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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

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Abstract A gene-dosage population was obtained by crossing two genotypes that were duplex for the GBSS allele. Nulliplex, simplex, duplex or triplex/quadruplex plants could be identified by monitoring the segregation of red and blue microspores after staining with iodine. GBSS activity was significantly different for all groups and showed an almost linear dosage effect for the wildtype GBSS gene. A dosage effect was found for amylose content that was not linear. The amylose content was similar for both the duplex and triplex/quadruplex group. Within the simplex group, differences in amylose content were found, which might be due to a different genetic background. There was no linear correlation between GBSS activity and amylose content. A certain level of GBSS activity led to a maximum amount of amylose, and further increase in GBSS activity did not result in a further increase in amylose content. The presence of one or more wildtype GBSS allele(s), and therefore the presence of amylose in the starch granules, had a great influence on the physico-chemical properties of the starch suspensions.

Key words Gene dosage · Granule bound starch synthase · *amf* mutant · Physico-chemical properties · *Solanum tuberosum*

Introduction

Starch is present in many plant species as a storage product and consists of two components: amylose (approximately 20%) and amylopectin (approximately 80%). These components are packaged in a specific order in the starch granules. When starch is used industrially either as a thickening agent, flavour carrier or binder in food systems, its effectiveness depends upon the ratio of amylose to amylopectin, as well as their organization within the starch granule (Zobel 1984), with the swelling of the granules in a heated aqueous starch suspension being influenced by the presence of amylose (Zobel 1984). Amylose is an essentially linear glucose polymer with α-1.4 glucosidic linkages. Its production is catalysed by the enzyme granule bound starch synthase (GBSS) (Shannon and Garwood 1984). Amylose is unstable in water (Zobel 1984) and depending on its concentration, it precipitates or forms a gel during cooling and ageing (Miles et al. 1985; Gidley 1989). Amylopectin is a branched glucose polymer with α-1.4 and α-1.6 glucosidic linkages, the last being responsible for the branched structure of amylopectin. The production of amylopectin is catalysed by soluble starch synthase (SSS) and the branching enzyme (BE). The BE produces the α-1.6 branches by cleaving a fragment from the linear chain, whose formation is catalysed by the SSS, and transferring it to the number 6 position of a glucose residue (Shannon and Garwood 1984). Gelation of amylopectin occurs at a much lower rate than that of amylose (Zobel 1984).

In many plant species, variation in the composition of starch is found due to a mutation in one of the genes involved in starch biosynthesis. An amylose-free (*amf*) potato mutant was isolated by irradiating a monohaploid with X-rays; it is a monogenic and recessive mutation (Jacobsen et al. 1989). The mutant lacks GBSS activity and protein in its starch granules (Hovenkamp-Hermelink et al. 1987). The mutant phenotype of the *amf* locus results from a point mutation in which a single base pair is deleted from the structural gene encoding the GBSS transit peptide (van der Leij et al. 1991). Although, the *amf* locus still produces a quantity of mRNA equivalent to that produced by the wildtype, no protein tightly linked to the starch granule is present

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(Hovenkamp-Hermelink et al. 1987). The difference in starch composition can easily be monitored by staining the starch with an iodine-potassium-iodine solution. The *amf* mutant has starch that stains red, whereas wildtype starch stains blue.

We describe here the development of a GBSS gene-dosage population of potato (*Solanum tuberosum* L.) and the dosage effect of wildtype GBSS alleles on amylose content, GBSS activity and the physico-chemical properties of the resulting starch.

Materials and methods

Plant material

The chromosome number of the original monohaploid *amf* mutant 86.040 of *Solanum tuberosum* L. was doubled by tissue culture. The sexually obtained fertile *amf* diploid was doubled by tissue culture again, resulting in 4 × *amf* plants. Since the original 4 × *amf* genotypes were partly sterile and lacked the ability to tuberize, these plants were crossed with wildtypes to improve their vigour, fertility and tuberization (Jacobsen et al. 1989). This resulted in two tetraploid potato plants, S90-1101-3 and S90-1102-7, with a duplex genotype for the wildtype GBSS allele (wildtype GBSS allele = *Amf* = *A*; mutant GBSS allele = *amf* = *a*), which were crossed to create the gene-dosage population J90-6031. Seeds of J90-6031 were sown, and seedlings were transferred to pots in the greenhouse where they were grown under standard conditions.

A field trial was set up with greenhouse-grown tubers. Six tubers per clone were placed in a row with two replications. In the field, the plant distances within a row was 40 cm and between rows 75 cm. The plot was surrounded with cv 'Cleopatra', a non-flowering, red-tuberizing cultivar. Tubers were harvested and bulked per clone and replication.

Staining for starch composition

The cut surfaces of harvested tubers were stained according to Kuipers et al. (1991). The individual starch granules and the microspores were stained as described by Flipse et al. (1994). Three flowers per genotype and two stamens per flower were stained. The segregation ratio of red and blue microspores was statistically determined using the χ^2 test with a 5% confidence limit.

Isolation of starch and determination of amylose content and GBSS activity

Several tubers, all originating from one field row, were taken randomly, and starch was isolated according to Kuipers et al. (1991). Amylose content was determined spectrophotometrically as described by Hovenkamp-Hermelink et al. (1988). This method is easy and fast but has the disadvantage that a certain background level of approximately 3% has to be taken into consideration, even in the amylose-free genotypes. The determination of GBSS activity was as described by Vos-Scheperkeuter et al. (1986). Two-milligram samples of isolated starch were used for measuring amylose content (three samples) and GBSS activity (six samples). A variance analysis test with a 5% confidence limit was used for statistically analysing the dosage effect on amylose content and GBSS activity. For a pairwise analysis of the group differences, a LSD test with a 5% confidence level was used.

Protein electrophoresis and immunoblotting

Protein samples were prepared by boiling 20 mg of starch for 1 min in 120 µl sample buffer (20 mM Tris-HCl pH 8.0, 2 m*M* EDTA, 20% glycerol, 2% SDS, 0.002% bromophenol blue, 10% β-mercaptoethanol). After boiling, the samples were kept on ice, and 15 µl of each sample was analysed on 10% polyacrylamide gels (Laemmli 1970). Immunoblotting was carried out as described by Hovenkamp-Hermelink et al. (1987) using antiserum raised against potato GBSS (Vos-Scheperkeuter et al. 1986). Alkaline phosphatase was used as a second antibody, and the antigens were detected by incubating the filters in the dark in 100 ml AF-buffer (100 m*M* Tris-HCl pH 9.5, 100 m*M* NaCl and 5 m*M* $MgCl_2$) with 200 µl NBT (75 mg/ml 4-nitro blue tetrazolium chloride in dimethylformamide) and 200 µl BCIP (50 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in H_2O). the reaction was stopped by incubating in AF-buffer.

Fractionation of amylose and amylopectin

For fractionation of amylose and amylopectin with the size exclusion chromatography procedure, 200–400 mg of pure native starch was solubilized in 1.5 ml of 0.1 *N* NaOH at 100 °C for 15 min. The sample was diluted to 0.01 *N* NaOH and applied to a Sepharose CL2B column (2.6 by 200 cm, Pharmacia). Fractions of 6–8 ml were collected after the addition of 0.01 *N* NaOH containing 0.001% sodium azide to the column at a flow rate of 25 $ml \cdot h^{-1}$. The optical density of 200 µl of each fraction was measured after each had been complexed with an iodine solution (1 Lugol: 4 water) by performing a wavelength scan from 450 to 700 nm. The wavelength showing the maximum optical density was taken as a point for the graphs. After the run was completed, the column was washed with 700–800 ml of 0.01 *N* NaOH before the next sample was applied.

Small deformation tests

Dynamic rheological properties of the 5% starch suspensions at small deformations were determined by applying a small oscillating shear deformation using a Bohlin VOR Rheometer as described by Keetels and van Vliet (1994). The Bohlin VOR Rheometer was equipped with concentric cylinders made of stainless steel. The radius of the inner cylinder was 14.00 mm and that of the outer cylinder 15.25 mm. The torque bar used for amylose-free starch was 0.17 $mN \cdot m^{-1}$ and for amylose-containing starch 0.38 $mN \cdot m^{-1}$.

The 5% starch suspensions were heated to approximately 65 °C under gentle stirring until the viscosity slightly increased. After transfer to the measuring body of the rheometer, which had a temperature of 50 °C, the starch suspensions were heated to 90 °C, kept at this temperature for 15 min and cooled to 20 °C at which temperature it was kept for 15 min. Heating and cooling were performed at a rate of 1 $°C \cdot min^{-1}$, with measurements being done every 60 s. Oscillations were performed at a frequency of 0.1 Hz and a strain amplitude of 0.01.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed with a Perkin Elmer DSC 2. Approximately 14 mg of starch and 56 mg of demineralized water were weighed into stainless steel cups. The suspensions were heated from 30 °C to 110 °C at a scanning rate of 5 $K \cdot min^{-1}$, and immediately after heating they were cooled to 30 °C at a rate of 40 $K \cdot min^{-1}$. An empty stainless steel cup was used as a reference.

Results

Development of a GBSS gene-dosage population

On the basis of iodine staining of microspores, genotypes corresponding to nulliplex (no wildtype GBSS allele), simplex, duplex and triplex/quadruplex for the

Table 1 The expected and obtained offspring when duplex plants (*AAaa* × *AAaa*) are crossed. The genotypes can generally be distinguished after iodine staining by their segregation of blue and red microspores the exceptions being the triplex (*AAAa*) and quadruplex (*AAAA*) plants, which have only blue staining microspores. Genotypes with enough tubers on which to perform a field trial were selected

Plant genotype	Chance	Microspore segregation blue:red	Number of genotypes found[a]	Number of genotypes selected
aaaa	1/36	0:1	3	2
Aaaa	8/36	1:1	20	10
AAaa	18/36	5:1	33	11
AAAa	8/36	1:0	19	6
AAAA	1/36	1:0		

[a] $\chi^2_{(1:8:18:9)} = 1.62 < 9.49$, which indicates that the offspring does not deviate from the expected 1:8:18:9 segregation of the gene-dosage genotypes for the wildtype GBSS allele

wildtype GBSS allele were selected. This selection corresponded well to the expected segregation presented in Table 1.

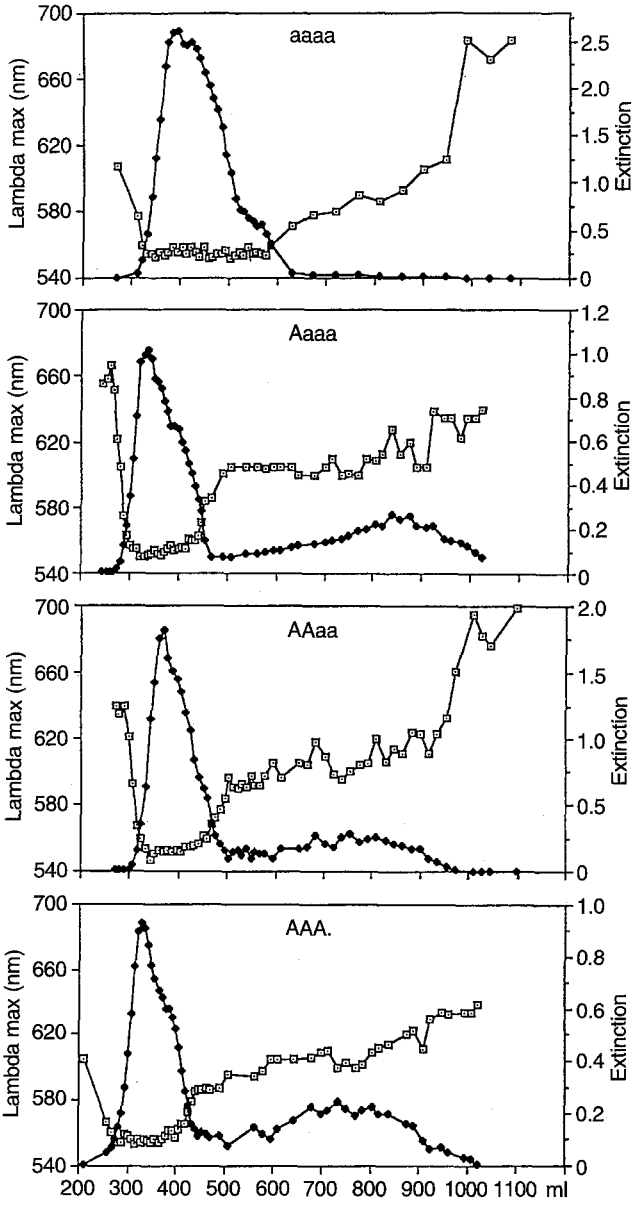
Starch granules of the duplex and triplex/quadruplex genotypes were completely blue. In some of the simplex genotypes, however, a small outer layer was red in a small percentage of the starch granules. A number of tuberizing plants belonging to each gene-dosage group was selected for further research in a field trial (Table 1).

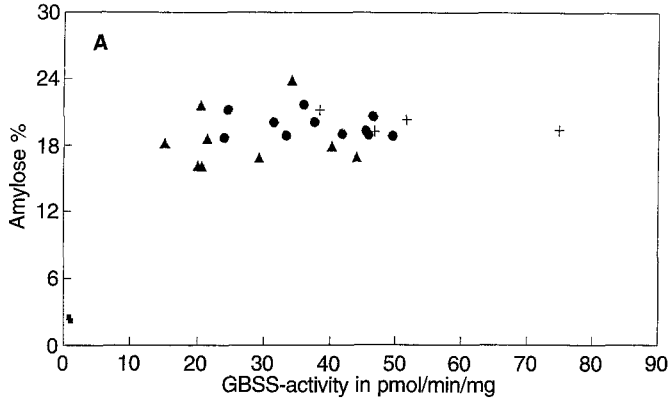
Amylose content and GBSS activity

A distinction between amylose-free and amylose-containing starch was made by size exclusion chromatography of starch isolated from field-grown tubers of all different genotypes. This test is the most frequently used separation technique for the characterization of polymer mixtures. In this technique, the polymer's physical size determines its separation profile. Larger molecules like amylopectin, which have a ten- to hundred-times higher weight than amylose molecules, elute first as they spend less time in the column's pores. It is clear from Fig. 1 that the starch of all groups contained amylopectin (first peak). The starch of all groups of plants possessing at least one wildtype GBSS allele contained, besides amylopectin, amylose (second peak), whereas the group with no wildtype GBSS allele was amylose-free. The λ_{max} for the amylopectin fraction was lower than that for the amylose fraction, and a slight increase in λ_{max} was observed in the amylopectin fraction of simplex, duplex and triplex/quadruplex genotypes compared to that of the nulliplex genotypes. This might indicate the presence of amylopectin molecules with longer, more amylose-like chains in the amylopectin fraction.

GBSS activity was determined for all of the different clones (Fig. 2A) and was shown to be significantly different for the gene-dosage groups after a statistical evaluation with an analysis of variance. When the differences are examined in more detail by means of the LSD test it is evident that all pairs of gene-dosage groups are significantly different (Table 2).

Fig. 1 Size exclusion chromatography results, indicating the λ_{max} (□–□) and extinction (■–■) of fractions eluting from the CL2B sepharose column





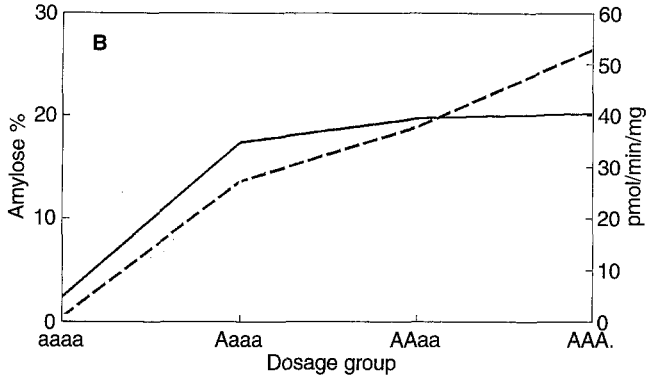


Fig. 2A The correlation between GBSS activity and amylose percentage for the different gene-dosage groups, which consisted of 2 nulliplex (*aaaa* ■), 9 simplex (*Aaaa*▲), 6 duplex (*AAaa* ●) and 4 triplex/quadruplex (*AAA.* +) plants. **B** The group means for GBSS activity (– –) and amylose content (——) against the number of wildtype GBSS alleles

Table 2 Differences in GBSS activity and amylose content between gene-dosage groups tested with the least significant difference test (LSD) at a 5% confidence level

	GBSS activity				Amylose content			
	aaaa	*Aaaa*	*AAaa*	*AAA.*	*aaaa*	*Aaaa*	*AAaa*	*AAA.*
aaaa		*	*	*		*	*	*
Aaaa			*	*			*	*
AAaa				*				NS
AAA.								

NS indicates that no statistical differences could be found
* = statistical differences between groups

The amylose content was estimated for all of the different clones (Fig. 2A) and replications investigated in the field trial. The method used to determine amylose content always shows a low background of amylose even in the *amf* genotypes, which showed an amylose content of less than 0.1% when amperometric titration was used (Kuipers, et al. 1994). The statistical evaluation with an analysis of variance showed that there was a significant difference in amylose content between the gene-dosage groups. Using the LSD test we tested differences between groups. There was a significant difference in amylose content between the nulliplex (*amf*) genotypes and the simplex, duplex and triplex/quadruplex genotypes. Also, the group of simplex genotypes had an amylose content significantly lower than that of the duplex and triplex/quadruplex genotypes. The duplex and triplex/quadruplex groups had an equivalent amylose content. The correlation between GBSS activity and amylose content is visualized in Fig. 2A. It is clear that a maximum amylose content was attained at a certain level of GBSS activity above which increases in activity did not lead to a higher amylose content. When the amylose content of the individual genotypes in the simplex group was studied in more detail, it became obvious that amylose content was not the same for all genotypes in this group. Three sub-groups with a significant difference in amylose content were found. It is furthermore clear that, when looking at the mean GBSS activities of the different gene-dosage groups, there is a linear relation between number of wildtype alles and GBSS activity. This was not observed for the amylose content (Fig. 2B). Four different sequences are known for the GBSS promoter (Rhode et al. 1988; van der Leij et al. 1991; Hofvander et al., 1992). All four sequences can be differentiated by the presence or absence of specific regions in the promoters. Assuming that different promoters could be present at different strengths their presence might account for the observed differences in amylose content between the simplex plants. Using the polymerase chain reaction (PCR) technique with specific primers, which are able to discriminate between the four different GBSS promoter sequences, we investigated the nature of the GBSS promoter in the simplex genotypes. However, no sequence differences were found for the GBSS promoters present in the simplex plants (data not shown).

GBSS protein content

The amount of GBSS protein present in the starch granule fraction of different genotypes was analysed. Figure 3 clearly shows that the amylose-free plants had no GBSS in their starch granules, however no difference could be observed in the GBSS protein level of the other groups, indicating that no dosage effect existed at the protein level.

No differences in starch granule size and starch and sucrose content of the tubers were found (data not shown).

Physico-chemical properties of starch-water systems

When starch granules are suspended in water and subsequently heated a series of processes known as gelatini-

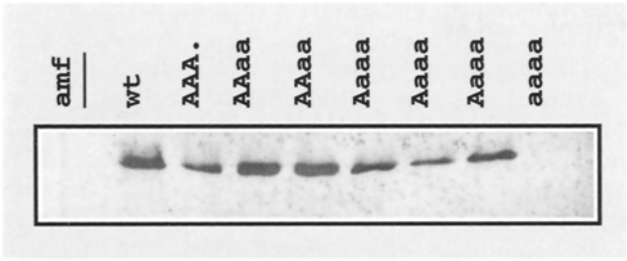


Fig. 3 Western blot of the starch granule fraction of plants with zero to four wildtype GBSS alleles using the antibody against GBSS as a probe

Table 3 Differential scanning calorimetry (DSC) data for 20 wt% potato starch suspensions originating from plants with different dosages of the wildtype GBSS allele. The starch samples used plants as in Fig. 4. Starch suspensions were heated at a rate of 5 K/min (T_o onset temperature, T_p peak temperature, T_m melting or termination temperature, ΔH melting enthalphy)

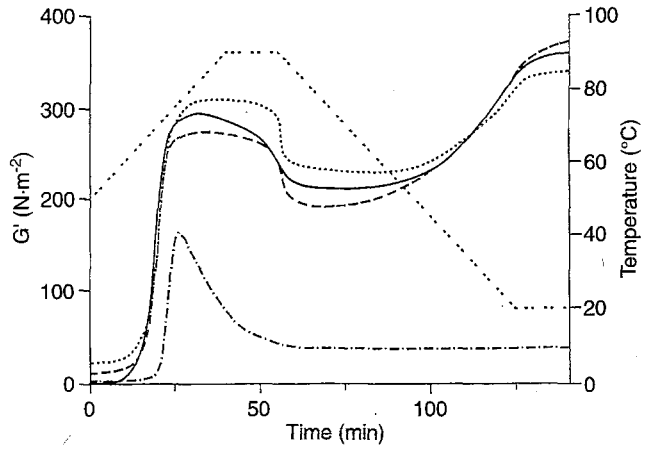
Starch	T_o(°C)	T_p(°C)	T_m(°C)	ΔH(J.g^{-1})
aaaa	68	71	69	18.2
Aaaa	65	68	66	18.1
AAaa	61	64	62	17.2
AAA.	63	66	64	17.9

zation occurs. It includes a drastic, irreversible swelling and a melting of the crystallites and is accompanied by a (partial) leaching of amylose from the granules. These processes occur over a temperature range which is characteristic for the type of starch and result in changes in rheological properties.

Dynamic rheological properties were determined by applying a small oscillating shear deformation using a Bohlin VOR Rheometer. The storage modulus (G') is then estimated, which is a measure of the energy stored and released per cycle of deformation and per unit of volume.

Figure 4 shows the changes in the storage moduli (G') of 5% starch suspensions that were measured during a heating and cooling cycle. It is clear that the changes in G' were highly different for amylose-containing and amylose-free starches. Hardly any differences were found between plants containing different numbers of wildtype GBSS alleles. The storage moduli of amylose-containing starches started to drastically increase in size at a temperature of about 63 °C, whereas the storage modulus of amylose-free starch showed an equivalent process at 69 °C. The increase in moduli size coincides with the first stages of crystallite melting (see T_o), as determined with DSC (Table 3). Moreover, the peak moduli were higher for amylose-containing starches and their moduli decreased less before the temperature of 90 °C was reached and during the time the starch system was at this temperature. During cooling the moduli of the amylose-containing starch systems increased in size whereas the moduli of the amylose-free starch remained constant.

Fig. 4 Changes in the storage moduli (G') of 5% potato starch suspensions during heating and cooling with — — — indicating the temperature against time. –·–·– *aaaa*: 2.50% amylose, ------- *Aaaa* 16.94% amylose, —— *AAaa*: 18.96% amylose, — — — *AAA.*: 20.32% amylose



Discussion

In this article we describe the development of a GBSS gene-dosage population that was obtained by crossing two different duplex potato genotypes. Staining of the microspores with an iodine solution enabled us to distinguish different classes in the progeny such as nulliplex, simplex, duplex and triplex/quadruplex genotypes (Table 1). Triplex and quadruplex genotypes had only blue microspores. It was already known from previous research that GBSS is responsible for the production of amylose (Shannon and Garwood 1984; van der Leij et al. 1991), one of the two components of starch. We could confirm the absence of amylose in the *amf* mutant (nulliplex) and its presence in all other gene-dosage groups using size exclusion chromatography (Fig. 1). The analysis of variance test demonstrated a gene-dosage effect of GBSS alleles on both GBSS activity and amylose content. A LSD test estimated that only one or two GBSS alleles had a dosage effect on the amylose content and that the presence of three or more GBSS alleles would not result in a further increase in amylose content (Table 2). With respect to the amount of amylose the gene-dosage effect was small and far from linear, as it was for GBSS activity (Fig. 2B). A gene-dosage effect of the GBSS allele for amylose content has been found in the endosperm of rice (Okuno 1978; Sano, 1984) and maize (Boyer et al. 1976). In crosses with rice plants containing low-amylose and high-amylose genes different dosage effects were found. For some alleles, only a single dose of the gene was necessary for a wildtype level of amylose and in other cases three doses

were necessary (Sano 1984). However, these results were all based upon qualitative data. The dosage effect on amylose content in the triploid endosperm of maize was not linear (Boyer et al. 1976), and no difference could be found when two or three wildtype alleles were present.

Although the *amf* mutant does not contain GBSS protein in the starch granules (Hovenkamp-Hermelink et al. 1987), no dosage effect could be found for the other groups because simplex plants were capable of producing wildtype levels of GBSS protein (Fig. 3). The differences in GBSS activity and amylose content seemed not to be caused by distinct differences in GBSS protein level. In rice endosperm a linear dosage effect of the GBSS allele was found with respect to GBSS protein level (Sano 1984). Also in maize, Tsai (1974) found that the amount of GBSS protein increases linearly with GBSS gene dosage. A dosage effect on GBSS activity in maize and rice endosperm was not investigated.

In all the potato plants except for a few simplex plants the starch granules were totally blue; in the latter a small percentage of starch granules had a small red outer layer, indicating that the granules were not completely filled with amylose. This phenomenon has also been observed by Kuipers et al. (1994) after GBSS gene expression was inhibited using antisense constructs and by Flipse et al. (1994) after incomplete complementation of the *amf* mutant with the potato GBSS gene. Kuipers et al. (1994) suggested that reduced GBSS gene expression results in amylose formation in a restricted zone of the granules in which wildtype levels of amylose are present. However, in general we can conclude from the results of this investigation that the whole granules are filled with amylose when only 87% of the wildtype level of amylose is present. The correlation between the GBSS activity and amylose content makes it clear that a maximum amylose content was caused by a certain level of GBSS activity after which an increased activity did not lead to a higher amylose content (Fig. 2). This would confirm the hypothesis that the starch granule consists of a crystalline organization of amylopectin molecules (Oostergetel and van Bruggen 1989) and that the empty places, with a restricted volume between these radially arranged amylopectin molecules, are filled with amylose to a maximal volume (Jane et al. 1992).

Sano et al. (1984) not only found that in rice the amylose content is affected by the number of wildtype GBSS alleles, but they also detected at least two different wildtype GBSS alleles that determine the level of the gene product as well as amylose content. Differences in amylose content were found within the simplex group constructed, suggesting that either different wildtype alleles with distinct expression levels were present, or that a difference in genetic background plays a determining role. No sequence differences were found with the PCR amplification method, indicating that the differences in expression levels observed here were due to the different genetic backgrounds of the simplex genotypes.

The effect of the presence of amylose in the starch granule on the mechanical properties at small deformations was studied. The results of the Bohlin test in Fig. 4 show that the gelation properties of amylose-free starch differ from those of amylose-containing starches.

The relation between structure and mechanical properties of starch systems during heating and cooling is discussed below. The increase in modulus is ascribed to the swelling of the starch granules that results from the melting of the crystalline regions in the granules (Keetels 1995). Presumably, the presence of amylose indirectly lowers the melting point of the crystalline regions, which would explain the lower temperature at which the moduli of amylose-containing starches started to increase; this was confirmed by the DSC thermogram of the starch suspensions (Table 3).

In addition to the melting of the crystalline regions and swelling of the granules, two other processes occur during the heating of a starch-water system. First amylose separates from amylopectin and (partly) leaches out of the granules and second, the amylopectin matrix within the swollen granules partly breaks down (Keetels and van Vliet 1994). The continued melting of the remaining crystallites and the breakdown of the amylopectin matrix would especially be involved in the decrease in G' at high temperatures.

Keetels (1995) observed that the 30% starch gels she investigated consisted of tightly-packed, only slightly swollen granules with a very thin amylose layer inbetween. In the 5% starch systems studied here, the granules had to swell to a greater extent before they filled the whole system. In the latter case, more amylose probably leached out of the granules. The volume fraction of the swollen granules was therefore somewhat lower than that observed in a concentrated starch system, but still high enough that the system may not be considered as an amylose gel with dispersed, non-interacting, granules. If it is assumed that the swollen granules filled almost or completely the whole available volume, the differences in the storage moduli at 90 °C between amylose-free and amylose-containing starches would be partly a result of the lower stiffness of the swollen granules in the amylose-free starch. The increase in G' of the amylose-containing starches during cooling would be explained by the fact that the leached-out amylose molecules rearrange, forming a thin amylose gel layer between the swollen granules.

This research shows that the wildtype GBSS allele has a gene-dosage effect on GBSS activity and amylose content in potato tubers, although the latter seems to reach a maximum. This optimum was also found for certain GBSS alleles in rice and for those in maize. Although a gene-dosage effect was found for protein level in rice and maize endosperm it was not found for potato. The presence of amylose has a large influence on the physico-chemical properties of starch suspensions.

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