased proportion of starch in the crystalline form as a result of a higher proportion of B1 chains. Wang *et al.* (1992, 1993a/b) described the structural characteristics of starches from 17 mutant maize genotypes and their effects on the physico-chemical behavior. They concluded that the precise relationship between starch structure and physical properties was not always clear, but found that the amylose content had a large influence on swelling and gelatinization. The amylose content was negatively correlated to the swelling power, light transmittance of starch solutions (%T) and peak viscosity and positively correlated to Blue Value and λmax. Other properties such as the intermediate size content and the ratio of short to longer chains were negatively correlated with peak viscosity. Jane and Chen (1992) conducted a study on the effects of the molecular size of amylose and the average side chain length of amylopectin on the paste properties of rice and maize starches. Rice amylopectin was found to have both higher viscosity and shorter

chain length compared to both high amylose maize and waxy maize, which contrasts with the results obtained by Wang *et al.* (1993b). However, the phosphorus content of the rice amylopectin was much higher and this could have contributed to the higher viscosity in addition to other structural characteristics of rice amylopectin, which also differed from those of the maize amylopectin.

Genetic modification of starch content and composition

Another way to obtain an altered starch composition, besides in mutants, is by using genetic modification. This work has mostly been carried out in potato, which is the most easily transformed among the important starch storing crops like maize, rice and cassave. By inhibiting Granule-Bound Starch Synthase gene expression via the so-called antisense RNA inhibition method, potato starch was obtained which was completely amylose-free (Visser *et al.*, 1991; Salehuzzaman *et al.*, 1993; Kuipers *et al.*, 1994a/b). The same approach was used to inhibit the endogenous branching enzyme gene, resulting in plants with reductions in branching enzyme RNA and protein. However, there were no detectable changes in amylopectin structure, such as the degree of branching, although some of the physico-chemical properties (mainly the viscosity) of the starch were changed (Flipse *et al.*, 1996a). Inhibiting the potato AGPase resulted in sugar-storing tubers with a decreased amount of starch and distorted tuber morphology, but not in structural changes of the starch (Müller-Rober *et al.*, 1992).

As an alternative to inhibiting gene functions, homologous or foreign genes can be introduced to change the starch composition. Reintroduction of GBSS into the *amf*-mutant of potato (van der Leij *et al.*, 1991; Flipse *et al.*, 1996b) resulted in tubers with up to wildtype levels of amylose in the starch. In contrast, insertion

of extra copies of the same gene into an amylose containing potato genotype, did not result in an increase of the amount of amylose. However, the opposite effect was sometimes observed. This phenomenon is called co-suppression. The amount of amylose was decreased to various degrees after insertion of extra GBSS gene copies (Flipse *et al.*, 1996b).

The introduction and expression of cyclodextrin glycosyl transferase (CGTase) from *Klebsiella sp.* resulted in an unique type of starch containing an appreciable amount of cyclodextrins (Oakes *et al.*, 1991). The genes from the glycogen synthesis operon of *E. coli*, glg A, B and C have also all been introduced and expressed in potato. Expression of a modified glgC gene, the ADP-glucose pyrophosphorylase, in the potato variety Russet Burbank, resulted in an increased amount of starch (Stark *et al.*, 1992) which displayed no structural changes. Expression of the glycogen synthase gene, glgA, led to an altered potato starch composition, with a reduced starch and amylose content and an increased degree of branching in the amylopectin (Shewmaker *et al.*, 1994). These altered structural features of the starch also had an effect on the physical properties such as viscosity and gelatinization.

Outline of the thesis

The main objective of this project was to obtain potato starch with a higher degree of branching. Since the starch structure determines the functional characteristics of the starch, it was expected that an increased branching degree would change the physico-chemical properties of the starch. Altered starch properties could lead to new, or facilitate existing applications of starch for industrial purposes. In this thesis the effects of the introduction of bacterial branching enzyme genes (glgB) on the starch of transgenic potato plants are

analyzed. Bacterial branching enzymes are involved in glycogen biosynthesis which is analogous to starch biosynthesis. Glycogen has no highly ordered structure like starch and is about five times more branched than plant starch. Expression of the glgB gene of *Escherichia coli* in the amylose-free potato mutant led to an increased branching degree of the amylopectin as is described in Chapter 2. The effects of introduction of the glgB genes of *E.coli* or *Anacystis nidulans* (*Synechococcus* sp.) on the starch from amylose-containing as well as amylose-free transgenic potato plants are described in Chapter 3. Indications were found that the bacterial branching enzymes were able not only to branch amylopectin more intensively, but in addition seemed to be able to use amylose as a substrate for branching as well. In Chapters 4 and 5 some physico-chemical properties of starches from transgenic plants are described. In Chapter 4 a comparison is made between starches from plants with a reduced amount of amylose as a result of a) antisense inhibited GBSS expression and b) expression of the *A.nidulans* glgB gene. In Chapter 5 the influence of an increased starch branching degree of amylose-free and amylose containing starches on viscosity and gelatinization is described. In Chapter 6 a general discussion of the research in this study is presented and a possible model that might explain the way that the bacterial branching enzyme is able to branch amylose and amylopectin is proposed. Also, the increased insight in the starch structural-functional relationship, gained by analysis of transgenic plant starch, is discussed.

2

Expression of *Escherichia coli* branching enzyme in tubers of amylose-free transgenic potato leads to an increased branching degree of the amylopectin

Anne J. Kortstee, Angela M.G. Vermeesch, Beja J. de Vries, Evert Jacobsen and Richard G.F. Visser.

The Plant Journal **10** (1), pp101-108, 1996.

Expression of *Escherichia coli* branching enzyme in tubers of amylose-free transgenic potato leads to an increased branching degree of the amylopectin

Abstract

In order to increase the branching degree of potato tuber starch, the gene encoding branching enzyme (glgB) of *Escherichia coli* was expresssed in the amylose-free potato mutant. The *E.coli* glgB was cloned in the binairy vector pBIN19 under the transcriptional control of the potato Granule Bound Starch Synthase (GBSS) promoter and transitpeptide sequence. The *E.coli* glgB was cloned behind the two N-terminal amino acids of the *GBSS* mature protein, creating a chimeric protein. Transgenic plants were obtained which expressed the *E.coli* branching enzyme as was shown by the presence of mRNA and protein in the tubers. Correctly processed protein was found both in the soluble and starch granule bound protein fraction. Analysis of the starch showed an increase in the branching degree (DE) of up to 25% more branchpoints. The increase in the number of branchpoints was due to the presence of more short chains, with a degree of polymerization (dp) of 16 glucose-residues or less in the amylopectin. Changes in other characteristics of the starch, such as average chainlength (CL) and λmax, indicated a more branched structure for starch of transformed plants as well.

Introduction

Starch is the major storage carbohydrate in higher plants and is found most often in special storage organs such as roots, seeds or tubers. Starch consists of a mixture of two glucose polymers : amylopectin (α-1,4 linked D-glucose residues with α-1,6 glucosidic branch points) and amylose (essentially linear chains of α-1,4 linked D-glucose) (Banks and Greenwood, 1975). The enzyme responsible for the formation of amylose is Granule Bound Starch Synthase (GBSS); amylopectin is synthesized by the combined actions of the enzymes Soluble Starch Synthase (SSS) and Branching Enzyme (BE). Within certain limits the branching degree of amylopectin and the distribution of side chains are specific for each species (Hizukuri 1986).

About the determination of branching characteristics of amylopectin different views exist. According to studies of Tolmasky and Krisman (1987) the branching degree of glucans is an intrinsic property of the branching enzyme. Glucans branched *in vitro* by a certain branching enzyme were almost identical to the native branched component of the origin of that branching enzyme. Work by Takeda (1993) and Burton (1995) on branching enzyme isoforms of respectively maize and pea support this view. Based on aminoacid homology branching enzymes can be divided into two groups; Type-I and Type-II (Burton 1995, Kram 1995). The group of Type-I branching enzymes contains maize BE-I, pea BE-II, rice BE-I, cassava BE and potato BE. Type-II branching enzymes form a group to which e.g. maize BE-II, rice BE-II, pea BE-I and *E.coli* glgB belong. Based on the differential enzymatic activity found for the maize BE isoforms, Guan and Preiss (1993) postulated that Type-I branching enzymes form a less branched amylopectin than Type-II branching enzymes. The other idea about the determining factor for amylopectin structure is that the branching degree of starch depends on the ratio branching:debranching enzymes and is supported by work of Pan and Nelson (1984) on the *Sugary-1* (*Su1*) mutant of maize which has a decreased

debranching enzyme activity (relative to branching enzyme activity) and an abnormally high branched starch (phytoglycogen).

In potato, where only one isoform of branching enzyme is known, an attempt was made to inhibit potato branching enzyme activity by the antisense RNA approach (Flipse *et al.* 1996a). A reduction in mRNA and protein level of potato branching enzyme was found but no detectable changes in amylopectin branching degree, although the physico-chemical properties of the starch had changed. Starch composition can be influenced by introduction of bacterial genes as was shown by Stark *et al.* (1992). Introduction of a mutant *E.coli* ADP-glucose pyrophosphorylase gene (glgC) in potato leads to an increase in tuber starch content. Expression of *E.coli* glycogen synthase (glgA) in potato leads to a number of changes in the starch composition, i.e a decrease in amylose content and a different branching pattern (Shewmaker *et al.* 1994).

In our attempt to modify the branching degree of potato tuber starch we introduced the gene encoding branching enzyme (glgB) of *E.coli* in potato, under the control of the tuber specific potato GBSS promoter. Targeting of the heterologous protein to the amyloplast was ensured by fusion of the glgB gene to the GBSS transitpeptide sequence. Activity of the bacterial branching enzyme in potato plants, which resulted in an amylopectin with a higher branching degree is described.

Experimental procedures

Subcloning of the Escherichia coli glgB gene

The *E.coli* glgB was subcloned from the plasmid pKVS-1 carrying part of the glycogen operon of *E.coli* (Kiel *et al.* 1987). The *E.coli* glgB was subcloned as a BglII fragment from pKVS-1 in the BamHI site of the vector pUC18, resulting in the plasmid pBWB. pWAM40, a pUC18 plasmid carrying an EcoRI fragment of

the genomic clone of GBSS of potato (van der Leij *et al.*, 1991) containing the promoter and transitpeptide region and part of the coding sequences was used to obtain the GBSS expression signals. Plasmid pWAM40 was cut with EcoRI and CelII to obtain the promoter and transitpeptide fragment. The glgB gene from *E.coli* was obtained as a PvuII-BglII fragment from the plasmid pBWB. A synthetic oligonucleotide (CelII-PvuII) was used as a linker between the glgB and the potato GBSS transitpeptide sequence preserving the splice-site for the mature protein and including the first two N-terminal amino acids from the GBSS coding region fused to the second codon of *E.coli* glgB. The linker contained the sequence (5'→3') TCAGCTACCATTGTT**TGTGGA**AAGGGATCCGAT (the splice-site typed in bold). The aminoacid sequence from GBSS splice site to the *E.coli* glgB has become **Cys-Gly**-Lys-Gly-Ser-Asp, splicing should occur between **Cys** and **Gly**, Lys and Gly are the GBSS codons, Ser and Asp being resp. the second and third codon of the *E.coli* sequence. The EcoRI-PvuII fragment was ligated to the linker and the PvuII-BglII fragment in EcoRI-BamHI cut vector pMTL23. The pMTL cloning vectors were described by Chambers *et al.* (1988). The entire DNA fragment carrying the chimeric gene fusion was ligated as a BglII-SalI fragment in the BamHI-SalI cut binairy vector pBIN19 (Bevan, 1984) to create plasmid $pB_{19}09EC$ (Fig 1). This plasmid was transferred to *Agrobacterium tumefaciens* strain LBA 4404 as described by Hoekema *et al.* (1983).

Complementation of glgB deficient E.coli KV 832

All non-binairy constructs carrying a branching enzyme encoding gene were tested for their ability to complement the branching enzyme deficient *E.coli* strain KV832 (Kiel *et al.*, 1987). Constructs were transformed to calcium chloride treated competent cells and plated on an antibiotics containing 0.5% glucose LB agar plate. Complementation was detected by staining the colonies with Lugols (I/KI 1:2 v/v) solution, complemented colonies with restored branching enzyme activity stained red whereas non complemented colonies stained blue.

Transformation of plant material

Stem segments of the diploid amylose-free potato mutant (*amf*) (which lacks GBSS activity) clone 1029-31 (2n = 2x = 24, Jacobsen *et al.*, 1989) were transformed with *Agrobacterium tumefaciens* strain LBA 4404 containing plasmid $pB_{19}09EC$ or AM8706 (the latter as described by Visser *et al.*,1989). Regenerating shoots were placed on selective Murashige & Skoog (1962) medium with 30 % sucrose containing kanamycin (50 mg/l) and claforan (100 mg/l). After root formation the plants were transferred to the greenhouse. Tubers were harvested 18 weeks after transfer of the plants to the greenhouse.

Starch isolation and RNA/DNA isolation and analysis

Starch and RNA were isolated from greenhouse grown tubers as described by Kuipers *et al.*(1994b). RNA gel blotting and hybridization were carried out using 40 µg of total RNA per sample as described by Sambrook *et al.* (1989). The blots were hybridized to ^{32}P labeled *E.coli* glgB probe.

DNA was extracted from leaves of greenhouse grown plants according to Dellaporte *et al.* (1983). By digesting the DNA with MluI or EcoRI and using the ^{32}P-labeled fragment of plasmid pM25EC as a probe, the minimum number of integrated T-DNA inserts could be estimated by scoring the number of bands in a Southern blot.

Preparation of polyclonal antibodies against E.coli branching enzyme

The coding sequence of the branching enzyme of *E.coli* was obtained from the *E.coli* glgB carrying plasmid pBWB using the primers 5'GAAGACAAC-CATGGCCGATC 3' and 5'GCCAATGTCGACTTGTGTC 3'. The PCR product was cloned in pMTL25 (Chambers *et al.*, 1988) in the NcoI-SalI sites to form plasmid pM25EC. Plasmid pM25EC was digested and the NcoI-BglII fragment was cloned in the expression vector pQE-60 (Qiagen GmbH, Dusseldorf,

Germany) carrying a 6*His affinity tag at the 3' site of the expression box. Overexpression of the glgB protein in *E.coli* M15[pREP]$_4$ host cells and purification of the protein by Ni-NTA resin column was carried out as described by the manufacturer (Qiagen).

Polyclonal antibodies were obtained using standard procedures (Maniatis *et al.* 1982). As soon as the antibody titer was high enough, all serum was collected and stored at -20 °C. The antiserum is referred to as "anti BE serum".

Western analysis

For Western analysis 10 mg of starch was boiled for 5 min. with 100 µl of 2*sample buffer (Laemmli, 1970). Samples were vortexed and spun in an Eppendorf microcentrifuge at 9000*g for 5 min. Of the supernatant 10 µl (approx. 10 µg protein) was electrophoresed on 10% polyacrylamide-SDS gels. For the soluble protein fraction 50 µl was boiled with 50 µl 2*sample buffer, vortexed and spun as for the starch protein fraction. Of the supernatant 10 µl (± 10 µg protein) was applied on gel. Immunoblotting was carried out as described by Hovenkamp-Hermelink *et al.* (1987) using 1:500 diluted anti BE serum. Alkaline phosphatase was used (2000 times diluted) as a second antibody and the antigen-antibody complexes were detected by incubating the membranes in the dark in 100 µl AF buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) with 200 µl NBT (4-nitro blue tetrazoliumchloride; 75 mg/ml in dimethylformamide) and 200 µl BCIP (5-bromo-4-chloro-3-indolyl-phosphate; 50 mg/ml in H_2O). The reaction was stopped by incubating the membranes in AF buffer.

Soluble sugar and starch content

The amount of soluble sugars (glucose, fructose and sucrose) and starch of transgenic tubers was determined as described by Kuipers *et al.*(1994a)

Analysis of starch branching degree

The branching degree of the isolated starch was determined after isoamylase digestion according to the Luff-Schoorl method with slight modifications (Schoorl, 1925).

100 mg starch was dissolved in 10 ml dH_2O by heating to 95 °C and 0.1 N HCL was added until pH 3.5 was reached. 10 mg of isoamylase (EC 3.2.1.68) was added (Hayashibara, Okayama 700, Japan) and the solution was incubated for two hours at 40 °C. From the debranched starch solution 8 grams were taken and added to 8.3 ml Luff-Schoorl reagens (12307 Boom BV Meppel) and boiled for exactly 10 min. After cooling 3.3 ml 1M KI and 6.7 ml 5N H_2SO_4 were added and finally 1.7 ml 1% soluble potato starch (Sigma S2630) was added as indicator. The dark purple solution was titrated with 0.050 N thiosulphate until the colour turned light purple. After addition of 1.7 ml 1M KSCN the solution was titrated with thiosulphate until the purple colour disappeared. The amount of added thiosulphate corresponded to the Luff-Schoorl number (LS number) which was used in the following equation to obtain the branching degree expressed in Dextrose Equivalents: DE = (LS/dw)*100 = % of reducing endgroups, expressed as glucose per dry weight.

Average chain length determination

The average chain length is the total carbohydrate equivalent to glucose divided by the number of reducing termini after isoamylase digestion; the mean length of the α–1,4-linked glucose chains.

The total carbohydrate equivalent to glucose was determined by acid hydrolysis of the starch into glucose. To 100 µl of 0.1% starch solution, 10 µl of 100% sulfuric acid was added and incubated at 95 °C for two hours. The sample was neutralised by addition of 2N Sodiumhydroxide until the pH was 7.0.

The number of reducing termini was determined after isoamylase digestion of a starch sample. To 1 ml 1% starch solution 150 µl 1M Sodiumcitrate pH 5.0 was

added and 350 U isoamylase (Hayashibara, Okayama 700, Japan). Digestion took place for 16 hours at 37 °C. Glucose and reducing termini were detected by the Park & Johnson method (1949) with slight modifications:
100 µl of solution I (Na_2CO_3 54 mM, $NaHCO_3$ 110 mM, KCN 10 mM) and 100 µl of solution II ($K_3[Fe(CN)_6]$) were added to 100 µl of sample (1.0% isoamylase digested or 0.1% acid-hydrolyzed starch sample). After incubation at 95 °C for exactly 15 min. the sample was cooled to room temperature and 500 µl solution III ($NH_4Fe(SO_4)_2.12H_2O$ 11 mM, H_2SO_4 50 mM, SDS 6%) was added. The formation of colored Fe- complex was completed after 20 min in the dark and spectrophotometerically measured at 690 nm. Beers law holds from 0.5-3.0 µg glucose with the A_{690} between 0.05-0.820 where a linear correlation exists between the molar amount of glucose and the absorbance.

λmax determination

From a 0.1% starch solution in 0.01 N NaOH 20 µl was added to 800 µl H_2O and 200 µlLugol : H_2O (1:4) and a scan was made from 400-700 nm using a spectrophotometer. The λmax was determined as the wavelength with maximum absorbtion.

Chain length distribution

Starch samples (2% w/v) were dissolved at 120 °C in dH_2O. The warm starch solution was adjusted to pH=3.5 with 0.1M HCL and isoamylase was added (Hayashibara) to a final concentration of 0.66 mg/ml. After 2 hrs of incubation at 40 °C the enzyme was inactivated at 100 °C for 30 min. After cooling to room temperature an equal volume of 0.1M NaOH was added and the digested starch solution was filtered through a 0.2 µM filter. The chain length distribution was determined with a Dionex series 45001 fitted with a Carbopac PA1 (250*4mm) column and a Carbopac PA1 guard column. The flow rate was 1.0 ml/min and 50 µl samples were injected with a Dionex automated sampler. Two eluents were

used. Eluent 1 (0.1M NaOH) and eluent 2 (0.5M Sodium acetate in 0.1M NaOH) were used according the following time-frame: 0-5 min. eluent 2, 5.1-20.1 min. eluent 1, 20.1-21.1 min. injection of sample, 21.1-61.6 min. gradient from eluent 1 to eluent 2 and 61.1-71.1 min. eluent 2. Detection of reducing sugars with a Dionex PED amperometric detector. Data were recorded with the Dionex A1-450 software. The data from the original HPLC chromatogram were processed according to a non-published method developed by Dr. J. Bergsma (AVEBE, Holland), which is freely available as a Lotus file.

The HPLC chromatogram represents the linear chain distribution of the debranched amylopectin. Each chain contains a reducing end group which is detected by the Dionex PED amperometric detector. The detector response however is not the same for all the different chains as was reported before (Larew and Jonhson, 1988). The response factor for chains with a degree of polymerization (DP) of 1 to 7 was determined for a standard mix of malto-oligosaccharides with known concentrations (1mM each). The response factor is the detector response in μCoulomb per ngram of oligosaccharide. The HPLC chromatogram of the standard mix was integrated according to a 100% method, meaning the area of each peak was expressed as the percentage of the sum of all peak areas. The molar sensitivity of the detector increased with each additional glucose to the polymer chain, up to DP ≈ 5. The relative peak areas of chains with a DP ≥ 5 were essentially the same. It was furthermore assumed that chains with a DP ≥ 7 had the same response factor as the oligosaccharide with DP = 7. The differences in response factors for the chains with a lower DP could be the result of the relative large influence of adding one or two glucose units on the redox status of the reducing group. The chromatograms of the debranched starch samples were integrated according to the 100% method and corrected for the response factors found for the standard malto-oligosaccharide mix to obtain a number percentage distribution. From the number percentage distribution a weight distribution could be calculated according to the following equation:

$$Wi = \frac{Ni * \{180 + (i-1) * 162\}}{\Sigma i\ Ni * \{180 + (i-1) * 162\}}$$

i = DP of the oligo

Wi = weight percentage of oligo i

Ni = number percentage of oligo with DP = i

The group of chains with a DP ≥ 35 were called the B-rest group and consisted of B_2, B_3 and B_4 chains. The chains which were represented in the HPLC chromatogram were the A and the B_1 chains. To divide the chains in A and B_1 chains three assumptions were made based on findings in the literature: the first assumption is that the mean length of the A chains was DP = 13. Secondly, no B_1 chains have a DP ≤ 13. And also the distribution of A chains was symmetrically around 13.

With these assumptions the chain length distribution was interpreted and the chains were divided in A and B_1 chains. The resulting A:B_1 chain ratios of 1.2:1.0 for starch from untransformed plants were consistent of those described in literature (Manners, 1989) where for potato amylopectin a ratio of A:B_1 chains was found of 1.0 to 1.5:1.0.

Finally the results of this method were compared with the results of GPC (Gel Permeation Chromatography according to the method of Hizukuri, 1986) which displays a weight distribution of the different fractions of chains of debranched amylopectin. Comparison between the two methods showed that in spite of the assumptions made in the here presented method both methods yield the same weight distribution of A, B_1, and B-rest chains for potato tuber starch.

Results

Molecular analysis of transgenic plants

After transformation of the diploid *amf* potato clone 1029-31 with the binary construct $pB_{19}09EC$ (Fig.1) by *Agrobacterium tumefaciens*, 40 independent shoots

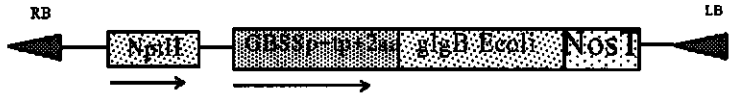


Figure 1. Construct $pB_{19}09EC$ used for transformation.
The glgB gene of *E.coli* was cloned behind the potato GBSS promoter plus transitpeptide sequence including the first two aminoacids after the splice site of GBSS protein and in front of the Nopaline synthase terminator sequence (NosT), in between the left-, and right border (LB and RB) sequences of vector pBIN19. Selection of transgenic plants was based on the Neomycinphosphotransferase (NPTII) gene conferring kanamycin resistance.

were harvested and tested for root growth on a kanamycin containing MS medium. The 34 transgenic root-forming shoots were multiplied and transferred to the greenhouse for tuber development. From each transformed plant tuber RNA was isolated to determine expression of the heterologous glgB gene. Northern blots were hybridized to the ^{32}P-labeled coding sequence of the *E.coli* glgB. In almost every plant tested, mRNA expression of the heterologous gene was detected (Fig.2).

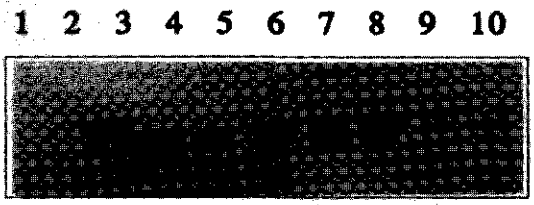


Figure 2. Northern blot hybridization of total tuber RNA of transgenic potato tubers. 40 µg of total tuber RNA was applied per lane, blotted on a hybond filter and hybridized to ^{32}P labeled *E.coli* branching enzyme probe. Lane 1 contains RNA from 1029-31, the untransformed control, lanes 2-10 contain RNA from the transformants EC-13, EC-15, EC-17, EC-20, EC-21, EC-22, EC-25, EC-26 and EC-28.

Western blots were prepared from soluble and starch fractions of the transformed tubers. The heterologous gene product was detected in the starch (Fig.3A) as well as in the soluble fraction of the transgenic tubers (Fig.3B)

The size of the protein found in the starch fraction of amyloplasts (approx. 85 kDa) suggested that processing occurred at the splice-site as expected and the transitpeptide was cleaved off at the splice-site. The multiple bands that could be observed in the soluble fraction could be non processed or partially degraded forms of the bacterial protein. If the GBSS transitpeptide has been cleaved of during amyloplast targeting the expected size of the mature *E.coli* branching enzyme is 84 kDa which was confirmed by Western analysis. The amount of

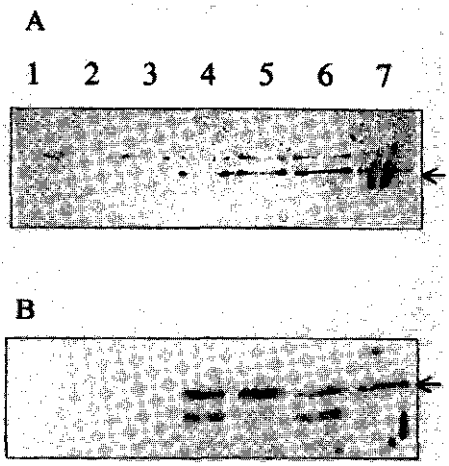


Figure 3. Western blot of soluble-, and starch protein fractions of transformed potato tubers.
A. Protein from 10 mg of starch was electrophoresed on an SDS-polyacrylamide gel, blotted and immunostained. Lane 1 contains protein from the untransformed control 1029-31, lane 2-6 contain protein from the following transformants; EC-2, EC-3, EC-7, EC-13 and EC-20. Lane 7 contains 10 pg of purified *E.coli* branching enzyme.
B. Protein from the soluble protein fraction of transformed tubers, the same numbers as mentioned in A. 10 µg of protein was applied in each lane.

heterologous branching enzyme in the starch fraction corresponding to the signal found was as high as 0.1% of both total soluble and starch protein.

Southern blots were prepared to determine the minimum number of T-DNA inserts integrated in the genome of transformed plants. The number of *E.coli* glgB carrying T-DNA inserts ranges from one to four (data not shown).

Branching degree of starch

The degree of branching of starch of the transgenic tubers was determined using the Luff-Schoorl method. The branching degree expressed in Dextrose Equivalent (DE) for untransformed potato starch was 3.8 ± 0.3, the DE of glycogen was 12 ± 0.5. The DE for the transformed plants ranged from 3.8 - 4.9

(Table 1). For several transformants the branching degree of the starch was significantly higher compared to the untransformed or GUS-transformed controls. The DE of starch of transformants EC-17 and EC-20 was 4.8 and 4.9 respectively which means a 25% increase in number of branchpoints, compared to the value of the control (3.8).

Table 1. Determination of branching degree of starch of transformed tubers expressed in Dextrose Equivalent (DE).
1029-31 is an untransformed control, 8706-3 is a control transformed with a GUS gene containing construct. The values are the average mean of at least two measurements of starch isolated from tubers grown in one (#) or two following years. Transformants EC-7, -17, -20, -21, and -25 have a significantly higher branching degree compared to the (un)transformed controls.

Transformant	DE
EC-3	4.1 ± 0.25
EC-7	4.6 ± 0.05
EC-13	4.6 ± 0.65
EC-15	$4.4 \pm 0.2^{\#}$
EC-17	4.8 ± 0.2
EC-20	4.9 ± 0.1
EC-21	$4.7 \pm 0.4^{\#}$
EC-22	$4.3 \pm 0.25^{\#}$
EC-25	$4.4 \pm 0.15^{\#}$
EC-28	$4.1 \pm 0.2^{\#}$
1029-31	3.8 ± 0.3
8706-3	3.8 ± 0.2

Further biochemical characterisation of transformed plants

After Western analysis and determination of branching degree of the starch three transformants were selected for further biochemical research. Transformant EC-20 was chosen because it showed the highest branching degree and transformant EC-3 was picked because it showed no increased branching degree of the starch

at all. Transformant EC-13 was picked at random.

λmax

As the branching degree of glucose polymers increases, the λmax of an iodine stained solution will decrease (Tolmasky & Krisman 1987). A decrease in absorbtion maximum could be seen when a starch solution of transformants was stained with Lugol and scanned on a spectrophotometer. A shift of the maximum absorbance to a lower wavelength (550 nm) could be seen for starch of transformant EC-20 and EC-13 compared to EC-3 and the (un)transformed control which both showed a λmax of 555 nm (Table 2).

Table 2. Average chain length and λmax determination of transgenic starches.
The λmax is determined after iodine staining of a 0.1% starch solution in 0.01N NaOH and scanning the spectrum from 400-700 nm. The average chain length is determined by dividing the number of reducing ends after isoamylase digestion by the number of reducing ends after total hydrolysis of starch to glucose. The reducing ends are determined by the Park and Johnson method (1949).

Transformant	λmax (nm)	$Cl_{av.}$
EC-3	555	26 ± 1.5
EC-13	551	23 ± 1.2
EC-20	550	22 ± 0.1
Control	555	25 ± 0.7

Average chain length

The average chain length of the α-1,6 linked side chains was determined as described. From the transformants tested, numbers EC-20 and EC-13 have a decreased value for the average chain length. The average chain length of EC-20 starch is 22 glucose residues whereas untransformed potato starch has an average chain length of about 25 glucose residues (Table 2).

Chain length distribution

Of the three transformants tested, EC-3, -13 and -20, only EC-20 showed a different side chain length distribution compared to the control(s) (Fig. 4).

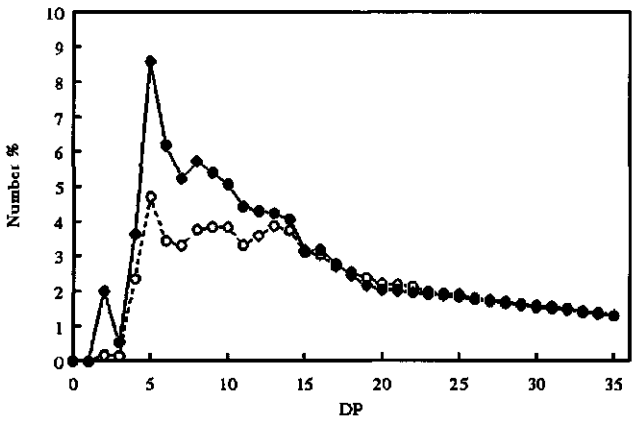


Figure 4. Chain length distribution of amylopectin of transformed potato clone EC-20 (-•-) expressing *E.coli* branching enzyme and control 8706 (-o-).
Amylopectin was debranched with isoamylase and separated on a Dionex series with a Carbopac PA1 column and guard column. A Dionex PED amperometric detector was used to detect the reducing sugars. Glucose, maltose-maltoheptose were used as standards. Glucan molecules which differ one glucose residue in length are separated up to a degree of polymerization (dp) of 36. The amount of chains of a certain length is given as the percentage of the total number of chains.

The amylopectin of EC-20 starch contained more chains with a degree of polymerization (dp) lower than 16 glucose monomers compared to the transformed control 8706-3, 1029-31 and industrial starch (data of the latter two not shown). According to the model for amylopectin structure proposed by Hizukuri (1986), side chains are designated A, B_1, B_2, B_3 and B_4 chains depending

on their size. The A chains wich carry no other chains have an average dp of 12-16, the B_1 chains which carry an A or another B chain have an average dp of 20-24 and the B_2, B_3 and B_4 chains (which extend in two or more clusters) have an average dp of respectively 45, 74 and 104. In this experiment the group of chains with a dp ≤ 16 are called the A chains, the B_1 chains are the chains with a dp from 16-36 and the chains with a dp ≥36 , containing the B_2, B_3 and B_4 chains are designated B-rest.

Because of the increase in chains with a dp of ≤ 16 from 43 to 62% of the total number of chains, the ratio A:B_1 chains is 2.3 for EC-20 starch, nearly twice as high compared to the control. The ratio short : long chains (A+B_1 : B rest) for amylopectin of EC-20 has also increased as a result of the increase in chains with a dp ≤ 16. For the control as well as for transformant EC-3, which do not show an increased branching degree, the ratio short : longer chains is 3:1 ; for transformant EC-20 this ratio is 9:1, so the ratio is three times higher.

Table 3. Chain length distribution of isoamylase debranched amylopectin separated by Dionex HPAEC-PAD.
As a control untransformed 1029-31 was used; EC-3 is a transformed plant with no increased branching degree and EC-20 is a transformed plant with a 25% increased branching degree of the amylopectin.

Plant	A	B_1	Ratio A:B_1	A+B_1	B-rest	Ratio A+B_1:B-rest
Control	43.1	31.8	1.3	74.9	25.1	3.0
EC-3	42.6	35.0	1.2	77.6	22.4	3.4
EC-20	62.8	27.3	2.3	90.1	9.9	9.1

Average chain length (in dp) of different types of chains according to Hizukuri's cluster model for amylopectin : dp of A-chains ≤ 16, dp of B_1 chains ≤ 36,
B-rest is the fraction of chains with a dp ≥ 36 (containing the B_2, B_3 and B_4 chains).

Soluble sugar and starch content

Mature tubers were taken two weeks after harvest from greenhouse grown plants and glucose, fructose, sucrose and starch were determined. The amount of starch in transformed plants is the same as for the non-transformed control, about 5-8% of the fresh weight is starch. The soluble sugar content also has not changed upon transformation or as a result from a higher branching degree (Table 4).

Table 4. Soluble sugar and starch content of mature transgenic tubers.
Glucose, fructose, sucrose and starch content are expressed as the percentage of fresh weight (w/w). The average percentage is the mean of two independant measurements from a mixture of grinded tubers from two different plants, grown under the same conditions.

Transformant	Glucose	Fructose	Sucrose	Starch
EC-3	1.8 ± 1.7	2.5 ± 0.4	2.6 ± 0.3	4.9
EC-13	1.7 ± 0.9	1.5 ± 0.6	1.9 ± 1.2	6.8
EC-20	2.4 ± 1.1	4.0 ± 2.0	2.4 ± 1.2	8.5
1029-31	4.6 ± 2.5	5.7 ± 3.2	2.1 ± 0.9	5.2
8706-3	1.3 ± 1.1	2.8 ± 1.3	2.3 ± 1.3	7.2

Discussion

Transgenic potato plants were obtained which expressed the *E.coli* gene encoding branching enzyme driven by the GBSS promoter of potato as was shown by the presence of mRNA for *E.coli* branching enzyme in tubers of transformed plants. Targeting of the *E.coli* branching enzyme was carried out by the potato GBSS transitpeptide. The chimeric branching enzyme was found in the tubers of

transgenic plants both in the soluble and in the granule bound protein fraction and seemed to be processed correctly.

Enzymatic activity of the *E.coli* branching enzyme in potato was confirmed by analysis of the starch branching degree. A 25% increase in branching degree of amylopectin of several transformants could be observed. The total soluble sugar and starch content of the transformed tubers did not seem to have changed, indicating no gross disturbances of the starch granule biogenesis. Other characteristics of the starch such as average chain length and λmax values, were found to be lower for the transgenic starches compared to the (un)transformed controls, indicating a more branched structure. As the potato genotype used for transformation was amylose free, all changes in starch structure concern only amylopectin. The increase in branching degree (DE) of starch of EC-20 could be explained by its chain length distribution as was seen after isoamylase digestion and HPLC separation. Up to 20% more short chains ($dp \leq 16$) were present in the amylopectin of EC-20 starch. The ratio $A:B_1$ chains had nearly doubled and the ratio $A+B_1$:B-rest chains had tripled. This ratio of 9:1 for short:longer chains was never before found to be this high for potato starch. The highest ratio found before ($A:B_1$), with a value of 5.7:1 was reported by Shewmaker *et al.* (1994) after transforming potato with the glycogen synthase (glgA) encoding gene of *E.coli.*

Our findings of an increase of the amylopectin branching degree by introduction of the glgB of *E.coli* are in contrast to earlier reports by Krohn *et al.* (1994) who expressed the glgB of *E.coli* in the amyloplast of wild-type potato. No effect could be seen in starch composition, except for a slightly higher amylopectin percentage and a small difference in granule surface morphology. In our transformants no change in granule surface morphology was detected but for the presence of more small granules (≤ 10 μm, data not shown). The two experiments differ in the expression signals used (GBSS versus Patatin) promoter, GBSS transitpeptide *versus* transitpeptide of the small subunit of Rubisco) and in the genetic background of the transformed potato (*amf*, 2n=2x *versus* wild-type variety

Russet Burbank 2n=4x) which may explain the differences, or a possible difference in expression level.

The highest branching degree found for transgenic starch is DE= 4.9, which is low compared to glycogen with a DE of 12. This relative low effect of the *E.coli* branching enzyme can possibly be attributed to a low activity of the protein. Low activity of the introduced protein may have been caused by post-translational modifications or explained by the assumed fact that complex formation of soluble starch synthase and branching enzyme is considered necessary for branching activity (Smith 1994). This complex formation may be difficult to realise with a heterologous protein such as *E.coli* branching enzyme due to possible differences in secondary structure. Aside from the possibly disfavorable properties of the *E.coli* branching enzyme there is the presence of the endogenous potato branching enzyme to consider, which will be most probably in competition with the heterologous protein for substrate and/or soluble starch synthase.

In this paper we have shown that as a result of introduction of the *E.coli* branching enzyme in potato the branching pattern of the amylopectin changed: an increased amount of α-1,6 branchpoints as a result of a shift in the distribution of side chains. These results agree with the proposed model for amylopectin synthesis (Kram, 1995) in which soluble starch synthase is stated to be responsible for side chain length, while branching enzyme determines the minimum distance between two branchpoints.

Further analysis of these types of starches with an increased branching degree will reveal what the effect is of the altered structure of the starch on the physical properties of the starch such as swelling and visco-elastic behaviour.

Acknowledgements

We would like to thank T. van de Brink and J. Rijksen for taking care of the plants in the greenhouse. We also would like to thank Dr. J.A.K.W Kiel (Department of Genetics, University of Groningen) for the gift of the glgB deficient *E. coli* strain KV832 and the *E.coli* glgB gene and Dr. J. Bergsma (AVEBE, Holland) for providing us with the method for the determination of the chain length distribution.

Branching of amylose and amylopectin after introduction of bacterial branching enzyme genes (glgB) in potato:

3

Substitution of amylose by an intermediate fraction.

Anne J. Kortstee, Angela M.G. Vermeesch, Luc C.J.M. Suurs, Evert Jacobsen and Richard G.F. Visser.

Submitted

Branching of amylose and amylopectin after introduction of bacterial branching enzyme genes (glgB) in potato: Substitution of amylose by an intermediate fraction.

Abstract

The branching enzyme genes (glgB) of *Escherichia coli* and *Anacystis nidulans* were introduced and expressed at the mRNA and protein level in potato clones with normal amylose-containing and with amylose-free starch. Four different constructs were tested after transformation which varied in targeting signals fused to the glgB sequence. The different constructs contained different glgB genes and signals to drive expression and targeting. The effect of the introduced heterologous branching enzymes was an increase of the branching degree of the starch. Construct pB_{19}tpssuEC, carrying the glgB gene of *E.coli* under the control of the potato GBSS promoter and the transitpeptide of the small subunit of Rubisco (SSU) of potato, was shown to be most effective in causing an increased branching degree of the starch in both the amylose-free and in the amylose containing background. The degree of branching of the starch, expressed as Dextrose Equivalent (DE) was increased but appeared never to be higher than approximately 5. This is an increase of 25-35% compared to the DE values of the untransformed controls. In some amylose-containing transformants with an increased starch branching degree, the amount of amylose had decreased as a result of branching of the amylose by the introduced heterologous branching enzymes. In these cases, gel permeation chromatography showed that amylose was replaced by an intermediate fraction with a higher degree of branching.

Introduction

In higher plants the major reserve polysaccharide, starch, is stored in a granular form. Starch consists of a mixture of α-D glucose polymer chains which are either essentially linear (amylose) or branched (amylopectin). Synthesis of starch in the amyloplast involves the combined action of a number of enzymes starting with AGPase which synthesizes ADP-glucose. In the next step, the glucosyl part of ADP-glucose is linked to the nonreducing end of a preexisting glucan chain by a starch synthase, forming an α-1,4 linkage while releasing ADP. Branching of linear α-1,4 chains is catalyzed by branching enzymes, which hydrolyze an α-1,4 linkage within a chain and then form an α-1,6 linkage between the reducing end of the chain (which was cut) and another glucose residue, probably from the hydrolyzed chain (Martin and Smith, 1995). Analogous to starch biosynthesis, glycogen synthesis occurs in bacteria and animals. Glycogen is a highly branched molecule consisting of α-1,4 linked chains with α-1,6 branches. The difference between starch and glycogen lies in the highly ordered structure of amylopectin, as opposed to glycogen, resulting in the crystalline nature of starch and in the much higher amount of α-1,6 branchpoints for glycogen (Preiss and Walsh, 1981). In addition to inhibiting gene functions involved in starch biosynthesis by antisense genes or co-suppression, introduction of foreign genes is also used to alter starch composition. The introduction and expression of the *Klebsiella sp.* cyclodextrin glycosyl transferase gene in potato resulted in a unique type of starch with a relatively high amount of cyclodextrins (Oakes *et al.*, 1991). The genes from the glycogen synthesis operon of *E. coli*, glg A, B and C have been, one by one, expressed in potato. Expression of a modified form of the glgC gene, the ADP-glucose pyrophosphorylase in potato variety Russet Burbank, resulted in an increased amount of starch (Stark *et al.*, 1992). Expression of the glycogen synthase gene, glgA, led to a reduction of starch content and an altered potato starch composition, like a lowered amylose content and an increased branching

degree of the amylopectin (Shewmaker *et al.*, 1994). An even higher branching degree of amylopectin (by HPLC assessment of the ratio short to longer amylopectin side chains) was obtained by the expression of the *E.coli* glgB gene, encoding glycogen branching enzyme, in the amylose-free potato mutant as was described in Chapter 2 of this thesis. However, expression of this heterologous branching enzyme was relatively low and the starch branching degree had not increased to the level observed in glycogen. To investigate the possibility of increasing the level of enzyme expression and starch branching degree more intensively, several plasmids were constructed carrying a glgB gene with different targeting signals. The transitpeptide of potato Granule-Bound Starch Synthase (GBSS) (van der Leij *et al*, 1991) and the small subunit of Ribulose bisphosphate carboxylase (Rubisco) (SSU) were used to direct targeting of the *E.coli* glgB gene product as well as that of the glgB gene of *A.nidulans*. The cyanobacterium *Anacystis nidulans* (or *Synechoccus sp.*) produces a glucan resembling phytoglycogen, a highly branched glucan (Weber and Wober, 1975). In this paper the expression of the glgB genes from *E.coli* and *A.nidulans* controlled by different targeting signals in both the amylose-free potato mutant and an amylose containing potato clone is described. The influence of the introduced constructs on the structure and composition of starch from transgenic tubers has been investigated.

Experimental procedures

Vectors used for transformation

The constructs used for transformation are shown in Figure 1.

$pB_{19}13AN$ is a pBIN19 (Bevan, 1984) derived plasmid carrying the glgB gene of *A.nidulans* under the control of the potato GBSS promoter plus transitpeptide sequence including the N-terminal part of GBSS consisting of box1, the substrate

binding site (van der Leij *et al.*, 1991). pB_{19}KG16EC is a pBIN19 plasmid carrying the glgB gene of *E.coli* under the control of the potato GBSS promoter and transitpeptide sequence including the first 16 amino acids of the mature protein after the splice site and the Nos terminator sequence. pB_{19}tpssuEC is a pBIN19 derived plasmid with the glgB gene of *E.coli* under the control of the potato GBSS promoter, and the transitpeptide of the Small Subunit of Rubisco of potato (SSU), including the first five amino acids after the splice site and the Nos terminator sequence. The pB_{19}09EC construct was described earlier in Chapter 2 of this thesis.

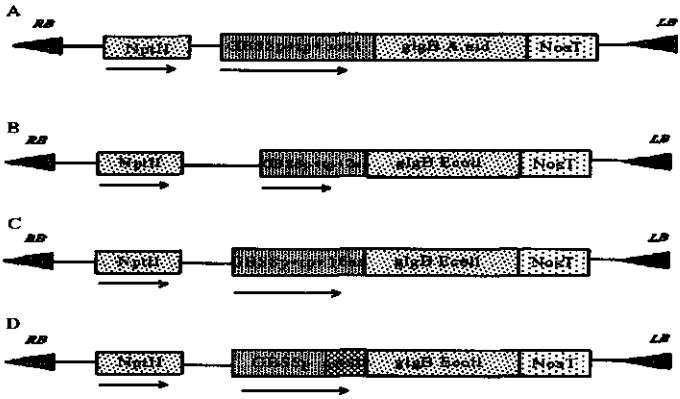


Figure 1. Constructs used for transformation, based on the binary vector pBIN19 and named: A, pB_{19}13AN; B, pB_{19}09EC; C, pB_{19}KG16EC; D, pB_{19}tpssuEC.

Plant material

The potato genotypes used for transformation were: **1029-31,** a diploid clone, 2n=2x=24, homozygous for the *amf* locus, with amylose-free starch, described by Jacobsen *et al.* (1989); **A16,** a diploid potato clone, 2n=2x=24, with normal amylose containing starch, described by El-Kharbotly *et al.* (1995); **Rv,** a diploid

potato clone, 2n=2x=24, with normal amylose containing starch, kindly provided by Ir. R. Eijlander of the department of Plant Breeding, WAU, NL.

Transformation procedure

Stem segments without axillary buds were used for transformation by cocultivation with *Agrobacterium tumefaciens* strain LBA 4404 according to the method described by Flipse *et al.* (1994) carrying one of the above mentioned plasmids. Regenerated shoots were placed on selective Murashige and Skoog (1962) medium with 30% sucrose containing kanamycin (50 μg/l) and claforan (100 μg/l) and were multiplied on the same medium. After root formation the plants were transferred to the greenhouse. Tubers were harvested 18-32 weeks after growth of the plants in the greenhouse.

Starch isolation and determination of the starch branching degree (DE)

Starch was isolated from mature greenhouse grown tubers as described by Kuipers *et al.* (1994b). The branching degree of the starch was expressed as Dextrose Equivalent (DE) after isoamylase digestion of the starch. The reducing endgroups were determined by the Luff-Schoorl method (Schoorl, 1925) with some modifications as earlier described in Chapter 2 of this thesis.

DNA/RNA isolation and blotting-hybridization.

DNA was isolated from leaves of greenhouse grown plants according to the method of Dellaporte *et al.* (1983), digested, blotted and hybridized with an appropriate probe according to methods described by Sambrook *et al.*(1989). RNA was isolated from tubers as described by Kuipers *et al.* (1994b), blotted and hybridized as described by Sambrook *et al.* (1989) subsequently to the glgB gene of *E. coli* or *A. nidulans*, the cDNA of potato branching enzyme and a ribosomal probe.

Western analysis

For Western analysis, 10 mg of starch was boiled for 5 min. with 100 µl of 2*sample buffer (Laemli, 1970). Samples were vortexed and spun in an Eppendorf centrifuge at 13.000 rpm for 5 min. Of the supernatant 1-2 µl (approx. 1 µg protein) was electrophoresed on 12,5% polyacrylamide-SDS Phastsystem gels. For the soluble protein fraction, 50 µl was boiled with 50 µl of 2*sample buffer, vortexed and spun as for the starch protein samples. Of the supernatant 1-2 µl (10 µg protein) was electrophoresed on Phastsystem SDS-PAA gels. Immuno blotting was carried out as described by the manufacturer using anti-serum raised against *E.coli* glgB (Chapter 2, this thesis).

λmax and Blue Value

5 mg of starch was dissolved in 1 ml of Dimethylsulphoxyde (DMSO) by boiling for 20 minutes and diluted to 10% DMSO. Of the 10% DMSO-starch solution, 20 µl was added to 180 µl demineralized H_2O and complexed with iodine by adding 800 µl KI/I_2 solution (Delrue *et al.*, 1992), final starch concentration is 0.01%. The λmax was determined as the wavelength with maximum absorption after scanning from 700-400 nm. The Blue Value was determined to be the absorption at 655 nm. The Blue Value (B.V.) was determined by boiling 10 mg of starch in 1 ml of 0.1N NaOH for 50 min. After cooling 9 ml of demineralized H_2O was added. Of this solution 15 µl was added to 285 µl 4 times diluted Lugol's solution, and scanned at 655 nm.

Amylose : amylopectin ratio

The apparent amylose content of starch samples was determined essentially as described by Hovenkamp-Hermelink *et al.* (1989). Three 2 mg starch samples were measured from each clone. The starch was dissolved in 50 µl $HClO_4$ (35%) and after addition of 100 µl of demineralized water was added, spun for 1 min. at 6500 rpm in an eppendorf micro centrifuge to remove debris. Of the starch

solution, 10 µl was added to 190 µl 35% $HClO_4$. After addition of 600 µl of demineralized H_2O and 100 µl of two times diluted Lugols' solution the sample was immediately scanned at 550 and 618 nm against a 35% $HClO_4$ solution. The amylose : amylopectin ratio was determined by comparing the values found for the samples to a standard curve. Amylose-free starch measured according to this method gave a value of ± 3% amylose, so values ≤ 3% were considered to be equal to zero for amylose content.

CL2B chromatography of native starch

Fractionation of amylopectin and amylose was performed with size exclusion chromatography by a sepharose CL2B column (2.6 by 200 cm, Pharmacia). A sample of 100 mg starch was dissolved in 1 ml 0.1M NaOH at 100 °C for 1 hour. The sample was diluted to 0.01 M NaOH, centrifuged at 3000*g for 5 min. and loaded on the column. The flow rate was 0.5 ml/min. and 0.01 M NaOH was the elution solution. Of each 10 ml fraction that was collected, 200 µl was added to 800 µl of Lugol's solution and scanned from 700-400 nm for determination of the λmax.

Results

For each construct/starch background combination twenty or more independent transgenic, kanamycin resistant, shoots were obtained and grown in the greenhouse for tuber production (Table 1). No differences were observed related to the constructs in obtaining transgenic plants. After determination of the starch branching degree, five to eight plants out of each population were chosen for further analysis. Each group of selected clones consisted of transgenic plants with the highest starch branching degree (DE) and those with a moderately altered or no altered starch branching degree. The clones of the last mentioned class were

picked at random. The individual transgenic clones were given names according to their original genetic background and the construct they carried (Table 1).

Table 1. Number and name of transformants obtained after introduction of different glgB containing constructs into amylose-free genotype 1029-31 or amylose containing genotypes A16 or Rv.

Construct	Amylose-free genotype Genotype	#	Name	Amylose containing genotype Genotype	#	Name
pB_{19}tpssuEC	1029-31	22	aTP-	Rv	24	vTP-
pB_{19}KG16EC	1029-31	32	aKG-	Rv	28	vKG-
pB_{19}13AN	1029-31	30	a13AN-	A16	40	z13AN-
pB_{19}09EC	1029-31	20*	aEC-	nd		

\# Number of independently obtained transgenic plants
* Analysis of some transformants was described before (Chapter 2)
nd not done.
The name of individual transgenic clones is followed by a number

Starch analysis (DE)

Starch was isolated from mature greenhouse grown tubers and subjected to structure analysis to determine whether the introduction and expression of the bacterial branching enzyme genes had an effect on the branching degree of the starch as was expected. The branching degree was expressed in DE, after isoamylase digestion of the starch, which is shown in Table 2. The DE of (un)transformed control plants was 3.6/3.7 for amylose containing and 3.9 for amylose-free starch. For the transgenic plants expressing the pB_{19}KG16EC construct in the amylose-free background the highest branching degree found varied between 5.0-5.2 for the transformants aKG-4, aKG-6, aKG-12, and aKG-34. The same pB_{19}KG16EC construct was transformed to a different genetic and

Table 2. Degree of branching (DE) of starch from transgenic clones transformed with bacterial branching enzyme genes and their controls.

Clone	DE	Clone	DE	Clone	DE
Amylose-free clones					
Construct pB_{19}KG16EC		pB_{19}tpssuEC		pB_{19}13AN	
aKG-4	5.0	aTP-4	4.8	a13AN-10	4.1
aKG–6	5.2	aTP-5	4.8	a13AN-15	3.9
aKG-9	4.7	aTP-8	4.2	a13AN-20	4.1
aKG-12	5.2	aTP-9	5.2	a13AN-25	4.0
aKG-19	4.8	aTP-13	4.8	a13AN-30	4.2
aKG-21	4.8	aTP-14	4.1	a13AN-35	4.2
aKG-27	3.9	aTP-28	5.0		
aKG-34	5.1	aTP-29	4.4		
Control 1029-31	3.9				
Amylose containing clones					
Construct pB_{19}KG16EC		pB_{19}tpssuEC		pB_{19}13AN	
vKG-1	3.7	vTP-15	3.7	z13AN-14	4.5
vKG -2	3.8	vTP -18	3.5	z13AN -24	4.5
vKG -9	3.7	vTP -22	4.9	z13AN -27	4.1
vKG -16	3.9	vTP -23	5.0	z13AN -38	3.7
vKG -20	3.9	vTP -26	3.9	z13AN -41	3.5
vKG -24	3.8	vTP -28	4.7	z13AN -43	3.7
vKG -25	3.8	vTP -36	3.6	z13AN -9	4.3
vKG -35	3.8	vTP -39	5.0		
vKG -36	3.6				
Control Rv	3.7			Control A16	3.6

amylose containing (Rv) background but none of the 32 tested transformants showed a clearly increased branching degree of the starch. The pB_{19}tpssuEC plasmid was transformed to both the amylose-free and the amylose containing background. In both series of transformants a number of plants were found with an increased branching degree of the starch. The DE had increased up to approximately 5 in both starch backgrounds. This meant an increase in branchpoints of approximately 25% of the starch for the amylose-free starches to almost 35% for the amylose-containing ones. The pB_{19}13AN construct in the A16 background resulted in several transformants with an increased branching degree (clones AN-9, AN-14, AN-24 and AN-27). The highest value found for the degree of branching was 4.5 (transformants AN-14 and AN-24), an increase of 25% compared to the value of the control starch A16 (DE = 3.6). The same construct was transformed to the amylose-free clone 1029-31. None of the 30 transformants showed a clearly increased starch branching degree compared to the control. These transgenic clones were excluded from further analysis.

DNA/RNA/protein analysis

Insertion of the heterologous branching enzyme gene in the potato genome was confirmed by Southern blot analysis of genomic DNA isolated from leaves. The number of minimally integrated T-DNA inserts ranged from 1 to 7 (Table 3).

Expression of the inserted chimerical bacterial branching enzyme genes was shown by Northern blot analysis of isolated tuber RNA (Table 3). The steady-state level of glgB expression for all the constructs was low, so only the visible presence (+) or absence (-) of RNA was noted. Expression of heterologous branching enzyme was analyzed by Western blotting using antiserum raised against the *E.coli* branching enzyme. Unfortunately, this antiserum did not cross-react to the *A.nidulans* branching enzyme as was expected. Attempts to raise a proper antiserum to the *A.nidulans* branching enzyme failed and, therefore, the plants transformed with the *A.nidulans* glgB could not be analyzed properly for

Table 3. Molecular characterization of selected, glgB transformed, amylose-free and amylose-containing potato clones.

Clone	T-DNA inserts	RNA*	Protein	Clone	T-DNA inserts	RNA	Protein
Amylose-free clones							
Construct $pB_{19}KG16EC$				$pB_{19}tpssuEC$			
aKG-4	6	+	++	aTP-4	4	+	+
aKG -6	1	+	++	aTP -5	1	+	+
aKG -9	7	+	++	aTP -8	nd	+	±
aKG -12	1	+	++	aTP -9	nd	+	++
aKG -19	4	-	+	aTP -13	1	+	++
aKG -21	nd	-	++	aTP -14	3	+	+
aKG -27	3	-	+	aTP -28	3	nd	++
aKG -34	2	nd	nd	aTP -29	1	nd	+
Amylose containing clones							
Construct $pB_{19}KG16EC$				$pB_{19}tpssuEC$			
vKG-1	nd	+	±	vTP-15	2	nd	±
vKG -2	2	-	±	vTP -18	3	+	±
vKG -9	nd	-	±	vTP -22	5	+	+
vKG -16	1	-	±	vTP -23	6	+	+
vKG -20	1	-	±	vTP -26	5	-	±
vKG -24	2	-	±	vTP -28	4	-	+
vKG -25	4	+	±	vTP -36	2	+	±
vKG -35	1	-	±	vTP -39	5	-	+
vKG -36	1	-	±				

nd = not determined
- = not detectable, ± = not clear or very low, + = present, ++ = >+

the protein expression of the heterologous branching enzyme gene in particular. In plants transformed with one of the *E.coli* glgB gene carrying plasmids the chimerical protein was found both in the soluble and in the starch bound protein fraction of the transgenic tubers. In Figure 2 the presence of the *E.coli* glgB protein is shown in the soluble and the starch-bound protein fractions of tubers of amylose-free plants transformed with the pB_{19}KG16EC construct. The lower arrow indicates the signal corresponding to the purified *E.coli* branching enzyme. The protein detected by the antiserum isolated from tubers of transgenic plants (upper arrow), migrated slower through the gel compared to the purified glgB protein, probably due to a slightly higher molecular weight than the purified *E.coli* glgB protein. This indicates that the transitpeptide is cleaved off at the splice site, leaving a chimerical protein with the 16 amino acid N-terminal addition. Expression levels of the heterologous *E.coli* BE were determined by signal intensity of Western dot blots (Table 3). Transformants carrying the pB_{19} KG16EC construct in the amylose-free background appeared to have higher expression levels compared to those with the amylose-containing background (where expression was very low or not detectable). In pB_{19t}pssuEC carrying transformants with an amylose-free background, expression of the *E.coli* protein was also more prominent. In more cases expression at the mRNA level could be detected as opposed to the transformants with the amylose-containing background. The highest expression levels of *E.coli* BE protein found for pB_{19}KG16EC carrying transformants were similar to those of pB_{19}tpssuEC carrying transgenic plants.

No obvious relationship was detected between the minimum number of integrated T-DNA copies and either mRNA or protein expression levels of the introduced *E.coli* BE gene. But, in general, the clones with the highest amount of *E.coli* BE protein also had the highest starch branching degree (Tables 2 and 3). The vKG- clones all showed low BE protein expression and no increased DE of the starch. In the amylose-free background however, higher protein expression

was found, especially in clones aKG-4, -6, -9 and -12, which are also among the clones with the highest starch branching degree (Table 2). The same was true for clones aTP-9, -13 and -28, and vTP-22, -23, -28 and -39, which displayed the highest level of *E.coli* BE expression and also the highest starch branching degree.

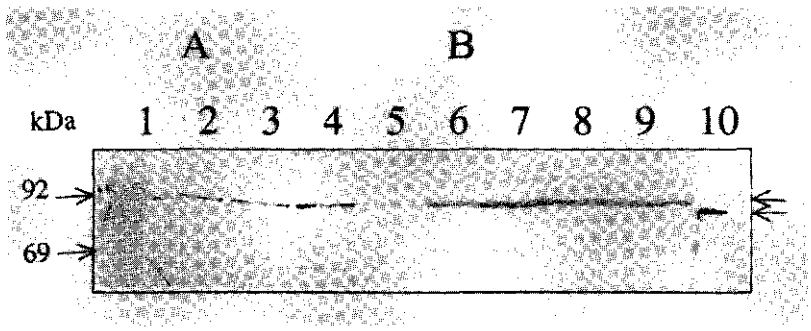


Figure 2. Western blot of the starch-bound (A) and soluble (B) protein fractions of transformants aKG-4 (lane 2,6), aKG-6 (lane 3,7), aKG-9 (lane 4,8) and aKG-12 (lane 5,9). Lane 1, rainbow marker. Lane 10, 5 pg of purified *E.coli* glgB protein.

Further analysis of starch (λmax, Blue Value and amylose content)

The λmax, Blue Value and amylose content of starch from most of the selected amylose containing transformants carrying the $pB_{19}13AN$ plasmid had not changed compared to the values of the control starch (data not shown). However, one of the transformants, AN-24, contained starch with a lowered amylose content (13 % compared to the control with 22%), a lowered λmax (565 nm instead of 575 nm) and the B.V. at 655 nm was reduced to 75% of the value for the control starch (see Chapter 5).

Table 4. Some characteristics of starch isolated from glgB transformed, amylose-free and amylose-containing transgenic plants and their controls.

Clone	λmax	B.V	AM%	Clone	λmax	B.V	AM%
Amylose-free clones							
Construct pB_{19}KG16EC				pB_{19}tpssuEC			
aKG-4	552	0.38	≤ 3	aTP-4	555	0.35	≤ 3
aKG-6	555	0.29	"	aTP -5	557	0.35	"
aKG -9	556	0.36	"	aTP -8	556	0.32	"
				aTP -9	555	0.28	"
aKG -19	554	0.34	"	aTP -13	555	0.31	"
aKG -21	555	0.35	"	aTP -14	557	0.32	"
aKG -27	555	0.27	"	aTP -28	554	0.35	"
aKG -34	556	0.35	"	aTP -29	554	0.35	"
Control 1029-31	555	0.31	"				
Amylose containing starches							
Construct pB_{19}KG16EC				pB_{19}tpssuEC			
vKG-1	579	0.49	20.0	vTP-15	577	0.54	18.5
vKG-2	581	0.48	20.1	vTP -18	560	0.35	3.9
vKG -9	584	0.53	16.4	vTP -22	563	0.38	7.4
vKG -16	573	0.52	18.4	vTP -23	557	0.32	≤3
vKG -20	570	0.48	19.0	vTP -26	583	0.50	18.0
vKG -24	574	0.48	18.7	vTP -28	555	0.32	≤3
vKG -25	571	0.44	17.9	vTP -36	578	0.44	17.2
vKG -35	574	0.47	19.5	vTP -39	556	0.30	≤3
vKG -36	572	0.44	19.0				
Control Rv	579	0.50	20.3				

B.V = Blue value at 655 nm, S.D.= ± 0.1. λmax S.D.= ± 3.0 AM% = Apparent amylose content, S.D.= ± 2.0.

The results for transformants carrying the pB_{19}KG16EC or the pB_{19}tpsEC plasmid are presented in Table 4. The transformants with either the pB_{19}tpssuEC or the pB_{19}KG16EC construct in the amylose-free background did not show a change in either λmax nor B.V. compared to starch from the control. The values of apparent amylose content for the transformants with the 1029-31 background were all less than 3 and considered to be negligible. Transformants in the amylose-containing background, carrying the pB_{19}KG16EC plasmid, showed variation in λmax and apparent amylose content. The differences in Blue Value were within the normally observed variation. More obvious were the changes in λmax, Blue Value and apparent amylose content for several amylose-containing transformants with the pB19tpssuEC construct. Starch from transformants vTP-18, vTP-22, vTP-23, vTP-28 and vTP-39 showed a decrease in λmax of up to 20 nm compared to the control, approximating amylose-free starch values. The same was seen for the Blue Values and amylose : amylopectin ratios of starch from these transformants, the values found resembled those of amylose-free starch.

Starch granule morphology

Starch granules of transformants were stained with iodine and examined by microscopy. Starch granules from amylose-free plants contained only red-staining granules. Starch with an amount of amylose ≥ 15% contained completely blue-staining granules. The granules of amylose containing transformants with a much reduced amylose content (≤ 3%) showed a blue-staining core surrounded by red-staining starch. The size of the blue staining core varied from medium to almost granule filling, but was larger than expected based on the amylose content and compared to starch granules from antisense inhibited GBSS plants with a similar amylose content. Granule shape and size did not seem to be affected for any of the transgenic plants.

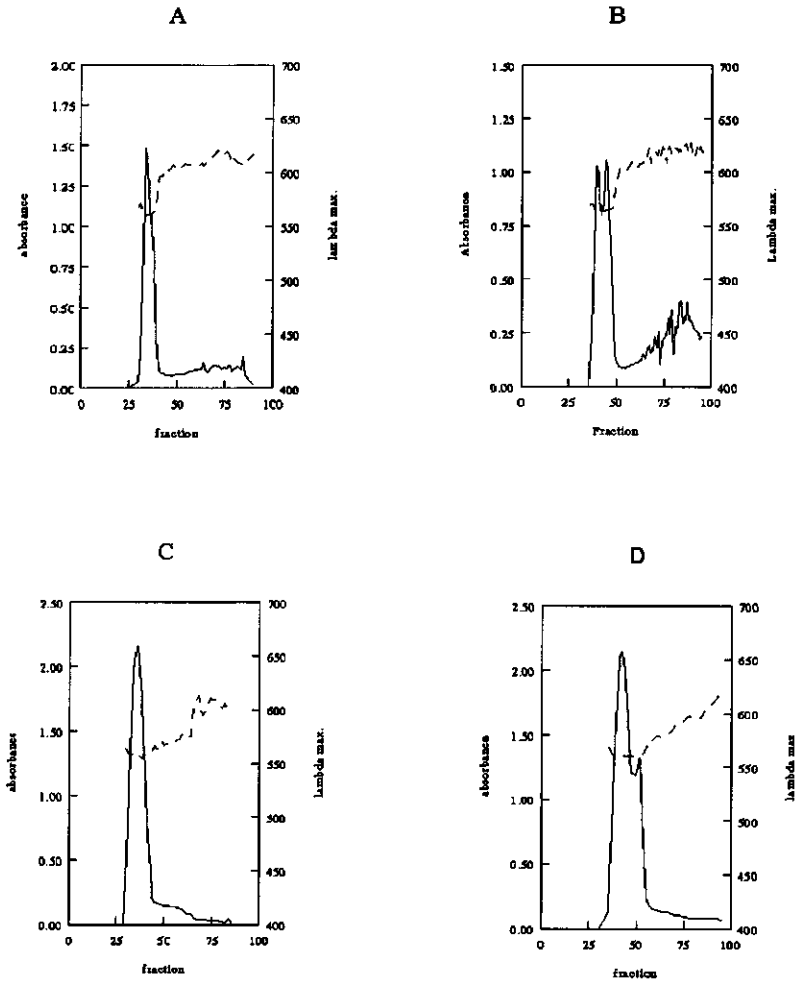


Figure 3. Size exclusion chromatography of native starches from amylose containing transformants. A, control starch Rv; B, starch from transformant vTP-15, C, starch from vTP-22 and D, starch from vTP-23. The left Y-axis represents the absorbance (unbroken line) at λmax and is an indication for the amount of glucan eluted from the column. At the right Y-axis the λmax value (dotted line) for each fraction is given.

Starch fractionation by CL2B chromatography

For the control wildtype starch as well as for the starch of transformant vTP-15 with a normal amount of amylose, amylopectin is eluted from the column as a narrow peak followed by the broader amylose peak (Fig. 3). The absorbance is a measure of the amount of glucan eluted from the column. The λmax values of the corresponding fractions show the branched nature of the eluted glucan. The narrow amylopectin peak showed a maximum absorption at 560 nm after complexation with iodine and the fractions of the amylose peak showed a lambda max of ≥ 600 nm. A sharp increase in λmax (from 560 to ≥ 600nm) of the fractions can be observed just after elution of amylopectin from the column. Starch from e.g. transformant vTP-22 and vTP-23 showed a large amylopectin peak and contained only a very small amount of amylose (λmax ≥ 600).

The amylose fraction decreased or disappeared and a new fraction emerged. This new fraction eluted from the column directly after the amylopectin peak and displayed a λmax between 560-600 nm and was termed intermediate fraction. In native cereal starches a similar fraction is sometimes found and called the intermediate fraction (Whistler and Duoane, 1961). This intermediate fraction is believed to contain long-chained low molecular weight amylopectin or branched amylose and displays λmax values of 560-600.

Comparison of the effects of the constructs in amylose-free and amylose-containing backgrounds

In Table 5 an overview is given of the results of transformation with the constructs used in the different backgrounds. For all the constructs which were used for transformation, transgenic plants could be found containing starch with an increased branching degree, although not always in every genetic background. It was observed that in the population of Rv plants transformed with the pB_{19}KG16EC construct, none of the transgenics contained starch with an increased branching degree. In the amylose-free background however, almost

Table 5. Overview of the effects of the different constructs in the amylose-free and the amylose containing background.

Construct	Starch composition (genotype)	No. of plants with higher DE#	Number of plants with DE max*
pB19tpsEC	amylose-free (1029-31)	9	2
" "	amylose containing (Rv)	4	2
pB19KG16	amylose-free (1029-31)	16	9
" "	amylose containing (Rv)	none	0
pB1913AN	amylose-free (1029-31)	none	0
" "	amylose containing (A16)	6	3

in amylose-free background DE of ≥ 4.6, in amylose containing background DE of ≥ 4.3
* DE max for transformants in 1029-31 and Rv background was 5.0, DE max for transformants in A16 background was 4.5.

half of the plants transformed with this construct showed an increase of the starch branching degree and 30% of the plants contained starch with a DE after isoamylase digestion of 5.0 or higher. The same branching enzyme gene but targeted by the transitpeptide of the SSU in the pB_{19}tpssuEC construct was found to have an effect on the starch branching degree in both the amylose-free and the amylose containing background. In both populations of transgenics, plants were found containing starch with an increased branching degree with a DE up to approximately 5. The pB_{19}13AN construct transformed to the amylose containing background A16 led in a number of plants to the increase of the branching degree of the starch. The highest DE found after isoamylase digestion of the starch was 4.5. The (un)transformed control starch showed a DE of 3.6, so the DE had increased approx. 25%. One of the starches with a higher branching degree (transformant AN-24) also showed a lowered λmax and Blue Value as well as a lowered amylose content as was observed for the Rv plants transformed with the pB_{19}tpssuEC-construct.

Discussion

Transgenic plants were obtained which expressed a chimerical glycogen branching enzyme with N-terminal addition of GBSS protein of varying size, or the 5 N-terminal amino acids of the SSU protein. Steady state mRNA level was low compared to the expression of the endogenous branching enzyme mRNA (data not shown), which could be due to high turnover or instable secondary structures of the mRNA. Heterologous branching enzyme expression was also low for the *E.coli* glgB gene in the soluble and starch bound protein fraction of the tubers for plants transformed with the pB_{19}09EC (shown in Chapter 2 of this thesis), the pB_{19}KG16EC as well as the pB_{19}tpssuEC construct. Based on the size of the protein detected on the blots (Fig. 2 of this Chapter and Fig. 3 in Chapter 2

of this thesis), processing of the heterologous branching enzyme seemed to occur correctly. From previous experiments (unpublished results) it was already concluded that an intact splice-site was essential for targeting a protein across the amyloplast membrane by the GBSS transitpeptide, but fortunately the influence of an N-terminal addition, creating a chimerical protein, on the enzyme activity is only partial or absent. In general, plants expressing most protein, as seen by Western dot blotting (Table 2), also showed the highest branching degree of the starch. Similar relations between branching degree and mRNA level or copy number of the inserted genes were not observed. Altered wildtype starches with a lowered amylose content displayed a lowered λmax and Blue Value as well, which was expected since these characteristics are all concerned with iodine binding properties of the starch. The same was described for the starch of maize mutants in a study by Wang *et al.*(1993b). Most starches with a lowered amylose content also had an increased starch branching degree, which could at least partly be explained by a relatively higher amount of amylopectin.

All constructs used for transformation showed the same effect on starch: an increased branching degree. The differences between the populations of transformants can be seen as the percentage of plants with a maximum effect (as shown in Table 5). Earlier it was reported that expression of the *E.coli* glgB gene in plants with an amylose-free background had an effect on the amylopectin structure (Chapter 2, this thesis). From the results presented here we conclude that the bacterial branching enzymes also act on the amylose as a substrate for branching, because the apparent amylose content decreased dramatically in some of the transformants (Table 4). Based on sequence homology, the bacterial branching enzymes belong to the group of Type-II branching enzymes, which were shown by Guan and Preiss (1993) to have a preference for a more branched glucan (like amylopectin) as a substrate. So, it was expected that the introduced branching enzyme would branch amylopectin, which indeed happened as was shown in this study (and in Chapter 2). However, in a number of cases a

disappearance of the amylose content was observed as well. Data from CL2B chromatograms indicated that the almost unbranched amylose was substituted by an intermediate fraction with a higher branching degree. Another indication for this was the blue-and-red staining pattern of starch granules from transgenic plants with an amylose content of ≤ 3%. According to the method used, an amylose content of ≤ 3% equals amylose-free. Therefore, it is assumed that the blue staining core of the granules does not contain amylose but blue-staining intermediate fraction derived from the amylose. This would also explain why the blue-staining core is larger than in starch granules from antisense inhibited GBSS plants with a similar amylose content. Confirmation of the nature of the intermediate fraction will have to be assured by further structural analysis.

Another explanation for the decrease in amylose content could be co-suppression of the endogenous GBSS gene, because the GBSS promoter and transitpeptide sequences of potato were used to direct expression and targeting of the heterologous branching enzyme. Supporting this idea were the findings that in some of the transgenic plants with a decreased amount of amylose, a decreased amount of GBSS protein and GBSS activity were measured (data not shown). However, this effect of decreasing the amylose content of the starch was most evident in transgenic plants carrying the pB_{19}tpssuEC construct which contained only the GBSS promoter sequence. The potato GBSS promoter sequence by itself was shown by Flipse *et al.*(1996b) not to be able to cause co-suppression of the endogenous GBSS gene in any of the over one hundred analyzed potato transformants. Here, we found almost a third of the plants to have a decreased amylose content.

No clear differences were observed between starch from transgenic plants carrying the glgB gene of *E.coli* and starch from those transformed with the *A. nidulans* branching enzyme. Although this was expected because of the differences in glucan produced by the two different prokaryotes. In each series of transformants the transgenic plants with the highest starch branching degree

contained starch with about 25-35% more branchpoints compared to their respective control. The highest DE found (after isoamylase digestion of the starch) was 5.2, regardless of the construct used for transformation or the genetic background. The reason for the minor increase in branching degree of the starch from transgenic plants could be the structure of the amylopectin and of the starch granule itself. As the amylopectin structure is evolutionary highly conserved, possibly a more branched amylopectin does not fit the granule structure without distorting its shape and will not readily be incorporated. A confirmation of this theory is the fact that some existing starch mutants display a different granule morphology as is shown in the pea mutant of the *r* locus (Bogracheva *et al*, 1995) and in the various maize mutants in which branching enzymes are involved (Wang *et al.*, 1993a). Another explanation for the relatively minor increase in starch branching degree could of course be the low RNA and protein levels found in the transgenic plants. Higher expression of the glgB gene would possibly lead to a more branched starch. Or maybe the N-terminal addition of GBSS/SSU sequences hinders the enzyme activity in some way, although it would be expected to find more variation between the populations of transformants because of the different N-terminal additions and genes used.

Similar to starch from glgA expressing potato plants (Shewmaker *et al.*, 1994), starch from glgB expressing plants showed a decreased amylose content and an increased branching degree of the amylopectin. However, starch from glgB expressing tubers contained a more intensively branched amylopectin and a lower amount of amylose (values sometimes similar to amylose-free) than starch from glgA expressing plants whereas the total starch and soluble sugar content of the tubers had not changed (data not shown).

Acknowledgements

We would like to thank Aletta Karsies, Alexandra Hillen, Emile Clerxks and Marja Roozeboom for transformation, transferring the plants into the greenhouse and helping with the analysis of the transgenic plants. Elly Janssen was responsible for the Southern blotting and Irma Straatman-Engelen prepared the Northern blots.

Physico-chemical properties of starches from genetically modified potato with a decreased amylose content

4

Anne J. Kortstee, Luc C.J.M. Suurs, Angela M.G. Vermeesch and Richard G.F. Visser.

Part of this Chapter was published in the Proceedings of the conference on Starch: Structure and Function. 15-17 April 1996, Cambridge, UK.

Physico-chemical properties of starches from genetically modified potato with a decreased amylose content

Abstract

Starch from a potato clone transformed with the *Anacystis nidulans* branching enzyme gene (glgB) with an increased degree of branching and a lowered amylose content (AN-24), was characterized. A comparison was made at the physico-chemical level between starch from AN-24 and its untransformed control A16, and starch from Granule-Bound Starch Synthase (GBSS) antisense gene transformed plants showing a similar decrease in amylose content. Some of the altered properties of starch from transformant AN-24 could be attributed to the lowered amylose content but others such as the lowered peak viscosity and swelling power were more likely the result of an increased branching degree.

Introduction

Starch is the most abundant storage carbohydrate found in higher plants. It consists of two types of glucose polymers; amylose an essentially unbranched α-1,4 linked glucose polymer and amylopectin, consisting of α-1,4 linked chains with α-1,6 branchpoints. Starch composition, like amylose to amylopectin ratio, molecular size and branching degree of amylose and amylopectin determines the physical properties of starch and therefore its specific industrial applications (Swinkels, 1985a/b). Each botanical source of starch has a unique composition

and the genetic variation within species for structural characteristics only adds to the diversity of starch thermal and functional properties (Campbell *et al.*, 1995). The enzyme responsible for the formation of amylose is Granule Bound Starch Synthase (GBSS), amylopectin is synthesized by the combined action of the enzymes Soluble Starch Synthase (SSS) and Branching Enzyme (BE) (Martin and Smith, 1995). Suppressing the expression of one or more of the starch synthesizing genes by means of antisense RNA technology results in starches with an altered composition as was reported before (Visser *et al.*, 1991, Müller-Rober *et al.*, 1992, Flipse *et al.*, 1996a/b). Partial inhibition of GBSS gene expression results in starch with a lowered amylose content (Kuipers *et al.*, 1994a). The reduced amount of amylose was found in a restricted zone in the granule. The amylose was present in a core of varying size at the hilum of each granule (Kuipers *et al.*, 1994). In our attempt to influence the starch branching degree, bacterial branching enzyme genes were introduced into potato. The glgB gene of *A.nidulans* was placed under the transcriptional control of the potato GBSS gene and transitpeptide sequence including box1, the substrate binding site, of the mature GBSS protein. Expression of this construct in potato tubers lead in some transformants to an increased branching degree of the starch. In one of the transformants, number AN-24, also a lowered amylose content was found. The questions arose whether the decreased amylose content was caused by the expression of the bacterial branching enzyme or by a side effect of the construct used and whether, apart from the lowered amylose content, the amylopectin structure had changed as well. It was decided to investigate some properties of altered starch and to compare it to starch with a lowered amylose content as a result of antisense expression of the GBSS gene and to starch with an increased branching degree as a result of the expression of the heterologous branching enzyme gene of *A.nidulans*. By comparing physico-chemical properties of AN-24 starch with those other two types it may be possible to determine whether AN-24 has an altered amylopectin structure, in addition to a lowered amylose content.

Experimental procedures

Plant material

Tetraploid, 2n=4x=48, potato clone K89.2002 (Karna, Valthermond, Holland) transformed with constructs pKGBA50 or pGB50 (Kuipers *et al.*, 1995). Plasmid pKGBA50 carried the GBSS cDNA of potato in anti-sense orientation under the control of the potato GBSS promoter and pGB50 carried the GBSS cDNA in anti-sense orientation under the control of the CaMV promoter. Individual transformants, selected for incomplete GBSS inhibition, used in this study were: tBK50-10, tBK50-34 and tB50-42. Diploid, 2n=2x=24, potato clone A16 was transformed with construct $pB_{19}13AN$, carrying the glgB gene of *A.nidulans* under the transcriptional control of the potato GBSS promoter sequence including the transitpeptide sequence and box1, the substrate binding site, of the mature protein as described in Chapter 3. Individually selected clones were AN-9, AN-14 and AN-24.

Isolation of starch, granule size/morphology, λmax and Blue Value

Starch from field-grown tubers from transgenic plants carrying the bacterial branching enzyme gene was isolated as described before (Chapter 4, this thesis). Starch from tubers of antisense inhibited GBSS plants was isolated as described by Kuipers *et al.* (1994b).

Average granule size and the size distribution of the starch granules were determined by a Coulter counter multisizer IIe. 10 mg of starch was dispersed in 160 ml of Isoton II as described by the manufacturer. Granule morphology was checked by microscopy after staining the granules with iodine. The apparent amylose content of starch was determined according to the method described by Hovenkamp-Hermelink *et al.*(1989).

The λmax and Blue Value of an 0.01% starch solution w-ere determined after staining with iodine as described by Delrue *et al.* (1992).

Determination of the branching degree (DE) of starch

The branching degree of starch was determined as the reducing power after isoamylase digestion of the starch and expressed as dextrose equivalent (DE), as described in Chapter 2 of this thesis by the Luff-Schoorl method (1925).

Western blot analysis

Protein isolation, gel electrophoresis, blotting and immuno detection were carried out according to the methods described in Chapter 2 and 3 of this thesis.

GBSS activity

The GBSS activity was measured as described by Vos-Scheperkeuter *et al.* (1986), two-milligram samples were measured in triplo for each clone.

Starch fractionation by CL2B chromatography

For fractionation of amylose and amylopectin with size exclusion chromatography, 100 mg of starch was dissolved in 10 ml 0.1 N NaOH, by boiling for 30 min. The dissolved starch was diluted to 0.01 N NaOH and loaded on the Separose CL2B column (2.6 by 200 mm). Elution with 0.01 N NaOH was at a flow rate of 30 ml/hr. Fractions of 10 ml were collected. The λmax of each fraction was measured after complexing 50 µl of fraction with 4 times diluted Lugols solution (1%KI/0.1%I2).

Swelling power of starch in water during heating

Swelling power and solubility were determined according to Leach *et al.* (1959) with some modifications. Amylose containing starch suspensions (0.5% w/v) were heated to 60, 65, 70, 75 and 80 °C respectively, and kept at that temperature for 30 min. at slow stirring at 100 rpm (MIRAK^{tm} thermolyne) just sufficient to keep the starch completely suspended and to minimize shearing of swollen granules. Amylose free starch suspensions (0.5% w/v) were heated to 66-80 °C

(with two degrees Celsius intervals) and kept at that temperature for 30 min. under slow stirring at 100 rpm. The swollen starch suspensions were poured in a measuring glass and allowed to precipitate overnight. The volume of sedimented swollen granules was estimated and a sample of the clear supernatant was removed to determine the amount of dissolved starch. The percentage of dissolved starch was determined with the Boehringer kit no. 207748 for measuring starch content, according to the manufacturers specifications but with smaller volumes to fit in a microtiterplate.

DSC

Differential Scanning Calorimetry was performed with a Perkin Elmer Pyris 1 equipped with a Neslab RTE-140 glyco-cooler. The instrument was calibrated with indium (mp= 156,6 °C) and zinc (mp= 419,47 °C). Approximately 10 mg of starch (dry weight basis) was weighed accurately into a stainless steel cup and 40 µl of dH2O was added. The cup was hermetically sealed and allowed to equilibrate at least 1 hour before analysis. The suspension was heated from 20 °C to 100 °C at a scanning rate of 10 °C/min. An empty stainless steel cup was used as a reference. For each endotherm the onset temperature (To) of melting and the enthalpy ΔH (J/g) were computed automatically. The results are the average of three scans. Enthalpies were calculated on a starch dry-weight basis. The dry matter content of the starch was determined at least in duplicate by oven drying of 30 mg samples for 4 hours at 120 °C.

Viscosity (RVA/Bohlin)

An 8% starch suspension in dH_2O (dwb) was placed in the RVA cup. The heating profile used was as follows: 2 min. at 45 °C, heating to 90 °C at a rate of 5 °C/ min., heating at 90 °C for 6 min., cooling from 90 °C to 30 °C at 14 °C/min. and keep at 30 °C for 6 min. The viscosity was measured in RVA units. For both the amylose containing and the *amf* starches the pasting profile of a 5% starch : water

(w:v) suspension was measured with a Bohlin VOR Rheometer (Mettler Toledo, Tiel, The Netherlands) as described by Flipse *et al.* (1996b). The pasting profile existed of heating to 90 C, keeping the paste at this temperature for 15 min., and cooling to 20 °C at a rate of 1 °C/min.

Gel strength

Starch pastes (20%, w:v) in water were prepared by adding 3.6 g of starch (20% moisture) to 14,4 ml of preboiled dH_2O in a straight side wide mouth polypropylene jar (Nalgene, 2118-9050). The jar was placed in a waterbath of 50 °C on a plate and stirred at 300 rpm by a 25X6 mm magnetic stirrer to disperse the starch. The sample was then heated to 80 °C while stirring, until the stirrer became trapped in the gel, what usually happened at about 70 °C, at the start of gelation. Immediately the sample was transferred to a 90 °C waterbath, incubated for one hour, cooled on ice and stored at 4 °C for five days. Gelstrenght was measured using a texture analyzer (T04, Etia, Compiegne, France) with a 20 N loadcell and a punch probe with a diameter of 6 mm. The gel was compressed at a speed of 2 mm/sec until breaking. For each sample, 6 separate gels were prepared and measured.

Results

Starch granule size and morphology

Granule size and morphology were determined by the Coulter Counter and by microscopically analysis of iodine stained starch. Granule size and shape did not seem to be altered in the transformed plants compared to their controls. Looking at iodine stained starch it was observed that the anti-sense inhibited GBSS plants had red staining starch granules with a blue core of varying size, from very small to granule filling as was reported before (Kuipers *et al.*, 1994, 1995a/b). The

untransformed control contained completely blue staining granules. Transformants with the pB1913AN construct all contained completely blue staining starch granules, except transformant AN-24 which contained red staining granules with a blue core, varying in size from very small to completely granule filling (Fig. 1).

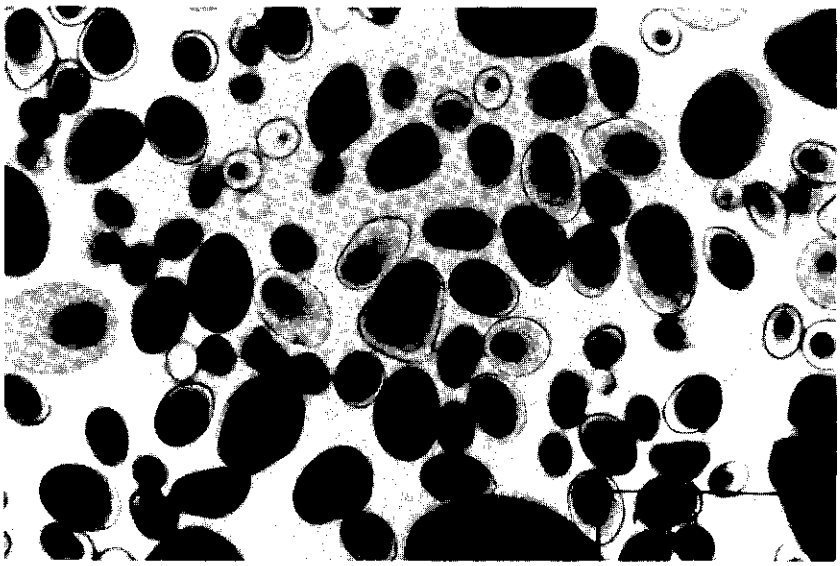


Figure 1. Starch granules from transformant AN-24 stained with iodine. The bar represents 20 μM.

Starch composition and structure

The apparent amylose content of the starches was determined by a spectrophotometrical method. Untransformed potato (=control) starch of clone K89.2002 contained about 20% amylose, the GBSS inhibited transformants showed a decreased amylose content ranging between 9 to 15% (Table 1). Some transformants with the $pB_{19}13AN$ construct showed a change in amylose content like transformant AN-24 which had a lowered amylose content of 10 %, and

transformant AN-14 which showed an apparent amylose content of 15%, although the granules were completely blue after staining with iodine.

Table 1. GBSS enzyme activity and apparent amylose content of starch granules isolated from tubers of transgenic plants.

Clone	GBSS activity* in % compared to the control	Amylose content* %
K89.2002[a]	100 ± 10	20 ± 1
tBK50-10[a]	90 ± 60	9 ± 3
tBK50-34[a]	" "	12 ± 7
tBK50-42[a]	" "	15 ± 4
A16	100 ± 4	21.4 ± 1
AN-9	114 ± 6	20.3 ± 1
AN-14	110 ± 6	14.6 ± 2
AN-24	66 ± 6	10.4 ± 2

* All values are the average of three independent measurements
[a] Data obtained from Kuipers *et al.* (1994b)

The λmax and Blue Value of solubilized starch were determined spectrophotometrically. The λmax of AN-24 starch with a lowered amylose content was lowered as was the Blue Value. The branching degree of the starch was determined by the Luff-Schoorl method. The Dextrose Equivalent (DE) after isoamylase digestion is expressed as the percentage of α-1,6 branchpoints per dry weight starch. The DE in the antisense transformants had probably increased as a result of the lowered amylose to amylopectin ratio. As amylose contains nearly no branchpoints compared to amylopectin the percentage of branchpoints per dry weight will increase if the amylose content is lowered. In transformant AN-9, expressing the glgB gene of *A.nidulans* the amylose content had remained the same, but the DE had increased. The amylopectin of this type of starch contained more short chains, the so-called A chains in their amylopectin (data not shown).

For transformants AN-14 and AN-24 a lowered amylose content was found and a higher DE, but also an increased ratio of short to longer chains after HPLC analysis of isoamylase digested amylopectin (data not shown). The increased DE after isoamylase digestion of AN-14 and AN-24 starch was probably caused by the lowered amylose content as well as the presence of more short chains in the amylopectin.

Table 2. λmax, Blue Value and branching degree (in DE) of starch isolated from tubers of transgenic plants.

Clone	λmax (nm)	Blue Value (at 680 nm) % of control	DE % of control
K89.2002	578	100	100
tBK50-10	558	64	117
tBK50-42	570	n.d	117
A16	574	100	100
AN-9	576	96	114
AN-14	575	98	125
AN-24	567	54	125

n.d = not determined

GBSS analysis

The selected transformants, tBK50-10 and tBK50-42 expressing the pKGBA50 construct, showed in our experiments a decreased expression of the GBSS protein (Fig 2) as was described before (Kuipers *et al.*, 1994b). Enzymatic activity of the GBSS protein had also decreased as can be seen in Table 1. The level of expression of the endogenous branching enzyme had not changed (data not shown). Tubers of the plants transformed with the pB1913AN construct, AN-9, AN-14 and AN-24, were found to have normal GBSS mRNA and protein levels.

One of them, number AN-24, showed a lowered GBSS activity, although the amount of GBSS protein present in the starch had not decreased as can be seen in Fig. 2. The endogenous branching enzyme mRNA and protein levels had not changed (data not shown).

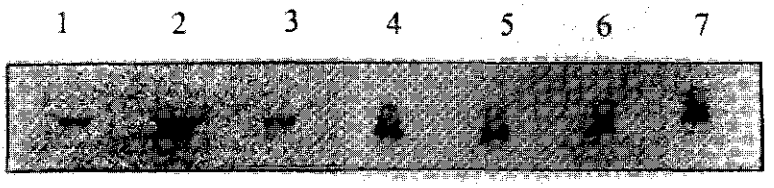


Figure 2. Western blot analysis of the starch protein fraction hybridized to antiserum raised against potato GBSS protein. Starch protein from lane 1= tBK50-42, lane 2=K89.2002, lane 3= tBK50-10, lane 4= A16, lane 5= AN-9, lane 6= AN-14, lane 7= AN-24.

CL2B chromatography

Amylopectin and amylose were separated by molecular size by CL2B gel permeation chromatography. The elution profiles are presented in Fig. 3 and were all very similar. The amylopectin elutes from the column as one peak, sometimes with a shoulder. The λmax increases sharply from 560 (amylopectin) to ≥ 600 nm, just after the amylopectin is eluted from the column. From the signal intensity of the absorbance it was concluded that starch from transformant tBK50-10 contained less amylose compared to the original background K89.2002. The same was true for starch from transformant AN-24 compared to its control A16.

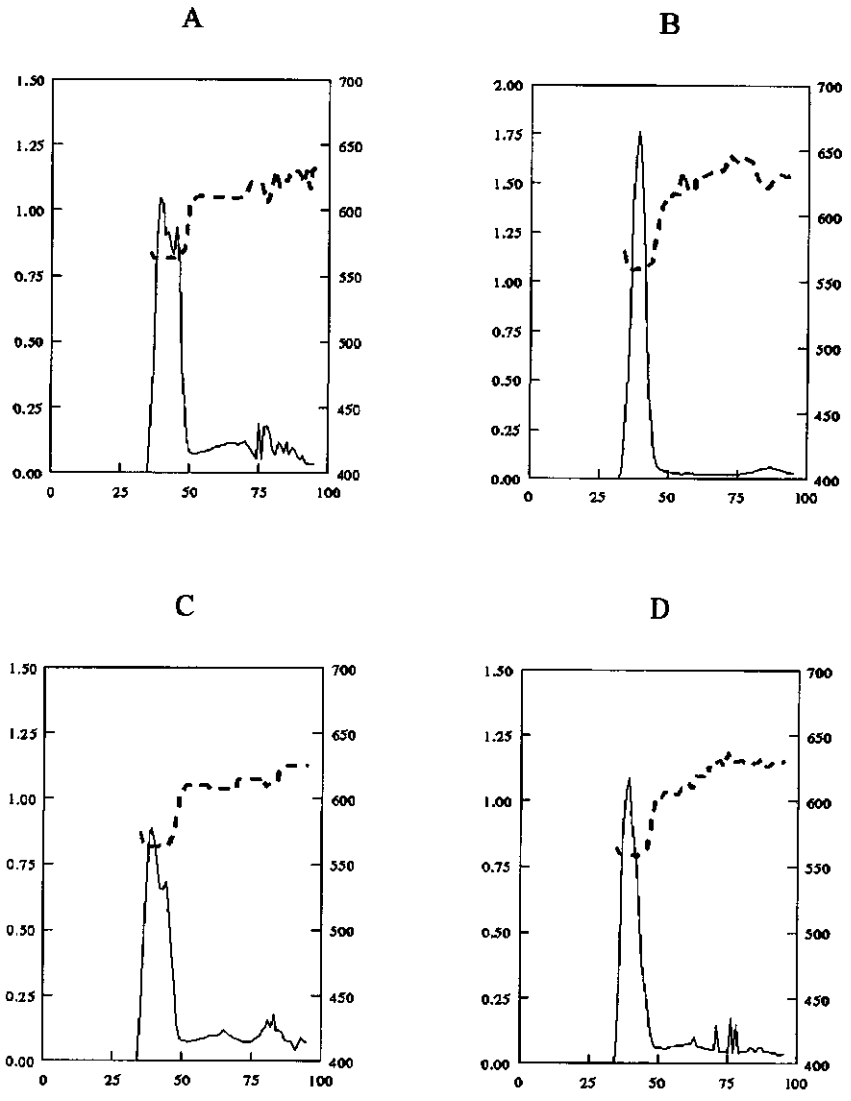


Figure 3. CL2B gel permeation profile of starches. The numbers on the X-axis represent the fractions, at the left Y-axis the absorbance at λmax is denoted and at the right Y-axis the λmax . ———, Absorbance at λmax, - - - -, λmax. A, K89.2002; B, tBK50-10; C, A16; D, AN-24.

Swelling power

The swelling power and solubility of starch in water were determined during heating and are presented in Fig. 4. Normal amylose containing potato starch swells linear in time with increasing temperature. Amylose-free starches swell much faster but usually begin to swell at a higher temperature. The starches of plants expressing the antisense GBSS gene showed a faster swelling pattern than the untransformed control so that at 70 °C the amount of free water becomes limiting for swelling. Solubility at 75 °C was lowered from 8% to 1-2% for the GBSS inhibited starches. This was also observed in amylose-free starch (data not shown). Starch from plants expressing the *A.nidulans* glgB gene showed a lowered swelling power compared to the untransformed control (data not

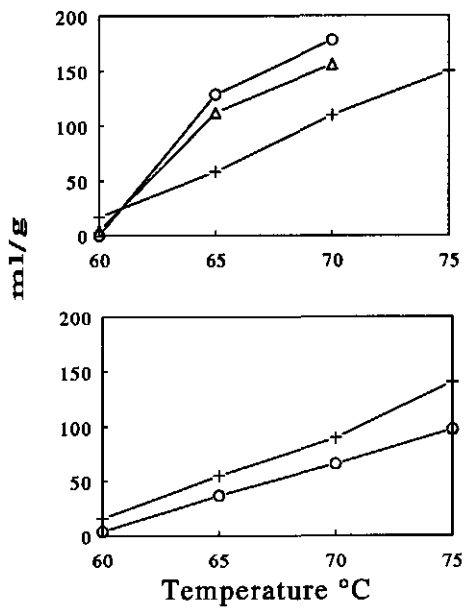


Figure 4. Swelling power of starch in water. Swelling power is determined as the volume of water taken up by 1 gram of starch and corrected for solubilized starch. Top: + = K89.2002, Δ = tBK50-42, O = tBK50-10. Bottom: += control A16, O = AN-24.

shown). The transformant with the lowest amylose percentage, AN-24, showed the characteristic *amf* lowered solubility (2% instead of 8%) as well as a sligthly lowered swelling power compared to the control A16.

Thermal analysis

DSC. The temperature of onset of gelatinization (To) and other gelatinization characteristics were measured by DSC. The results of the DSC analysis are presented in Table 3.

Table 3. Thermal analysis by DSC of starch from transgenic plants.

Clone	To (°C)	Tp (°C)	ΔH (KJ/g)
K89.2002	60.3	64.5	16.5
tBK50-10	64.0	68.8	21.7
tBK50-34	62.9	67.2	24.2
tBK50-42	63.5	69.5	21.3
A16	59.3	63.3	16.7
AN-9	59.7	64.2	19.4
AN-14	60.8	65.5	21.1
AN-24	60.9	66.6	18.4

Starch of transformants AN-14 and AN-24 showed a slight increase in the temperature of onset of gelatinization (To) compared to the untransformed control (+ 1 °C). Likewise the peak temperature (Tp) had shifted to a higher temperature, and a slightly higher enthalpy (ΔH) but a similar gelatinization range (Tp-To) were found. Starches from tubers from antisense GBSS inhibited plants with a lowered amylose content showed an increase in the temperature of onset of gelatinization (To) of about 3 °C. Likewise the Tp had shifted to a higher temperature and the enthalpy had increased slightly.

Bohlin. Dynamic rheological properties were determined by applying a small oscillating shear deformation using a Bohlin VOR Rheometer as described by Keetels (1995). The results are shown in Fig. 5. The storage modulus (G') of the starch/water suspensions increased sharply upon heating. After a maximum was reached the viscosity decreased during further heating. After cooling the viscosity increased again as a result of retrogradation of, presumably, amylose. The difference between the control K89.2002 and the transformant with partial GBSS inhibition and a lowered amylose content tBK50-42 can be seen in Fig 5.

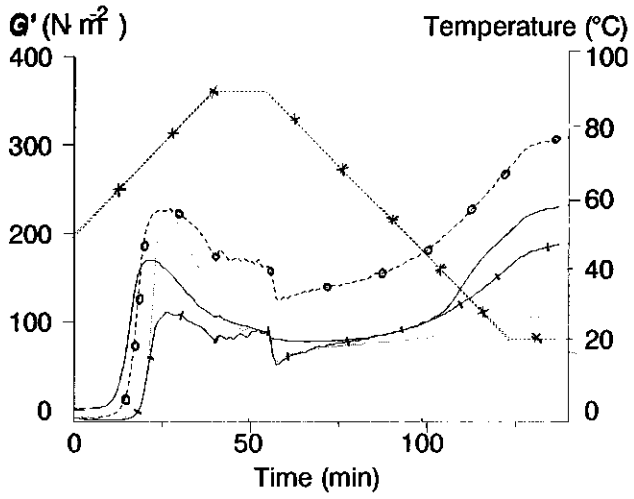


Figure 5. Changes in the storage moduli (G') of 5 % (w/v) starch suspensions against time and temperature (-*-), -O- = K89.2002, ... = tB50-42, ——= A16, —+—= AN-24.

The viscosity of the transformant at the onset of gelatinization, starts to increase at a higher temperature compared to the untransformed control. The maximum viscosity however is reached at the same time/temperature and is of similar height. The viscosity as a result of retrogradation after cooling is almost absent in the antisense GBSS transformant with the lowered amylose content. Starch from transgenic plants expressing the glgB gene of *A.nidulans* showed the same viscosity profile as the untransformed control except for a lowered peak viscosity for the starch with an increased branching degree described in Chapter 5 of this thesis. Starch from transformant AN-24 not only had a lowered peak viscosity but also showed a higher temperature of onset of gelatinization. The increase in viscosity as a result of retrogradation of amylose was observed for all amylose containing starches.

Relative compression

Relative compression studies were carried out with starch gels from AN-24 and A16 plants. There was not enough starch available to do the same with starch from the antisense inhibited GBSS plants. A sample of amylose-free starch, from the mutant potato clone 1029-31 (Jacobsen *et al.*, 1989), lacking GBSS activity and amylose, was measured instead and can be thought of as most resembling starch from antisense inhibited GBSS plants. The results of the measurements can be seen in Fig. 6. The starch gel from starch of transformant AN-24 broke at an applied stress of 1.5 N, which was considerably lower compared to the untransformed starch gel of A16, which required almost twice as much force before breaking. The gel from starch of the amylose-free potato mutant showed more similarities with the AN-24 starch gel in terms of force required to break the gel and the slope of the curve. Both were much weaker than the A16 control.

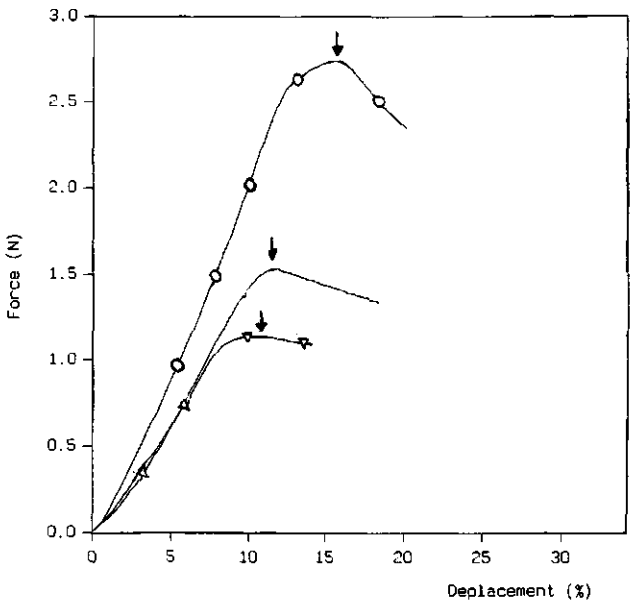


Figure 6. Relative compression study (gelstrength) of starch gels (20 % w:v) of transformant AN-24 (——), its untransformed control A16 (—o—), and an amylose-free potato mutant 1029-31 (—Δ—).

Discussion

In this study we compared some physico-chemical properties of starch from transgenic potato plants. Plants transformed with a construct carrying a bacterial branching enzyme gene showed an increased branching degree of the starch. One of the transformants, number AN-24, had a halved amylose content. To

determine whether the effect on the starch in AN-24 concerned only the amylose or also the amylopectin fraction of the starch, starch of GBSS inhibited plants (containing a decreased amount of amylose) was also analyzed and compared to starch from the glgB expressing plants.

The differences in absolute values of the measurements between the two control starches K89.2002 and A16 can be attributed to minor differences in structure and composition which are the result of the genetic variation. On top of that some of the differences may be due to the difference in ploidy level as A16 is a diploid and K89.2002 is a tetraploid cultivar.

All transformants contained starch granules of normal size and morphology. Microscopic analysis of iodine stained starch granules showed the presence of amylose in a restricted area of the starch granules for transformant AN-24 and the antisense GBSS expressing plants. The reduced amount of amylose was present in a core around the hilum of the granule. In plants with a reduced amount of amylose also a reduction of the GBSS activity could be observed as was reported before (Kuipers *et al.*, 1994a). In contrast to the antisense GBSS inhibited plants, AN-24 starch contained a similar amount of GBSS protein compared to the control as was detected by Western blotting. Starch with a lowered amount of amylose had, when dissolved, a lower λmax and Blue Value compared to the normal amylose containing starches, as was expected from the established relationship between amylose content and λmax and Blue Value (Wang *et al*, 1993b). The exception was the starch from transformant AN-14 with a lowered amylose content which showed the same λmax and B.V as the control starch. Possibly the amount of amylose in these granules is high enough to behave like normal amylose containing starch for most determinations. The DE after isoamylase digestion was found to be higher in starch with a lowered amount of amylose on account of relatively higher amount of amylopectin per dry weight starch. The DE (after isoamylase digestion) of starches with a normal amylose content, but expressing the glgB gene of *A.nidulans* ,had also increased.

This was the result of the presence of more short chains in the amylopectin. No large differences in elution profiles were observed between A16 and AN-24, except for a lowered amount of amylose present in AN-24 starch. The same was seen for starch from K89.2002 and the transformant tBK50-10 (and for tBK50-42, data not shown). The elution profile of AN-24 starch resembled mostly that of starch from antisense inhibited GBSS plants like tBK50-10 with very little amylose.

Not only structural, but also swelling and gelation properties of the starches were examined. The swelling power measured during heating of all the starches from plants expressing the glgB gene was essentially the same, including transformant AN-24. The solubility at 75 °C was about half for starch from transformant AN-24 compared to A16, AN-9 and AN-14. The antisense GBSS starches with a decreased amylose content showed a different swelling power from the controls. These starches showed very fast and almost unrestricted swelling, comparable to amylose-free mutant starch (data not shown). In case of AN-24 starch, the faster swelling as a result of less amylose is probably complemented by the increase in branching degree of the starch resulting in a similar swelling pattern as the (un)transformed control. The influence of amylose on swelling behaviour of starch was studied by Tester and Morrison (1990). From their research it was concluded that amylose acts simply as a starch diluent during swelling, but can in some cases actively inhibit swelling, which seems to be the case in our experiments. Starch from antisense inhibited GBSS plants showed an increase in To of almost three degrees Celsius compared to the untransformed amylose containing control. The influence of the presence of amylose on the temperature of the onset of gelatinization (To) was shown before (Flipse *et al.* 1996c) and is apparently not only a property of the amylopectin as is widely believed. Starch from transformant AN-24 had an increased To, but this increase was less than in the starch from antisense inhibited GBSS plants. Transformant AN-14 with the highest branching degree of the amylopectin

showed an increase in the To compared to the untransformed control. This could be the result of tighter stacking of the more branched amylopectin or more entanglement between the more branched amylopectin molecules.

The dynamic rheological properties of the starches were determined by Bohlin analysis. Starch from the antisense GBSS expressing transformants showed gelatinization at a higher temperature, maximum viscosity was reached at the same temperature but an increase in G' after cooling as a result of retrogradation was almost absent. The increase in G' for starch from transformant AN-24 was just as the starch from antisense transformants at a higher temperature compared to the control. However, the peak viscosity was lower for transformant AN-24 compared to the control. This decrease in peak viscosity could also be seen in transformants with a normal amylose content but a higher branching degree of the amylopectin. The increase in the storage modulus after cooling was similar for all starches from the glgB carrying transformants. From the relative compression studies it could be concluded that AN-24 starch resembled the amylose-free starch more then the original background A16. Gels from the amylose-free starch and from AN-24 starch were weaker and needed lesser force to break than the A16 starch gel.

Starch from transformant AN-24 seemed to express some physico-chemical properties typically related to the amylose content such as the change in λmax and B.V., the increased temperature of onset of gelatinization as could be seen from the DSC and Bohlin analysis and the lowered solubility in water during heating. The force needed to break a starch gel from AN-24 starch was much lower then the force needed to break the control starch gel or the gels from starch of any other A16 transformant (data not shown). In this aspect the AN-24 starch gel resembled the amylose-free starch gel. Other characteristics of AN-24 starch could not simply be attributed to the decreased amount of amylose. These characteristics, like the decrease in swelling power and the decreased peak viscosity seem to be related to structural changes in the amylopectin. They are

similar to the changes in properties seen for transformants with a normal amylose content and an increased branching degree of the amylopectin. Furthermore it seems that the faster swelling pattern caused by the lowered amount of amylose is completely neutralized by the increase in the branching degree of the amylopectin (and possibly the amylose as well). So, probably, transformant AN-24 has an altered amylopectin structure as the result of the expression of the bacterial branching enzyme. The lowered amylose content could be the result of the action of the heterologous branching enzyme, synthesizing amylopectin-like material from amylose. Another possible explanation for the lowered amylose content could be co-suppression, a phenomenon often seen in transgenic plants (Finnegan and McElroy, 1994). Co-suppression can cause the inhibition of gene expression of both inserted and endogenous genes, especially if they share homology (Matzke and Matzke, 1995). Since we used the potato GBSS promoter and transitpeptide sequence including a substantial part of the GBSS coding region to drive the expression of the glgB of *A. nidulans*, the possibility of co-suppression of the endogenous GBSS gene can not be excluded.

Acknowledgements

We like to thank T. van de Brink and J. Rijksen for taking care of the plants in the greenhouse, Dr. A.G.J. Kuipers for the gift of starch from the antisense GBSS plants, Dr. C.J.A.M. Keetels for the Bohlin analysis and helpful discussions and Nik Boer of Perkin & Elmer (Holland) for helping with the DSC analysis.

The influence of an increased degree of branching on the physico-chemical properties of starch from genetically modified potato.

5

Anne J. Kortstee, Luc C.J.M. Suurs, Angela M.G. Vermeesch, Christel J.A.M. Keetels, Evert Jacobsen and Richard G.F. Visser

Submitted

The influence of an increased degree of branching on the physico-chemical properties of starch from genetically modified potato.

Abstract

Transgenic potatoes were studied which contained starch with an increased degree of branching of the amylopectin as a result of the expression of the glycogen branching enzyme gene (glgB) of *Anacystis nidulans* or *Escherichia coli*. These trans-genes were expressed in a normal amylose-containing wildtype and in an amylose-free (*amf*) potato mutant. The degree of branching of these starches had increased up to 25%. This increase in degree of branching could be partly explained by the presence of 5 to 15% more short chains in the amylopectin, the so called A chains. The influence of the altered degree of branching on the physico-chemical properties of the starches was investigated. No change in granule size or granule morphology could be observed for the altered starches of these transgenic plants. Regardless of the presence or absence of amylose, starches with an increased degree of branching showed a shift towards more short chains of the amylopectin, a lower peak viscosity and for the amylose-free starch a tendency to form weaker gels. These results show that increasing the degree of branching of amylopectin leads to specific changes in the physico-chemical properties of the starch.

Introduction

Starch is the most abundant storage carbohydrate in higher plants. It can be found in storage organs such as roots and tubers in a granular form. Size and shape of the storage starch granules are specific for each starch crop (Jane *et al.* ,1994). Starch consists of two types of glucose polymers; amylose, an essentially unbranched α-1,4 linked glucose polymer and amylopectin, consisting of α-1,4 linked chains with α-1,6 branches. Common starches contain about 15-30% amylose. Native starch and starch derivatives are widely used in the manufacturing of food, paper, textiles, adhesives, pharmaceuticals and building materials. The physical properties of starches, and therefore the applications, depend on the starch composition (amylose : amylopectin ratio, size and degree of branching of amylose and amylopectin) and are unique for each botanical source (Swinkels, 1985a/b). Many studies were undertaken to clarify the relationship between structural characteristics and the physico-chemical properties of starch (Howling, 1980; Sanders *et al.*, 1990; Tester and Morrison, 1990; Jane and Chen, 1992; Wang *et al.*, 1993a/b). In these studies native starches from different botanical origins and mutants with an altered starch composition were studied. Most mutants are found in maize where they are known for almost all of the isoforms of the enzymes involved in starch biosynthesis, resulting in starches with different composition and characteristics. The best studied example of mutant starch is that of the *waxy* mutant of maize (Echt and Schwartz, 1981). This mutation has also been identified and studied in other crops like potato (Hovenkamp-Hermelink *et al.*, 1989), rice, barley and wheat (Shannon and Garwood, 1984; Nakamura *et al.*, 1995; Sano, 1984). These mutants contain amylose-free starch as a result of a defect in the gene encoding Granule Bound Starch Synthase (GBSS) and a subsequent absence of activity (Smith *et al.*, 1995). Another group of mutants contains a relatively high amount of amylose in the starch as a result of a mutation in one of the isoforms of starch branching enzyme

and can be found in maize and pea: *amylose-extender* maize (Boyer and Preiss, 1981, Hedman and Boyer, 1982;) and *wrinkled* pea (Edwards *et al.* 1988, Smith, 1988). Several examples of genetically modified potato starches are described after the introduction of bacterial genes into the plant. The production of unique carbohydrates such as cyclodextrins by introduction and expression of the cyclodextrin glycosyltransferase gene from *Klebsiella* sp. in potato was described by Oakes *et al.* (1991). After introduction of the *E. coli* glgA gene, encoding glycogen synthase, in potato, a change in the starch composition could be observed. An increased degree of branching (measured by HPLC analysis of isoamylase debranched starch) and a decreased amylose content were described for starch of glgA expressing potatoes (Shewmaker *et al.*, 1994). Some physical properties of the starch like gelatinization and thermal behavior had also changed: the onset temperature (To) of gelatinization measured by Differential Scanning Calorimeter (DSC) analysis was lower and Rapid Visco Analysis (RVA) showed a higher paste temperature and a lower peak viscosity for the starch of the transgenic potatoes.

In this paper we describe the physico-chemical analysis of starch from genetically modified potatoes in both the normal amylose containing as well as in the amylose-free mutant (*amf*) background. The degree of branching of these starches had increased after the introduction and expression of the glycogen branching enzyme encoding gene (glgB) of *E.coli* (Chapter 2, this thesis) or *A. nidulans* (Chapter 3, this thesis). The effect of the increased degree of branching on the thermal behavior such as swelling and gelatinization of the starch will also be described.

Experimental procedures

Plant material

Transgenic plants were obtained in previous research which showed an increased degree of branching of the starch as the result of the expression of the bacterial branching enzyme of *E. coli* or *A. nidulans*. Molecular biological and biochemical analysis of these plants has partly been described before (Chapters 2 and 3 of this thesis). The individually selected clones in the diploid amylose-free (*amf*) background of clone 1029-31 (Jacobsen *et al.*,1989), transformed with the *E.coli* branching enzyme, were: EC-13, EC-17 and EC-20 with clone 1029-31 as the untransformed control. Clones with amylose containing starch and transformed with the branching enzyme of *A.nidulans* were: AN-9 and AN-14, with A16 (El-Kharbotly *et al.*, 1995) as the untransformed control. Plants were grown in the greenhouse and in the field. Mature tubers were harvested for starch isolation.

Isolation of starch

Starch was isolated from 5 kg (fresh weight) of field grown tubers. Tubers were grinded with 1 gram (± 200 ppm) $Na_2S_2O_5$ in a Robot Coupe model R15 (series 5, Spangenberg BV, Vianen, Holland) for 5 min. at position 1 followed by 5 min. at position 2. The sludge was mixed with 5 l. demineralized water (dH_2O) and sieved through a shaking sieve (PERFLUX, N.V. TEMA, Den Haag, Holland) with 122 µM pores. The sludge was washed with dH_2O two more times and sieved to extract most starch and the granules were allowed to precipitate for at least two hours. After the granules had settled, the potato juice was decanted and the starch was resuspended in 1 l. dH_2O and filtered through filter cloth with pores of 112 µM. The starch was washed at least 3 more times with dH_2O to remove small dirt particles and coagulated protein. After the last wash, the starch was filtered through Whatmann paper (3 mm) on a Buchner funnel. The starch was air dried on filter paper for at least 72 hours to a moisture content of

about 20%. The dried starch was passed through a sieve with pores of 508-635 μM, collected and stored at room temperature. Isolation of starch from greenhouse grown tubers was as described by Kuipers *et al.*(1994a/b).

Starch granule size and morphology

Average granule size and size distribution of the isolated starch were determined by a Coulter counter multisizer IIe. 10 mg of starch was dispersed in 160 ml of Isoton II, according to the manufacturers instructions. The diameter of the tube was 200 μm and 50.000 particles were counted with a coincidence of 10%. Starch granules were stained with 3 times diluted Lugols solution (an 1% I_2/KI solution, 1:2, w/w) and examined under the microscope (magnification 100 times) to check granule morphology.

λmax and Blue Value

5 mg of starch was dissolved in 1 ml of Dimethylsulphoxyde (DMSO) by boiling for 20 minutes and diluted to 10% DMSO. Of the 10% DMSO-starch solution, 20 μl was added to 180 μl dH_2O and complexed with iodine by adding 800 μl KI/I_2 solution (Delrue *et al.*, 1992), the final starch concentration was 0.01%. The λmax was determined as the wavelength with maximum absorption after scanning from 700-400 nm. The Blue Value was determined by boiling 10 mg of starch in 1 ml of 0.1N NaOH for 50 min. After cooling, 9 ml of dH_2O were added. Of this solution 15 μl was added to 285 μl 4 times diluted Lugol's solution, and scanned from 700-400 nm with a Beckman DU-64 spectrophotometer. The Blue Value was determined as the absorption at 655 nm.

CL2B chromatography

100 mg starch was dissolved in 1 ml 0.1N NaOH by boiling for 60 min. The sample was diluted by adding 9 ml of dH_2O, centrifuged for 5 min. at 3000g at roomtemperature and loaded on a CL2B column (2.6 by 200 cm). Fractions of 10

ml. were collected during elution of the column with 0.01 N NaOH at a flow rate of 30 ml/hr. Of each fraction 200 µl was added to 800 µl of an iodine/potassium-iodine solution (Delrue *et al.*, 1992) and scanned from 700-400 nm to determine the λmax and the maximum absorption.

Amylose content

The apparent amylose content was determined according to the method described by Hovenkamp-Hermelink *et al.* (1989). For the amylose-free starches amylose percentages of ≤ 3 were found and considered to be negligible.

Determination of starch degree of branching

The degree of branching of starch was determined with the Luff-Schoorl method (Schoorl, 1925) with slight modifications as described in Chapter 2 of this thesis.

Chain length distribution

Isoamylase debranched starch was separated by HPLC (a Dionex Carbopac PA 100 series). The peaks in the original chromatogram were reintegrated and corrected for the response factor to obtain a number percentage chain length distribution as was described before (Chapter 2, this thesis).

Swelling power in H_2O

Swelling power was determined according to Leach *et al.* (1959) with some modifications. Amylose containing starch suspensions (0.5% w/v) in water (preboiled seralpure) were heated to 60, 65, 70, 75 and 80 °C respectively, and kept at that temperature for 15 min. at slow stirring by hand, just sufficient to keep the starch completely suspended and to minimize shearing of swollen granules. Amylose-free starch suspensions (0.5% w/v) were heated to 66-80 °C (with two degrees Celsius intervals) and kept at that temperature for 30 min. under slow stirring. The swollen starch suspensions were poured in a measuring

glass and allowed to precipitate overnight. The volume of sedimented swollen granules was determined and a sample of the clear supernatant was removed to determine the amount of dissolved starch. The percentage of dissolved starch was determined with the Boehringer kit no. 207748 for measuring starch content, according to the manufacturers specifications but with smaller volumes to fit in a microtiterplate. The swelling power was corrected for the amount of solubilized starch.

DSC

Differential Scanning Calorimetry was performed with a Perkin Elmer Pyris 1 equipped with a Neslab RTE-140 glyco-cooler. The instrument was calibrated with indium (mp= 156,6 °C) and zinc (mp= 419,47 °C). Approximately 10 mg of starch (dry weight basis) was weighed accurately into a stainless steel cup and 40 µl of dH2O was added. The cup was hermetically sealed and allowed to equilibrate at least 1 hour before analysis. The suspension was heated from 20 °C to 100 °C at a scanning rate of 10 °C/min. An empty stainless steel cup was used as a reference. For each endotherm the onset temperature (To) of melting and the enthalpy ΔH (J/g) were computed automatically. The results are the average of three scans. Enthalpies were calculated on a starch dry-weight basis. The dry matter content of the starch was determined at least in duplicate by oven drying of 30 mg samples for 4 hours at 120 °C.

Viscosity (RVA/Bohlin)

An 8% starch suspension in dH_2O (dwb) was placed in the RVA cup. The heating profile used was as follows: 2 min. at 45 °C, heating to 90 °C at a rate of 5 °C/ min., heating at 90 °C for 6 min., cooling from 90 °C to 30 °C at 14 °C/min. and keep at 30 °C for 6 min. The viscosity was measured in RVA units. For both the amylose containing and the *amf* starches the pasting profile of a 5% starch : water (w:v) suspension was measured with a Bohlin VOR Rheometer (Mettler Toledo,

Tiel, The Netherlands) as described by Flipse *et al.* (1996b). The pasting profile existed of heating to 90 °C, keeping the paste at this temperature for 15 min., and cooling to 20 °C at a rate of 1 °C/min.

Gel strength

Starch pastes (20%, w:v) in water were prepared by adding 3.6 g of starch (20% moisture) to 14.4 ml of preboiled dH_2O in a straight side wide mouth polypropylene jar (Nalgene, 2118-9050). The jar was placed in a waterbath of 50 °C on a plate and stirred at 300 rpm by a 25 by 6 mm magnetic stirrer to disperse the starch. The sample was then heated to 80 °C while stirring, until the stirrer became trapped in the gel, what usually happened at about 70 °C, at the start of gelation. Immediately the sample was transferred to a 90 °C waterbath, incubated for one hour, cooled on ice and stored at 4 °C for five days. Gelstrenght was measured using a texture analyzer (T04, Etia, Compiegne, France) with a 20 N loadcell and a punch probe with a diameter of 6 mm. The gel was compressed at a speed of 2 mm/sec. until breaking. For each sample, 6 separate gels were prepared and measured.

Results

Granule size and morphology

Starch isolated from greenhouse-, and field-grown tubers was stained with iodine and examined at the microscopic level. No changes in granule morphology could be observed for the starches of the transgenic potatoes. All transformants had red or blue staining starch according to their original background. The median (d50) granule size by volume of the field-grown *amf* starches was between 22-32 µm. Starch of transformant EC-13 appeared to contain slightly larger granules than normal *amf* starches, but all values were

within the range of normal variation. The d50 of the field-grown amylose containing starches was 19-27 μm by volume. Starch of transformant AN-9 consisted of more smaller granules, the median granule size for these granules was 19 μm compared to the other amylose containing starches with a d50 of 26-27 μm. Starch from greenhouse grown transgenic plants showed similar results; no change in granule size or shape compared to the control starch was observed.

Composition and finestructure

The apparent amount and structure of amylose in starch with an increased degree of branching did not seem to have changed, according to spectrophotometrical analysis as can be seen in Table 1, except for starch of tubers from field-grown transformant AN-14, which displayed a lowered amylose : amylopectin ratio of about 0.15, although the granules stained completely blue with iodine. The same clone but grown in the greenhouse, displayed an apparent amylose content of 20%, identical to the untransformed control.

The λmax of the starch of the ...sgenic plants had remained the same or was lowered. The latter was an indication of a higher degree of branching since the iodine binding capacity is inversely related to the degree of branching as was shown by Krisman *et al.* (1962). The Blue Value for the amylose-free starches was lower than for the amylose containing starches as was expected. Within the groups no significant changes were found between the controls and the transformants.

Table 1. Apparent amylose content (AM%), λmax and Blue Value (B.V.) at 655 nm of starches from greenhouse grown and field grown plants.

Clone	Greenhouse grown tubers			Field grown tubers		
	AM%	λmax (nm)	B.V.	AM%	λmax (nm)	B.V.
Amylose-free clones						
1029-31	-	555	-	≤ 3	555	0.34
EC-13	-	555	-	≤ 3	552	0.31
EC-17	-	-	-	≤ 3	555	0.33
EC-20	-	550	-	≤ 3	550	0.31
Amylose containing clones						
A16	22.0 ± 3.1	576	0.75	24.1 ± 0.5	573	0.50
AN-9	21.7 ± 1.5	579	0.91	20.3 ± 0.6	573	0.43
AN-14	21.3 ± 2.7	574	0.89	14.6 ± 0.4	573	0.47

Separation of amylose and amylopectin by size exclusion chromatography with a CL2B column is shown in Figure 1. Amylopectin is eluted from the column as a single narrow peak, sometimes with a shoulder. The λmax of the fractions of this peak was approx. 560 nm, confirming the identity of the eluted glucan. The amylose-free starches from 1029-31 and EC-20 eluted from the column as one large peak, with a λmax of the fractions of 560 nm. Behind the amylopectin bulk a few glucan containing fractions eluted from the column with a λmax of up to 600. The glucan in those fractions is called the intermediate material and is believed to consist of long chained amylopectin and/or branched amylose similar to to such fraction sometimes found in cereal starch (Whistler and Doane, 1961). Whether this intermediate fraction exists *in vivo* in the starch granule or if it signifies starch breakdown is still debated (Tester and Karkalas, 1996). Amylose is eluted from the column in a much broader peak, the λmax of these fractions were higher than 600 nm. In the case of starch from AN-14, however, the fractions eluted from the column immediately after amylopectin had a λmax of 580 nm. Finally fractions with a λmax ≥ 600 nm, indicating amylose, were eluted from the column. The presence of fractions with a λmax of 580 nm suggests that they consist of the so-called intermediate material. The control starch did not contain this type of glucan.

The degree of branching of the starch expressed as DE (after isoamylase digestion) is presented in Table 2. The degree of branching (DE) of greenhouse-grown untransformed *amf* tuber starch was 3.8 ± 0.2. Starch from transgenic *amf* plants expressing the *E.coli* branching enzyme had a degree of branching of up to 5.0 ± 0.2. For field grown tubers of these transformants similar values were observed. The degree of branching of starch of the untransformed wildtype clone A16 was 3.6 ± 0.2. Transgenic plants carrying the $pB_{19}13AN$ construct, containing the glgB gene of *A.nidulans*, showed a degree of branching expressed in DE in the range from 3.6 to 4.5. Starch from greenhouse-grown and field-grown tubers showed a similar degree of branching. For the *amf* as well as for the wildtype

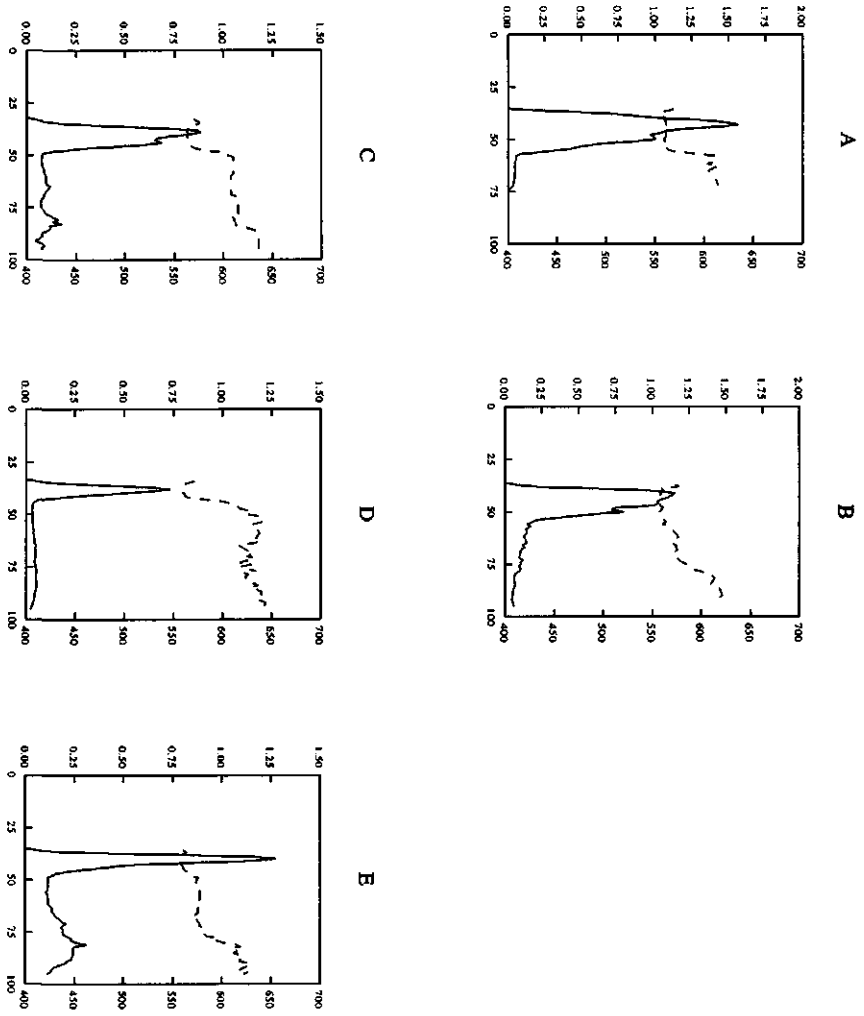


Figure 1. Gel permeation chromatogram of *amf* control 1029-31 and amylose containing control A16 starch and their transformants. The optical density of the iodine-polysaccharide complex was measured for each 10 ml fraction at λmax, where λmax is displayed as a broken line (- - -). Starch from A: untransformed control 1029-31, B: EC-20, C: untransformed control A16, D: AN-9 and E: AN-14.

based altered starches the degree of branching (DE) had increased maximally up to 25%. Starch from the greenhouse-grown *amf* tubers from transformant EC-20 displayed a shift in chain length distribution compared to the (un)transformed control. About 10-20% more short chains were present, shifting the ratio short to long chains from 3:1 to 9:1 as reported before (Chapter 2, this thesis). The wildtype based starches of transgenic plants were analyzed in the same way. Starch from greenhouse grown tubers of transformant AN-14 contained about 10% more short chains (dp ≤ 16) compared to the controls. In the same series starch from transformant AN-9 contained about 5% more short chains. Similar results were obtained with starch from field grown tubers which showed also a shift towards more short chains, but to a lesser degree.

Table 2. Degree of branching (DE) and percentage of A chains of starch from greenhouse grown plants.

Clone	DE	% A chains
Amylose-free clones		
1029-31	3.8 ± 0.2	29
EC-13	4.4 ± 0.4	30
EC-17	5.0 ± 0.2	nd
EC-20	4.9 ± 0.1	45
Amylose-containing clones		
A16	3.6 ± 0.2	30
AN-9	4.3 ± 0.2	35
AN-14	4.5 ± 0.2	40

nd = not determined

Gelatinization characteristics

The thermal properties of the amylose-free and wildtype based starches of transgenic potatoes were measured in starch:H_2O suspensions (20 % starch w/v, 80% H_2O v/v) using differential scanning calorimetry. The results are presented

in Table 3. From the greenhouse grown *amf* tubers only the untransformed control and transformant EC-20 were analyzed. EC-20 appeared to have a higher temperature of onset of gelatinization compared to the control (To 69.2 and 68.3 °C, respectively). The peaktemperature (Tp) had likewise shifted to a higher temperature, but a similar enthalpy (ΔH) and gelatinization range (Tp-To) were found for both the transformant and the control. Results from field-grown tuber starch revealed no significant differences in temperature of onset of gelatinization between the control starch and starch from transgenic plants (data not shown).

Table 3. Differential Scanning Calorimetry measurements of starch:water suspensions from greenhouse-grown plants, 20% (w:w); temperature of onset of gelatinization (To), temperature of gelatinization peak (Tp) and enthalpie (ΔH) released.

Transformant	To (°C)	Tp (°C)	ΔH KJ/g starch
Amylose-free clones			
1029-31	68.3	72.2	18.7
EC-20	69.2	73.9	18.6
Amylose containing clones			
A16	62.5 ± 0.3	66.0 ± 0.3	11.4 ± 0.2
AN-9	61.5 ± 0.4	65.1 ± 0.2	10.5 ± 0.3
AN-14	64.1 ± 0.2	67.5 ± 0.4	12.0 ± 0.2

Average of 3 measurements

Starch from wildtype based transformants from greenhouse grown tubers did not display a change in temperature of onset of gelatinization, enthalpy or gelatinization range. Starch from the fieldgrown tubers of the same group of transformants was also analyzed by DSC. Transformant AN-14 appeared to have a higher temperature of onset of gelatinization, a higher Tp but the same enthalpy and gelatinization range compared to the control. In general only the

starch from the amf and wildtype transformants with the greatest change in degree of branching showed a shift in the temperature of onset of gelatinization and peaktemperature. In those cases both the To and the Tp had increased approximately one degree Celsius compared to their respective controls.

Swelling

The swelling power of starch from greenhouse grown tubers was measured in excess water (Fig. 2). The swelling curve of the *amf* starches differed radically from that of the amylose containing starches. The *amf* starches swell very fast, within a temperature increase of only a few degrees Celsius they are completely swollen instead of over an extended temperature range like the amylose-containing starches. Starch of transformant EC-20 displayed a shift in the swelling curve, the whole swelling curve was shifted to a higher temperature (+2 °C) as can be seen in Fig 2A.

For the amylose containing starches (Fig 2B) a lowered swelling power could be detected for the transformants with an increased degree of branching compared to the control. In a temperature range from 60-75 °C the volume of swollen granules increased linearly. The swelling curve of the starch with the increased degree of branching (AN-9 and AN-14) was parallel but lower than that of the control.

For the starches from field-grown plants no differences could be detected in swelling power between the transformants and their respective controls.

Viscosity (Bohlin/RVA)

Because of limited sample size, starches from greenhouse-grown tubers were subjected to Bohlin analysis to determine changes in storage modulus (G') during heating and subsequent cooling. Starch from field-grown tubers was analyzed by RVA to measure the pasting properties. The difference between both methods lies in the applied shearing forces. In the Bohlin assay starch granules remain intact during heating and cooling, whereas they are degraded by shearing forces

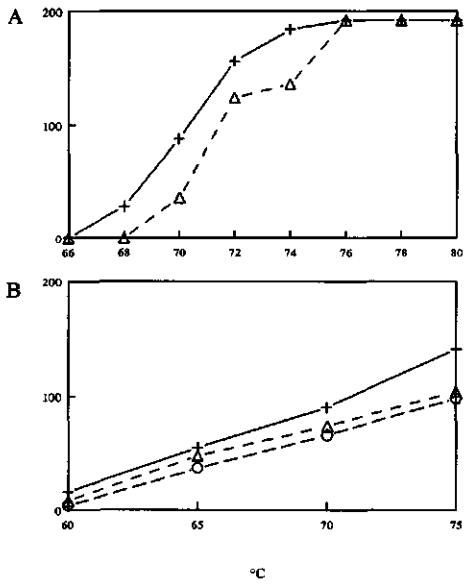
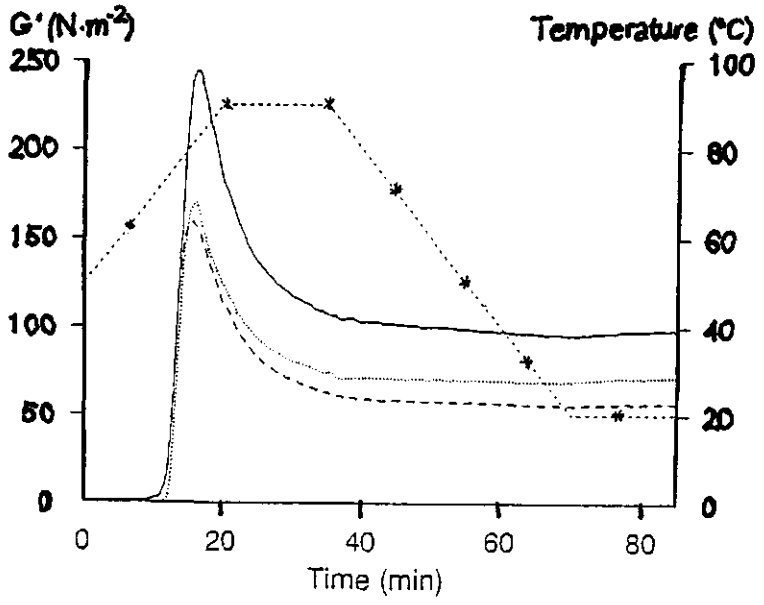


Figure 2. Swelling power of starch from *amf* (A) and wildtype (B) greenhouse-grown transgenic plants.
The swelling power of a 0.5% (w:w) starch suspension was measured in water. The swelling power was corrected for the amount of solubilized starch. A: +, 1029-31; Δ, EC-20. B: +, A16; O, AN-14; Δ , AN-9.

during RVA.

Bohlin analysis. Starch from greenhouse grown tubers of the *amf* transformants was analyzed by Bohlin. During heating an increase in storage modulus (G') was seen for control and transformants at the same temperature. The transformants however, reached a lower peak viscosity before the storage modulus (G') dropped and G' remained lower during cooling as can be seen in Fig. 3A. The

A



B

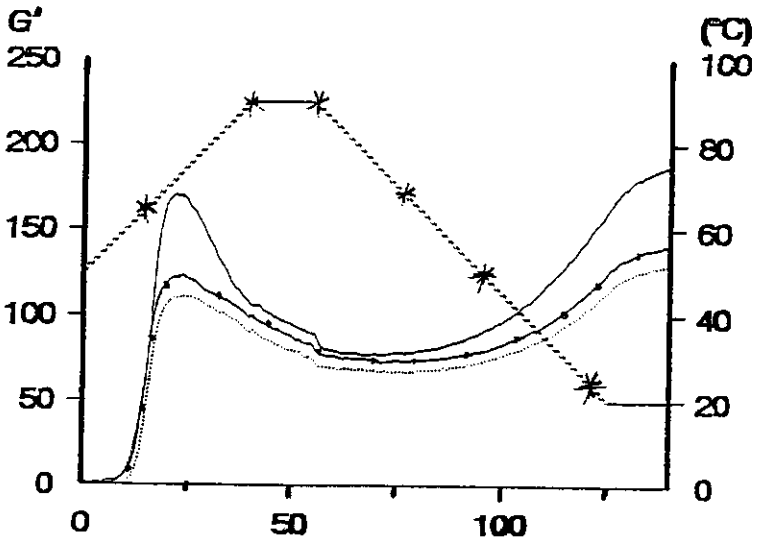


Figure 3. Bohlin gelation profile of 5% starch suspensions.
A. Changes in the storage moduli of 5% (w:w) amylose-free starch suspensions during heating and subsequent cooling. - -* - -, temperature against time; ——, amylose free control starch; . . . . ., transformant EC-13; - - - -, transformant EC-20.
B. Changes in the storage moduli (G') of 5% starch suspensions during heating and subsequent cooling. - -* - -, temperature against time; ——, control starch of A16; . . . . ., transformant AN-9; —•—, transformant AN-14.

amylose-containing starches from the greenhouse-grown tubers were also tested by Bohlin and were found to have the same temperature at which G' increased for transformants and control starch. The transformants with a higher degree of branching of the starch however, had a lowered peak viscosity compared to the (un)transformed control, and after cooling G' stayed lower for the starch from the transformants as can be seen in Figure 3B.

RVA analysis. The results from the RVA analysis of starch from field-grown tubers are displayed in Fig. 4 The *amf* starches all showed the same RVA profile like the one displayed in Fig 4A The starches showed an increase in viscosity at the same time/temperature, reached a maximum which was much lower than that for the amylose containing starches and hardly displayed any setback viscosity after cooling. This RVA profile of the *amf* starches with the irregular line, may possibly be due to a higher sensitivity of the *amf* starch granules to shear.

From Fig. 4B can be seen that transformant AN-9 and AN-14 displayed a lower peak viscosity compared to the control. The rest of the pasting profile is similar for transformants and control. All amylose-containing starches showed a high peak viscosity followed by a rapid decrease in viscosity which is attributed to the thinning effect caused by mechanical shearing. The setback viscosity after cooling and an increase in viscosity as a consequence of retrogradation could be observed for all amylose-containing starches.

Gel strength

In Fig. 5 the results of the relative compression studies can be seen. The curves that are displayed are representatives of the six measurements made for each sample. For the amylose-free starches it is noted that starch gels from transformant EC-13 and EC-17 are as strong as the gel of the untransformed control 1029-31. They all break at the same applied force of 1 N, and showed similar displacement of gel material at the breaking point. The gel prepared

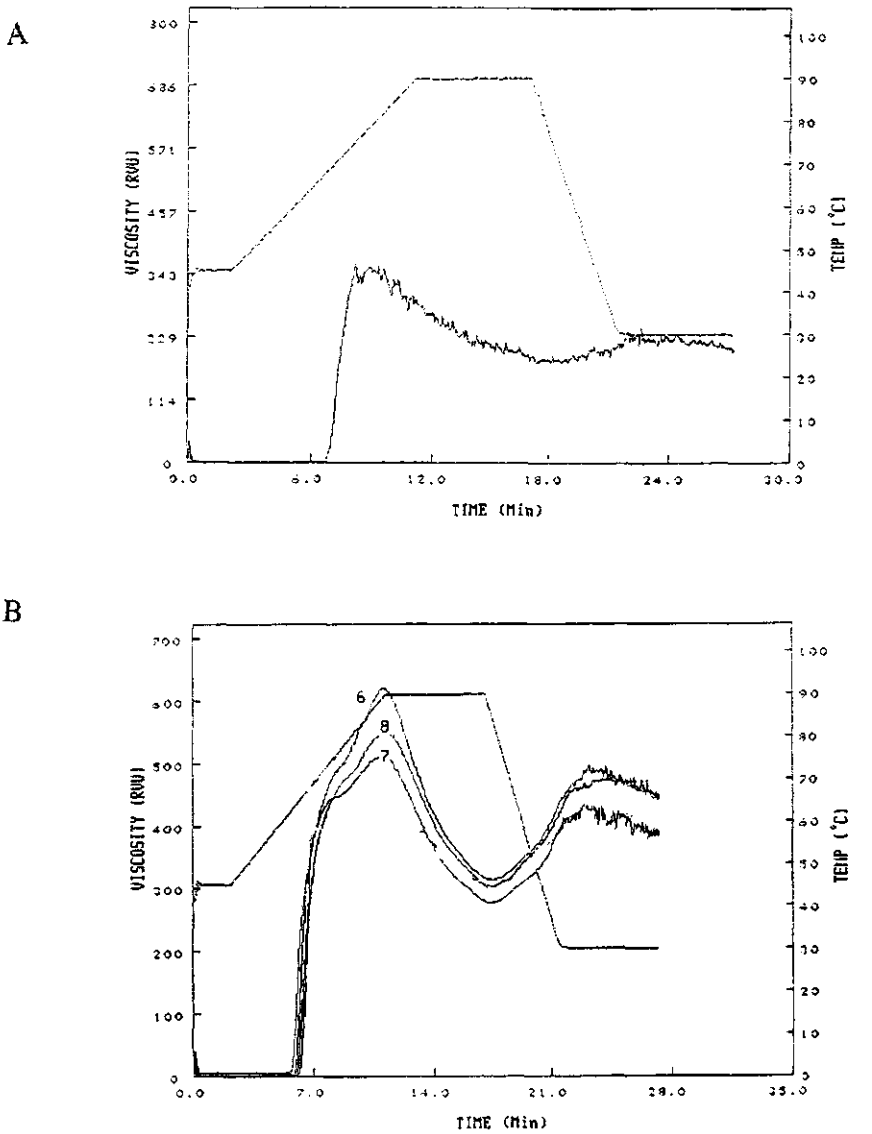


Figure 4. Rapid Visco Analysis of 8% starch suspensions.
A. Changes in viscosity of 8% (w:v) amylose-free starch suspensions during heating and subsequent cooling measured by RVA.
B. Changes in viscosity of 8% (w:w) amylose containing starch suspensions during heating and subsequent cooling measured by RVA. ———, temperature against time; - - - -7, transformant AN-9; - - - 8, transformant AN-14; - - - 6, untransformed control A16.

from EC-20 starch breaks at the applied force of less then 1 N, and the displacement of gel by the probe is higher than that for the control. Starch from transformant EC-20 forms much weaker gels compared to the untransformed control. Amylose-free gels with a starch percentage of lower than 20% were to weak to be measured. From the amylose containing starches gels were prepared and measured. The results displayed in Fig. 5B show no clear differences between the control starches and the starches with the increased degree of branching with respect to their gel strength. This may be due to the large variation between the different gels of the same starch sample.

Discussion

Some physico-chemical properties of starches with an increased degree of branching of the amylopectin were described. Both amylose containing and amylose-free mutant starches with an increased degree of branching were used. For starches with an increased degree of branching the same tendencies were found in the physico-chemical characteristics, despite the presence or absence of amylose. The same clones were grown in the greenhouse and on the field. The isolated starch was iodine stained and examined under the microscope for morphology of the starch granules. No change in granule morphology was seen for the transgenic starches. Granule size and size distribution remained similar for transformants and their controls except for AN-9 which had slightly smaller granules. The starch composition as far as amylose content was concerned had not changed for most of the transformants except for AN-14, which had a lowered amylose content but a similar λmax and Blue Value compared to the control. From the CL2B profile of AN-14 starch it was observed that the starch contained an additional fraction to amylose and amylopectin, the so-called intermediate fraction. Whether this fraction consisted of highly branched

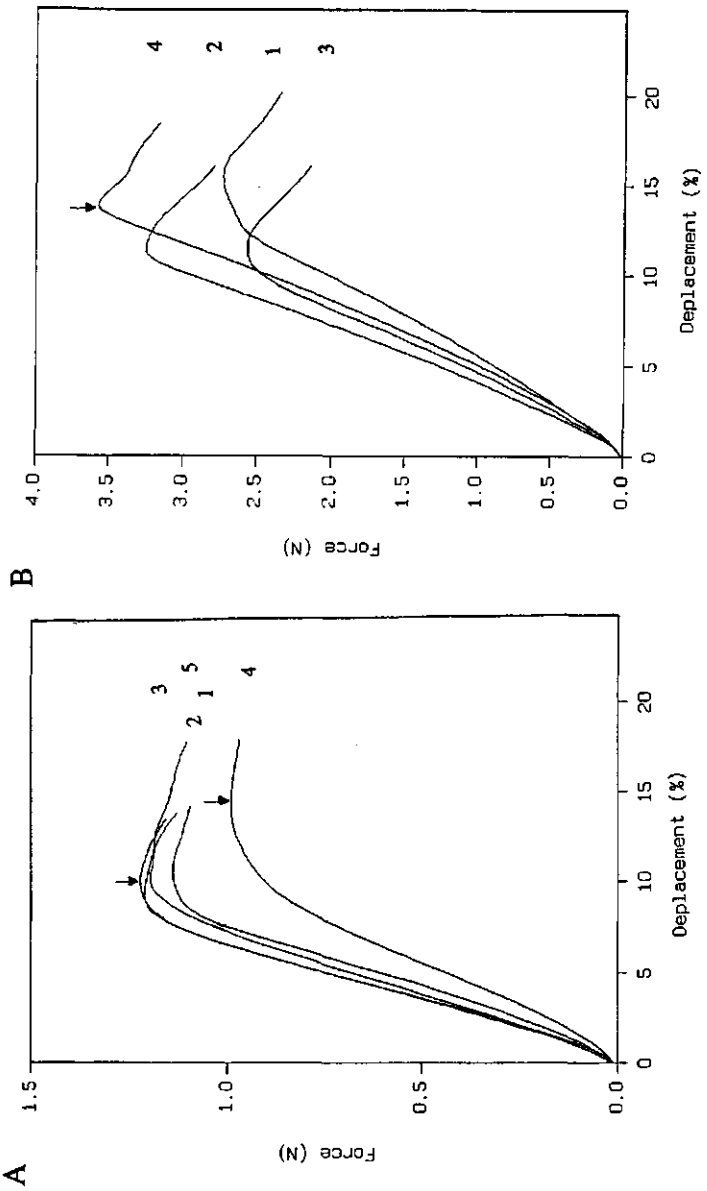


Figure 5. Relative compression of 20% (w:v) starch gels, stored at 4 °C for five days.
A. Gels from amylose-free starches, 1029-31 is the untransformed control (1), with transformants EC-3 (2), EC-13 (3), EC-20 (4) and EC-17 (5).
B. Gels from amylose containing starches, A16 is the untransformed control (1) and the transformants AN-9 (2), AN-14 (3) and a transformed control (4).

amylose or long chained amylopectin is not evident from our results. The degree of branching of the starch of transgenic tubers expressed in DE had increased by 15-25% compared to the controls. The difference in DE between *amf* and wildtype control starch could probably be attributed to the fact that the DE was expressed as reducing power per dry weight starch. Because of the unbranched nature of amylose the overall degree of branching would have been lower in amylose containing starches. The increased degree of branching of the amylopectin of the starches could be, at least partly, explained by the presence of more short chains, the so-called A-chains. The ratio short to long chains had increased as a result of the presence of more short chains. To investigate the influence of the higher branched starches on the physical properties of the starches, thermal behavior and gelatinization were measured. For the *amf* and amylose containing starches the Bohlin pasting profile showed a lower peak viscosity and a lower storage modulus (G') after cooling of starches with an increased degree of branching. These results were in accordance with results obtained by Flipse *et al.* (1996) on their work on plants with an inhibited expression of the endogenous branching enzyme. Inhibition of potato branching enzyme in the *amf* mutant background lead (after iodine staining) to red staining granules with a small blue core. The Bohlin pasting profile of this type of starch showed an increased storage modulus (G') and a higher peak viscosity compared to the untransformed control. So the addition of a heterologous branching enzyme increased the degree of branching and the ratio short to long chains and lowered the top viscosity. In addition to this the inhibition of the endogenous branching enzyme lead to a type of starch with presumably longer chains, blueish staining starch and a higher top viscosity. The ratio short: longer chains apparently influences the physical properties of starch as was also shown by Wang *et al.* (1993a/b). They described the influence of structural properties of 17 mutant maize genotypes on the physico-chemical behavior of those starches. It was concluded in their study that the relationship between starch structure and physical

properties was not always clear. However, it was found that the amylose content had a large effect on swelling and gelatinization. The amylose content was negatively correlated to the swelling power, %T and peak viscosity and positively correlated to Blue Value and λmax. Other structural properties such as intermediate size content and the ratio short to long chains were found to be negatively correlated with peak viscosity.

In our experiments we obtained genetically modified potato plants with an increased degree of branching of the starch. The starch with the increased degree of branching (both amylose-free and amylose containing) showed a higher temperature of onset of gelatinization, a lower peak viscosity and a decreased swelling power compared to the untransformed controls. Starch gels from starch with an increased degree of branching were as strong as or weaker compared to the gels made from starch of the untransformed control.

So we conclude that the lowered peak viscosity of the starches from our transgenic plants can be attributed to the increased degree of branching as a result of the shift in chain length distribution towards the presence of more short chains. In contrast to this were the observations of Jane and Chen (1992). They found rice amylopectin (with average shorter chain length) to have greater viscosity compared to both high amylose and waxy maize. However, the observed higher phosphorus content in rice amylopectin could have resulted in the higher viscosity.

The other characteristics of the transgenic potato starches with an increased degree of branching were not always found to be the same for both the greenhouse-grown and field-grown starches of both the amylose-free and amylose containing altered starches. Among the field-grown tuber starches less differences were found between controls and transformants, which may be due to the less favorable circumstances in the field, for the introduced gene.

Generally, the temperature of onset of gelatinization seemed to have increased. This could be the result of more entanglement between the more branched

amylopectin molecules. Sanders *et al.* (1990) studied the relationship between amylopectin structure and thermal behavior in four maize inbred lines. They found a higher To for *aewx* starch compared to the other studied starches (*wx, duwx,* and *aeduwx*) and an increased high molecular weight peak in HPLC chromatograms of isoamylase debranched starch. They concluded that variation in the proportions of short and longer chains could explain the difference in thermal behavior. Their findings are directly opposite to ours in the fact that they found a higher To of gelatinization as a result of more longer chains whereas we found an increased To with an increase of the shorter chains. Perhaps the influence of the genetic background or the presence of other substances besides the starch, like protein and phosphate is stronger here. Starches with an increased DE (after debranching) also showed a shift in swelling pattern. The swelling curves were similar to those of their respective controls but at a lower rate. Work by Tester and Morrison (1990) showed swelling to be a property exclusively of the amylopectin; amylose and lipids actively inhibit swelling or sometimes only act as a diluent. The uptake of water at higher temperatures, or, a decreased swelling power for potato starches with an increased degree of branching would have to be due to changes in the structure of the amylopectin, possibly the result of more entanglement between amylopectin molecules.

In this paper we have described a (first) step in the direction of the production of naturally (granule based) starches with properties analogous to chemically modified starches now in use. In the future it may even be possible to produce "tailor made" starches for each thinkable application or even totally new applications, by the use of biotechnology. From the data we showed it also becomes clear how biotechnology can be of use in helping to clarify the relationship between starch fine structure and starch properties.

Acknowledgements

We would like to thank Dr. A.M. Janssen of the analytical department of AVEBE R&D, Holland, for analysis, helpful discussions and critical reading of the manuscript. Dr. Ir. H. Masselink of Unifarm, Wageningen, took excellent care of the plants in the field.

General discussion

6

General discussion

This study describes the introduction and expression of bacterial branching enzyme genes into potato. Different classes of transgenic plants were obtained with a modified starch composition. In an amylose-free background, expression of the branching enzyme gene (glgB) of *E.coli* led to an increased starch branching degree (Chapter 2). In an amylose-containing background also an increased starch branching degree was observed, which was sometimes accompanied by a decreased amylose content. The amylose appeared to have been substituted by a so-called intermediate fraction with a higher branching degree than amylose itself (Chapter 3). Changes in the starch composition were expected to influence the physico-chemical properties of the starch. In Chapters 4 and 5 the results are presented from the analysis of starch from transgenic plants with a higher starch branching degree and/or with a lowered amylose content. Some of the aspects with regard to the activity of the introduced bacterial branching enzymes and the influence of the modified starch composition on the physico-chemical properties of the starch will be discussed here.

Branching of amylose and amylopectin by bacterial branching enzymes

Branching of amylopectin by the branching enzyme of E.coli

Introduction of the bacterial branching enzyme gene of *E.coli* in amylose-free potato was observed to increase the branching degree (DE) of the amylopectin. Analysis of isoamylase debranched starch by HPLC showed that the increase in

DE coincided with a higher amount of short side chains of the amylopectin, the so-called A chains. Especially the amount of chains up to dp ≤ 8 had increased almost twofold (see Chapter 2). These additional side chains were not expected to take part in the crystalline structure of the amylopectin molecule since the minimum chainlength to form two helices is reported to be ≥ dp 14 (Hizukuri, 1985; Gidley and Bulpin, 1987)). Supporting this view were the results from DSC analysis of this type of starch which showed no altered melting temperatures (Chapter 4) which would have been expected if the amount of crystalline material had increased or if the nature of the crystalline part of the starch had been changed.

Branching of amylose by bacterial branching enzymes

Introduction and expression of the glgB genes of *E.coli* and *A.nidulans* in an amylose containing background also resulted in a higher starch branching degree of up to 25-35%. In some of the transformants however, the higher starch branching degree was accompanied by a decrease in amylose content (Chapter 3). The amylose to amylopectin ratio, determi ed by iodine complexation of the starch, had decreased to values comparable to those of amylose-free starch. Iodine staining of the starch granules showed a blue staining core of varying size surrounded by red staining starch. The size of the blue-staining core however, was much larger than found in granules from antisense GBSS inhibited plants with a similar amylose content. It was suggested that the decreased amylose content was a result of co-suppression of the endogenous GBSS gene, because sequences homologous to GBSS were used to make the constructs. Supporting this view were the findings of a decreased amount of GBSS protein detected by Western blotting and a decreased GBSS activity of the starch granule fraction from some of the transformants. However, although the potato GBSS promoter sequence was used to drive expression of the introduced gene, results obtained by Flipse *et al.* (1996b) showed that the GBSS promoter sequence by itself alone

was not sufficient to suppress the endogenous GBSS gene expression. The decrease in amylose content was found with the highest frequency in populations transformed with the pB_{19}tpssuEC construct. This construct carries the glgB gene of *E.coli* driven by the GBSS promoter, but targeted by the transitpeptide sequence of the Rubisco Small Subunit. So, co-suppression of the GBSS gene was considered unlikely. More likely, the amylose synthesized by GBSS is branched further by the introduced bacterial branching enzyme, in a way which will be outlined below. The occasionally found decrease in GBSS protein amount and activity could be the result of some (yet unknown) feedback mechanism.

From reports on *in vitro* experiments with branching enzyme it was known that bacterial branching enzymes, although they prefer amylopectin over amylose as a substrate, are perfectly able to branch amylose more intensively (Takeda *et al*, 1993, Guan and Preiss, 1993). Evidence supporting the idea of amylose being branched by the bacterial branching enzymes in potato was shown by gel permeation chromatography of native starches (Chapter 3). The amylose fraction decreased or disappeared in the starch from e.g. transformant vTP-22 and a new fraction emerged. This new fraction was called the intermediate fraction because it displayed λmax values of 560-600 nm, in between those for amylose and amylopectin. A similar fraction was sometimes found in native cereal starches like maize (Wang et al, 1993a/b) and oat (Tester and Karkalas, 1996) displaying a λmax of 560-600 nm. This additional fraction found in cereal starch is believed to contain long-chained amylopectin and/or branched amylopectin. The intermediate fraction reported in the starch of the transgenic potatoes is believed to be derived solely from the amylose portion of the starch. Confirmation of the indicated structural nature of the intermediate fraction reported here will have to be assured by determination of the average chainlength of the glucan and further structural analysis. Determination of e.g. the α- and β-limit dextrin number would elucidate the structure of the backbone of the amylopectin molecule,

number of clusters per molecule and distribution of branchpoints over the cluster.

Possible mechanisms for branching of amylose and amylopectin by the introduced bacterial branching enzymes

The obvious question arises why amylose is branched by a heterologous branching enzyme and not by the endogenous branching enzyme of potato. The endogenous BE is located in the stroma at the surface of the growing granule. The fact that it can be extracted from granules (Flipse *et al,* 1996a) is probably due to entrapment of the protein during granule growth. The newly synthesized amylopectin molecule contains a region with nearly all the branchpoints, and a zone containing the unbranched parts of the chains. Crystallization of the linear chains is supposed to occur at the same time as synthesis (French, 1984), dividing the amylopectin molecule in an amorphous and a crystalline phase (Fig. 1A). It is generally assumed that amylose is synthesized just below the granule surface, in the amylopectin matrix because of the need for ADP-glucose which is formed in the stroma. The location of GBSS, inside the *e* ıorphous part of the amylopectin matrix, makes the newly formed amylose inaccessible for branching enzyme (Fig. 1A). The GBSS becomes trapped in the granule during this process (Kram, 1995).
For the bacterial branching enzymes to have access to amylose they must be active in the amorphous region of the growing starch granule. This process is possible either by diffusion of the bacterial BE and becoming trapped like GBSS is believed to be, or by the formation of a complex with GBSS like the assumed BE/SSS (Martn and Smith, 1995), or possibly because of a higher affinity of the bacterial BE to amorphous structures like glycogen (Fig 1B).
If the bacterial BE becomes trapped and/or is active in the amorphous part of the starch granule it not only has access to the amylose (which is in the amorphous phase), but also to the amorphous part of the cluster of the amylopectin molecule. The amylopectin can be branched more intensively by transferring

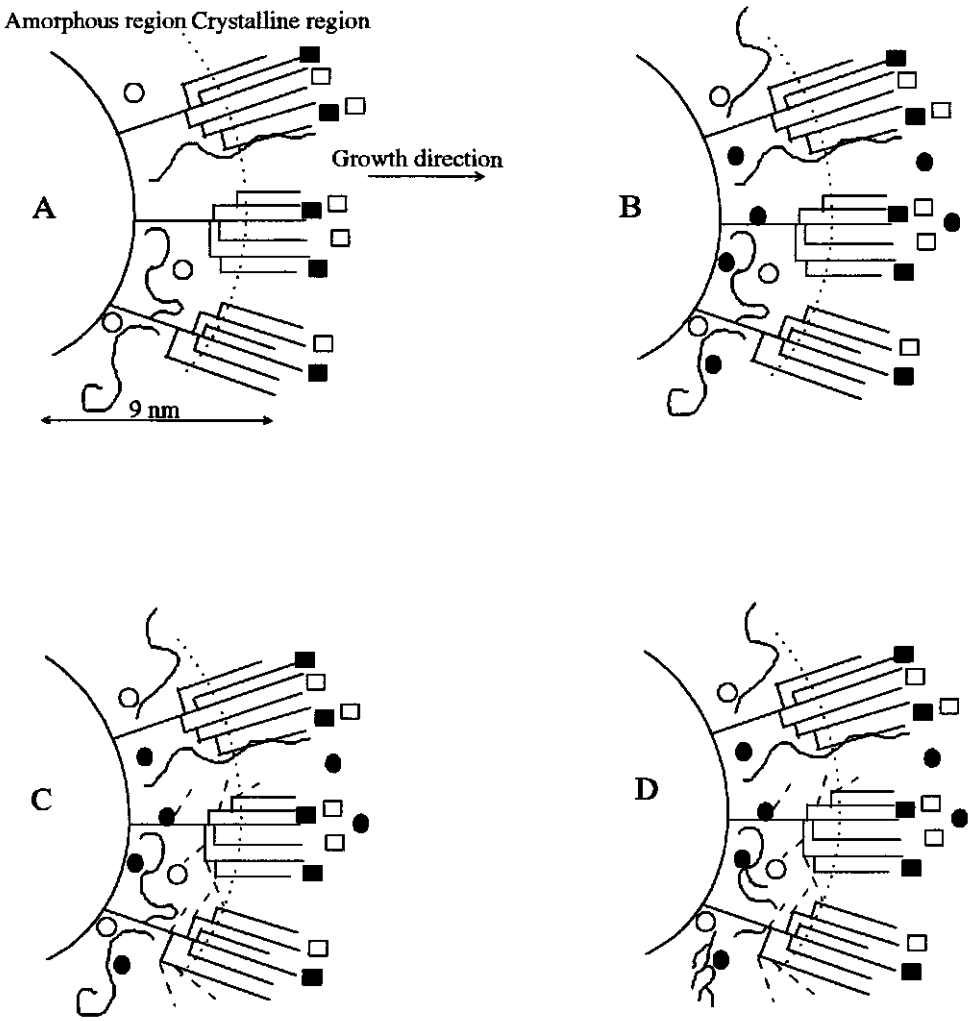


Figure 1. Putative model for the branching of amylopectin and amylose by bacterial branching enzymes.Fig.1A depicts the surface of the growing starch granule, divided in an amorphous and a crystalline region, spanning approximately 9 nm. In Fig. 1B the bacterial branching enzymes have access to the amorphous region of the starch granule, either by diffusion or active transport. Fig. 1C shows the further branching of the amorphous part of the amylopectin by the bacterial branching enzyme by transferring short chains to the outerside of the clusters. In Fig. 1D is shown that the amylose becomes branched as well.

short A-chains to the outside of the cluster (Fig. 1C) at the cost of some of the longer B-chains, or the amylose (if present). There is of course a limit to which the amorphous part of the starch granule can be branched, probably dictated by the amount of free space in the amylopectin backbone.

This limit of free space could answer the question why in our experiments the branching degree (expressed as DE) of the starch was never found to be higher than 5.2. Initially the bacterial branching enzyme probably introduces branches in the part of the amylopectin clusters where all the other branchpoints are located, the amorphous part of the molecule. After the amylopectin is branched to a certain degree, amylose in turn can be used as a substrate by the bacterial branching enzyme (Fig. 1D). Maybe if expression and activity are high enough, the glgB enzyme can somehow branch even the crystalline part of the amylopectin as the gel permeation chromatogram profile of starch from one of the transgenic plants (vTP-18, Chapter 3) did suggest. One of the consequences of the proposed mode of action of the bacterial BE is that the activity of the introduced glgB is lagging just behind amylopectin synthesis. The glgB protein should be found throughout the entire granule by *in situ* hybridization.

Considering the structure of starch and the starch granule, we wondered to what extent starch can be branched theoretically. The amylopectin molecule consists of clusters of chains along the axis. The clusters can be divided in two parts; a part which contains nearly all the branchpoints (in the amorphous part of the molecule) and the part consisting of the linear A and B chains which can form helices and form the crystalline part of amylopectin and starch. For potato starch, about 80% of the starch consists of amylopectin and about 75 % of the amylopectin consists of A/B1 chain clusters. The A/B1 helices are generally assumed to have an average length of at least dp 14, the minimum length needed to form two helix turns (Hizukuri, 1985; Gidley and Bulpin, 1987). For potato the average length of A chains was reported to be 14 (Hizukuri, 1986), which will probably be the length of the A/B1 double helix. Assuming each of these A/B1

helices obtained one extra branchpoint as was observed by *in vitro* experiments with bacterial branching enzymes and amylopectin as a substrate (pers. comm. J. Bergsma, AVEBE, Holland), then 75% of the to the DE contributing glucan becomes twice as branched. The branching degree could increase from DE 3.7 (the value for starch of the untransformed control) to maximally (3.7 + 0.75*3.7=) 6.5. Amylose can also be used to branch amylopectin or can itself be branched simultaneously. Normally, amylose molecules contain less than 1% α-1,6 branchpoints and this contribution to the overall starch branching degree is negligible (the DE value represents the percentage of α-1,6 branchpoints). To contribute significantly to the starch branching degree, the amylose fraction of the starch has to be branched intensively. If all of the 20% starch in the form of amylose is branched maximally by a bacterial branching enzyme, to a level comparable to glycogen, with average side chain length of dp 7 (meaning one in every 7 glucosidic bonds is an α-1,6 branchpoint) it could add maximally 2.8 DE units (20% of the starch with relative DE of 14, so the increase for the total amount of starch will be 0.2*14=2.8) to the starch branching degree. So, one additional branchpoint in each A/B1 helix of the amylopectin and maximum branching of amylose would result in a starch branching degree with a DE value of 9.3.

	Starch granule:	
Amylopectin (80%)	A/B1 clusters (60%) (¾ of amylopectin)	Amylose (20%)
Contribution to the DE:		
3.7 (100%)	(75%)	none

If each A/B1 clusters obtains an extra branchpoint, the total DE of the starch will be:
3.7 + 2.8 = 6.5
The addition of a maximally branched amylose (with a relative DE of 14) to the total DE of the starch can be (0.2 * 14) = 2.8
resulting in a DE of the total starch, if both amylopectin and amylose are maximally branched of:
3.7 + 2.8 + 2.8 = 9.3

The increase in DE values found for starch from transgenic plants is nowhere near the theoretically maximum value. Therefore it is unlikely to assume that all the amylose is branched to the extent of glycogen or that all the A/B1 chain pairs would obtain an extra branchpoint, whether in the crystalline part of the cluster or in the amorphous region of the molecule. An increase in DE was observed for amylose containing starch from 3.7 to 5.2, or an 35% increase in DE. In the amylose-free background a 25% increase in DE was found, a difference of about 10%. So, if heavily branched amylose in the wildtype background would contribute the additional 10% of the increase in DE it would mean that the amylose is about 5 times more branched (20% of the starch accounts for an addition of DE ± 0.4 on the total starch, indicating that every 2 in 100 of the glucosidic bindings in the former amylose are α-1,6 branchpoints). Branching the amylose about five times would explain the loss of blue iodine staining capacity. For the development of blue-staining after complexation with iodine, a minimal linear chainlength of dp 50-80 is required (Thorn and Mohazzeb, 1990). Normal potato amylose has an average dp of 200-250 (Cheng-Yi Lii *et al.*, 1987) and if this becomes five times more branched, the average dp will be 50 or lower and thus loose the blue-staining capacity.

From our experiments it could not be concluded directly whether the blue-staining core in the starch granules contained amylose or lesser branched amylopectin. However, from the gel permeation chromatography profiles it was shown that there is hardly any amylose left ($\lambda max \geq 600$ nm) and that the amylose has been replaced by an intermediate fraction (λmax between 560-600 nm). Since the amylopectin fraction is unaffected it can only be concluded that amylose is the source for this intermediate fraction. Why the blue-staining starch is only found in the core of the granule remains a question to be answered. Possibly this has to do with the granule structure. Initially space can be a limiting factor for further branching of this glucan in the core by the glgB enzyme while during further granule growth more space becomes available. Jane and Chen

(1992) described that more amylose is present in the outer regions of the granule. The amylose content increased during granule growth, and thus the relative amount of amorphous region. Expression of the introduced glgB gene was regulated by the GBSS promoter so it was expected to be activated simultaneously with GBSS. The possibility of the glgB arriving to late in the granule core in order to have access to the blue-staining starch seems not a very likely scenario.

Another possibility could be that the glgB protein acts at the surface of the growing starch granule like the endogenous BE instead of becoming trapped (or maybe both). The bacterial branching enzyme could either form a complex with one of the SSS or maybe the bacterial branching enzyme acts completely on its own, not regulated by any mechanism at all. Branching of amylopectin can occur by adding extra short (A) chains to the cluster or by initiating clusters more often. If more clusters are initiated per amylopectin molecule (which remains the same size), α-limit dextrin will posses a higher number. This would mean a change in the distance between amylopectin clusters. However, this distance of 9 nm (Jenkins *et al.*, 1993) is evolutionary very conserved. This action would visibly affect the granule structure and morphology, a phenomenon which was not observed. More clusters would also mean a relatively increased amount of crystalline material in the starch granule, which should be measurable by DSC analysis which was not observed.

Influence of starch structure on physico-chemical properties

In Table 1 examples are given of the effects of genetic modification on potato starch composition. Effects on the starch composition range from those affecting a single trait e.g amylose content, to severe disturbance of the pathway of starch biosynthesis and tuber development. Severe reduction of the major soluble

starch synthase SSS-III, which was recently identified, seemed to have no effects on the amylopectin to amylose ratio nor on the total starch content but did affect the granule shape. Two types of granules were present in the starch from transgenic tubers: simple granules with deep, often T-shaped cracks centered on the hilum, and granules that appeared to be large clusters of tiny, spherical granules (Marshall *et al.*, 1996; Abel *et al.*, 1996). After introduction of the *Escherichia coli* glgA gene, encoding glycogen synthase, into potato a change in the starch composition could be observed. An increased branching degree (as measured by HPLC analysis of isoamylase debranched starch) and a decreased amylose content were described for starch of glgA expressing potatoes (Shewmaker *et al.*, 1994). Some physical properties of the starch, such as gelatinization and thermal behavior, were also changed. The onset temperature of gelatinization measured by DSC analysis was lower and Rapid Visco Analysis (RVA) showed a higher paste temperature and a lower peak viscosity for the starch of the transgenic potatoes. The higher paste temperature could possibly be attributed to the lowered amylose content as could the lowered peak viscosity. The lowered temperature of onset of gelatinization (To), however, could not be attributed to the decrease in amylose content and may have resulted from the increased branching from amylopectin or from a change in another structural characteristic. The much reduced starch content and increased sugar content of tubers indicated a disturbance of the starch biosynthesis in these transgenic tubers.

Table 1. Genetic modification of potato starch

Trait	Effect	Reference
Antisense inhibition:		
GBSS	Amylose-free starch	Visser *et al.*, 1991, Kuipers *et al.*, 1994
BE	Blue staining core (in *amf* granules)	Flipse *et al.*, 1996a
SSSIII	Compound granules	Marshall *et al.*, 1996; Abel *et al.*, 1996
AGPase	Sugar storing tubers, distorted tuber formation	Müller-Rober *et al.*, 1992
Sense (over)expression:		
GBSS potato	Restored amylose content in *amf*-mutant	Leij *et al.*, 1991; Flipse *et al.*, 1996b)
glgA	Less amylose, higher branching degree amylopectin Reduced starch biosynthesis	Shewmaker *et al.*, 1994
glgB	Higher branching degree amylopectin Amylose replaced by intermediate fraction	Chapter 2, this thesis Chapter 3, this thesis
$glgC^{M}$	Increased amount of starch	Stark *et al.*, 1992
CGTase	Cyclodextrin production	Oakes *et al.*, 1991

AGPase: ADP-glucose pyrophosphorylase; BE: Branching Enzyme; GBSS: Granule-Bound Starch Synthase; SSSIII: Soluble Starch Synthase III.
glgA and -B: Genes from the glycogen synthesis operon of *E.coli*, CGTase: Cyclodextrin glycosyltransferase gene from *Klebsiella sp.*
$glgC^{M}$: modified glgC gene of the glycogen synthesis operon of *E.coli*

Relationship between amylose content, λmax, BV and degree of branching

As is shown in Figure 2A, B and C, where the results from analysis of starch from transgenic potato plants is represented (data from Chapter 3), the amylose content is positively correlated to the B.V. and the λmax. These relations were a confirmation of those observed for starch from maize mutants (Wang *et al.*, 1993a/b). From the results in Fig. 2C it can be concluded that in the amylose containing background an increase in DE can occur either with or without accompanied by a decrease in amylose content.

Influence of amylose on physico-chemical properties of starch

From literature, the influence of amylose on the physico-chemical properties of starch was known to be considerable (Swinkels, 1985; Jane and Chen, 1992; Wang et al., 1993a/b,). Combined with the results which were obtained by analyzing starch from transgenic potato plants with a reduced amylose content (Chapter 4), it can be concluded that amylose reduces the swelling power of starch in water during heating. Amylose decreases the temperature of onset of gelatinization of starch-water suspensions, probably by lowering the relative amount of crystalline material (amylopectin) in the starch granule (Chapters 4 and 5). The presence of amylose accounts for retrogradation and amylose increases gelstrength. These findings were in accordance to reports of other researchers (Sanders et al., 1990; Tester and Morrison, 1990; Jane and Chen, 1992; Wang et al., 1993a/b).

Influence of amylopectin branching degree on physico-chemical properties of starch

Increasing the degree of branching of the starch by introduction and expression of a bacterial glycogen branching enzyme gene had no dramatic effects on the physico-chemical behaviour of the starch. Small differences were detected between starch with a 25% increased degree of branching and its untransformed control. (Chapters 4 and 5). Analysis of changes in the storage modulus (G') of

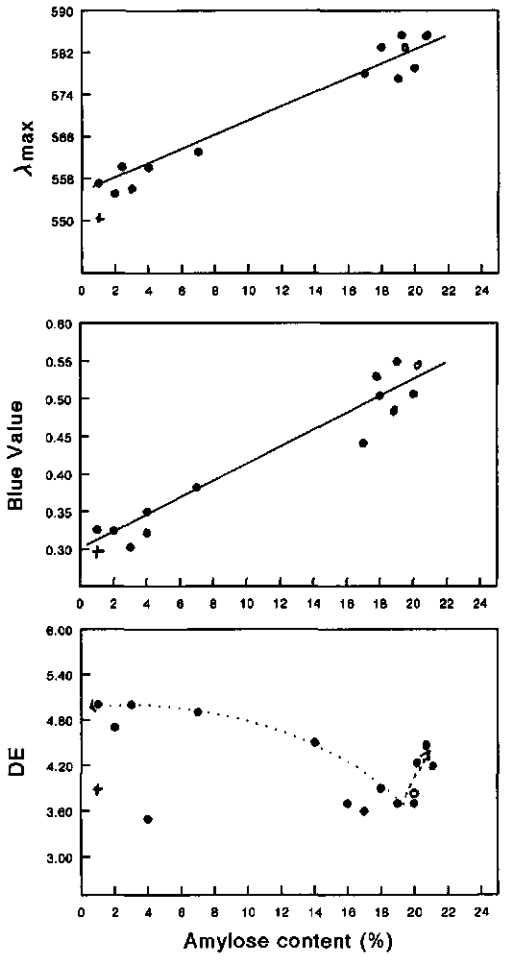


Figure 2. Relation between amylose content, λmax, Blue Value and DE of starch from amylose containing transgenic potato tubers. The 0 indicates the value of the normal amylose containing control starch. The + indicates the value for an amylose-free starch control.

5% starch suspensions showed a decreased peak-viscosity for starch with an increased degree of branching. This was supported by the results of experiments in which the expression of endogenous branching enzyme was inhibited. This type of starch, which presumably had more longer chains, showed an increase in peak viscosity when compared with control material (Fig. 3; data obtained from Flipse *et al.*, 1996a).

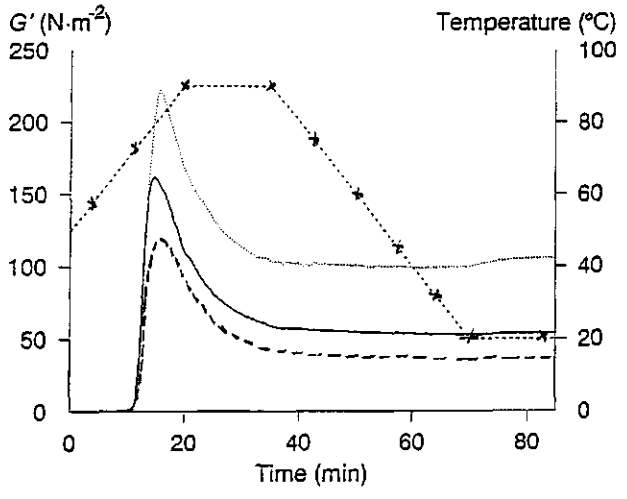


Figure 3. Changes in storage moduli (G') of 5% starch suspensions during heating and subsequent cooling. Temperature against time (.*.); amylose-free control starch (——); starch from an antisense BE inhibited plant (....); starch from an *E.coli* glgB expressing plant with an increased starch branching degree (- - - -).

An increase in the amount of shorter chains therefore led to a lowered peak viscosity and an increase in the amount of longer chains to a higher peak viscosity. These results with transgenic potato are in accordance with the results obtained by Wang *et al.* (1992, 1993a/b) with starch from comparable maize mutants. They also found a negative correlation between the ratio of short to longer chains and the peak viscosity of starch suspensions. Correlation's between the amount of shorter chains and other properties were less clear, but there were indications that starches with a higher degree of branching had a decreased gel strength and a decreased swelling power (Chapters 4 and 5). Similar relations were reported for starches of maize mutants (Wang *et al.*, 1992, 1993 a/b).

In this thesis a (first) step is described in the direction of the production of naturally, granule based, starches with a modified composition. These genetically modified starches have properties analogous to chemically modified starches now in use. In the future it may even be possible to produce "tailor made" starches for each thinkable application by the use of plant biotechnology. From the data we presented it also becomes clear that genetic modification can help to clarify the relationship between starch fine structure and starch properties.

Summary

Starch consists of two major components; amylose and amylopectin. Amylose is synthesized by the enzyme Granule-Bound Starch Syntase (GBSS) and consists of essentially linear chains of α-1,4 linked glucose residues. Amylopectin is synthesized by the combined activity of the enzymes Soluble Starch Synthase (SSS) and Branching enzyme (BE) and consists of linear α-1,4 linked glucosidic chains with α-1,6 linked branchpoints. The amount and fine structure of each of the components determine the starch physico-chemical properties and, therefore, the possibilities for industrial applications. Modification of starch composition by genetic engineering can be a way to improve starch quality or design starches with characteristics for new industrial purposes. In order to obtain potato starch with a higher degree of branching, prokaryotic genes encoding branching enzyme were introduced into the potato. These genes are involved with glycogen biosynthesis in bacteria which is analogous to amylopectin biosynthesis in higher plants. The differences between amylopectin and glycogen (both α-1,4 α-1,6 branched glucan) are found in the ordered structure of amylopectin with clusters of branches as opposed to the random branching in glycogen. Furthermore, glycogen is much more branched compared to amylopectin.

The branching enzyme encoding genes (glgB) from *Escherichia coli* and *Anacystis nidulans* were placed under the control of the tuberspecific GBSS promoter of potato in the binary plasmid $pBIN_{19}$. Targeting was ensured by fusing the glgB genes to the transitpeptide sequence of either GBSS or to that of the potato small subunit of Ribulose bisphosphate carboxylase (Rubisco) including a N-terminal addition of variable size, thus creating chimeric proteins. The plasmids were transformed by *Agrobacterium tumefaciens* to a diploid amylose-free potato

mutant clone, lacking GBSS activity, as well as to diploids with normal amylose containing starch. Transgenic plants were obtained which expressed the heterologous branching enzyme as was shown by the presence of mRNA and protein in the tubers, although expression levels were relatively low. All four constructs used for transformation were found to change the starch of transgenic tubers of amylose-free and/or wildtype plants in the same way and to a similar degree. Analysis of the starch structure showed an increase in the branching degree (DE), representing up to 25% more branchpoints in the amylose-free mutant background. An increase of up to 35% more branchpoints was observed in the amylose-containing background. The increase in the number of branchpoints was partly caused by the presence of more short chains, the so called A chains, with a degree of polymerization of $\leq$ 16 glucose-residues. Changes in other characteristics of the starch, such as average chainlength and λ_{max}, indicated a more branched structure for starch from transgenic plants as well. Among all the transgenic plants, the starch branching degree was never found to be higher than approximately DE 5. Values for the untransformed control starches were 3.6 for amylose-containing and 3.9 for amylose-free starch. Apart from low expression levels or activity of the introduced branching enzyme, this restricted increase in DE might be due to the structure of the starch granule itself, allowing little space for changes in amylopectin structure. Especially if the activity of the introduced branching enzyme is lagging behind normal starch biosynthesis as was proposed in a model to explain the possible mechanism of branching of starch by the introduced bacterial branching enzymes. The introduced bacterial branching enzymes were not only capable of branching amylopectin, but amylose in wildtype starch as well. It was assumed that the bacterial branching enzymes are located in the amorphous region of the growing starch granule to branch amylose and simultaneously transfer short A chains to the outerside of the amylopectin clusters in the region where all the other branchpoints are located. In some of the transgenic plants with an

increased starch branching degree, the amylose content had decreased to a level comparable to that of amylose-free starch (Chapter 3). Starch granules of those transformants showed after iodine staining a blue core surrounded by red-staining starch. Gel permeation chromatography showed that the essentially unbranched amylose was replaced by a so-called intermediate fraction, with a higher degree of branching.

For a number of transgenic plants with an increased starch branching degree, in the amylose-free as well as in an amylose-containing background, enough starch was available to determine some physico-chemical characteristics (Chapters 4 and 5). No change in granule size or morphology could be observed for the altered starches of these transgenic plants compared to their (un)transformed controls. Regardless of the presence or absence of amylose, starches with an increased branching degree showed similar results: a shift towards more short chains in the amylopectin and a lowered peak viscosity of starch suspensions during heating. For the amylose-free starch gels it was also found that the starches with an increased branching degree showed a tendency to form weaker gels.
The presence of amylose was shown to be the most important characteristic for determining the physico-chemical properties of the starch, as was expected. Amylose had a negative effect on the swelling power of starch in water and it lowered the temperature of the onset of gelatinization. Our results in potato confirmed the structural-functional relationships described in literature for starch from maize mutants.

More research is needed to establish the extend of changes in rheological properties of the starches from the transgenic plants and to investigate the possible new applications of the altered types of starches.

Samenvatting

Zetmeel is een van de belangrijkste vormen van reserve koolhydraten in planten en wordt opgeslagen in de vorm van korrels in knollen, wortels en zaden. Zetmeel bestaat uit amylopectine (± 80%) en amylose (± 20%). Bij de synthese van zetmeel zijn een aantal enzymen betrokken. Het enzym Korrel-Gebonden Zetmeel Synthase (KGZ) is verantwoordelijk voor de synthese van amylose, een bijna geheel lineair molecuul bestaande uit α-1,4 gekoppelde glucose eenheden. Amylopectine is een vertakt molecuul dat bestaat uit lineaire ketens (α-1,4 gekoppelde glucose) met α-1,6 vertakkingspunten. De enzymen betrokken by de synthese van amylopectine zijn Oplosbaar Zetmeel Synthase (OZ) en Vertakkings Enzym (VE). De verhouding tussen de hoeveelheden amylose en amylopectine en de structuur van deze componenten, is bepalend voor het fysisch-chemisch gedrag van het zetmeel. De industrieele toepassingen van zetmeel zijn op hun beurt weer afhankelijk van de fysisch-chemische eigenschappen. Genetische modificatie kan bijdragen aan een verandering van de zetmeel samenstelling en leiden tot een hogere kwaliteit van het zetmeel of zelfs tot geheel nieuwe toepassingen. Met als doel een hogere vertakkingsgraad van aardappel zetmeel te verkrijgen werden bacteriële vertakkingsenzymgenen tot expressie gebracht in aardappel. Deze genen zijn betrokken bij de glycogeen synthese in bacteriën die analoog verloopt aan de amylopectine biosynthese in planten. Het verschil tussen glycogeen en amylopectine is dat glycogeen geen clusters van vertakkingspunten heeft maar willekeurig vertakt is. Daarnaast heeft glycogeen een hogere vertakkingsgraad. De vertakkingsenzymgenen van *Escherichia coli* en *Anacystis nidulans* werden voorzien van de juiste expressie en transport signalen en gekloneerd in de binaire vector pBIN19. Voor hoge

expressie in knollen werd gebruik gemaakt van de promoter van het aardappel KGZ gen. Om transport van het heterologe eiwit over de amyloplast membraan te verzekeren werd het transitpeptide van het KGZ of het transtitpeptide van de kleine subunit van het Ribulose bisfosfaat carboxylase (Rubisco) voor het glgB gen geplaatst. De transitpeptides bevatten een gedeelte van het eiwit achter de splice-site, waardoor chimere eiwitten ontstonden. De constructen met de glgB genen werden naar aardappel getransformeerd met behulp van *Agrobacterium tumefaciens*. De constructen werden zowel naar diploide aardappel klonen met normaal amylose bevattend zetmeel als naar een kloon met amylose-vrij zetmeel getransformeerd. Tijdens het onderzoek werden transgene planten verkregen die het heterologe vertakkingsenzym tot expressie brachten op zowel mRNA, als eiwit niveau. Over het algemeen was de expressie relatief laag. Elk van de vier constructen die werden gebruikt voor transformatie, resulteerde in een veranderde zetmeelsamenstelling in of amylose-vrije en/of amylose bevattende planten. Het algemene effect van de constructen op het zetmeel van transgene planten was een verhoging van de vertakkingsgraad. De vertakkingsgraad van het zetmeel in de amylose-vrije achtergrond was toegenomen met maximaal 25%. In de amylose bevattende achtergrond was de vertakkingsgraad maximaal verhoogd met 35% (Hoofdstukken 2 en 3). De hogere vertakkingsgraad van het zetmeel kon gedeeltelijk worden toegeschreven aan een toename van het aantal korte ketens, de zogenaamde A-ketens met een polymerisatiegraad van ≤ 16 glucose eenheden. Ook andere eigenschapppen zoals een lagere gemiddelde ketenlengte en een lagere λmax wezen op een verhoogde vertakkingsgraad van het zetmeel in de transgene planten. De zetmeelvertakkingsgraad werd uitgedrukt in DE (Dextrose Equivalent). De DE waarde voor amylose-vrij zetmeel van ongetransformeerde planten is 3.9 en voor amylose bevattend zetmeel 3.6-3.7. De vertakkingsgraad van het zetmeel in transgene planten varieerde van 3.7 (onveranderd) tot maximaal 5.2. Behalve door lage expressieniveaus en lage activiteit van het ingebrachte eiwit wordt deze beperkte

verhoging van de vertakkingsgraad wellicht veroorzaakt door de structuur van de zetmeelkorrel zelf. De opbouw van het amylopectine is misschien wel zodanig dat er weinig ruimte is voor veranderingen van de structuur zonder veranderingen in de korrel. Zeker als de activiteit van het ingebrachte enzym achterloopt op de zetmeel biosynthese (zoals werd verondersteld in een model om het mechanisme te verklaren op welke manier de bacteriele vertakkingsenzymen in staat zijn om zetmeel te vertakken). De heterologe vertakkingsenzymen bleken niet alleen in staat om amylopectine verder te vertakken maar ook amylose. Er werd aangenomen dat om amylose te kunnen vertakken, de bacterieële vertakkingsenzymen zich in de amorfe regio van de korrel moeten bevinden. In deze regio zouden de enzymen zowel amylose, als het gedeelte van het amylopectine waarin zich alle vertakkingspunten bevinden verder kunnen vertakken. Waarschijnlijk worden korte A-ketens aan de buitenzijde van de amylopectine clusters gehangen. In een aantal van de planten met een hogere zetmeelvertakkingsgraad was de hoeveelheid amylose verlaagd tot een niveau vergelijkbaar met dat van amylose-vrij zetmeel (Hoofdstuk 3). Zetmeel korrels vertoonden na kleuring met jodium een blauwe kern omringd door rood kleurend zetmeel. Fractionering van dit zetmeel liet zien dat het onvertakte amylose was vervangen door een zogenaamde intermediaire fractie met een hogere vertakkingsgraad. Verder onderzoek zal moeten uitwijzen in welke mate het vroegere amylose vertakt is.

Van een aantal planten met een verhoogde zetmeelvertakkingsgraad was voldoende zetmeel beschikbaar om een aantal fysisch-chemische eigenschappen te bepalen (Hoofdstukken 4 en 5). De vorm en grootte van de zetmeelkorrels was niet veranderd vergeleken met die van korrels van de ongetransformeerde controles. Zetmeel met een hogere vertakkingsgraad bleek, ongeacht de aan-, of afwezigheid van amylose, tijdens het verhitten van zetmeel-water suspensies een lagere piekviscositeit te vertonen. Amylose-vrij zetmeel met een hogere vertakkingsgraad vormde ook zwakkere gelen.

De aanwezigheid van amylose bleek verreweg de grootste invloed op het fysisch-chemische gedrag van zetmeel uit te oefenen, zoals al verwacht werd. Amylose verlaagt het zwelvermogen van zetmeelkorrels in water en de aanwezigheid van amylose verlaagt de temperatuur waarbij gelering begint. Onze resultaten met zetmeel van transgene aardappels bevestigen de structuur-functie relaties die in de literatuur voor zetmeel van diverse mais mutanten zijn beschreven.

Verder onderzoek zal nodig zijn om te bepalen in hoeverre de fysisch-chemische eigenschappen van zetmeel met een hogere vertakkingsgraad veranderd zijn en wat voor mogelijke toepassingen deze zetmelen met een gemodificeerde samenstelling kunnen bieden.

References

Abel G.J.W., Springer F., Willmitzer L. and Kossmann J. (1996) Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (*Solanum tuberosum*). *The Plant Journal* **10** (6),981-991.

Ball S., Guan H.P., James M., Myers A., Keeling P., Mouille G., Buleon A., Colonna P. and Preiss J. (1996) From glycogen to amylopectin: a model for the biogenesis of the plant starch granule. *Cell* **86**, 349-352.

Banks, W. and Greenwood, C.T. (1975) *Starch and its components* University Press: Edinburgh, UK, pp 242-305.

Bevan, M. (1984) Binairy *Agrobacterium* vectors for plant transformations. *Nucl. Acids Res.* **12**, 8711-8721.

Bogracheva T. Ya., Davydova N.I., Genin Ya. V. and Hedley C.L. (1995) Mutant genes at the *r* and *rb* loci affect the structure and physico-chemical properties of pea seed starches. *Journal of Experimental Botany* **46** (293), 1905-1913

Boyer C.D. and Preiss J. (1981) Evidence for independent genetic control of the multiple form of maize endosperm branching enzymes and starch synthases. *Plant Physiol.* **67**, 1141-1145

Burton, R.A., Bewley, J.D., Smith, A.M., Bhattacharyya, M.K., Hamilton, W.D.O. and Martin, C. (1995) Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. *Plant Journal* 7, 3-15

Campbell M.R., Pollak L.M. and White P.J. (1995a) Genetic variation for starch thermal and functional properties among nonmutant maize inbreds. *Cereal Chem.* 72 (3), 281-286

Campbell M.R., White P.J. and Pollak L. (1995b) Properties of Sugary-2 maize starch: Influence of exotic background. Cereal Chem **72** (4), 389-392

Chambers, S.P., Prior, S.E., Barstow, D.A. and Minton, N.P. (1988). The pMTL nic- cloning vectors.I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* **68**, 139-149.

Cheng-Yi Lii, Yi-Yuan Shao and Kuo-Hsuen Tseng (1995) Gelation mechanism and rheological properties of rice starch. Cereal Chem **72** (4), 393-400

Dellaporte, S.L., Wood, J. and Hicks, J.B. (1983) A plant minipreparation version II *Plant. Mol. Biol. Rep.* **1**, 19-21.

Delrue B., Fontaine T., Routier F., Decq A., Wieruszeski J.M., Van Den Koornhuyse N., Maddeleis M.L., Fournet B. and Ball S. (1992) Waxy *Chlamydonomas reinhardti*: Monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase accumulate a structurally modified amylopectin. *Journal of Bacteriology* **174** (11): 3612-3620

Echt C.S. and Schwartz D. (1981) Evidence for the inclusion of controlling elements within the structural gene at the *waxy* locus in maize. *Genetics* **99**, 275-284

Edwards J., Green J.H. and ap Rees T. (1988) Activity of branching enzymes as cardinal features of the *ra* locus in *Pisum sativum*. *Phytochemistry* **27**, 1615-1620

El-Kharbotly A., Jacobsen E., Stiekema W.J. and Peirera A. (1995) Genetic localisation of transformation competence in diploid potato. *Theor. Appl. Genet.* **91**, 553-706

Finnegan J. and McElroy D. (1994) Transgene inactivation: plants fight back! *Bio/technology* **12**, 883-888.

Flipse E., Huisman J.G., de Vries B.J., Bergervoet J.E.M., Jacobsen E. and Visser R.G.F. (1994) Expression of a wild-type GBSS-gene introduced into an *amylose-free* potato mutant by *Agrobacterium tumefaciens* and the inheritance of the inserts on microsporic level. *Theor. Appl. Genet.* **88** , 369-375

Flipse E., Suurs L., Keetels C.J.A.M., Kossmann J., Jacobsen E. and Visser R.G.F. (1996a) Introduction of sense and anti-sense cDNA for branching enzyme in the *amylose-free* potato mutant leads to physico-chemical changes in the starch. *Planta* **198**, 340-347

Flipse E., Straatman-Engelen I. Kuipers A.G.J., Jacobsen E. and Visser R.G.F. (1996b) GBSS T-DNA inserts giving partial complementation of the *amylose-free* mutant can also cause co-suppression of the endogenous GBSS-gene in a wildtype background. *Plant. Mol. Biol.* **31**, 731-739

Flipse E., Keetels C. J. A. M., Jacobsen E. and Visser R. G. F. (1996). The dosage effect of the wildtype GBSS allele is linear for GBSS activity but not for amylose content: absence of amylose has a distinct influence on the physico-chemical properties of starch. *Theor. Appl. Gen.* **92**, 121-127.

Fontaine T., D'Hulst C., Maddelein M., Routier F., Pepin T.M., Decq A. Wieruszeski J-M., Delrue B., van den Koornhuyse N., Bossu J-P., Fournet B. and Ball S. (1993) Toward an understanding of the biogenesis of the starch granule: Evidence that *Chlamydonomas* soluble starch synthase II controls the synthesis of intermediate size glucans of amylopectin. *Journal of Biological Chemistry* **268** (22), 16223-16230.

French D. (1984) Organization of starch granules. In: Starch: Chemistry and Technology, 2nd edition, edited by R.L. Whistler, J.N. Bemiller, E.F. Paschall. Academic Press, Orlando, pp. 183-247.

Gidley M.J. and Bulpin P.V. (1987) Crystallization of malto-oligosachharides as models of the crystalline forms of starch: Minimum chain-length requirement for the formation of double helices. *Carbohydrate Research* **161**, 291-300.

Guan, H.P and Preiss, J. (1993) Differentiation of the properties of the branching isozymes from maize *(Zea mays). Plant Physiol.* **102**, 1269-1273.

Hedman K.D. and Boyer C.D. (1982) Gene dosage at the amylose extender locus of maize: effects on the levels of starch branching enzymes. *Biochem. Genet.* **20**, 483-492

Hizukuri S. (1985) Relationship between the distribution of the chain length of amylopectin and the crystalline structure of starch granules. *Carbohydrate Research* **141**, 295-306.

Hizukuri S. (1986) Polymodal distribution of the chain lenghts of amylopectins, and its significance. *Carbohydrate Research* **147**, 342-347

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoort (1988) A binary vector based on separation of vir and T-region of the *Agrobacterium tumefaciens* Ti plasmids. *Nature* **303**, 179-181.

Hovenkamp-Hermelink J.H.M., Jacobsen E., Ponstein A.S., Visser R.G.F., Vos-Scheperkeuter G.H., Bijmolt E.W., de Vries J.N., Witholt B. and Feenstra W.J. (1987) Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.) *Theor. Appl. Genet.* **75**, 217-221

Hovenkamp-Hermelink J.H.M., de Vries J.N., Adamse P., Jacobsen E., Witholt B. and Feenstra W.J. (1989) Rapid estimation of the amylose/amylopectin ratio in small amounts of tuber and leaf tissue of potato. *Potato Research* **31**, 241-246

Howling D. (1980) The influence of the structure of starch on its rheological properties. *Food Chem.* **6**, 51-61

Jacobs H., Eerlingen R.C., Clauwaert W. and Delcour J.A. (1995) Influence of annealing on the pasting properties of starches from varying botanical sources. *Cereal Chem.* **72** (5), 480-487

Jacobsen, E., Hovenkamp-Hermelink, J.H.M., Krijgshed, H.T., Nijdam, H., Pijnacker, L.P., Witholt, B. and Feenstra, W.J. (1989) Phenotypic and genotypic characterization of an amylose-free starch mutant of potato. *Euphytica* **44**, 43-48.

Jane J. and Chen J. (1992) Effect of amylose molecular size and amylopectin branch length on paste properties of starch. *Cereal Chem.* **69** (1), 60-65.

Jane J., Kasemsuwan T., Leas S., Ames I.A., Zobel H., Il D. and Robyt J.F. (1994) Anthology of starch granule morphology by scanning electron microscopy. *Starch* **46**, (4), 121-129.

Jenkins P.J., Cameron R.E. and Donald A.M. (1993) A universal feature in the structure of starch granules from different botanical sources. *Starch* **45**, 417-420.

Keetels C.J.A.M. (1995) Retrogradation of concentrated starch systems; mechanisms and consequences for product properties. PhD thesis, University of Wageningen, The Netherlands, Chapter 3, pp 55-82.

Kiel, J.A.K.W., Vossen, J.P.M.J. and Venema, G. (1987) A general method for the construction of *Escherichia coli* mutants by homologous recombination and plasmid segregation. *Mol. Gen. Genet.* **207**, 294-301.

Kossmann J., Visser R.G.F., Muller-Rober B., Wilmitzer L. and Sonnewald U. (1991) Cloning and expression analysis of a potato cDNA that encodes branching enzyme: evidence for co-expression of starch biosynthetic genes. *Mol. Gen. Genet.* **230**, 39-44.

Kram A.M. (1995) Structure and biosynthesis of starch. PhD thesis. University of Groningen, Groningen, The Netherlands. pp 87-104.

Krisman C.R. (1962) A method for the colorimetric estimation of glycogen with iodine. *Analytical Biochemistry* **4**, 17-23.

Krohn, B.M., Stark, D.M., Barry, G.F., Preiss, J. and Kishore, G.M. (1994) Modification of starch structure in transgenic potato. In *Supplement to Plant Physiology* **105**. Abstracts of posters of the 1994 Annual Meeting of the American Society of Plant Physiologists, pp 37.

Kuipers G.J., Jacobsen E. and Visser R.G.F. (1994a) Formation and deposition of amylose in the potato tuber starch granule are affected by the reduction of granule-bound starch synthase gene expression. *The Plant Cell* **6**, 43-52

Kuipers, G.J., Soppe, J.J., Jacobsen, E. and Visser, R.G.F. (1994b) Field evaluation of transgenic potato plants expressing an antisense granule-bound starch synthase gene: increase of the antisense effect in starch granules during tuber development. *Plant. Mol. Biol.* **26**, 1759-1773.

Kuipers A. G. J., Soppe W. J. J., Jacobsen E. and Visser R. G. F. (1995) Factors affecting the inhibition of granule-bound starch synthase gene expression in potato via antisense RNA. *Mol.Gen.Genet.* **246**, 745-755.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Leach H.W., McCowen L.D. and Schoch T.J. (1959) Structure of the starch granule. I. Swelling and solubility patters of various starches. *Cereal chemistry* **6** (36), 534-544.

van der Leij F.R., Visser R.G.F., Oosterhaven K., van der Kop D.A.M., Jacobsen E. and Feenstra W.J. (1991) Complementation of the amylose-free starch mutant of potato (*Solanum tuberosum* L.) by the gene encoding granule-bound starch synthase. *Theor. Applied Genetics* **82**, 289-295

van der Leij F.R., Visser R.G.F., Ponstein A.S., Jacobsen E. and Feenstra W.J.(1991) Sequence of the structural gene for granule-bound starch synthase of potato (*Solanum tuberosum* L.) and evidence for a single point deletion in the *amf* allele. *Mol Gen. Genet* **228**, 240-248

MacDonald F.D. and Preiss J. (1985) Partial purification and characterization of granule bound starch synthases from normal and *waxy* maize. *Plant Physiol.* **73**, 849-852.

Maddelein M., Libessart N., Bellanger F., Delrue B., D'Hulst C., van den Koornhuyse N., Fontaine T., Wieruszeski J., Decq A. and Ball S. (1994) Towards an understanding of the biogenesis of the starch granule: Determination of granule-bound and soluble starch synthase functions in amylopectin synthesis. *The Journal of Biological Chemistry* **269**, 25150-25157.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular cloning: a laboratry manual.* Cold Sping Harbor Laboratry press, Cold Spring Harbor, New York.

Manners D.J. (1989) Recent developments in our understanding of amylopectin structure. *Carbohydrate Polymers* **11**, 87-112

Marshall J., Sidebottom C., Debet M., Martin C., Smith A.M. and Edwards A. (1996) Identification of the major starch synthase in the soluble fraction of potato tubers. *The Plant Cell* **8**, 1121-1135.

Martin C. and Smith A.M. (1995) Starch biosynthesis. *The Plant Cell* **7**, 971-985

Matzke A.M. and Matzke A.J.M. (1995) How and why do plants inactivate homologous (trans)genes? *Plant Physiol.* **107**,679-685.

Mouille G., Maddelein M., Libessart N., Talaga P., Decq A., Delrue B. and Ball S. (1996) Preamylopectin processing: a mandatory step for starch biosynthesis in plants. *The Plant Cell* **8**:1353-1366.

Müller-Rober B., Sonnewald U. and Wilmitzer L. (1992) Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar storing tubers and influences tuber formation and expression of tuber storage protein genes. *EMBO Journal* **11**, 1229-1238

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497.

Nakamura Y., Takeichi T., Kawaguchi K. and Ymanouchi H. (1992) Purification of two forms of starch branching enzyme (Q enzyme) from developing rice endosperm. *Phys. Plants* **84**, 329-335.

Nakamura Y., Yamamori M., Hirano H., Hidaka S. and Nagamine T. (1995) Production of waxy (amylose-free) wheats. *Mol. Gen. Genet.* **248**, 253-259

Nakamura Y., Umemoto T., Takahata Y., Komea K., Amano E. and Satoh H. (1996) Changes in structure of starch and enzyme activities affected by *sugary* mutations in developing rice endosperm. Possible role of starch debranching enzyme (R-enzyme) in amylopectin biosynthesis. *Physiol. Plant* **97**: 491-498.

Oakes J.V., Shewmaker C.K. and Stalker D.M. (1991) Production of cyclodextrins, a novel carbohydrate, in the tubers of transgenic potato plants. Biotechnology **9**, 982-986

Pan, D. and Nelson, O.E. (1984) A debranching enzyme deficiency in endosperms of the *Sugary-1* mutant of maize. *Plant. Physiol.* **74**, 324-328.

Park, J.T. and Johnson, M.J. (1949) A submicrodetermination of glucose. *J. Biol. Chem.* **181**, 149-152.

Preiss J. and Walsh D.A. (1981) The comparative biochemistry of glycogen and starch. In Biol. of Carbohydrates vol. 1, John Wiley Press, V. Ginsbury eds.

Preiss J. and Sivak M. (1996) Starch synthesis in sinks and sources. In: Photoassimilate distribution in plants and crops: Source-sink relationships. E. Zanski and A.A. Schaffer, Eds (New York;Marcel Dekker, Inc), pp 63-96.

Salehuzzaman S.N.I.M., Jacobsen E. and Visser R.G.F. (1992) Cloning, partial sequencing and expression of a cDNA coding for branching enzyme in cassava. *Plant Mol. Biol.* **20**, 809-819.

Salehuzzaman S.N.I.M., Jacobsen E. and Visser R.G.F. (1993) Isolation and characterization of a cDNA encoding Granule-Bound Starch Synthase (*Manihot esculenta* Crantz) and its antisense expression in potato. *Plant. Mol. Biol.* **23**, 947-962.

Sambrook J., Fritsch E.F., and maniatis T. (1989) Molecular cloning: A laboratory manual, 2nd edition, Cold Spring Harbour Laboratory. (Cold Spring harbour, NY, USA).

Sanders E.B., Thompson D.B. and Boyer C.D. (1990) Thermal behavour during gelatinization and amylopectin fine structure for selected maize genotypes as expressed in four inbred lines. *Cereal Chem.* **67** (6), 594-602

Sano Y. (1984) Differential regulation of *waxy* gene expression in rice endosperm. *Theor. Appl. Genet.* **68**, 467-473

Shannon J.C. and Garwood D.L. (1984) Genetics and physiology of starch development. In: Whistler R.L., BeMiller J.N. and Paschall E.F. (Editors) Starch: chemistry and technology, 2nd edition. Academic Press, Orlando, pp 25-86.

Shewmaker C.K., Boyer C.D., Wiesenborn D.P., Thompson D.P., Boersig M.R., Oakes J.V. and Stalker D.M. (1994) Expression of *Escherichia coli* glycogen synthase in the tubers of transgenic potatoes (*Solanum tuberosum*) results in a highly branched starch. *Plant Physiol.* **104**, 1159-1166

Schoorl, N. (1925) Suiker-titratie. *Chemisch Weekblad* **22**, 132-133 and 285-286.

Smith A.M. (1988) Major differences in isoforms of starch branching enzyme between developing embryos of round- and wrinkled-seeded peas (*Pisum sativum* L.) *Planta* **125**, 270-279

Smith A.M., Denyer K. and Martin C.R. (1995) What controls the amount and structure of starch in storage organs. *Plant Physiol.* **107**, 673-677.

Stark D.M., Timmerman K.P., Barry G.F., Preiss J. and Kishore G.M. (1992) Regulation of the amount of starch in plant tissue by ADP-glucose pyrophosphorylase. *Science* **258**, 287-292

Swinkels J.J.M. (1985a) Composition and properties of commercial native starches. *Starch* **37** (1), 1-5

Swinkels J.J.M. (1985b) Starch conversion technology, chapter 2: sources of starch, its chemistry and physics. Eds. G.M.A. van Beynum and J.A. Roels. Marcel Dekker, inc. New York and Basel.

Takata H., Takaha T., Okada S., Takagi M. and Manaka T. (1996) Cyclization reaction catalysed by branching enzyme. *J. of Bact.* **178** (6), 1600-1606.

Takeda C., Takeda Y. and Hizukuri S. (1989) Structure of amylomaize amylose. *Cereal Chem.* **66**, 22-25.

Takeda, Y., Guan, H.P. and Preiss, J. (1993) Branching of amylose by the branching isozymes of maize endosperm. *Carbohydrate Research* **240**, 253-263.

Tester R.F and Morrison W.R. (1990) Swelling and gelatinization of cereal starches. I. Effects of amylose, amylopectin and lipids. *Cereal Chem.* **67** (6):551-557.

Tester R.F. and Karkalas J. (1996) Swelling and gelatinization of oat starches. *Cereal Chem.* **73** (2), 271-277

Thorn W. and Mohazzeb S. (1990) α-D-Polyglucane-Iodine Complexes. *Starch* **42** (12), 455-459.

Tolmasky, D.S. and Krisman, C.R. (1987) The degree of branching in (α-1.4)-(α-1,6)-linked glucopolysaccharides is dependent on intrinsic properties of the branching enzymes. *Eur. J. Biochem* **168**, 393-397.

Visser, R.G.F., Hesseling-Meinders, A., Jacobsen, E., Nijdam, H., Witholt, B. and Feenstra, W.J. (1989) Expression and inheritance of inserted markers in binairy vector carrying *Agrobacterium rhizogenes* transformed potato (*Solanum tuberosum* L.). *Theor. Appl. Genet.* **78**, 705-714.

Visser R.G.F., Somhorst I. Kuipers G.J., Ruys N.J., Feenstra W.J. and Jacobsen E. (1991) Inhibition of expression of the gene for granule-bound starch synthase in potato by anti-sense constructs. *Mol. Gen. Genet.* **225**, 289-296

Vos-Scheperkeuter G. H., de Boer W., Visser R. G. F., Feenstra W. F. and Witholt B. (1986) Identification of granule-bound starch synthase in potato tubers. *Plant physiol.* **82** 411-416.

Wang Y.J., White P. and Pollak L. (1992) Thermal and gelling properties of maize mutants from the Oh43 inbred line. *Cereal Chem.* **69** (3), 328-334

Wang Y.J., White P., Pollak L. and Jane J. (1993a) Characterization of starch structure of 17 maize endosperm mutant geneotypes with Oh43 inbred line background. *Cereal Chem.* **70** (2), 171-179

Wang Y.J., White P. and Pollak L. (1993b) Physicochemical properties of starches from mutant genotypes of the Oh43 inbred line. *Cereal Chem.* **70** (2), 199-203

Weber M. and Wober G. (1975) The fine structure of the branched α-D-glucan from the blue-gree algae *Anacystis nidulans*: comparison with other bacterial glycogens and phytoglycogen. *Carbohydrate Res.* **39**, 295-302

Whistler R.L. and Doane W.M. (1961) *Cereal Chem.* **38**, 251-256

Nawoord

Na vijf jaar experimenteren en schrijven is het proefschrift dan eindelijk klaar. Het feit dat het er nu ligt is niet in de laatste plaats te danken aan de hulp van een heleboel mensen waarvan ik er een aantal met name wil noemen. Op de eerste plaats natuurlijk mijn begeleider Richard Visser. Hij had altijd tijd voor me, of maakte die. Resultaten werden altijd positief bekeken, voor alle problemen was wel een oplossing te vinden en hij had altijd meer ideeën dan dat ik tijd had om ze uit te voeren. Stukken werden met een bijna angstaanjagende snelheid doorgelezen. Mijn andere begeleider, Evert Jacobsen werd in een later stadium van het onderzoek meer betrokken bij het project. Door zijn "helicopter view" ging ik zelf ook heel anders tegen bepaalde zaken aankijken.

De begeleidingscommissie bestaande uit Peter Bruinenberg, Paul Heeres, Jan van Loon en Will Feenstra wil ik bedanken voor hun belangstelling voor het onderzoek wat tot uiting kwam op de halfjaarlijkse vergaderingen. Het dwong mij ook om de zaken minstens halfjaarlijks op een rijtje te zetten. Dankzij Peter Bruinenberg werden ook veel van de analyses aan het zetmeel uitgevoerd bij de AVEBE en konden Luc en ik zelfs een hele week op het AVEBE lab terecht om zetmeel van de veldproef te isoleren. De mensen van het biotechlab waren zeer belangstellend en hadden veel goede raad en duldden ons op het lab terwijl we veel troep en afgrijselijke herrie maakten.

De verzorging van de planten in de kas was aan de goede zorgen toevertouwd van Teus en Jan. Herman Masselink plukte iedere dag de knoppen van de planten op het veld, gaf ze stuk voor stuk water en toen het weer heel erg warm werd maakte hij zelfs afdakjes om ze tegen de zon te beschermen.

Frits Zwaal, Aletta Karsies, Alexandra Hillen en Emile Clerkxs hebben in de vorm van een afstudeervak meegewerkt aan het onderzoek. Hun hulp bij de transformatie en analyse van de transgene planten was meer dan welkom. Ook Marja Schippers-Roozeboom heeft een flink deel van de transformaties voor haar rekening genomen.

De "starch sisters" Elise, Beja en Anja en alle andere meiden van het moleculaire lab hebben ervoor gezorgd dat ik me snel ingeburgerd voelde op de vakgroep. Zij voorzagen me van alle benodigde informatie, hetzij op praktisch gebied, hetzij op het gebied van de "human interest", tot aan kledingadviezen toe. Dankzij hen heb ik ook alle kantines van de Universiteit leren kennen. Collega AIO's Anja en Elise voorzagen me van allerlei specifieke AIO informatie en ook kon ik bij hen altijd terecht om lekker te klagen over alle AIO-, en andere probleempjes.

Mijn paranimfen Angela en Luc wil ik bedanken voor al het werk dat ze voor me gedaan hebben. Met name in het laatste deel van het project toen we steeds meer de kant opgingen van de zetmeelanalyse, en later ook nog toen ik officieel al lang weg was. We hebben veel gediscussieerd over de manier waarop we de proeven zouden uitvoeren en natuurlijk de resultaten die niet altijd even duidelijk waren. We hebben ook veel gepraat over de rheologie in het algemeen, wat een nieuw terrein voor ons was.

Het laatste half jaar heb ik doorgebracht op "de Terp", met een aantal andere schrijvende AIO's, Peter, Hanneke, Heleen en Ronald. Dat had ik al veel eerder moeten doen. Een rustige omgeving zodat je lekker kunt schrijven en toch heel gezellig met ons eigen koffiehoekje. Ook veel goede tips van mensen die verder waren met hun proefschrift, of gewoon iets handiger met de computer.

Mijn ouders en natuurlijk mijn zussen wil ik bedanken voor hun belangstelling tijdens mijn studie en het AIO-schap. Het allerleukste was wel om samen met mijn vader een proefje op het lab te bedenken en uit te voeren, ook al zijn de resultaten daarvan niet in dit proefschrift opgenomen.

En dan Sjon, die bang was dat ik mijn proefschrift aan hem zou opdragen, nee dus. Wel bedankt voor alle hulp bij de spreekwoordelijke laatste loodjes. Ik weet dat ik je bijna tot wanhoop heb gedreven met de manier waarop ik tabellen maak en mijn nonchalante manier van layouten.

Iedereen, bedankt!