The *amylose-free* potato mutant as a model plant to study gene expression and gene silencing

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The *amylose-free* potato mutant as a model plant to study gene expression and gene silencing

Proefschrift

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Stellingen

1 Partiële complementatie van een diploïde *amylose-vrije* aardappelmutant kan veroorzaakt worden door co-suppressie.

(dit proefschrift)

2 "High-amylose" aardappelgenotypen kunnen met behoud van de huidige zetmeelkorrel structuur niet worden verkregen.

(dit proefschrift)
(Kram AM, (1995) PhD thesis)
(Jane J *et al.*, Cereal Chem (1992) 69: 405-409)

3 De verschillende aspecten van "transgene-inactivation", beschreven in een grote variatie aan studies, maken het onderling vergelijken en zoeken naar een gemeenschappelijk oorzaak van dit fenomeen zeer moeilijk.

4 De *amylose-vrije* aardappelmutant biedt als uitgangsmateriaal voor fundamenteel onderzoek veel voordelen.

(dit proefschrift)
Pereira A, *et al.* (1991) Maydica 36: 323-327
Jacobsen E, *et al.* (1992) Theor Appl Genet 85: 159-164
Jacobsen E, *et al.* (1993) Euphytica 69: 191-201
Jacobsen E, *et al.* (1995) Heredity 74: 250-257

5 Genetische modificatie vergroot de genetische variatie ten behoeve van de plantenveredeling.

6 Financiering van een vaste oever verbinding tussen Zuid-Beveland en Zeeuwsch-Vlaanderen is voor de zeeuwen een tunnel te ver.

7 Het feit dat in vele documentaires en films over geestelijk gehandicapten hun prestatieniveau en mogelijkheden worden gerelateerd aan reguliere maatstaven toont aan dat het met hun acceptatie in de maatschappij nog steeds slecht gesteld is.

8 In tegenstelling tot fietsen, wandelen en hardlopen, waarbij het aantal kilometers wordt vermeld verdient het aanbeveling de trainingsarbeid voor een rugzaktrektocht weer te geven in kilouren (= aantal kilo's bepakking x aantal wandeluren) met als toevoeging "bij stevig wandeltempo".

9 Het is de kunst van het fotograferen, kunst te scheppen.

10 Een toename van het door bedrijven gefinancierd biotechnologisch onderzoek bevordert de vrije toepassing van de verworvenheden hiervan door de kleine boer in ontwikkelingslanden onvoldoende.

(maandblad Mininsterie van Ontwikkelingssamenwerking, april 1995)

11 De door John Hatt ontworpen proef om de invloed van vitamine B1 inname op de resistentie tegen aanvallen van muskieten te bestuderen getuigt van een zwak statistisch inzicht.

(O'Hanlon R, (1986) Into the heart of Borneo)

Stellingen behorende bij het proefschrift "The *amylose-free* potato mutant as a model plant to study gene expression and gene silencing" door Elise Flipse, in het openbaar te verdedigen op dinsdag 14 november 1995, te Wageningen.

Abstract

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Key words: *amylose-free* mutant, Branching Enzyme, gene-expression, gene silencing, starch, transgenic inheritance.

In this thesis, gene-expression and gene silencing were examined for Granule Bound Starch Synthase (GBSS) which catalyses the formation of amylose and Branching Enzyme (BE) which catalyses the formation of amylopectin. The GBSS deficient, with iodine, red staining *amylose-free* (*amf*) potato mutant was used in order to facilitate the experiments; GBSS-gene expression and BE-gene silencing resulted in blue staining starch.

A dosage effect of the wild-type GBSS-allele on GBSS-activity and amylose content was found in a tetraploid dosage population. The presence of amylose had a distinct influence on the physico-chemical properties of the starch.

Insertion of the wild-type GBSS-gene in the *amf*-mutant resulted in fully and partially complemented plants. The minimum number of independently segregating active GBSS-inserts was estimated by genetic analysis after microspore staining. The complemented phenotype was normally transmitted to the F1 for the fully complemented, but not for the partially complemented plants. For one plant the partial complementation was correlated with the presence of a block of five inserts. This block was also capable of inhibiting the endogenous GBSS-gene in a wild-type. This indicates that partial complementation can be caused by co-suppression, besides low transgene expression. Co-suppression was also obtained when the full size GBSS sequence or GBSS cDNA were introduced in a wild-type potato.

Introduction of the distal 1.5 kb cDNA coding for BE in both sense and antisense orientation resulted in several transgenic plants with a small blue core in these starch granules. This could indicate the presence of loosely branched amylopectin in the core of the starch granules. The expression of the endogenous BE-gene was largely or fully inhibited as judged by the absence of BE mRNA and protein. This did not result in a measurable effect on the branching degree, but resulted in altered physico-chemical properties of the starch compared to *amf*-starch.

It is concluded that this study shows that the *amf*-mutant is successfully used as a model plant to examine different aspects of gene expression.

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Chapter 1

General introduction

Use of starch in food and non-food applications

Starch is the most important carbohydrate storage product in higher plants. Two types of starch can be distinguished according to their role in plant metabolism. Transitory starch is synthesized in chloroplasts as a short time storage of photosynthetic products. For long time storage, so called reserve starch is accumulated in amyloplasts of tubers, roots and seeds (Shannon and Garwood, 1984). As such, starch is, besides cellulose, one of the main carbohydrates present in food. Isolated starch can, depending on the source (potato, maize, wheat, cassava) and modification technique (Kraak, 1993), be used for many specific purposes. Approximately two thirds of the starch production in Western Europe is used in food and beverage industries (Koch and Röper, 1988) as thickener, or after enzymic hydrolysis as sweetener or confectionary. The other one third is used in a wide range of non-food products i.e. paper, packaging, textile, chemical industry, cosmetics, pharmaceuticals. More recently starch is also used in biodegradable plastics or as a fat replacer.

Isolated starch forms the basis of a large variety of industrial applications. A lot of effort has been made to unravel the structure and formation of starch, in order to understand the specific properties of starch and to apply more specific modifications.

Starch composition and starch structure

Starch is organized in granules that vary in size and shape, depending on the source and the developmental stage of the plant (French, 1984). The granules consist predominantly of carbohydrates but also contain proteins, lipids and phosphorus (Swinkels, 1985). The molecular structure of starch is simple, being a polymer built solely of glucose units, linked by no more than two different types of bonds. In normal starch granules two polymers are present: amylose and amylopectin. In potato 18 to 23 % of the tuber starch

consists of amylose (Shannon and Garwood, 1984). Amylose is an essentially linear polymer of 100-10,000 glucose units linked by α(1.4)-linkages. The few branch points present are the result of α(1.6)-linkages. Amylopectin is built of short linear α(1.4)-linked chains, with an average length of 25 glucose residues and connected by α(1.6)-linkages. In amylopectin three types of linear chains are distinguished (Fig. 1; Hizukuri, 1986; Manners, 1989).

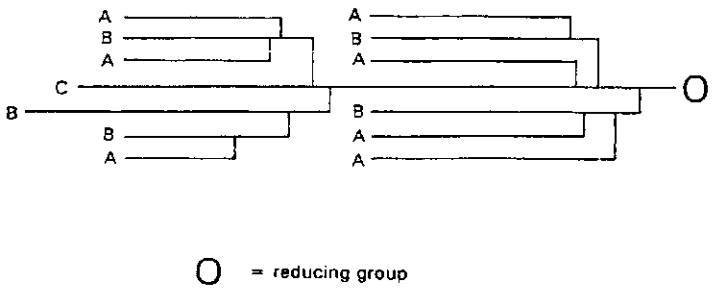

Fig. 1. The molecular structure of amylopectin. The branch points are located in an amorphous zone. The linear parts of the chains form crystalline domains. Both the amorphous and crystalline phase are approximately 5 nm. (according to Hizukuri, 1986).

A and B-chains are both linked to the molecule by their reducing group. The B-chains carry one or more A-chains. The C-chains carry the sole reducing group of the molecule and are further comparable to B-chains. The chain length composition and distribution of different chain types are characteristic for amylopectin of a given botanical source. Due to the difference in structure, amylose and amylopectin have distinctive characteristics. So can the long chain of amylose form an inclusion complex with iodine resulting in a blue colour, whereas the short chains of amylopectin stain red. This provides us with a simple and good screening method for the presence or absence of amylose.

Although the molecular structure is simple, more complex is the ultrastructure and the starch granule organization. In starch granules, concentric growth rings can be observed under the light or electron microscope. They represent semi-crystalline and amorphous layers (French, 1984). The semi-crystalline layers originate from the organization of amylopectin

molecules (Oostergetel and van Bruggen, 1989). In a cluster model, proposed for amylopectin of potato and maize, the crystalline lamellae represent the linear chains that are organized in double helices (Oostergetel and van Bruggen, 1993). The starch molecules are radially oriented in the granule with the growing non-reducing ends pointing towards the surface of the granule. Amylose and the branching regions of amylopectin form the amorphous regions of the starchgranule. It can be said that amylopectin forms the framework of the starch granule, whereas amylose fils the 'empty' spaces. Cross-linking experiments with maize and potato starches have demonstrated that the amylose molecules are interspersed among the radially arranged amylopectin (Jane *et al.*, 1992). Chemical gelatinization of potato starch granules and subsequent separation and analysis of the gelatinized periphery and the remaining core of the granules showed that amylose was more concentrated at the periphery than at the core of the granule (Jane and Shen, 1993).

Not much is known with respect to the initiation of starch granule formation. The first step is thought to be the accumulation of amorphous starch followed by crystallization giving the centre or hilum of the starch granule (French, 1984). The hilum is usually less organized than the rest of the granule and contains an amylose-lipid complex. A starch granule grows by apposition (Badenhuizen and Dutton, 1956). Chains, already incorporated in the structure of the starch granule are elongated (French, 1984; Smith and Martin, 1993).

Physico-chemical properties of starch

When suspended in water at room temperature, starch granules can reversibly take up a small amount of water, resulting in a minor swelling while the original shape and birefringence stays intact. When the suspension is heated, starch undergoes a series of processes known as gelatinization. The drastic swelling, which occurs in all directions, is not reversible and takes place nearly simultaneously with a melting of the crystalline structure and is accompanied with a partly leaching out of amylose from the granules (Keetels, 1995). The leaching out of amylose is due to amylose and amylopectin being incompatible in a concentrated solution and the higher mobility

of amylose compared to amylopectin. The amount of amylose that leaches out depends on the starch concentration in the suspension. When the starch concentration is more than 4 %, the swollen granules fill almost the whole volume, which reduces leaching out of amylose. Within the granules, separation of amylose and amylopectin takes place. This results in amylose-rich and amylopectin-rich domains. After all crystallites are melted, the swelling of the granules and the leaching of amylose continues. These processes occur over a temperature range which is characteristic for the type of starch. The structural changes that occur during gelatinization, like swelling of granules and melting of crystallites, result in changes in the rheological properties. The dynamic rheological properties can be determined by applying a small oscillation shear deformation by using for example a Bohlin Vor Rheometer. The storage modulus G' (Fig. 2) is a measure for the elastic response.

The increase in G' indicating an increased stiffness of the starch-water system, is the result of swelling and melting. A further melting of the crystalline structure results in a decrease of G' (Fig. 2). The energy (temperature) necessary for melting, can be determined using Differential Scanning Calorimetry.

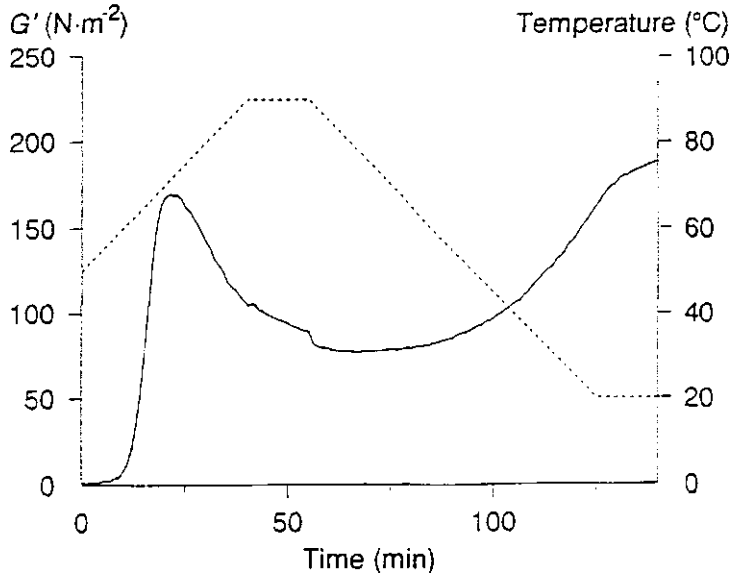

Fig. 2. Changes in the storage modulus (G') of a wild-type starch suspension during heating and cooling. The dashed line indicates the temperature against time.

During cooling and storage of concentrated starch suspensions the starch molecules rearrange which increases the stiffness of the suspension. Short term crystallization is caused by crystallization of amylose. Reordering of amylopectin occurs at a much lower rate than of amylose.

Starch synthesis

Several enzymes play a role in starch biosynthesis. ADPglucose pyrophosphorylase (AGPase), Granule Bound Starch Synthase (GBSS), Soluble Starch Synthases (SSS) and Branching Enzyme (BE) are the last in the pathway and therefore, directly involved in the formation of amylose and amylopectin (Fig. 3).

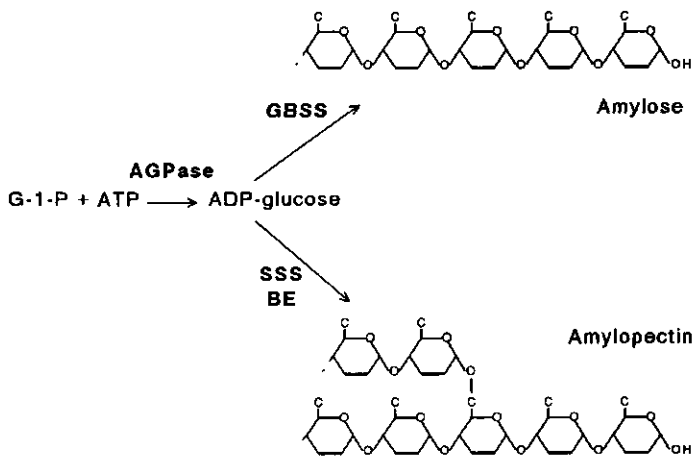

Fig. 3. The structure of amylose and amylopectin and the enzymes involved in their biosynthetic pathway.
AGPase, ADP-glucose phyrophosphorylase; BE, Branching Enzyme; G-1-P, glucose-1-phophate; GBSS, Granule Bound Starch Synthase; SSS, Soluble Starch Synthase (Kuipers, 1994).

ADPglucose pyrophosphorylase

Inside the amyloplast, ADPglucose pyrophosphorylase (AGPase, E.C. 2.7.7.27) catalysis the formation of ADPglucose from glucose-1-phosphate. ADPglucose is preferred as substrate for starch formation *in vivo*. The AGPase catalysed formation of ADPglucose is the regulatory step in starch synthesis (Preiss, 1991). The enzyme activity in most plants is increased by 3-P-glyceraldehyde (present during a high rate of photosynthesis) and decreased by inorganic phosphate (present during a low rate of photosynthesis). The central role of AGPase in the biosynthesis of potato starch has been demonstrated by Stark *et al.* (1992) and Müller-Röber *et al.* (1992). Antisense inhibition of expression of the gene encoding subunit B of AGPase resulted in a decreased starch content and an increase in soluble sugars (Müller-Röber *et al.*, 1992). Plant AGPases isolated so far consist of two small and two large subunits that are encoded by different genes. Both subunits are needed for full activity (Müller-Röber *et al.*, 1992).

Granule Bound Starch Synthase

Starch synthase (E.C. 2.4.1.21) catalysis the formation of linear chains by the addition of ADPglucose to the non-reducing end of the glucan chain. Two types of starch synthase can be distinguished: Granule Bound Starch Synthase (GBSS) and Soluble Starch Synthase (SSS). Although both enzymes catalyse the same reaction, they play distinct roles in starch synthesis. GBSS is, unlike SSS, tightly bound to the starch granule. Because it was shown that GBSS and SSS were immunologically different in potato it could be concluded that GBSS is not just a SSS which is tightly bound to the granule (Ponstein, 1990).

GBSS is responsible for the production of amylose. This is demonstrated by the absence of amylose in *waxy* mutants, which lack the GBSS-protein and GBSS-activity. A *waxy* mutant is obtained in potato, were it was called *amylose-free* (*amf*) (Hovenkamp-Hermelink *et al.*, 1987), in maize (Tsai, 1974; Echt and Schwartz, 1981), rice (Sano, 1984), amaranth (Konishi *et al.*, 1985) and sorghum (Hseih, 1988).

A combination of several studies led to the evidence that the *waxy* locus of maize is the structural gene encoding GBSS (Echt and Schwartz, 1981; Nelson *et al.*, 1978). In pea the identity between the GBSS-activity and

waxy (or GBSS) protein has been proven by immunoblotting (Sivak *et al.*, 1993).

Several findings support the idea that GBSS is responsible for the production of amylose in potato. The GBSS-activity is reduced in the *amf*-mutant (Hovenkamp-Hermelink *et al.*, 1987) and this is correlated with a loss of the 60 kDa GBSS protein in the starch granules and the formation of amylose-free starch in tubers, stomatal guard cells, columella cells and microspores (Hovenkamp-Hermelink *et al.*, 1987; Jacobsen *et al.*, 1989). The *amf*-mutation has been identified by sequence analysis as caused by a point deletion in the region encoding the GBSS transit peptide (van der Leij *et al.*, 1991b). This transit peptide plays a role in the transport of GBSS into the plastids. The *amf*-mutation could be complemented by the introduction of the wild-type GBSS-gene via transformation with *Agrobacterium rhizogenes*, resulting in a complete restoration of GBSS-activity and formation of amylose in all starch synthesizing tissues (van der Leij *et al.*, 1991a). Antisense RNA mediated inhibition of GBSS-gene expression resulted in a reduced expression of GBSS-activity in starch, reduced amounts of 60 kDa GBSS protein and a reduced amount of amylose in starch synthesizing tissues (Visser *et al.*, 1991).

The presence of a second Granule Bound Starch Synthase (GBSS II; the *waxy* protein is called GBSS I) was reported for pea, potato (Dry *et al.*, 1992) and maize (McDonald and Preiss, 1985). GBSS II is expressed early in the granule development in pea and potato. It is not likely to have much influence on amylose synthesis (Dry *et al.*, 1992; Edwards *et al.*, 1995). The influence of the *waxy* mutation on amylose synthesis indicates that GBSS I is crucial for amylose synthesis. The role of GBSS II in amylose biosynthesis is still unknown.

Soluble Starch Synthase

In contrast with the Granule Bound Starch Synthase, Soluble Starch Synthase is mainly involved in the synthesis of amylopectin. In different plant species SSS occurs in two isoforms with a different specificity for primer molecules. Both isoforms were also found in potato (Ponstein, 1990). Their native molecular weight is 220 kD. They are built of 78 kD and 85 kD subunits (Ponstein, 1990). Baba *et al.* (1990) partly purified SSS II from

potato tubers and estimated a native weight of 70 kD. Only recently (Abel *et al.*, 1995) two different SSS were isolated named SSS I and SSS III which were quite distinct both immunologically and based upon sequence data. From the same study the suggestion is coming that GBSS II and SSS II might be identical enzymes. At present the relation between the different isolated potato SSS's is unclear. Knowledge of the SSS is trailing behind that of GBSS.

Branching Enzyme

Branching Enzyme (BE; E.C. 2.4.1.18) catalyses the formation of branch points in the linear $\alpha(1.4)$ chains formed by SSS. The branches are created by hydrolysing an $\alpha(1.4)$-bond and ligating the severed chain to another chain by an $\alpha(1.6)$-bond (Smith and Martin, 1993). Multiple isoforms have been found in several plant species. In maize BE I, BE IIa and BE IIb are described (Boyer and Preiss, 1978; 1981). Differential branching activity has been reported for maize BE I and BE II (Guan and Preiss, 1993). BE I has the highest activity in branching linear starch chains leading to a slightly branched structure. Further branching is catalysed by BE II. In developing pea embryo's two isoforms were found (Matters and Boyer, 1981; Smith, 1988): BE I and BE II. With respect to immunological and sequence similarities two groups of BE's can be identified. BE I of maize resembles mostly BE II of pea (called type I), whereas BE II of maize is more related to BE I of pea (Called type II). In potato tubers so far only one BE isoform could be identified (Drummond *et al.*, 1972, Borovsky *et al.*, 1975, Vos-Scheperkeuter *et al.*, 1989, Kossmann *et al.*, 1991). This potato BE was cloned (Kossmann *et al.*, 1991). At the amino acid level it has 67 % homology with maize BE I (Poulsen and Kreiberg, 1993) and is therefore called a type I BE.

A few mutants with increased amylose content due to a mutation in a gene encoding BE have been found in pea and in maize. In the *wrinkled* pea mutant BE I is absent (Matters and Boyer, 1982; Edwards *et al.*, 1988). In maize the *amylose extender* and *dull* mutants are known. They are both mutated in a gene encoding one of the type II BE isoforms. In *amylose extender* BE IIb and in *dull* BE IIa are absent (Hedman and Boyer, 1982). Both mutants have a decreased starch and dry weight production and an increased sugar content compared to the wild-type. The starch has a blue

colour when stained with iodine. In maize a double mutant was created by crossing the *waxy (wx)* and the *amylose-extender (ae)* mutant. It resulted in the double mutant *aewx*. The starch of this mutant consists of *amylose-free* loosely branched amylopectin, which stains blue with iodine. No potato mutant with reduced BE-activity is known.

Inhibition of gene expression

Inhibition of gene expression can be useful to determine its function as a single gene or its influence in a metabolic pathway. Several strategies for gene inhibition are at hand. Endogenous gene expression can be decreased by introducing antisense sequences of the target gene. The application of the antisense technique in potato was demonstrated by Visser *et al.* (1991), Müller-Röber *et al.* (1992), Zrenner *et al.* (1993) and Kuipers (1994). An inhibition in expression level up to 100 % is possible, although it generally varies among and within transgenic clones (Kuipers, 1994). Antisense genes can be based on the full length cDNA or on the 5' or 3' part of the cDNA (Smith *et al.*, 1988; Kuipers *et al.*, 1995b). Results with antisense genomic, intron-containing, sequences are also described. Both the 35S CaMV promoter, the promoter of the target gene or tissue specific promoters can be successfully used (Kuipers *et al.*, 1995b).

Besides by using antisense genes, inhibition of gene expression can also be obtained by insertion of additional copies of the target gene in sense orientation. This phenomenon is called co-suppression and has been described in different plant species (petunia, tobacco and tomato) for genes encoding different enzymes (chalcone synthase, polygalacturonase) (van der Krol *et al.*, 1990, Napoli *et al.*, 1990, Smith *et al.*, 1990). The exact mechanism of antisense and sense inhibition is not fully understood (reviewed by Jorgensen, 1990; Matzke and Matzke, 1993; Matzke *et al.*, 1994; Mol *et al.*, 1994). In the case of double transformation of two different T-DNAs in tobacco, inhibition was correlated with increased methylation of the transgenes (Matzke *et al.*, 1989). Not all gene silencing caused by sense transgenes is correlated with an increased methylation. Inhibition of chalcone synthase activity by sense genes seems to be a post-transcriptional process (Mol *et al.*, 1994).

Transgene expression and inheritance.

The production of transgenic plants is becoming a routine procedure for a number of species. Transgenic crops will only have economical value when the new phenotype is stably expressed and transmitted to the next generation. The level of transgene expression is unpredictable and varies between transformants. This variability is usually thought to be caused by the number of transgenes inserted (Stockhaus *et al.*, 1987; Hobbs *et al.*, 1990) or the place of insertion (Jones *et al.*, 1985; Eckes *et al.*, 1985; Nagy *et al.*, 1985; Pröls and Meyer, 1992) and can be correlated with methylation of the transgene (reviewed by Finnegan and McElroy, 1994). Up to now expression studies have mainly been focused on non-plant transgenes like the NPT II gene coding for kanamycin-resistance (Matzke *et al.*, 1989) or heterologous genes like the A1 gene of maize coding for one of the enzymes involved in flower pigmentation which was inserted into petunia (Meyer *et al.*, 1987; Linn *et al.*, 1990; Meyer *et al.*, 1992).

The problem of variability can be overcome by regenerating a sufficient amount of independent transformants to select for those with the appropriate expression level, supposing a good screening procedure is available. Of more concern for their commercial prospects is the fact that instability of the expression of the transgene can be observed after a sexual cycle (Meyer *et al.*, 1992). Inheritance of expression of trangenes has mostly been studied for antibiotic resistance markers in plant species like tomato (Sukhapinda *et al.*, 1987), petunia (Deroles and Gardner, 1988 a,b), arabidopsis (Feldmann, 1991; Kilby *et al.*, 1992, Scheid *et al.*, 1991), tobacco (Matzke and Matzke, 1991; Matzke *et al.*, 1993), pea (Puonti-Kaerlas *et al.*, 1992), maize (Walters *et al.*, 1992) and rice (Schuh *et al.*, 1993). The transgenics had either a normal or abnormal segregation pattern in the sexual offspring. The transmission of traits complementing recessive mutations have been poorly investigated. Only Vaucheret *et al.* (1990) described the complementation of a nitrate reductase deficient mutant of *Nicotiana plumbagonifolia* and the level of complementation in the offspring of a few partly complemented transgenics.

Outline of this thesis.

In this thesis the *amylose-free* mutant of potato is used as a model plant to examine gene-expression and gene-silencing. The recessive mutation in the GBSS gene leads to *amylose-free* (*amf*) starch which can easily be scored in tubers and microspores by staining with iodine. A gene-dosage population for the wild-type GBSS-allele has been created by making use of genetic analysis of staining of microspores with iodine (Chapter 2). The dosage effect and the effect of different wild-type GBSS-alleles on GBSS-activity and amylose content is described. In the Chapters 3, 4 and 5 expression of wild-type GBSS transgenes introduced in the *amf*-mutant (Chapters 3 and 5) and in a wild-type (Chapter 5) are investigated. The variation in complementation level of the transgenics is determined (Chapter 3). The minimum number of independently segregating T-DNA inserts is estimated using microspore staining and Southern hybridisation. Several transgenics are crossed with either the *amf*-mutant (Chapter 4) or a wild-type (Chapter 5) to examine the transgene expression in the F1 offspring. In order to create a situation as in the double mutant *aewx* of maize, the expression of the gene encoding Branching Enzyme is inhibited by introducing sense and antisense cDNA for the potato BE in the *amf*-mutant (Chapter 6). The influence of the inhibition of the BE gene expression on the starch colour and several starch characteristics like branching degree and physico-chemical properties of the starch is described. A general discussion is given in Chapter 7.

Chapter 2

The dosage effect of the wild-type GBSS-allele is linear for the GBSS-activity but not for the 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. The GBSS-activity was significantly different for all groups and showed an almost linear dosage effect for the wild-type GBSS gene. A dosage effect was found for the amylose content, which was not linear. The amylose content was similar for both the duplex and triplex/quadruplex group. Within the simplex group, differences in the amylose content were found, which might be due to a different genetic background. There was no linear correlation between the GBSS-activity and amylose content. A certain level of GBSS-activity already led to a maximum amount of amylose. A further increase of GBSS-activity did not lead to a higher amylose content.

The presence of one or more wild-type 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.

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 packed in a specific order in 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). The swelling of granules in a heated aqueous starch suspension is 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, which formation is catalysed by the SSS, and transferring it to the number six 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.*, 1991b). Although, the *amf*-locus still produces mRNA equivalent to that of the wild-type level no protein is present tightly linked to the starch granule (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 red staining starch, whereas wild-type starch stains blue.

We describe here the development of a GBSS gene-dosage population of potato (*Solanum tuberosum* L.) and the dosage effect of wild-type GBSS-alleles on the amylose content, GBSS-activity and 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 4x *amf*-plants. Since the original 4x-*amf* genotypes were partly sterile and lacked the ability to tuberise, these plants were crossed with wild-types 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 wild-type GBSS allele (wild-type 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-tuberising 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 colour of the individual starch granules and the microspores were stained as described by Flipse *et al.* (1994). Three flowers per genotype and two stamen per flower were stained. The segregation ratio of red and blue microspores was statistically determined by using the χ^2 test with a 5 % confidence limit.

Isolation of starch and determination of amylose content and GBSS-activity

Several tubers, originating from one field row were taken randomly and starch was isolated according to Kuipers *et al.* (1991). The amylose content was determined spectrophotometrically as described by Hovenkamp-Hermelink *et al.* (1988b). This method is easy and fast, but has the disadvantage that a certain background level of approximately 3 % is calculated, even in the *amylose-free* genotypes. The determination of GBSS-activity was as described by Vos-Scheperkeuter *et al.* (1986). For measuring the amylose content, three samples, and for measuring the GBSS-activity, six samples of 2 mg of isolated starch were used. A variance analysis test with 5 % confidence limit was used for statistically analyzing the dosage effect on the amylose content and GBSS-activity. For a pairwise analysis of the group differences, a LSD test with 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 mM EDTA, 20% glycerol, 2% SDS, 0.002% bromophenolblue, 10% ß-mercaptoethanol). After boiling, the samples were kept on ice and 15 µl was used for analyzing on 10% polyacrylamide gels (Laemmli, 1970). Immunoblotting was carried out as described by Hovenkamp-Hermelink *et al.* (1987) by 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 mM Tris.HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) 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 to 400 mg of pure native starch was solubilised 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 200cm, Pharmacia). Fractions of 6 to 8 ml were collected after adding 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 complexation with an iodine solution (1 Lugol: 4 water) by performing a wavelenght scan from 450 to 700 nm. The wavelenght 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 before the next sample was applied.

Small deformation tests

Dynamic rheological properties of the 5 wt.% 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 wt.% starch suspensions were heated to approximately 65°C under gentle stirring until the viscosity slightly increased. After transferring them 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 $1°C \cdot min^{-1}$. Measurements were done every 60 sec. 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 using 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 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

Based on iodine staining of microspores, genotypes corresponding to nulliplex (no wild-type GBSS-allele), simplex, duplex and triplex/quadruplex for the wild-type GBSS allele were selected. This selection was according to the expected segregation presented in Table 1.

Table 1. The expected and obtained offspring when duplex plants (AAaa x AAaa) are crossed. These genotypes can be distinguished after iodine staining by their segregation of blue and red microspores; triplex (AAAa) and quadruplex (AAAA) plants where taken in one group. Genotypes with enough tubers 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: $\chi^2_{(1:8:18:9)} = 1.62 < 7.82$ which indicates that the offspring is not deviating from the expected 1:8:18:9 segregation of the gene-dosage genotypes for the wild-type GBSS-allele.

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 tuberising 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 with starch, isolated from field grown tubers of all different genotypes. This test is the most frequently used separ-

ation 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 wild-type GBSS allele contained, besides amylopectin, amylose (second peak) whereas the group with no wild-type 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 the nulliplex genotypes. This might indicate the presence of amylopectin molecules with longer, more amylose-like chains in the amylopectin fraction.

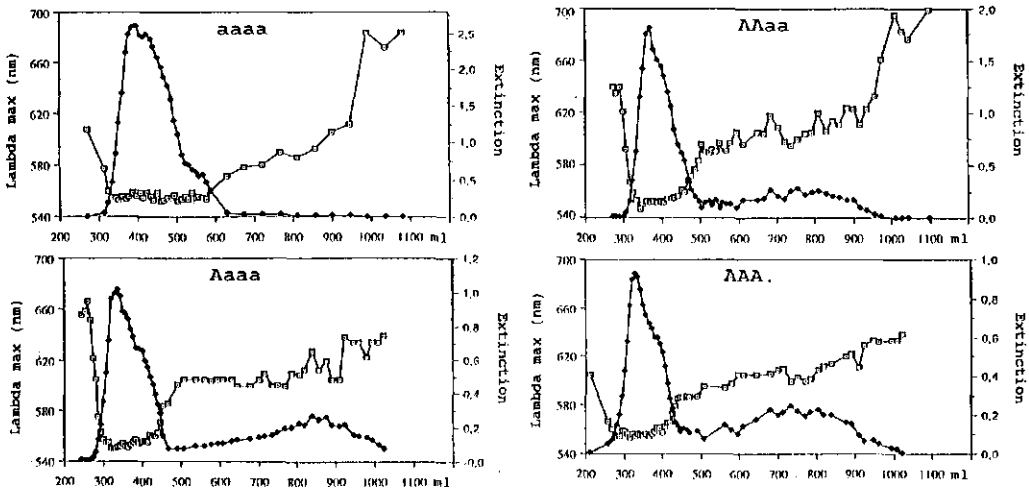

Fig. 1. Size exclusion chromatography results, indicating the λ_{max} (□-□) and extinction (■-■) of fractions eluting from the CL2B sepharose column.

The GBSS-activity was determined for all 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. Looking at the differences in more detail using the LSD test it is evident that all pairs of gene-dosage groups are significantly different (Table 2).
The amylose content was estimated for all different clones (Fig. 2A) and replications in the field trial. The used method to determine the amylose content always shows a low background of amylose even in the *amf*-genotypes which show an amylose content of less than 0.1 % when amperometric titration was used (Kuipers, 1994). The statistical evaluation with an analysis of variance showed a significant difference in amylose content between the gene-dosage groups. Using the LSD test differences between groups were tested. 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.

Table 2. Differences in GBSS-activity and amylose content between gene-dosage groups tested with Least Significant Difference test (LSD) with a 5 % confidence level.

	GBSS-activity				amylose content			
	aaaa	Aaaa	AAaa	AAA.	aaaa	Aaaa	AAaa	AAA.
aaaa		*	*	*		*	*	*
Aaaa			*	*			*	*
AAaa				*				NS
AAA.								

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

The correlation between the GBSS-activity and amylose content is visualised in Fig. 2A. It is clear that a maximum amylose content was attained at a certain level of GBSS-activity after which an increased activity did not lead to a higher amylose content.

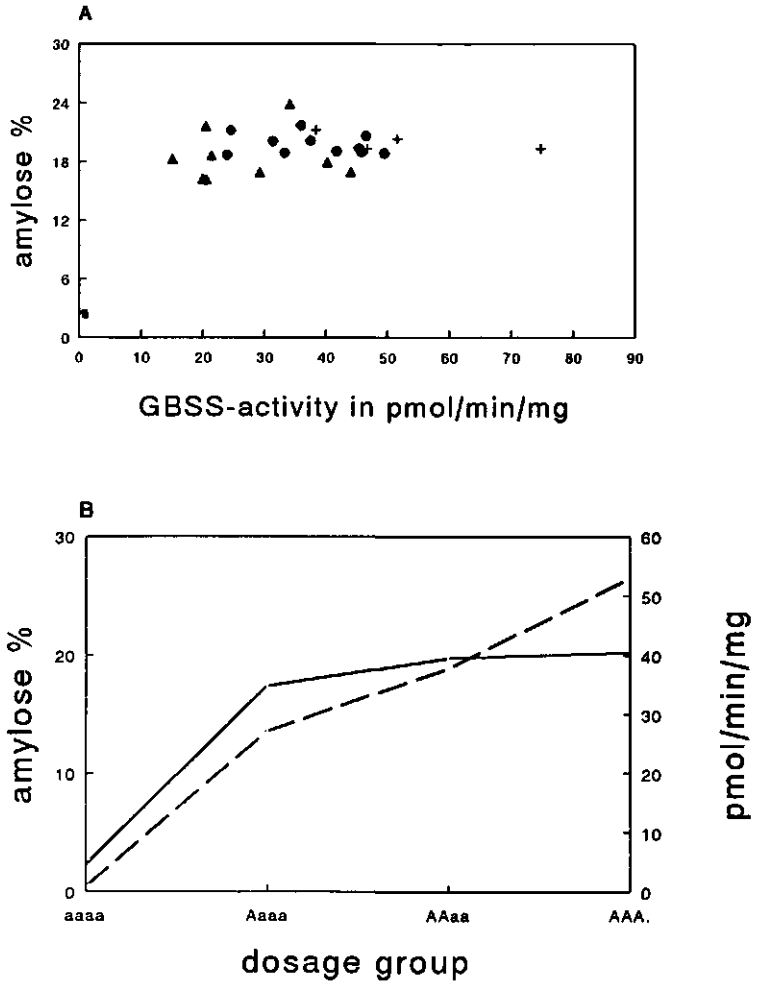

Fig. 2.
A. The correlation between the GBSS-activity and amylose percentage for the different gene-dosage groups consisting of 2 nulliplex (aaaa ■), 9 simplex (Aaaa ▲), 6 duplex (AAaa ●) and 4 triplex/quadruplex (AAA. +) plants.

B. The group means for GBSS-activity (— —) and amylose content (——) against the number of wild-type GBSS alleles.

Studying the amylose content of the individual genotypes in the simplex group in more detail, it was obvious that the 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 the number of wild-type 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.*, 1991b; 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 of different strength, their presence might account for the observed differences in amylose content between the simplex plants. Using the PCR technique with specific primers, that 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 in the simplex plants (data not shown).

GBSS-protein content

The amount of GBSS-protein in the starch granule fraction of different genotypes was analyzed. Fig. 3 clearly shows that the *amylose-free* plants had no GBSS in the 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 no shown).

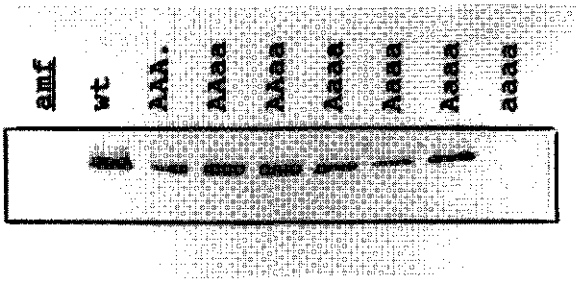

Fig. 3. Western blot of the starch granule fraction of plants with none to four wild-type GBSS-alleles by using the antibody against GBSS as a probe.

Physico-chemical properties of starch-water systems

When starch granules are suspended in water and heated a series of processes known as gelatinization occurs. It includes a drastic, irreversible

swelling and a melting of the crystallites and is accompanied with 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. Estimated is the storage modulus (*G'*) which is a measure of the engergy stored and released per cycle of deformation and per unit of volume.

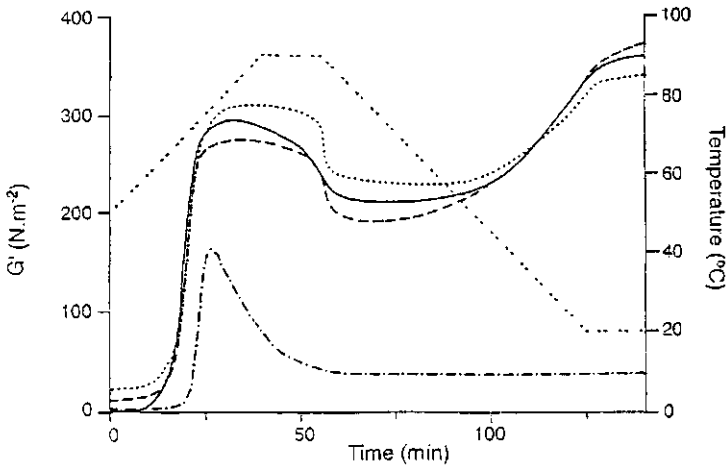

Fig. 4. Changes in the storage moduli (*G'*) of 5 wt.% potato starch suspensions during heating and cooling with -- -- -- indicating the temperature against time (— — .aaaa: 2.50 % amylose; ····· Aaaa: 16.94 % amylose;——— AAaa: 18.96 % amylose; — — AAA.: 20.32 % amylose).

Fig. 4 shows the changes in the storage moduli (*G'*) of 5 wt. % starch suspensions, measured during heating and cooling. 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 wild-type GBSS-alleles. The storage moduli of amylose containing starches started to increase strongly at a temperature of about 63 °C, whereas the storage modulus of *amylose-free* starch started to increase strongly at 69 °C. The increase in moduli coincides with the first stages of crystallite melting (see T_o) as determined with DSC (Table 3).

Table 3. Differential scanning calorimetry (DSC) data for 20 wt.% potato starch suspensions originating from plants with different dosages of the wild-type GBSS-allele. Starch samples were used from similar plants as in Fig 4. Starch suspensions were heated with 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

Moreover, it was shown that the peak moduli were higher for amylose containing starches and that 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, whereas the moduli of *amylose-free* starch remained constant.

Discussion

In this study the development of a GBSS gene-dosage population by crossing two different duplex potato genotypes is described. By staining microspores with an iodine solution it was possible to distinguish different classes in the progeny such as nulliplex, simplex, duplex and triplex/quadruplex genotypes (Table 1). 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.*, 1991a,b), one of the two components of starch. We could confirm the absence of amylose in the *amf*-mutant (nulliplex) and the presence in all other gene-dosage groups by using size exclusion chromatography (Fig. 1). Using the analysis of variance test a gene-dosage effect of the GBSS-alleles on both the GBSS-activity and amylose content was found. 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 did not count for a further increase in amylose content (Table 2). For the

amount of amylose the gene-dosage effect was small and far from linear as it was for the GBSS-activity (Fig. 2B). A gene-dosage effect of the GBSS-allele for the amylose content was earlier 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 and in other cases three doses of the gene were necessary for a wild-type level of amylose (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). No difference could be found when two or three wild-type alleles were present.

Although the *amf*-mutant did 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 wild-type 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 in the GBSS-protein level (Sano, 1984). Also in maize, Tsai (1974) found that the amount of GBSS-protein increases linear with the GBSS gene-dosage. A dosage effect on the GBSS-activity in maize and rice endosperm was not investigated.

The starch granules of all the potato plants were totally blue except for a few simplex plants in which a small percentage of starch granules had a small red outer layer, indicating that the granules were not completely filled with amylose. This phenomenon was found before by Kuipers *et al.* (1994) after inhibiting the GBSS-gene expression 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 wild-type 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 wild-type level of amylose is present. By looking at the correlation between the GBSS-activity and amylose content, it was 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

idea that the starch granule is formed due to a crystalline organization of the 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 with a maximal volume (Jane *et al.*, 1992).

Sano (1984) has not only found in rice that the amylose content was affected by the number of wild-type GBSS-alleles, but could also detect at least two different wild-type GBSS-alleles which determine the level of gene product as well as amylose content. Within the constructed simplex group, differences in amylose content were found suggesting that different wild-type alleles with distinct expression levels were present, or that a difference in genetic background is of influence. Using the PCR-amplification method no sequence differences were found indicating the the differences in expression level observed here were due to the different genetic background for 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 showed that the gelation properties of *amylose-free* starch differed 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 swelling of the starch granules, which is a result of melting of the crystalline regions in the granules (Keetels, 1995). Presumably, the presence of amylose indirectly lowered the melting of the crystalline regions, which would explain the lower temperature at which the moduli of amylose containing starches started to increase and this was confirmed with the DSC thermogram of the starch suspensions (Table 3).

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

It was described by Keetels (1995) that 30 % starch gels consist of tightly-packed, only slightly swollen granules with a very thin amylose layer in

between. In 5 wt. % starch systems, which were studied here, the granules had to swell to a greater extent before they filled the whole system. Probably, more amylose leached out of the granules. The volume fraction of the swollen granules was therefore somewhat lower than in a concentrated starch system, but still so high that the system may not be considered as an amylose gel with dispersed, non interacting, granules. Assuming 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 showed that the wild-type GBSS-allele has a dosage effect on the 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 there was a dosage effect found on the GBSS-protein level in rice and maize endosperm this was not found for potato. The presence of amylose has a large influence on the physico-chemical properties of starch suspensions.

Chapter 3

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

Flipse E, Huisman JG, de Vries BJ, Bergervoet JEM, Jacobsen E and Visser RGF.

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Abstract

Granule Bound Starch Synthase (GBSS) catalyses the synthesis of amylose in starch granules. Transformation of a diploid *amylose-free* (*amf*) potato mutant with the gene encoding GBSS, leads to the restoration of amylose synthesis. Transformants were obtained which had wild-type levels of GBSS-activity and amylose content. It proved to be difficult to increase the amylose content above that of the wild-type potato by the introduction of additional copies of the wild-type GBSS-gene. Staining of starch with iodine was suitable for investigating the degree of expression of the inserted GBSS-gene in transgenic *amf*-plants. Of the nineteen investigated transformants, four had only red staining starch in tubers indicating that no complementation of the *amf*-mutation had occurred. Fifteen complemented transformants had only blue staining starch in tubers or tubers of different staining categories (blue, mixed and red), caused either by full or partial expression of the inserted gene. Complementation was also found in the microspores. The segregation of blue and red staining microspores was used to analyze the inheritance of the introduced GBSS-genes. Comparing the results from microspore staining and Southern hybridisation indicated that in three tetraploid transgenics, the gene was probably inserted before (duplex) and in all others after chromosome doubling (simplex). The partial complementation was not due to methylation of the *HPA* II/*MSP* I site in the promoter region. Partial complemented plants had low levels of mRNA as was found when the GBSS expression levels were inhibited by the antisense technology.

Introduction

Starch is the major storage carbohydrate in plants. In potato tubers it consists of about 20 % amylose, an unbranched glucose-polymer and of 80 % amylopectin, a branched glucose-polymer (Shannon and Garwood, 1984). Amylopectin stains red with iodine-potassium iodine, whereas pure amylose stains blue. The monogenic recessive *amylose-free* potato mutant (*amf*) lacks Granule Bound Starch Synthase (GBSS) activity and GBSS-protein and the starch stains red with iodine (Hovenkamp-Hermelink *et al.*, 1987). The *amf*-mutant is similar to the *waxy*-mutant of maize but the phenotypic expression of the former has different tissue specificity. The *waxy*-mutation in maize is expressed in microspores, endosperm and embryosac (Echt and Schwartz, 1981), whereas the *amf*-mutation in potato is expressed in all tissues containing transitory and reserve starch, like tubers, stomatal guard cells of leaves, columella cells of root tips and microspores (Jacobsen *et al.*, 1989). For several plant species it was shown that the *waxy*-mutation, resulting in loss of GBSS-activity, was due to an alteration in the gene encoding GBSS (Wessler and Varagona, 1985; Okagaki and Wessler, 1988; Okagaki *et al.*, 1991). In potato, van der Leij *et al.* (1991a) were able to complement the *amf*-mutant with the cloned wild-type GBSS-gene by using *Agrobacterium rhizogenes*. Transformants with a blue starch colour in the roottips indicated restored amylose synthesis. Sequence analysis of both the mutated and wild-type allele of GBSS showed a single basepair deletion in the transitpeptide region of the gene coding for this protein (van der Leij *et al.*, 1991b). Stable expression of an introduced gene is of considerable importance for the application of the gene transfer technique in plant breeding. Because the expression of the wild-type GBSS-gene is easily monitored in an *amylose-free* background by staining with an iodine solution, this system can be used as a model to examine the stability of expression of an inserted gene. The inheritance of the inserted gene can be examined directly by iodine staining of the starch in microspores. Furthermore, the consequences of chromosome doubling either before or after the insertion of T-DNA into the genome can be critically monitored through the detection of either simplex or duplex segregation at the microspore level. The expression of the, by *Agrobacterium tumefaciens*, inserted gene in greenhouse and field grown tubers and the inheritance of the inserted gene according to the segregation pattern of red and blue staining microspores is presented.

Materials and methods

Plant material

Stem segments were obtained from a diploid (2n = 2x = 24) homozygous *amf*-genotype 87.1029-31 of *Solanum tuberosum* L. This mutant, called genotype B hereafter, was the result of a crossing program between the original *amf*-mutant 86.040 and two different diploid wildtypes. Crosses were made with the aim to improve the fertility and tuber production of the original transformant (Jacobsen *et al.*, 1989). Several wild-types which were genetically related to 87.1029-31 were used as the wild-type control. Plants were grown in jars containing MS medium (Murashige and Skoog, 1962) with 30 g/l sucrose (MS 30) and 8 % agar, at 22°C and 16 h light. Transgenic shoots were grown and multiplied on MS 30 medium supplemented with 100 mg/l kanamycin. Microtubers were obtained by transferring stem segments with one or more nodal buds to MS medium with 60 g/l sucrose and 1.5 mg/l BAP. A transformant with T-DNA containing the GUS and NPT-II gene was called BAM and transformants obtained after transformation with the construct pWAM 100 containing the complete genomic GBSS sequence were called B followed by the clone number. Ploidy levels were determined by counting the number of chloroplasts in stomatal guard cells (x: 7-9, 2x: 11-14, 4x: 16-22, Frandsen, 1968).

In April 1991 the *in vitro* plants were planted in pots in the greenhouse, where they were grown under standard conditions. In May, twenty plants per genotype were transferred to the field. In the field the plant distance within a row was 40 cm. and between rows 75 cm. The plot was surrounded with cv Cleopatra a red tuberising, non flowering potato cultivar. The field experiment was set up according to the rules of the government (Anonymous, 1990). Flower buds were removed every 1-3 days. Two plants per genotype remained in pots in the greenhouse in order to evaluate starch composition, amylose content and GBSS-activity in the tubers, the segregation pattern of blue and red staining starch in the microspores and the number of inserted GBSS-genes.

Vectors and transformation procedure

The vector pWAM 100 was used in this experiment. This construct possesses the genomic GBSS sequence driven by its own promoter in opposite orientation in relation to the gene coding for kanamycin resistance in the plant (van der Leij

et al., 1991a). pWAM 100 was transferred to *A. tumefaciens* LBA 4404 using the direct transformation procedure described by Höfgen and Willmitzer (1988). To test the integration of the mobilized plasmid into *A. tumefaciens*, plasmid DNA, isolated from *A. tumefaciens* was analyzed (Holmes and Quigly, 1981). For the control experiment *A. tumefaciens* strain AM 8706 was used harbouring a binary plasmid, which contains the gene encoding ß-glucuronidase and the gene encoding kanamycin resistance (Visser *et al.*, 1989b). Transformation was performed essentially as described by Visser (1991), but two or three days after inoculation, explants were transferred to MS 20 medium with 1 mg/l zeatin, 100 mg/l kanamycin, 200 mg/l cefotaxime and 200 mg/l vancomycin and transferred to fresh medium every 3 weeks. On this medium shoot outgrowth took place after 3 to 4 months. Shoots were isolated and placed on MS 30 medium with 75 mg/l kanamycin. Only transgenic shoots were able to root on kanamycin containing medium.

Staining for starch composition in different tissues

Tubers were stained according to Kuipers *et al.* (1991). The colour of the individual starch granules was determined by spreading a small sample of starch on a microscopic slide, staining it with a water/Lugol's solution (1:1) and screening it under a microscope. Microspores were stained with the same solution to determine the segregation ratio. Two flowers per genotype and three stamen per flower were stained in each case.

Starch isolation, determination of amylose content and GBSS-activity

Starch from greenhouse and field grown tubers was isolated according to the method described by Kuipers *et al.* (1991). Amylose content was determined as described by Hovenkamp-Hermelink *et al.* (1988b) and GBSS-activity as described by Vos-Scheperkeuter *et al.* (1986). For measuring amylose content, 3 samples and for measuring GBSS-activity, 6 samples of about 2 mg isolated starch were used. A two-sided t-test with 5 % confidence level was used for the statistical analysis.

Molecular analysis of the transformants

DNA was extracted from leaves of greenhouse grown plants according to

Dellaporta *et al.* (1983). By digesting the DNA with the restriction enzyme *Eco*RI and using the ^{32}P random prime labelled, distal 1.3 kb cDNA of the GBSS cDNA as a probe the minimum number of integrated T-DNA copies could be found by scoring the number of additional bands in a Southern blot (van der Leij *et al.*, 1991a). DNA and RNA of red and blue staining tubers were extracted according to Salehuzzaman *et al.* (1992). To check whether the inserted GBSS-gene was methylated the DNA was digested with *HPA*II or *MSP*I and the proximal part of the GBSS gene which includes the promoter was ^{32}P labelled and used as a probe. Equal amounts of RNA (checked by hybridisation with potato 28s rDNA as a probe) were loaded to allow a comparison between different transformants and between different tuber parts of one transformant. The RNA was hybridised with a 2.4 kb GBSS cDNA. DNA and RNA blot hybridisation and labelling were performed as described previously (Visser *et al.*, 1989a,b). RNA transcript levels were densitometrically quantified using the Cybertech CS-1 Processing system.

Results

The starch phenotype of in vitro transformants

After inoculation of stem explants with *Agrobacterium tumefaciens*, 36 regenerated shoots were harvested from the explants and rooted on MS-medium with kanamycin. From each explant only one shoot was isolated to ensure that all transgenic shoots resulted from independent transformation events. Sixteen transformants (44 %) were diploid like the original genotype and twenty (56 %) were tetraploid. Microtubers appeared on induction medium after two to three weeks. Analysis of cut surfaces after staining with iodine showed that twenty nine transformants (81 %) had blue staining starch in the microtubers similar to the wild-type potatoes (complemented type). Seven transformants (19 %) had microtubers with red staining starch like the transformed *amf*-control (BAM) and the untransformed *amf*-mutant (B) (Table 1). Nineteen transformants and controls were selected for further analysis.

Table 1. Analysis of microtubers and greenhouse grown tubers of transgenic clones and their controls for starch colour after Lugol staining, amylose content and GBSS-activity.

clone	colour				amylose starch[a] granules	GBSS-content[b]		activity[b] nmolmin^{-1}mg^{-1}	
	microtubers	number of greenhouse tubers				%	S.D.		S.D
		red	mixed	blue					
controls									
B	red	13 (100%)	-	-	red	3.0	(0.7)	0.8	(0.3)
BAM	red	12 (100%)	-	-	red	3.2	(0.9)	0.5	(0.3)
wildtype	blue	-	-	15 (100%)	blue	23.5	(5.5)	73.4	(18.5)
transformants									
non-complemented									
B5	red	24 (100%)	-	-	red	2.5	(0.4)	2.4	(0.8)
B11	red	7 (100%)	-	-	red	3.9	(0.4)	4.6	(4.8)
B18	red	11 (100%)	-	-	red	4.5	(0.1)	0.2	(3.1)
B19	red	9 (100%)	-	-	red	3.8	(0.4)	1.2	(3.1)
partially complemented									
B1	blue	-	17 (68%)	8 (32%)	blue/red	V		V	
B6	blue	1 (6%)	9 (56%)	6 (38%)	blue/red	V		V	
B10	blue	15 (100%)	-	-	blue/red	V		V	
B15	blue	10 (67%)	1 (6%)	4 (27%)	blue/red	V		V	
B17	blue	-	-	9 (100%)	blue/red	V		V	
fully complemented									
B2	blue	-	-	17 (100%)	blue	23.9	(4.2)	105.1	(27.3)
B3	blue	-	-	4 (100%)	blue	23.2	(1.6)	112.2	(4.0)
B4	blue	-	-	11 (100%)	blue	24.7	(1.6)	74.1	(15.8)
B7	blue	-	-	17 (100%)	blue	25.5	(1.1)	113.6	(4.3)
B8	blue	-	-	9 (100%)	blue	22.2	(1.3)	124.8	(20.5)
B9	blue	-	-	19 (100%)	blue	22.0	(1.0)	125.6	(10.2)
B12	blue	-	-	7 (100%)	blue	21.1	(0.7)	82.7	(16.4)
B13	blue	-	-	17 (100%)	blue	17.5	(0.7)	108.7	(21.1)
B14	blue	-	-	7 (100%)	blue	22.2	(2.4)	33.2	(12.0)
B16	blue	-	-	22 (100%)	blue	22.7	(1.2)	ND	

[a] : the starch granules were isolated from greenhouse grown tubers.
[b] : The amylose content (n = 3) and GBSS-activity (n = 3) was measured in starch isolated from greenhouse grown tubers. S.D. is standard deviation.
ND : not determined.

Characterisation of greenhouse grown tubers

The two classes of non-complemented and complemented transformants above described were also found in greenhouse grown tubers when analyzed by Lugol staining. However, the class of complemented transformants could be divided into a partially and a fully complemented subclass (Table 1). The transformants B1, B6, B10, B15 and B17 belong to the group of visually partially complemented transformants. For the transformants B1, B6 and B15 this was shown by the variation in starch colour of greenhouse grown tubers. The blue and red colour in the mixed tubers was not separated into clear sectors. The red colour was mostly found in the middle of the tuber, but the presence of blue staining starch was not absent from these regions. Their starch granules had a red coloured outer layer, showing up as a red coloured outer ring around a blue core of varying size. The size of the core was related to the macroscopically observed tuber colour. All greenhouse grown tubers of B10 stained red, but their starch granules contained a very small blue core indicating partial complementation. All greenhouse grown tubers of B17 stained blue, but starch granules were found which contained red outer rings.

The amylose contents and GBSS-activities of the untransformed control B and the transformed control BAM were similar (Table 1), indicating that the transformation event itself had no direct influence on starch composition and GBSS-activity. The amylose contents and GBSS-activities of the non-complemented transformants were like those of the *amf*-mutant. For each of the partially complemented transformants the amylose content and GBSS-activity was variable throughout the tuber depending on the size of the blue core in the starch granules (data not shown). The amylose contents and GBSS-activities of the fully complemented transformants were in the range of that of the wild-type controls. The GBSS-activities were highly variable as was also seen for the wild-type controls (Table 1). A significant ($p < 0.05$) correlation was found between the amylose content and the GBSS-activity using the Spearman rank correlation test.

Characterisation of field grown tubers

The staining results of the field grown tubers (Table 2) resembled those of the greenhouse grown tubers. Only B17 had more red staining tuber parts and some tubers of B10 had blue staining parts. The blue and red colour in

the mixed tubers was in contrast to greenhouse grown ones more clearly concentrated in separated parts and different staining patterns could be observed.

Table 2. The number and percentage of tubers for the different staining categories after staining with Lugol.

	colour field tuber					
	red		mixed		blue	
clones	number	%	number	%	number	%
controls						
B	325	(100)	-		-	
BAM	116	(100)	-		-	
wildtype	-		-		372	(100)
transformants						
non-complemented						
B5	151	(100)	-		-	
B11	125	(100)	-		-	
B18	232	(100)	-		-	
B19	157	(100)	-		-	
partially complemented						
B1	29	(17)	60	(36)	80	(47)
B6	24	(20)	48	(40)	48	(40
B10	141	(80)	28	(16)	7	(4)
B15	102	(70)	40	(28)	3	(2)
B17	-		3	(2)	163	(98)
fully complemented						
B2	-		-		227	(100)
B3	-		-		77	(100)
B4	-		-		125	(100)
B7	-		-		151	(100)
B8	-		-		140	(100)
B9	-		-		229	(100)
B12	-		-		130	(100)
B13	-		-		167	(100)
B14	-		-		143	(100)
B16	-		-		259	(100)

Molecular analysis

No difference in restriction pattern could be found when restricting the DNA with *MSP* I or *HPA* II followed by hybridi-sation analysis, which indicates that methylation in the promoter region of the GBSS-gene was not found (data not shown). However, this is not a proof that methylation outside the promoter region does not interfere with the expression of the inserted GBSS-gene. RNA was extracted from tubers of different transformants and North-

ern blots were made. Almost all transformants showed a band of the correct size (Fig. 1A), among the different transformants no correlation was found between the amount of GBSS mRNA and the starch colour. However, when RNA was extracted from blue and red staining tuber parts of a partially complemented transformant, there seemed to be a relation between the degree of complementation and the amount of mRNA found. A clear GBSS mRNA signal was found in blue staining parts, a weak signal in red staining parts and an intermediate signal in the mixed staining parts (Fig. 1B). Quantification of the signals showed that there could be three times more transcript present in a blue staining part of a tuber than in a red staining part of the same tuber.

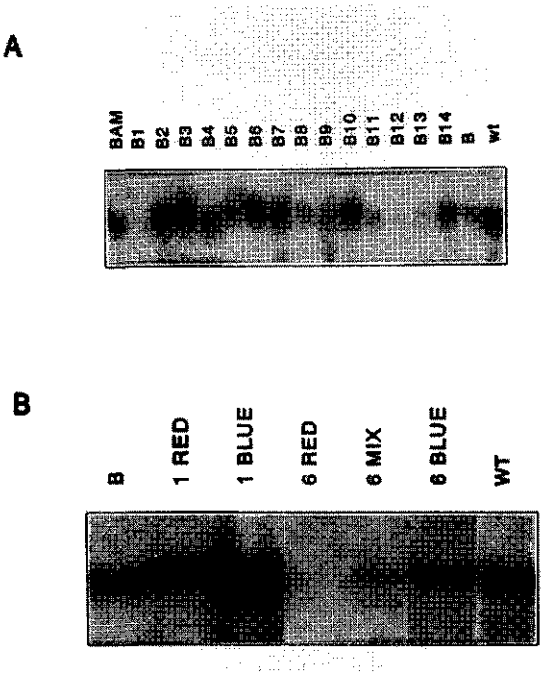

Fig. 1 Northern hybridisations of different transformants and controls with a 2.4 kb GBSS cDNA.
A) Expression of GBSS mRNA in greenhouse grown tubers.
B) GBSS mRNA expression in red, mixed and blue staining tubers parts of the partially complemented transformants B1 and B6; the values given below were determined by densitometrical scanning of the autoradiograph and the values are in comparison with the red staining tuber part: 1 red = 1, 1 blue = 2.5, 6 red = 1, 6 mixed = 1.6 and 6 blue = 3.0.

Genetical analysis and determinatation of copy number

In Table 3 the transformants that flowered were classified according to their segregation of blue and red staining microspores. The transformants B5 and

B11 without GBSS expression in their micro-, greenhouse and soil grown tubers also lacked expression in their microspores which stained red with iodine like the controls. Southern hybridisation (Fig. 2) of the non-complemented clone B11 indicated that one inactive GBSS-gene was present in the genome. For B5 no inserts could be detected. A segregation according to one active insertion was found in the diploid transformants B7 and B9 (GBSS -) and in the tetraploid B12 and B14 (GBSS - - - ; simplex). According to the Southern hybridisation one or two inserted genes appeared to be present. Because of this monogenic inheritance the second gene in B7, B9 and B14 is expected to be inactive or closely linked to the first one. A segregation of 3:1, indicating that two genes are segregating independently, was found for the diploid B2 ($GBSS_1$ -, $GBSS_2$ -) and the tetraploid B8 ($GBSS_1$ - - -, $GBSS_2$ - - -) and confirmed by Southern analysis (Fig. 2). B8 contained a truncated GBSS-gene as well. The tetraploid transformants B1, B4 and B6 had a segregation of 5:1 indicating a duplex situation (GBSS GBSS - -). One band was found by Southern hybridisation (Fig. 2) for B1 and B6 supporting this duplex situation. For B4 three bands were found.

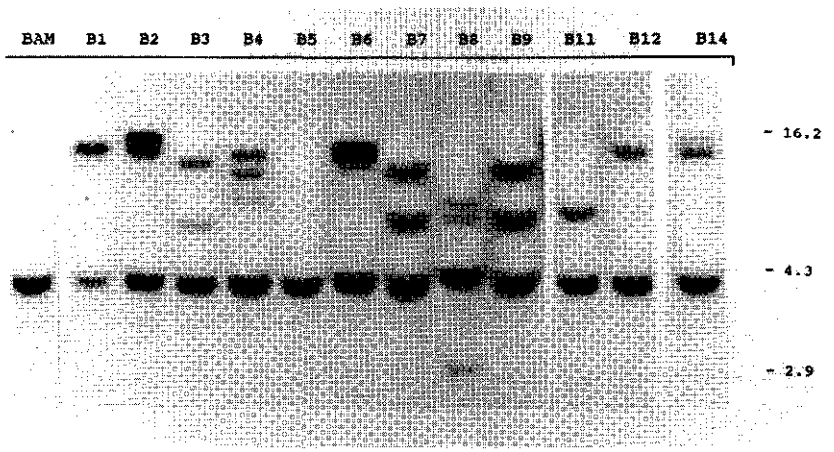

Fig. 2 Southern hybridisation of total DNA from transformants with the distal part of the GBSS cDNA as a probe. The plant DNA was cut with *Eco*RI, which gives an endogenous band of 4.3 kb with this probe. The band of 2.9 kb in lane B8 indicates the insertion of a truncated GBSS-gene

Table 3. The ploidy level, microspore segregation after staining with Lugol, the number of inserts found by Southern hybridisation and colour of field grown tubers of different transgenic potato clones.

clone	ploidy level	segregation ratio blue : red	T-DNA* copies	starch colour field tubers
		Class I: No alleles; - - or - - - - (0:1)		
B	2 X	0 : 825	0	red
BAM	2 X	0 : 1000	0	red
B5	4 X	0 : 1324	0	red
B11	4 X	0 : 987	1	red
		Class II: One allele, simplex; GBSS - or GBSS - - - (1:1)		
B7	2 X	445 : 413 (χ^2 = 0.08)	2	blue
B9	2 X	250 : 235 (χ^2 = 0.35)	2	blue
B12	4 X	234 : 210 (χ^2 = 1.30)	1	blue
B14	4 X	337 : 333 (χ^2 = 0.02)	2	blue
		Class III: Two alleles independently segregating, double simplex; $GBSS_1$ -, $GBSS_2$ - or $GBSS_1$ - - -, $GBSS_2$ - - - (3:1)		
B2	2 X	583 : 218 (χ^2 = 2.10)	2	blue
B8	4 X	500 : 139 (χ^2 = 3.59)	2	blue
		Class IV: One allele in duplex; $GBSS_1$ $GBSS_1$ - - (5:1)		
B1	4 X	188 : 30 (χ^2 = 1.32)	1	mixed
B4	4 X	347 : 80 (χ^2 = 1.30)	3	blue
B6	4 X	887 : 181 (χ^2 = 0.06)	1	mixed

*: The number of additional inserts according to the Southern hybridisation.

The duplex segregation suggests that two of them are inactive, or placed on the same chromosome as the gene in duplex.

Discussion

Using the *Agrobacterium tumefaciens* system, the introduction of the GBSS-gene into the *amf*-mutant lacking GBSS-activity leads to a restoration of

GBSS-activity and amylose synthesis (Table 1). This is in agreement with the observations of van der Leij *et al.*, (1991a) after using *Agrobacterium rhizogenes* as a vector. With respect to starch colour in subterranean tubers three different groups of transformants were found: non-complemented, fully complemented and partial complemented. Transformants of the latter class contained starch granules which had a blue core of varying size and a red outer ring. This had also been observed after transforming a wild-type potato with an antisense GBSS-gene (Kuipers *et al.*, 1994). The amylose contents and GBSS-activities of these transformants varied depending on the size of the blue core in the starch granules.

The GBSS-gene was also expressed in microspores. According to the segregation of red and blue staining microspores up to two independently segregating active inserts were present. Comparison of the results of the microspore segregation and the Southern hybridisation indicated that sometimes more than one insert could be present closely linked on one chromosome or that not all inserts were active (Table 3). The tetraploid transformants B1 and B6, with unstable expression of the inserted gene probably were duplex (Table 3). This means that here insertion had taken place before the chromosome doubling, leading to a duplex situation. The active inserts in the fully complemented tetraploid plants were either situated on two non-homologous chromosomes or on homologous chromosomes on different positions.

The amount of GBSS-mRNA varied between the different transformants and was not correlated with the expression of the inserted GBSS-gene (Fig. 1A). This could be expected because Visser *et al.* (1989b) reported the presence of normally sized GBSS-mRNA in the *amf*-mutant. However, within a single transformant, a relation between the degree of expression and amount of mRNA could be found based on a densitometric quantification of the bands. A small amount of mRNA was found in the *amylose-free* red staining parts of the tubers and this amount did raise with the increase in amylose content (Fig. 1B).

Unstable expression of an inserted gene has been described earlier and, amongst others, methylation or sense inhibition are mentioned as possible reasons. Unstable gene expression was found in a petunia mutant transformed with the A1 cDNA of maize (Meyer *et al.*, 1987) and was due to methylation of the promoter, which was positively correlated with the number of integrated genes and also influenced by the chromosomal position

of the inserted genes (Linn *et al.*, 1990). Similar results were reported by Matzke *et al.* (1989), who found methylation and inactivation of a first inserted T-DNA after the integration of a second T-DNA gene and therefore presumed that the methylation was the result of an interaction of homologous sequences. In this research however, no indication was found for methylation of the GBSS-gene in the red sectors of the mixed staining tubers.

Another possible explanation for the unstable gene expression is sense or co-suppression. Co-suppression is the phenomenon that the mutant phenotype can be obtained after introduction of one or more copies of the wild-type gene into wild-type plants. Grierson *et al.* (1991) suggested that the anti-sense RNA was formed because of a simple read through of the kanamycin resistance gene which was placed in opposite orientation. The GBSS gene expression in wild-type potato has been inhibited in previous research by using antisense RNA (Visser *et al.*, 1991) or by introducing an extra GBSS gene (van der Leij *et al.*, 1990; van der Leij, 1992). In the present research the construct pWAM 100 was used which possesses the gene for kanamycin resistance in opposite orientation towards the GBSS-gene, so the kanamycin resistance gene could have read through. Another explanation could be that a strong promoter in the plant DNA near the place of T-DNA insertion did cause the formation of anti-sense RNA. However, the main difference with the above mentioned previous research on antisense genes is that we introduced wild-type genes in a mutant background. Although the observations in this investigation were similar to those obtained after inhibiting the GBSS-activity in wild-type potato with antisense GBSS RNA, we were not able to detect antisense RNA in the transformants (data not shown). The fact that the partially complemented plants showed a decrease in GBSS mRNA upon the increase of red staining starch in tubers might indicate that co-suppression plays a role in these plants.

This research shows that a wild-type gene inserted into the mutant lacking the activity of this particular gene is not always fully expressed. For the application of gene manipulation in plant breeding, stable expression of an inserted gene is important. Selection of plants with stable gene expression should be performed before using them in plant breeding.

Chapter 4

Expression of wild-type GBSS transgenes in the offspring of partially and fully complemented *amylose-free* transformants of potato.

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Submitted

Abstract

The *amylose-free* (*amf*) potato mutant can easily be complemented by introduction of the wild-type gene coding for Granule Bound Starch Synthase (GBSS). After iodine staining the starch of the *amf*-mutant is red whereas that of the wild-type and the complemented *amf*-mutant is blue. The level of complementation of selected transformants and their sexual offspring after backcrossing with *amf*, was investigated using sporophytic tuber cells and gametophytic microspore cells. Two diploid and two tetraploid transformants with normal complementation showed the expected segregation patterns of 1:1 (one active insert) or 3:1 (two independently segregating active inserts) in the microspores and in the F1 offspring based on staining of tubers. All expected genotypes in the F1 generation were found, based on microspore segregation patterns of the individual F1 plants. Two transformants with partial complementation (mixed phenotypes) were investigated. One of them, B1, was tetraploid and duplex for the GBSS-insert, which had originated through mitotic doubling of transformed diploid cells. In the F1 generation three phenotypic classes were found: *amf*, fully complemented and partially complemented. The latter two exist independently of a simplex or duplex situation. The second transformant with partial complementation, B10, appeared to have a complex molecular composition. One cluster of five transgenes caused the partial complementation. Fully and partially complemented phenotypic classes were found after crossing B10 with the *amf*-mutant. Partial complementation was found after transformation of diploid and not of tetraploid *amf*-genotypes. Beside that the level of complementation was higher in less polyploidised tissue.

Introduction

The introduction of foreign DNA into plants in order to create new desirable phenotypes has become a routine procedure in a number of plant species. In potato, selected transgenics can sometimes directly be used as a new variety and/or as a parent. Therefore, it is important to know more about the expression of the introduced character in sexual offspring. After transformation of diploid potato, diploid and tetraploid transgenics can be obtained. These tetraploids can be the result of mitotic doubling ocurring before or after the transformation event, resulting in transgenic plants carrying the introduced transgenes in simplex or duplex (Flipse *et al.*, 1994). Inheritance of expression of trangenes has mostly been studied for antibiotic resistance markers in plant species like tomato (Sukhapinda *et al.*, 1987), petunia (Deroles and Gardner, 1988 a,b), arabidopsis (Feldmann, 1991; Kilby *et al.*, 1992, Scheid *et al.*, 1991), tobacco (Matzke and Matzke, 1991; Matzke *et al.*, 1993), pea (Puonti-Kaerlas *et al.*, 1992), maize (Walters *et al.*, 1992) and rice (Schuh *et al.*, 1993). The transgenics had either a normal or abnormal segregation pattern in the sexual offspring. In certain cases these abnormalities could be explained. The transmission of traits complementing recessive mutations have been poorly investigated in plants. Vaucheret *et al.* (1990) described the complementation of a nitrate reductase deficient mutant of *Nicotiana plumbagonifolia* and the presence of this character in the offspring of a few partially complemented plants. Van der Leij *et al.* (1991a) after transformation with *A. rhizogenes* and Flipse *et al.* (1994) after transformation with *A. tumefaciens* described partial and full complementation of an earlier described *amylose-free* (*amf*) potato mutant (Hovenkamp-Hermelink *et al.*, 1987) by one or more transgenes of the wild-type GBSS-gene. In these transgenic plants, the level of complementation could be investigated in the sporophytic phase in tubers as well as in the gametophytic phase in microspores after staining starch granules with iodine. *Amylose-free* starch stains red and wild-type starch stains blue with iodine. It means that expression of wild-type GBSS-transgenes can easily be investigated in different phases of development, not only in the original transformant, but also in offspring plants. Here, the level of complementation is evaluated in tubers and microspores of offspring plants. The segregation of genotypes is compared with the segregation in the microspores of the original transformant. A phenomenon described in literature is trans-

inactivation. It can be found when two or more inserts are present on homologous or non-homologous chromosomes (Matzke and Matzke, 1992). In the present study a similar phenomenon was examined by diluting the number of inserts of transformants in the sexual offspring. In addition, it was investigated whether partial complementation, mainly observed in field tubers of diploid or tetraploidised transformants could be caused by the phenomenon of trans-inactivation on homologous chromosomes after mitotic doubling.

Materials and methods.

Transgenic plant material

The (partially) complemented diploids (2n = 2x = 24) B2, B7, and B10 and tetraploids (2n = 4x = 48) B1, B8 and B14 were described previously (Flipse *et al.*, 1994) and were obtained by transforming the diploid *amf*-genotype 87-1029-31 (Jacobsen *et al.*, 1989) with the vector pWAM 100. This vector possesses the wild-type GBSS (wildtype GBSS = Amf = A; mutant GBSS = *amf* = a) sequence driven by its own promoter in opposite orientation in relation to the NPT II gene coding for kanamycin resistance in the plant (van der Leij *et al.*, 1991a). The plants were screened for the expression of the inserted GBSS-gene by staining the starch in tubers and microspores with Lugol (an iodine-potassium-iodine solution). The segregation of red and blue microspores gave an impression of the inheritance of the inserted GBSS-genes (Flipse *et al.*, 1994). The ploidy level was determined by counting the number of chloroplasts in stomatal guard cells (Frandsen, 1968). A summary of these results is given in Table 1, whereas the basic information for B10 is new.

More transgenics obtained after transforming the diploid *amf*-mutant 87-1029-31 and the tetraploid *amf*-mutant 90-6009-8 with the vector pWAM 100 were screened for the expression of the inserted GBSS gene by staining micro- and greenhouse grown tubers.

Crossings

Transgenic plants were grown in a biosafety greenhouse in which flowering of transgenic potato plants was allowed. Flower buds were emasculated and polli-

nated with an *amf*-phenotype 1-3 days later. The diploid *amf* Hb 92-7007-7 was used for fertilizing the diploid transgenic potato plants. This plant resulted from a crossing between 880004-3 (Jacobsen *et al.*, 1991) and 87-1031-9 (Jacobsen *et al.*, 1989). As a tetraploid *amf* clone J90-6016-11 was used (Jacobsen *et al.*, 1989).

Embryo rescue

Three weeks after pollination embryos were collected and placed on embryo growth medium (Neal and Topolewski, 1983; 1985). They were transferred to fresh medium every fortnight, until outgrowth of the embryos occurred. The seedlings were transferred to MS medium (Murashige and Skoog, 1962) with 30 g/l of sucrose (MS 30) and 8 % agar, without using kanamycin as a selective antibiotic. *In vitro* culture was done at 22 °C and 16 h light. After two to three months plants were transferred to the greenhouse for flowering and tuberisation.

Staining for starch phenotype in different tissues

Cut surfaces of tubers were stained with iodine according to Kuipers *et al.* (1991) showing red, blue (full complementation) and mixed (partial complementation) phenotypes. Individual starch granules and microspores were stained according to Flipse *et al.* (1994). Based on observation of individual starch granules plants with partial complementation were detected by the variable size of the blue core. Some transformants with a red staining cut surface showed partial complementation at the individual starch granule level.

Molecular analysis

DNA was extracted from the leaves of greenhouse-grown plants according Dellaporta *et al.* (1983). By digesting the DNA with the restriction enzyme *Eco*R1 and using the ^{32}P-random-prime-labelled, distal 1.3 kb GBSS cDNA and NPT II gene as a probe, the minimum number of T-DNA inserts was determined by scoring the number of additional bands in a Southern blot (van der Leij *et al.*, 1991a).

Results

The level of complementation in the sexual offspring of several fully complemented genotypes

Four transformants with full complementation, B2, B7, B8 and B14 were investigated. These were respectively two diploids, B2 and B7 and two tetraploids B8 and B14.

Of these, the diploid transformant B2 contained two inserts. A 3:1 segregation of blue and red microspores was found, indicating that the GBSS-genes were inserted in the genome allowing independent segregation. Based on this view, it was expected that complementation in the microspores was caused by one or both active inserts. After staining tubers of the F1 offspring a 3:1 segregation of plants with blue or red staining starch granules in their tubers was found (Table 1). Most of the F1 plants produced flowers and the starch colour in their microspores indicated that the blue staining plants, as expected, had a microspore segregation of 1:1 or 3:1 (Table 2). This clearly shows that both transgenes either separately or together are capable of complementing the mutant in both tubers and microspores.

Table 1. Some characteristics of the original transformants and the segregation of the GBSS-trangenes in the offspring when the original transformants were crossed with an *amf*-genotype.

Clone	Ploidy level	microspore segregation ratio blue:red	number of inserts	Starch colour in tuber		Tubers of F1 plants blue:mixed:red
				micro	field	
B2	2x	$3:1_{(\chi2=2.10)}$	2	blue	blue	$16:0:5_{(\chi2\ 3:1=0.02)}$
B7	2x	$1:1_{(\chi2=0.08)}$	2	blue	blue	$15:0:13_{(\chi2\ 1:1=0.14)}$
B10	2x	1:0[a]	7	blue	mixed	$12:7:0_{(\chi2\ 1:1=1.32)}$
B1	4x	$5:1_{(\chi2=1.32)}$	1	blue	mixed	$25:11:6_{(\chi2\ 5:1=0.2)}$[b]
B8	4x	$3:1_{(\chi2=3.59)}$	2	blue	blue	$27:0:7_{(\chi2\ 3:1=0.35)}$
B14	4x	$1:1_{(\chi2=0.02)}$	2	blue	blue	$13:0:11_{(\chi2\ 1:1=0.17)}$

[a]: in several anthers a small percentage of red staining microspores was found during the end of the growing season.

[b]: $\chi^2_{5:1}$ was calculated for the classes blue and mixed combined. $\chi^2_{4:1:1}$ for all three classes is 2.79.

Table 2. The microspore segregation in the flowering F1 plants and the starch composition by staining the cut surfaces with iodine.

Clone	F1 segregation	number of plants	colour
B2	3:1	3	blue
	1:1	10	blue
	0:1	5	red
B7	1:1	7	blue
	0:1	6	red
B10	3:1[a]	1	mix
	3:1[b]	1	blue
	1:1[b]	2	blue
	1:1[c]	1	blue
	variable[a]	2	mix
	variable[c]	2	blue
B1	5:1	1	blue
	1:1	5	blue
	1:1	1	mixed
	0:1	2	red
B8	3:1	2	blue
	1:1	5	blue
	0:1	1	red
B14	1:1	7	blue
	0:1	4	red

[a]: The block of five and 2 inserts (b and f) are present.
[b]: Number of inserts is unknown.
[c]: One insert (b or f) is present.

B7 is a diploid transformant (Table 1) with two GBSS-inserts but despite this observation a 1:1 segregation of red and blue microspores was found. In the F1 offspring also a 1:1 segregation was found for plants with blue (full complementation) or red (mutant phenotype) staining starch granules in the tubers. As expected, plants with red staining starch granules had only red staining microspores, whereas the blue ones had a 1:1 segregation (Table 2). This proves that at least one inserted GBSS-gene is well expressed in both the tubers and microspores of the F1 plants, or that both are active but closely linked.

Transformant B8, a tetraploidised plant with two GBSS-inserts gave a segregation of 3 blue : 1 red microspores indicating that both transgenes were expressed and segregated independently. The tubers of the sexual

offspring showed a segregation of 3:1 with either blue or red starch granules. These fully complemented F1 plants had a segregation of 1:1 or 3:1 in the microspores. It can, therefore, be concluded that in the original transformant both inserts were expressed in tubers as well as in microspores. Their expression was sufficient to complement the mutation when they were present separately as well as together. Introduction of both inserts into the genome occurred after chromosome doubling.
The tetraploidised transformant B14 contained two inserts but in the microspores a 1:1 segregation of blue and red was found. As expected, the F1 offspring had also a 1:1 segregation for plants with complemented or non-complemented starch in the tubers (Table 1). All flowering F1 plants with blue staining starch granules had a 1:1 segregation (Table 2). This indicates that only one of the inserted GBSS-genes is active in both tubers and microspores as was already found in the original transformant or that both transgenes are closely linked. This means that the cells of this transformant were already doubled before transformation occurred.

The level of complementation in the sexual offspring of partially complemented genotypes

Two transformants with a normal complementation in microtubers and a partial complementation in greenhouse grown tubers were used for more detailed analysis. They were selected for their segregation pattern of complementation in the microspores and their ploidy level. The tetraploidised transformant B1, which had produced red, mixed and blue staining tubers under both field and greenhouse conditions, was thought to have the chromosome number doubled after one GBSS-gene was inserted (Table 1). The results of Southern hybridisation (1 insert) and microspore staining (5 blue:1 red) indicated a duplex segregation. This means that two inserts were present at the same position on two homologous chromosomes. Table 1 shows the segregation in the sexual offspring derived from crossing B1 with an *amf*-genotype. Three types of plants with blue, mixed or only red staining starch granules in the tubers were found with a segregation of 5:1 after combining blue and mixed plants in one class. The three phenotypic classes, blue, mixed and red, fit the segregation ratio 4:1:1 assuming that two T-DNA inserts (duplex condition) did cause trans-inactivation of the full

complementation and one insert did not. In order to investigate whether the F1 offspring plants contained one or two inserts, microspores were stained. Due to environmental conditions in the greenhouse only a few plants were flowering. The results presented in Table 2 showed that microspores of F1 plants with a normal complementation had a 1:1 or 5:1 segregation indicating that these plants were either simplex or duplex. Only one of the offspring plants with a decreased complementation flowered and showed a segregation ratio of 1:1. This plant appeared to be simplex with one active GBSS-transgene. These results suggest that in this case trans-inactivation is not dependent on duplex constitution and that the earlier proposed 4:1:1 segregation is not supported. This means that in the F1 offspring unstable complementation is also found in plants with only one T-DNA insert. The fact that a high frequency of fully complemented F1 plants was found indicate that alteration of the genetic background can allow a better expression of transgenes in field grown tubers of both simplex and duplex plants.

The diploid transgenic plant B10 showed such a low degree of complementation in tuber starch that no tubers with a blue staining cut surface were formed under both field and greenhouse conditions. This was in contrast with the staining results from microtubers of this plant, where only blue staining cut surfaces were found. Staining of individual starch granules of greenhouse grown tubers showed a small blue core and a large red outer layer. However, all microspores stained blue, indicating presence and expression of transgenes in all starch containing microspores. Only during late season flowering, a low percentage of microspores with red staining starch was found indicating some instability of expression of one or more transgenes in these cells. Southern hybridisation showed a complex situation (Figure 1). At least seven inserts were observed. In order to investigate the sexual transmission of this transgenic phenotype, B10 was crossed with a diploid *amf*-genotype. Nineteen F1 plants produced tubers (Table 1). These tubers were stained with iodine and a segregation of 12 blue : 7 mixed was found. Southern hybridisation results of several of these F1 plants are shown in Fig. 1. Band a was the endogenous *amf*-allele which was capable of hybridising with the probe as well. Bands c, d, e, g and h were closely linked, as they were all either present or absent.

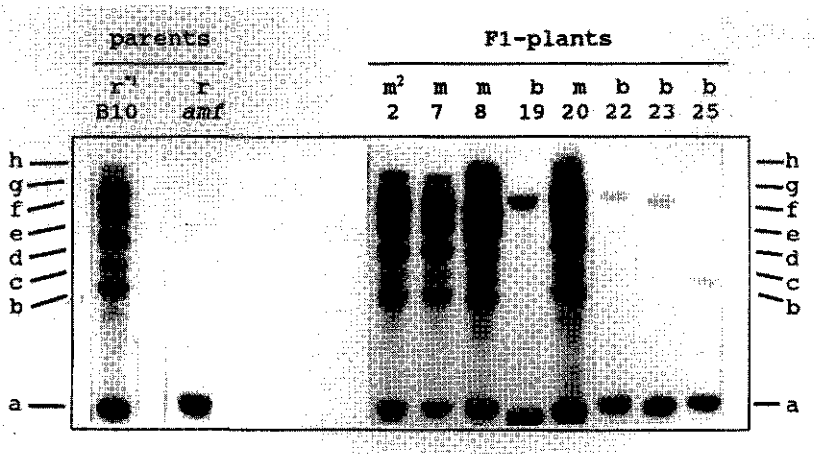

Fig 1. Southern analysis of the partially complemented *amf*-mutant B10, an *amf*-genotype and their progeny after crossing. The DNA was digested with restriction enzyme *Eco*RI. The distal 1.3 kb GBSS cDNA was used as a probe. The bands b and f are independently segregating and can cause full complementation of the *amf*-mutant. No partially complemented plants were found without the inserts b and/or f. By using the NPT II as a probe the same bands were found.
[1]: The partially complemented *amf*-mutant B10 has red staining tubers. The starch granules are red with a small blue core.
[2]: The tubers of several F1 plants have a mixed staining pattern. The starch granules of these mixed F1 plants have a blue core of varying size. No plants with red staining tubers as the original B10 transformant were found among the segregating population.

Bands b and f were segregating independently. The unstable complementation was correlated with the presence of the block of inserts representing bands c, d, e, g and h. This observation and the fact that blue or mixed F1 plants were found to be not deviating from a 1:1 segregation ratio, suggests that this block of transgenes behaves like a single locus suppressing the expression of the loci of the other inserts in tuber tissue. The class of F1 plants with blue staining starch in the tubers appeared to be connected with band(s) b or f in the absence of block c,d,e,g,h. Nine flowering F1 plants were investigated for the segregation of microspores. Indications were found for normal 1:1 and 3:1 segregations, suggesting the presence of one or two independently segregating active inserts. A number of F1 plants showed

large variation in segregation between different samples of different anthers, suggesting that environmental factors are influencing expression of GBSS-transgenes within these cells as well and that these negative environmentally caused effects are less frequently compensated by well expressed inserts.

The level of complementation in a tetraploid amf-mutant

In total, 36 transformants of the diploid *amf* clone 87-1029-31 were investigated (Table 3). Seven of them gave at microtuber level red staining starch and were not complemented. The microtubers of the others stained blue. However, seven of these blue staining clones showed mixed staining greenhouse grown tubers, indicating only partial complementation.

Table 3. Number of transformants in different staining categories after transforming the diploid *amf*-mutant 88-1029-31 and tetraploid *amf*-mutant 90-6009-8 with the construct pWAM 100.

	diploid *amf*		tetraploid *amf*	
	microtubers	greenhouse grown tubers	microtubers	greenhouse grown tubers
red	6	6	2	2
mixed	0	7	0	0
blue	37	30	25	25

Transformants with mixed starch comprised of five normal diploids besides two, which were mitotically doubled. The doubled plants were duplex because integration took place before doubling occurred. A comparison was made between complementation of a diploid and a tetraploid *amf*-clone. Twenty-seven transgenics whose tubers were obtained after transforming the 4x *amf*-clone J90-6009-8 with the wildtype GBSS-gene. Two of them showed absence of complementation whereas the other 25 were fully complemented, not only in microtubers but also in greenhouse grown tubers. This observation clearly shows a difference in frequency of partial complementation between the diploid *amf* 87-1029-31 (24%) and the tetraploid *amf*-clone (<4%).

Discussion

In this study the phenomenon of expression of transgenes in the gametophytic and sporophytic phase of potato development was investigated. Several possibilities involved in the unstable expression of an inserted gene like methylation, sense inhibition, trans-inactivation, read-through of the kanamycin resistance gene or a strong promoter in the plant DNA which is depending on the site of insertion have been discussed earlier by Flipse *et al.* (1994).

The genetic interpretation of all four transformants with normal segregation patterns in the microspores, could be checked in tubers and microspores of F1 plants. All predicted classes for these diploids: --; GBSS-; $GBSS_1$- $GBSS_2$-; and tetraploids: ----; GBSS---; GBSSGBSS-- or $GBSS_1$--- $GBSS_2$--- were found based on segregating microspores of F1 plants.

A percentage of F1 offspring plants originating from B1, with mixed starch in tubers of the parental plants, showed a blue starch colour. The genetic analysis on microspores of a number of these plants showed no indication for the phenomenon of inactivation correlated with tetraploidisation before insertion (duplex situation). It must therefore be suggested that expression of the insert in highly polyploidised cells of fieldgrown tubers of the original transformant B1 is not-optimal. This expression could probably be improved easily after crossing as was evident in several sexual F1 offspring plants. The partly complemented transformant, B10 had F1 in its progeny with microspores segregating into 1:1 or 3:1 ratios which is expected when one or two active inserts are present. The decreased expression in tubers of the F1 offspring derived from B10 was correlated with the presence of a block of five inserts. This block was trans-inactivating the other inserts with normal expression (co-suppression) as could be seen in complemented F1 plants which lacked this block. Only in transformant B10 indications for unstable complementation in microspores were found. Some red staining starch granules were only found in anthers of late season flower buds. In the F1 offspring the variability of segregation was not restricted to late season flower buds. In literature, examples are described in which environmental and developmental factors influence the expression of transgenes (Walter *et al.*, 1992).

It is known that the ploidy level of cells in microtubers is comparable with that of other sporophytic cells (Hovenkamp-Hermelink *et al.*, 1988a).

However, in tubers of glasshouse or field grown plants, where the GBSS promoter is also very active, almost all cells are highly polyploidised up to 16 x, 32 x and 64 x. This situation is found in tubers of diploids as well as tetraploids (Hovenkamp-Hermelink *et al.*, 1988a).

It is expected that expression of transgenes can be different in normal and highly polyploidised cells. A strong indication has been found in the transformant B10. This plant showed almost always normal expression of the wild-type GBSS-transgenes in microspores and microtubers, whereas in field grown tubers with highly polyploidised cells a high degree of inactivation was found. This could be due to or determined by the high sucrose content in microtuber induction medium which stimulates the GBSS promoter activity. The second indication comes from the comparison of the phenotypes found after transformation of diploid and tetraploid *amf*-mutants. The mixed class is only found among diploid and tetraploid trangenics, doubled after the transformation event of the 2x *amf* parental plant and not in transgenics originating from a 4x *amf*-mutant. This could mean that sufficient expression of transgenes in 4x cells is a reliable prediction for expression in tuber cells with a much higher degree of polyploidisation.

The sexual offspring clearly showed that all fully complemented transformants only segregated into two classes i.e. complemented and non-complemented and the partially complemented ones into one class more i.e. mixed.

More research is required to understand the mechanism underlying the partial complementation. The potential use of transgenes in agriculturally important crops, like potato is highly dependent on a predictable transmission of gene expression to the offspring, to enable the use of transgenes in normal plantbreeding programmes.

Chapter 5

GBSS T-DNA inserts giving partial complementation of the *amylose-free* mutant can also cause co-suppression of the endogenous GBSS-gene in a wild-type background.

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Submitted

Abstract

The wild-type gene encoding Granule Bound Starch Synthase (GBSS) is capable of both complementing the *amylose-free* (*amf*) potato mutant and inhibiting the endogenous GBSS-gene expression in wild-type potato. Co-suppression of the endogenous GBSS-gene, easily visualised by staining the starch with iodine, occurred when the full size GBSS sequence (genomic) or GBSS cDNA were introduced into the wild-type potato. Even the mutant *amf*-allele, which yields a protein that cannot be transported over the amyloplast membrane, caused co-suppression, though in a lower frequency. The GBSS promoter alone did not cause co-suppression. The orientation of the GBSS-gene towards the kanamycin resistance gene did not influence the frequency of plants with inhibited expression, as was also the case for the presence of an enhancer in front of the GBSS-promoter. After crossing a partially complemented *amf*-mutant with a homozygous wild-type plant, the F1 offspring segregated into plant phenotypes with normal and decreased expression of the GBSS-gene. Using Southern hybridisation the decreased expression was found to be correlated with the presence of a block of five inserts which was previously shown to be correlated with partial complementation of the *amf*-mutant. This crossing experiment indicates that co-suppression can cause inhibition of gene expression of both inserted and endogenous wild-type GBSS-genes. The frequency of partially complemented *amf*-plants was equal to the frequency of co-suppressed wild-types, when the construct pWAM 101E, with an enhancer in front of the GBSS-promoter, was used. This suggests that partial complementation of the *amf*-genotype caused by unstable expression of the transgene can be overcome by inserting an enhancer in front of the GBSS-promoter.

Introduction

Starch accumulates as a storage carbohydrate in amyloplasts of cells of potato tubers. Its main components are amylose, a linear glucose polymer with $\alpha(1.4)$ bonds and amylopectin, a branched glucose polymer containing $\alpha(1.4)$ and $\alpha(1.6)$ bonds. Formation of amylose is catalysed by Granule Bound Starch Synthase (GBSS) whereas amylopectin is catalysed by Soluble Starch Synthase (SSS) and Branching Enzyme (BE). The latter is responsible for the presence of branching points (Shannon and Garwood, 1984). Stained with an iodine solution, amylose has a blue colour which is dominant over the red colour of amylopectin in wild-type starch. For the starch industry the quality of starches depends on the amylose and amylopectin content.

A decreased amylose content in potato starch can be obtained by mutation induction of the GBSS-gene as in the *amf*-mutant (Hovenkamp-Hermelink *et al.*, 1987) or by antisense genes coding for GBSS as described by Visser *et al.* (1991). Indications for a third possibility were found during complementation studies of the *amf*-mutant with a wild-type GBSS-gene of potato (van der Leij *et al.*, 1991a; Flipse *et al.*, 1994). When complementing the potato *amf*-mutant, transformants were found with normal and partial complementation. The sexual progeny of partially complemented plants, in which T-DNA inserts with different effects could be seperated, consisted of partially and fully complemented plants. Clear indications for an interaction between different T-DNA inserts were found. So was the expression of certain inserts, showing the ability to fully complement in the presence of other GBSS-inserts, decreased (This thesis; Chapter 4).

The phenomenon of inhibition caused by T-DNA inserts containing sense genes is called co-suppression and has been described in many other examples in wild-types but not in complemented mutants. In the present study, a partially complemented *amf*-mutant was crossed with a wild-type potato, in order to investigate the effect of co-suppression on complementation in the presence of an endogenous wild-type GBSS-allele. Simultaneously with the complementation experiments (Flipse *et al.*, 1994), the cloned wild-type GBSS-gene was introduced in a wild-type potato genotype. The characteristics of these transgenic plants and those resulting from transformation with other GBSS-sequences containing constructs will be outlined.

Material and methods.

Plant material

For transformation experiments stem segments were used from the diploid (2n = 2x = 24) homozygous *amfamf* genotype 87.1029-31 and the diploid wild-type AmfAmf genotype R5 of *Solanum tuberosum* L. The mutant 87.1029-31 derived from crossing the original *amf*-mutant 86.040 and two different diploid wild-types. Crosses were made in order to improve the fertility and tuber production of the original mutant (Jacobsen *et al.*, 1989). Plants were grown in jars containing MS medium (Murashige and Skoog, 1962) with 30 g/l sucrose (MS 30) and 8 % agar, at 22 °C and 16 h light. Trangenic shoots were grown and rooted on MS 30 medium containing 100 mg/l kanamycin. Ploidy level was determined by counting the number of chloroplasts in stomatal guard cells (Frandsen, 1968).

Vectors and transformation procedure

The constructs pWAM 100, pWAM 101, pWAM 101E, pGB 60, pAMF 110 and pGB 121s were used in the experiments (Fig. 1). The constructs pWAM 100, pWAM 101 and pWAM 101E possessed the genomic wild-type GBSS sequence driven by its own promoter. For pWAM 100 the GBSS gene was placed in opposite orientation to the NPT II gene coding for kanamycin resistance in plants (van der Leij *et al.*, 1991a). For pWAM 101 (van der Leij *et al.*, 1991a) and pWAM 101E the GBSS-gene was placed in the same orientation as the NPT II gene. The construct pWAM 101E was formed by ligating the 0.7 kb enhancer fragment of the CaMV-promoter in the *Xbal* - *Sall* restriction site in front of the GBSS-promoter. The construct pGB60 contained the GBSS cDNA driven by the CaMV-promoter (Visser *et al.*, 1991). The construct pAMF 110 contained the *amf*-allele (mutant GBSS) driven by its own promoter (van der Leij *et al.*, 1991b), whereas pGB 121s contained solely the GBSS promoter.

The constructs were transferred to *A. tumefaciens* LBA 4404 using the direct transformation procedure described by Höfgen and Willmitzer (1988). To test the integration procedure of the mobilized plasmid into *A. tumefaciens*, plasmid DNA, isolated from *A. tumefaciens* was analyzed (Holmes and Quigly, 1981).

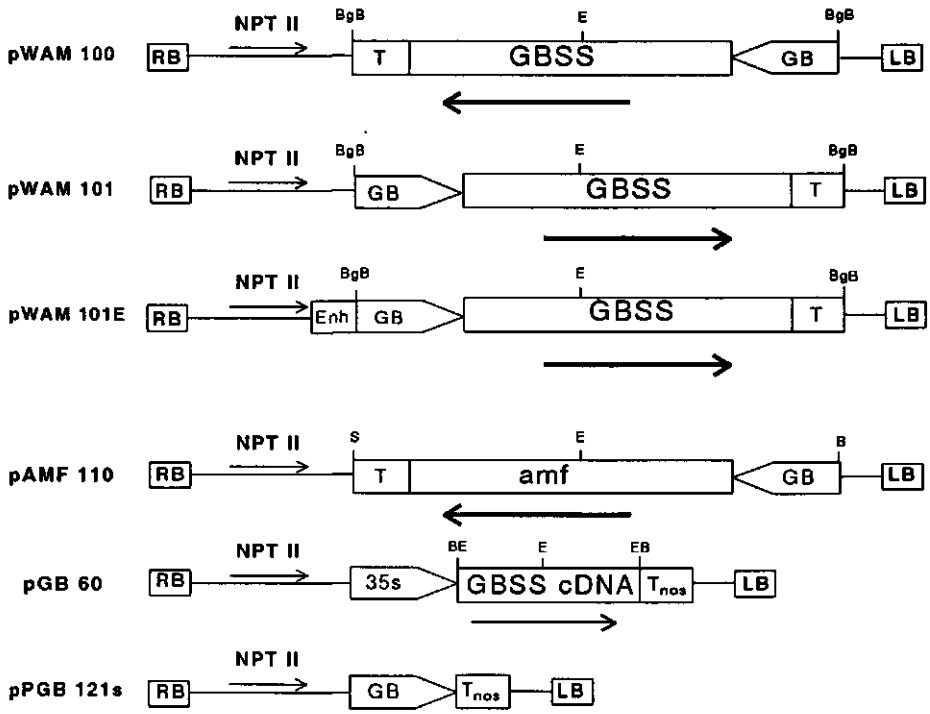


Fig. 1. The constructs pWAM 100, pWAM 101 and pWAM 101E possess the genomic wild-type GBSS sequence driven by its own promoter. For pWAM 100 the GBSS gene was placed in opposite orientation to the NPT II gene coding for kanamycin resistance in plants (van der Leij *et al.*, 1991a). For pWAM 101 (van der Leij *et al.*, 1991a) and pWAM 101E the GBSS-gene (6.5 kb) was placed in the same orientation as the NPT II gene. The construct pWAM 101E was formed by ligating the 0.7 kb enhancer fragment of the CaMV-promoter in the *Xbal* - *Sall* restriction site in front of the GBSS-promoter. The construct pGB 60 contains the GBSS-cDNA (2.3 kb) driven by the CaMV-promoter (Visser *et al.*, 1991). The construct pAMF 110 contains the *amf* allele (mutant GBSS) driven by its own promoter (van der Leij *et al.* 1991b), whereas pPGB 121s contains solely the GBSS-promoter. amf = *amf*-allele; B = *Bam*HI; Bg = *Bgl*II; E = *Eco*RI; GB = GBSS-promoter; GBSS = genomic GBSS-sequence; LB = left border; NPT II = neomycin phosphotransferase; RB = right border; S = *Sst*I; T = GBSS-terminator; T_{nos} = nopalinesynthase terminator; 35S = 35s CaMV promoter

For the control experiments *A. tumefaciens* strain AM 8706 was used harbouring a binary plasmid containing the gene encoding ß-glucuronidase and the gene encoding kanamycin resistance (Visser *et al.*, 1989a). Transformation was performed as described by Flipse *et al.* (1994).

Determination of the starch composition and GBSS-activity

The cut surfaces of harvested tubers were stained and starch was isolated from several tubers according to Kuipers *et al.* (1991). The colour of the individual starch granules was investigated as described by Flipse *et al.* (1994). The amylose content was determined spectrophotometrically as described by Hovenkamp-Hermelink *et al.* (1988b) and the determination of GBSS-activity was as described by Vos-Scheperkeuter *et al.* (1986). For measuring the amylose content, three samples, and for measuring the GBSS-activity, six samples were analyzed and a t-test with a 5 % confidence limit was used for statistical analysis.

Crossings

The partially complemented genotype B10 originated from the diploid *amf*-mutant 87.1029-31 after transformation with the construct pWAM 100 (Flipse *et al.*, 1994; This thesis; Chapter 4). Although the cut surfaces of greenhouse grown tubers were red when stained with iodine, a small blue core was observed in the starch granules indicating partial complementation. In order to get flowers, B10 was grown in a biosafety greenhouse in which flowering of transgenic potato plants was allowed in containers. Flower buds were emasculated and pollinated with the wildtype diploid SHU3711 1-3 days later.

Embryo rescue

Three weeks after pollination embryos were collected and placed on embryo growth medium (Neal and Topolewski, 1983; 1985). They were transferred to fresh medium every fortnight, until outgrowth of the embryos occurred. The seedlings were transferred to MS 30 medium. After two to three months plants were transferred to the greenhouse for flowering and tuberisation.

Molecular analysis of the transformants

DNA was extracted from the leaves of greenhouse-grown plants transformed with the construct pWAM 100 according to Dellaporta *et al.* (1983). The DNA was digested with the restriction enzyme *Eco*RI. Using the ^{32}P-random-prime-labelled distal 1.3 kb of the GBSS cDNA and NPT II gene as a probe, the minimum number of integrated T-DNA copies were estimated by scoring the number of additional bands in a Southern blot (van der Leij *et al.*, 1991a).

RNA was extracted from tubers according to Salehuzzaman *et al.* (1992). Equal amounts of RNA (checked by hybridisation with potato 28*s*rDNA as a probe) were loaded to allow a comparison between different transformants. The RNA was hybridised with a 2.4 kb GBSS cDNA. DNA and RNA blot-hybridisation and labelling were performed as described previously (Visser *et al.*, 1989 a,b).

Results

Phenotypical and biochemical observations on starch

After transforming the diploid wild-type R5 with the construct pWAM 100, thirty kanamycin resistant shoots were isolated on selective medium, multiplied and transferred to the greenhouse. Starch was isolated from harvested tubers of each of the transgenics and analyzed for GBSS-activity and amylose content. The GBSS-activity of most of these transformants was similar or increased as compared to the untransformed and transformed controls. This increase in GBSS-activity could, however, not account for an increased amylose content, which was still similar to that of the controls (Fig. 2A,B).

For the three transgenics, A4, A21 and A40, the GBSS-activity was highly decreased compared to the controls (Fig. 2A). This was in two cases clearly correlated with a reduction in amylose content to 5-7% (Fig. 2B).

As expected, in the case of clearly reduced GBSS-activity and amylose content, the colour of cut tuber surfaces and of individual starch granules after staining with iodine was altered. The transformants with normal amylose content had, as expected, blue staining starch in the tubers.

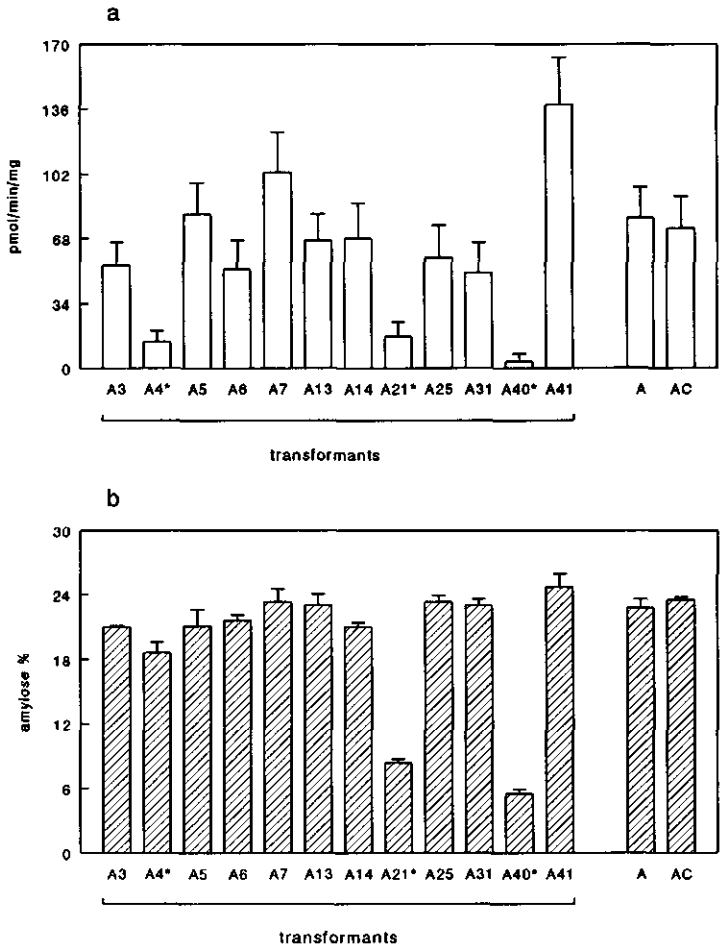


Fig. 2. The GBSS-activity (a) and amylose content (b) of the untransformed wild-type R5 (here named A), the transformed control AC and several transformants after introduction of the construct pWAM 100, containing the GBSS-gene, in the wild-type R5. The * marked genotypes have mixed or red staining tubers and starch granules with a blue core and red outer layer and are therefore considered co-suppressed.

The three transgenics had a starch colour of the tuber which was dependent on the level of reduction in amylose content of that specific tuber sector. The stained cut surfaces were red for A40, red to mixed for A21 and mixed to blue for A4. The presence of red and blue staining sectors causing the mixed pattern was related to the staining pattern of individual starch gran-

presence of a small blue core surrounded by a large red outer layer whereas in blue staining tuber sectors the size of the blue core was increased up to granules with only a small red outer layer.
Although we can state that a reduction in amylose content was found in the tubers of these transformants no indication of such a reduced amylose content, based on iodine staining was found in the stomatal guard cells and roottips. This result was comparable with the observations on transformants containing the antisense oriented GBSS-gene.

A reduced amylose content is correlated with a reduced GBSS-gene expression

Steady state mRNA was isolated from tubers, blotted and hybridised with a 2.4 kb GBSS-cDNA. Transgenic wild-type plants with a decrease in GBSS-activity and amylose content showed a reduced amount of steady state GBSS mRNA as compared to either the control or the transgenic plants without inhibition (Fig. 3). The reduced amylose content was also correlated with a reduced amount of GBSS-protein in the starch fraction (data not shown).

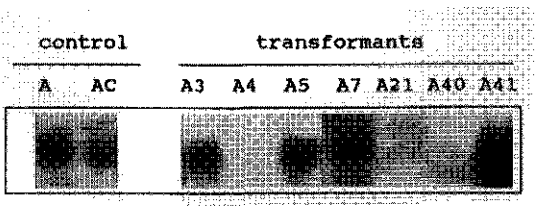


Fig 3. The steady state GBSS mRNA level of the control R5 (here named A), the transformed control (AC) and a selection of the with pWAM 100 transformed R5 plants. The RNA was hybridised with a 2.4 kb GBSS cDNA as a probe.

The introduction of additional copies of the GBSS-gene infrequently inhibited the expression of the endogenous GBSS-gene causing a reduced mRNA content finally resulting in a lower amylose content. The average number of additional inserts for plants with a reduced amylose content was higher (3.7) than it was for plants without inhibition (1.6) (data not shown).

Co-suppression in transgenic potato plants using different constructs

Several constructs containing either the full length GBSS (genomic) sequence, the *amf*-sequence, the GBSS-cDNA, or only the GBSS-promoter (Fig 1) were analyzed for their capacity to inhibit the expression of the endogenous GBSS-gene in a wild-type potato clone and partly for their capacity to complement an *amf*-mutant. Per construct-genotype combination at least 22 kanamycin resistant shoots were selected, multiplied and transferred to the greenhouse. All transgenic clones were screened for the expression of the inserted GBSS-genes by staining the cut surfaces of tubers with iodine. Based on the colour of the tuber and the individual starch granules, the transgenic *amf* and wild-type clones were classified into red (total red staining starch granules), red* (red staining tuber with red staining starch granules containing a small blue core), mixed (mixed staining pattern of the tubers with red starch granules containing a blue core of varying size) and blue (total blue starch granules) (Table 1A, B).

All three constructs with full length genomic GBSS-sequence were capable of complementing the *amf*-mutant (Table 1A). In pWAM 100 the GBSS-gene is located opposite towards the kanamycin resistance (Kan') gene. This creates the possibility that the reading through of Kan' leads to the formation of antisense GBSS RNA. However, this does not seem to be important for the partial complementation, because unexpectedly, the construct pWAM 101 results in even a higher frequency of plants (48 % instead of 16 %) with partial complementation. The lower frequency of plants with no or a reduced expression, using pWAM 101E is expected to be caused by the enhancer which is placed in front of the GBSS-promoter.

Introduction of the four constructs containing either the full length GBSS-gene with its own promoter or the GBSS-cDNA with CaMV promoter in a wild-type plant (Table 1B) resulted sometimes in transgenics with mixed staining tubers. This reduced amount of amylose content was found in 10 % of the transgenics. The position of the GBSS-gene towards the Kan'-gene and the presence of an enhancer in pWAM 101E had no influence on the frequency of such transgenic plants. Forty-five transgenics were selected after transformation with the construct pAMF 110. Although this mutant *amf*-allele is not able of catalysing the formation of amylose in the amyloplasts, mRNA can be formed leading to the synthesis of GBSS-protein in the soluble fraction, which cannot be transported into the amyloplasts.

Table 1. Number of transformants in different categories according to the constructs used and the colour of the tubers and starch granules. For a description of the constructs see material and methods and Fig. 1. Table 1A shows the results of complementing the *amf*-mutant 87-1029-31, by which four classes of transgenic plants were formed. Table 1B shows the inhibition effect on the GBSS-gene expression when different constructs are introduced in the wildtype R5. ND = not determined.

A) *amf*-mutant

		non complemented	fully complemented	partially complemented		
construct	total	red[1]	blue[2]	red*[3]	mixed[4]	total
pWAM 100	43	6 (14%)	30 (70%)	1 (2%)	6 (14%)	7 (16%)
pWAM 101	23	1 (4%)	11 (48%)	2 (9%)	9 (39%)	11 (48%)
pWAM 101E	22	0 (0%)	20 (91%)	0 (0%)	2 (9%)	2 (9%)
pGB 60			ND			
pAMF 110			ND			
pPGB 121s			ND			

B) wild-type

		non-inhibited	inhibited		
construct	total	blue[2]	red*[3]	mixed[4]	total
pWAM 100	30	27 (90%)	1 (3%)	2 (7%)	3 (10%)
pWAM 101	37	33 (90%)	1 (3%)	3 (7%)	4 (10%)
pWAM 101E	51	46 (90%)	1 (2%)	4 (8%)	5 (10%)
pGB 60	45	39 (87%)	2 (4%)	4 (9%)	6 (13%)
pAMF 110	45	44 (98%)	0 (0%)	1 (2%)	1 (2%)
pPGB 121s	47	47 (100%)	0 (0%)	0 (0%)	0 (0%)

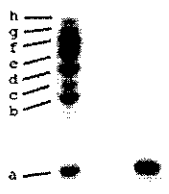
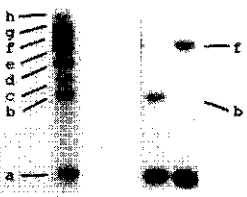
[1]: red staining tubers; starch granules completely red.
[2]: blue staining tubers; starch granules completely blue.
[3]: red* staining tubers; starch granules red with small blue core.
[4]: mixed staining tubers; starch granules red with blue core of varying size.

Iodine staining of tubers showed one transgenic with reduced amylose con tent. When only the GBSS-promoter (pGB 121s) was inserted none of the 47 plants had an inhibited GBSS-gene expression. This indicates that the coding part of the GBSS-gene is needed for co-suppression. It was found for all constructs except for pAMF 110 that plants showing co-suppression contained multiple inserts (data not shown).

Inhibition of an endogenous GBSS-gene by transgenes involved in partial complementation of the amf-mutant

The diploid transformant B10 was partially complemented for the *amf*-mutation (Flipse *et al.*, 1994). It contained at least seven GBSS-inserts. This plant was crossed with the diploid wild-type SUH3711. Embryo's were isolated, grown on embryo outgrowth medium and transferred to the green-house. Finally, tubers of fifty-three F1 offspring plants were screened for their starch composition by staining with iodine (Table 2).

Table 2. The segregation in mixed and blue staining phenotypes in the F1 progeny of the partially complemented *amf*-mutant B10 after crossing with the wild-type SUH3711. The DNA was restricted with the restriction enzyme *Eco*RI. The distal 1.3 kb GBSS cDNA was used as a probe. When the NPT II gene was used as a probe the same bands were found. Band a is the endogenous band of 4.3 kb. The sizes of the other bands are: b: 6.2 kb, c: 6.7 kb, d: 7.5 kb, e: 9.2 kb, f: 11.2 kb, g: 13.6 kb and h: ca. 17 kb

	Control		F1-progeny of B10	
clone	B10	SHU3711	-	
number of plants	1	1	28	25
starch colour	red[1]	blue	mixed[2]	blue
Southern				

[1]: The partially complemented *amf*-mutant B10 has red staining tubers. The starch granules are red with a small blue core.
[2]: The tubers of the F1-progeny have a mixed staining pattern. No plants with red staining starch as the original B10 transformant were found among the F1 segregating population. The starch granules are red with a blue core of varying size, in all cases the blue core is larger than in the starch granules of the original B10 transformant.

Normally, after crossing a non-transgenic *amf*-mutant with a wild-type, all F1 plants contain blue staining starch, because the wild-type allele is dominant. In the crossing with B10, twenty-five F1 plants had tubers with the expected blue staining cut surface. However, twenty-eight F1-plants had mixed staining tubers. The starch colour of the F1-plants differ from the original transformant B10 which had red staining tubers. The partial complementation in the F1-offspring after crossing B10 with an *amf*-mutant (This thesis; Chapter 4) was correlated with the presence of a block of five inserts. In order to estimate the number of inserts here we isolated DNA from leaves. Southern hybridisation results of several of these F1 plants are shown in Table 2.

Band a was the endogenous *amf*/wild-type GBSS-allele. Bands c, d, e, g and h were closely linked, as they were all present or absent. Bands b, and f were segregating independently. Table 2 clearly shows that also in this case the inhibition of the endogenous wild-type GBSS-gene was correlated with the presence of the block of five inserts, which probably acts as a single locus.

Discussion

Increased GBSS-activity gives only wild-type levels of amylose

Introduction of additional copies of the GBSS-gene into a wild-type potato did not increase the amylose content. Although, it is clear that the presence of amylose was correlated with the GBSS-activity, an increase in GBSS-activity above a certain level could not account for more amylose. The positive correlation between GBSS-activity and amylose content was not linear but led to a maximum amount of amylose as was already obtained in wild-type plants. This phenomenon was observed and described in earlier situations with complemented *amf*-plants (Flipse *et al.*, 1994) and in a GBSS gene-dosage population (This thesis; Chapter 2). Carbohydrate synthesis is a complex process, in which different enzymes are involved in the formation of several products. It is likely to assume that one of these enzymes or intermediate products are becoming limited for amylose production instead of the GBSS enzyme. Kuipers *et al.* (1994) found that the amylose content of the

blue cores of starch granules from antisense GBSS-plants was similar to that of wild-type starch and so the amylose content of starch granules was correlated with the size of the blue core in the starch granules. This strongly suggests that only a restricted amount of amylose can be stored in the starch granules. This assumption is supported by the findings of Jane *et al.* (1992) that the amylose molecules are interspersed among the radially arranged amylopectin molecules. We suggest that an increase in amylose content above wild-type levels is only possible when more amylose can be stored in the granules due to a change in the crystalline structure of the amylopectin molecules or when the granule size increases.

Co-suppression of the endogenous GBSS-allele

An inhibition of the endogenous GBSS-allele was found in several transgenic plants containing additional GBSS sequences. The inhibition, resulting in a changed starch composition, could easily be visualised and was correlated with a loss in GBSS-activity and a lower level of steady state GBSS mRNA and GBSS protein present in the starch granules. It is, therefore, conceivable that the transcription of the endogenous GBSS-allele was affected. In several reports the phenomenon of co-suppression of plant genes has been described (Napoli *et al.*, 1990; van der Krol *et al.*, 1990; Smith *et al.*, 1990) and possible mechanisms causing co-suppression have been put forward (reviewed by Jorgensen, 1990; Matzke *et al.*, 1994; Mol *et al.*, 1994), like: inactivation of genes by DNA methylation introduced by the presence of homologous DNA sequences (Matzke *et al.*, 1989; Hobbs *et al.*, 1990; Linn *et al.*, 1990) and allelic interactions which change the gene expression. It has been suggested that co-suppression is caused by antisense RNA, transcribed from the introduced gene (Grierson *et al.*, 1991). This antisense RNA can be formed when the T-DNA is integrated close to a plant promoter, or alternatively the promoter of the sense gene converges with the promoter of the antibiotic resistance gene in the T-DNA. A duplex formation between sense and antisense RNA should explain the gene inhibition.

In case of co-suppression of the GBSS-allele the position of the sense gene towards the Kan^r gene appeared not to influence the frequency of co-suppression. The fact that the *amf*-allele (pAMF 110) is capable of inhibiting the GBSS-gene expression suggests that the presence of GBSS mRNA and

perhaps GBSS protein in the soluble fraction is sufficient for the gene inhibition. The observation that the frequency of plants with co-suppression is lower when the *amf*-allele than when wild-type GBSS sequences are used might suggest that co-suppression is not caused by one mechanism, in which the GBSS mRNA plays a role, but that also an interaction with GBSS protein in the starch granules can be involved. The fact that no co-suppression was found when solely the GBSS promoter is inserted might support the idea that for co-suppression at least a part of the transcriptional sequence must be present.

Inserts giving partial complementation of the amf-mutant can also cause co-suppression of the endogenous wild-type GBSS-gene

The crossing experiment of the partially complemented transformant B10 with an *amf* and a wild-type respectively showed that the block of five inserts is capable of inhibiting the expression of introduced wild-type GBSS-inserts (This thesis, Chapter 4) as well as the endogenous wild-type GBSS-alleles (Table 2). One can therefore propose that co-suppression is in part responsible for partial complementation, something which has not been described before.

The constructs pWAM 100, pWAM 101 and pWAM 101E caused co-suppression in 10 % of the wild-types. This frequency is equal to the 10 % of the partially complemented *amf*-genotypes using the construct pWAM 101E. This suggests that the partial complementation with pWAM 101E is mainly due to a co-suppression like phenomenon whereas the partial complementation after introducing pWAM 100 and pWAM 101 (which is the case in more than 10 % of the transgenics) is due to co-suppression or a low expression of the transgenes. The latter can be overcome by using an enhancer in front of the promoter.

A comparison between co-suppression, antisense inhibition and complementation

The fact that co-suppression only occurred in 10 % of the transgenics and that this percentage was equal for all four constructs with GBSS sequences makes co-suppression an interesting phenomenon. The reason for variation

in inhibition of the gene expression between tubers and tuber sectors is unclear but was also observed in flowers of petunia (van der Krol *et al.*, 1990). The phenotype of co-suppressed plants was very similar to the phenotype of transgenics resulting from antisense GBSS inhibition experiments using wild-type genotypes (Visser *et al.*, 1991; Kuipers *et al.*, 1994) and experiments in which *amf*-mutants were partly complemented (van der Leij *et al.*, 1991a; Flipse *et al.*, 1994). The inhibited GBSS gene-expression in the transgenic wild-type led to the production of mixed staining starch in greenhouse grown tubers. The starch granules possessed a blue core and red outer layer as in antisense inhibited and in partially complemented plants. The steady state GBSS mRNA level was reduced in plants with an inhibited amylose production caused by either sense (this research) or antisense (Kuipers *et al.*, 1991) constructs and was also observed in the red staining tuber parts of a partially complemented *amf*-plant (Flipse *et al.*, 1994).
The frequency of wild-type plants showing sense inhibition using the full lenght GBSS-DNA and GBSS-promoter was lower (10 %) compared to antisense inhibition (52 %; Kuipers *et al.*, 1995b). The frequency of plants with a complete inhibition was similar (3 %) as was its positive correlation with the number of T-DNA inserts. The stability of inhibition and the inheritance should be investigated in field trials and crossing studies to determine whether sense or antisense inhibition of the GBSS-gene in potato is the most promising approach for commercial purposes.

Chapter 6

Introduction of sense and antisense cDNA for Branching Enzyme in the *amylose-free* potato mutant leads to physico-chemical changes in the starch.

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Abstract

One isoform of the Branching Enzyme (BE; EC 2.4.1.18) of potato (*Solanum tuberosum* L.) is known and catalyses the formation of α-1.6 bonds in a glucan chain, resulting in the branched starch component amylopectin. Constructs, containing the antisense and sense orientated distal 1.5 kb part of a cDNA for potato BE, were used to transform the, with iodine, red staining *amf*-mutant. The expression of the endogenous BE-gene was inhibited either largely or fully as judged by the decrease or absence of the BE mRNA and protein. This resulted in a low percentage of starch granules with a small blue core and large red outer layer. There was no effect on the amylose content, branching degree and λ_{max} of the starch. However, when the physico-chemical properties of the different starch suspensions were assessed, small differences were observed. Though small, these differences indicated that starch in the transformants was different from that of the *amf*-mutant.

Introduction

Starch is the major form of carbohydrates stored in plants and consists for 20-25 % of amylose and 75-80 % of amylopectin. Amylose is an essentially linear polymer, consisting of α(1.4)-linked α-D-glucopyranosyl units. Amylopectin is a branched polymer of α-D-glucopyranosyl units linked by α(1.4) bonds with branches resulting from α(1.6)-linkages (Shannon and Garwood, 1984).

Mutants with an altered starch composition are known. They lack the activity of at least one of the several enzymes involved in starch synthesis. These mutants are very important for understanding the role of these enzymes. For instance the *waxy* maize (Echt and Schwartz, 1981) and *amylose-free* (*amf*) potato mutant (Hovenkamp-Hermelink *et al.*, 1987) have starch granules with almost 100 % amylopectin and lack Granule Bound Starch Synthase (GBSS) activity. *Amf*-starch can be easily distinguished from wild-type starch by the red colour when starch is stained with an iodine-potassium-iodine solution (Shannon and Garwood, 1984).

Amylopectin is produced by the interaction of two enzymes; Soluble Starch Synthase (SSS) and Branching Enzyme (BE). The latter is capable of hydrolysing an α(1.4)-bond of a glucan chain and linking the separated chain segment to an acceptor chain via an α(1.6)-bond. Although multiple forms of (BE) have been found in maize (BE I, BE IIa and BE IIb; Boyer and Preiss, 1978) and pea (BE I and BE II; Smith, 1988), only one subunit could be identified in potato (Drummond *et al.*, 1972; Borovsky *et al.*, 1975; Vos-Scheperkeuter *et al.*, 1989), despite intensive research. This potato enzyme is closely related to the BE I of maize and the BE II of pea (Salehuzzaman *et al.*, 1992).

Several mutants like the *amylose-extender* (BE IIb) and *dull* (BE IIa) of maize and the *wrinkled* pea (BE I) have an increased amylose content (Hedman and Boyer, 1982; Edwards *et al.*, 1988), due to the lack of one of the multiple forms of the branching enzyme (Boyer and Preiss, 1981; Smith, 1988). These mutants have a decreased starch and dry weight production and an increased sugar content compared to wildtype seeds. This starch has, as expected, a blue colour, when stained with an iodine solution. No mutants are known for the other isoforms of the BE in maize and pea. Crossing the red colouring *waxy* (*wx*) maize mutant with the blue colouring *amylose-extender* (*ae*) resulted in a double mutant *aewx* plant with blueish staining

starch. This *aewx* starch consists solely of loosely branched amylopectin with long external chains (Boyer *et al.*, 1976).

In potato no mutation for BE is known and the influence of this enzyme on the starch composition in potato is, therefore, unknown. The fact that BE is probably present in only one isoform and that the cDNA coding for this BE has been cloned (Kossmann *et al.*, 1991) makes the antisense RNA technique very attractive for effectively decreasing or inhibiting the BE expression and to determine its role in starch biosynthesis. This antisense procedure has been used succesfully for other genes (van der Krol *et al.*, 1988; Smith *et al.*, 1988; Visser *et al.*, 1991). Other studies have shown that also introduced "sense" genes can interact with the homologous genes in the plant, leading to a decreased expression of both genes (Napoli *et al.*, 1990; van der Krol *et al.*, 1990; Smith *et al.*, 1990). This phenomenon is called co-suppression.

In this paper, experiments are described to create transformants with a decreased expression of the gene for BE in order to study its function in carbohydrate synthesis. In an attempt to create a situation comparable with that of the *aewx* maize mutant, an *amf*-potato genotype was transformed with *Agrobacterium tumefaciens* containing the distal 2.8 or 1.5 kb cDNA of BE in sense and antisense orientation. A decreased expression of the BE-gene in an *amf*-background of potato should be expected to produce loosely branched amylopectin, staining blue with iodine. To test this hypothesis, the starch of transformants was stained with iodine and analysed for its physicochemical properties.

Materials and methods

Construction of binary plasmids

A partial potato BE clone of 1.5 kb and 2.8 kb cDNA isolated by screening a phage lambda ZAP II potato tuber cDNA library (Kossmann *et al.*, 1991) was used to clone in the binary vector pROK-1 (Baulcombe *et al.*, 1986). The cDNA was digested with *Eco*RI and cloned into the *Eco*RI site of pMTL 25 (Chambers *et al.*, 1988), digested out of the plasmid pMTL 25 by *Bam*HI and cloned into

the *Bam*HI site of pVU 1012, resulting in the recombinant plasmids pCVE 1.5 A (antisense) and pCVE 1.5 B (sense) with the CaMV promoter in front of the cDNA (Fig. 1). The pCVE 1.5 and 2.8 plasmids were transformed into *Agrobacterium rhizogenes* LBA 1333 by the direct transformation procedure as described by Höfgen and Willmitzer (1988). The *A. rhizogenes* strain AM 8703 containing pBI 121 was used as a control strain (Visser *et al.*, 1989b). Mobilization of the pCVE 1.5 and 2.8 plasmids to *A. tumefaciens* LBA 4404 was done with the helper plasmid pRK 2013 (Ditta *et al.*, 1980). Here the *A. tumefaciens* strain AM 8706 was used as a control. To test the integration of the mobilized plasmids into the *Agrobacterium* strains, isolated plasmid DNA was analyzed (Holmes and Quigley, 1981).

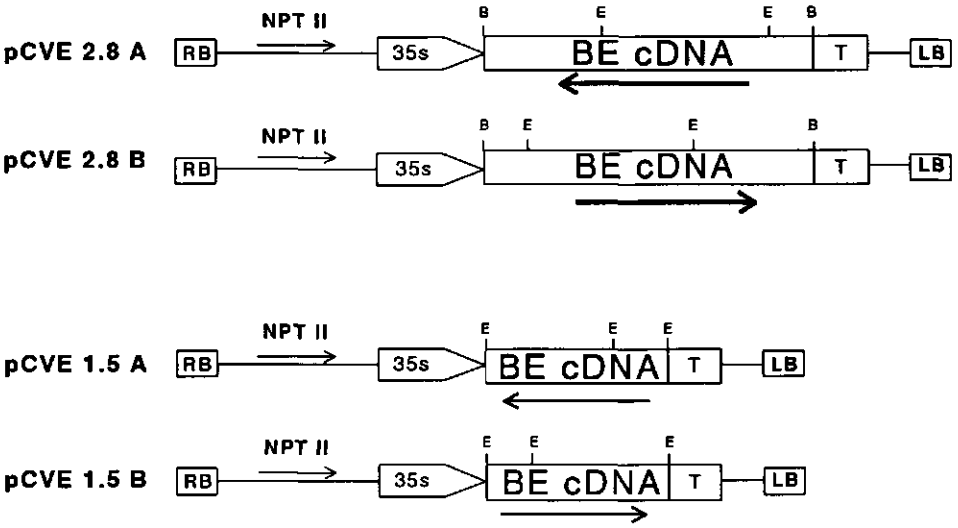

Fig. 1. Constructions of antisense and sense genes based on the 3.1 kb full lenght BE cDNA. pCVE 1.5 A = antisense 1.5 kb cDNA; pCVE 1.5 B = sense 1.5 kb cDNA; pCVE 2.8 A = antisense 2.8 kb cDNA; pCVE 2.8 B = sense 2.8 kb cDNA; B = *Bam*HI; E = *Eco*RI = I; LB = left border; NPT II = neomycin phosphotransferase; RB = right border; T = nopalinesynthase terminator; 35S = 35S CaMV promoter.

Plant material

Stem segments, without axillary buds, of a diploid (2n = 2x = 24) homozygous *amf*-mutant genotype 87.1029-31 of *Solanum tuberosum* L. were used for transformation experiments. This mutant was the result of a crossing program between the original *amf*-mutant 86.040 and two different diploid wildtypes (Jacobsen *et al.*, 1989). Plant growth, transformation with *A. tumefaciens* and microtuber induction was as described by Flipse *et al.* (1994), transformation with *A. rhizogenes* as described by Visser *et al.* (1989c).

Determination of starch characteristics

Screening of hairy roots for the presence of amylose was performed by staining the root tips with a freshly prepared mixture of chloralhydrate (2 mg in 2 ml water) and Lugol's solution (iodine-potassium-iodine) in a ratio of 1:3 (v/v). Starch granules were stained as described by Flipse *et al.* (1994). The cut tubers were stained and starch was isolated according to the method described by Kuipers *et al.* (1991). Amylose content was determined as described by Hovenkamp-Hermelink *et al.* (1988b). The Luff-Schoorl method was used to measure the branching degree of the starch (Schoorl, 1925). For this purpose 100 mg of starch was diluted in water and cooked until a clear solution was obtained. This soluble starch was debranched by adding 2 drops of 0.1 N HCl and 10 mg isoamylase and incubated at 40°C for 2 h. To determine the amount of reduced ends 8.3 ml Luff-Schoorl solution (50 $g \cdot l^{-1}$ citric acid; 25 $g \cdot l^{-1}$ cupric sulphate; 143.8 $g \cdot l^{-1}$ $CaCO_3$) was added, the mixture was boiled for exactly 10 min and quickly cooled. To the solution 3.3 ml KI, 6.7 ml 5 N H_2SO_4 and 1.7 ml starch solution (0.5 g hydrolysed starch in 10 ml water plus 40 ml boiled water) was added and the mixture was titrated with 0.05 N thiosulphate until a pink colour appeared. The used amount of thiosulphate was a measure for the amount of reducing ends. The branching degree of the starch was determined by searching for the correct values in the Luff-Schoorl table.

Starch and sugar content were determined as described by Kuipers *et al.* (1995a).

Western analysis

Western analysis was done using the PhastSystem procedure. To examine the presence of BE protein in the soluble fraction, tubers were pressed and 50 μl of

supernatant was diluted with 50 μl sample-buffer with a final concentration of 20 mM Tris·HCl pH 8.0, 2 mM EDTA, 20 % glycerol, 2 % SDS, 0.002 % bromophenolblue and 10 % ß-mercaptoethanol and boiled for 2 min. Denyer *et al.* (1993) have proven, in a study with developing pea embryos that both BE isoforms are not only present in the soluble fraction, but also tightly bound to the starch granules. To examine the presence of BE protein in the starch fraction, a starch suspension of 50 mg·ml^{-1} sample-buffer was boiled for 2 min and centrifuged (for 5 min at 10.000 rpm in an eppendorf centrifuge. The supernatant was 10 times concentrated by placing in vacuum inside an exicator. From each sample of the soluble or granule fraction, 4 μl was analyzed on 12 % polyacrylamide gels which were provided with the system. Blotting on nitrocellulose was done in the PhastSystem for 15 min by 20 V using transfer buffer (25 mM Tris, 0.2 M glycine, 200 ml methanol in 1 l). After blocking for at least 1 h with 3 % gelatine in TBS (20 mM Tris·HCl; pH 7, 0.5 M NaCl in 2.5 l) the blots were washed twice for 5 min in TTBS (TBS with 0.05 % Tween). After hybridising the blots with an antiserum raised against a denatured preparation of potato BE (Vos-Scheperkeuter *et al.*, 1989) the blots were washed again in TTBS. Alkaline phosphatase was used as a second antibody and the antigens were detected by incubating the blots for 30 min in the dark in 100 ml buffer (0.1 M $NaHCO_3$ and 1 mM $MgCl_2$, pH 9.8) with 200 μl NBT (4-nitro blue tetrazolium chloride; 30 mg·ml^{-1} solution of 7 demethylformamide: 3 H_2O) and 200 μl BCIP (5-bromo-4-chloro-3-indolyl-phosphate; 15 mg·ml^{-1} in demethylformamide). The reaction was stopped with water.

Molecular analysis

The RNA was extracted from tubers as described by Salehuzzaman *et al.* (1992). Equal amounts of RNA were loaded to allow a comparison between different transformants. The RNA was hybridised with the full length cDNA of the branching enzyme. RNA blot hybridisation and labelling were performed as described by Visser *et al.* (1989a,b).

Starch granule size determination

Starch granule size distribution was determined of a starch-water solution, using a Coulter Laser LS 130 particle size analyzer. The determination with the Coulter Laser is based on analyses of the forward light scattering by the

particles. The patterns were converted into particle size distributions by use of Fraunhofer theory.

Small deformation tests

Dynamic rheological properties of the 5 wt% 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. A torque bar of 0.17 $mN \cdot m^{-1}$ was used. The 5 wt.% starch suspensions were heated to approximately 65°C under gentle stirring until the viscosity slightly increased. After transferring them to the measuring body of the rheometer, which had a temperature of 50°C, the starch suspensions were heated to 90°C, at which temperature they were kept for 15 min and cooled to 20°C. They were allowed to stay at this temperature for several hours. Heating and cooling were performed at a rate of 2 $K \cdot min^{-1}$. Measurements were made 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 was performed using 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 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 scanning rate of 40 $K \cdot min^{-1}$. An empty stainless steel cup was used as a reference.

Results.

Testing of the constructs in an Agrobacterium rhizogenes system by making use of the amf-mutant

Four different binary plasmids were constructed containing a 2.8 or 1.5 kb

part of the BE cDNA in sense (pCVE 2.8 B; pCVE 1.5 B) or antisense (pCVE 2.8 A; pCVE 1.5 A) orientation (Fig. 1). The effect of these plasmids, when inserted in potato was quickly screened by examining the starch colour in hairy roots after iodine staining.

A red starch colour, as for the control, was always found in the hairy roots resulting from the transformation events with the plasmids containing the 2.8 kb cDNA in sense or antisense orientation (Table 1). A low frequency of hairy roots with blue starch was obtained when the antisense 1.5 kb cDNA part of the branching enzyme was used. Also a low frequency of hairy roots with blue colouring starch was found with the sense 1.5 kb cDNA part. Since none of the hairy roots with the control plasmid had this blue colouring starch in the roottips, the inserted 1.5 kb cDNA was thought to be responsible for the change in starch colour.

Table 1. The number of hairy roots obtained after *Agrobacterium rhizogenes* transformation, classified according to the inserted plasmid and the starch colour in their columella cells, after staining with iodine

	Colour		
Construct[a]	red	blue	total
pCVE 1.5 A	135	4	139
pCVE 1.5 B	287	4	291
pCVE 2.8 A	138	0	138
pCVE 2.8 B	110	0	110
control			
8703	152	0	152

[a] pCVE 1.5 A: 1.5 kb cDNA for BE in antisense orientation;
pCVE 1.5 B: 1.5 kb cDNA for BE in sense orientation;
pCVE 2.8 A: 2.8 kb cDNA for BE is antisense orientation;
pCVE 2.8 B: 2.8 kb cDNA for BE in sense orientation;
8703: control plasmid

Selection of A. tumefaciens transformants with differently colouring starch after iodine staining

Because a test of the constructions with *A. rhizogenes* showed that introduction of the distal part of the 1.5 kb cDNA in both sense and antisense

orientation had influence on the starch composition of hairy roots, transformation with *A. tumefaciens* was performed using the constructs containing 1.5 kb cDNA. Shoots were multiplied and transferred to microtuber induction medium and finally to the greenhouse. The tubers of all the individual transformants had red coloured cut surfaces, when stained with iodine. For a more detailed view of the starch colour, starch granules were isolated, stained with iodine and screened under a microscope.
Of the 46 regenerated transformants with the pCVE 1.5 A, 12 had a blue core in the small starch granules of both, the micro and greenhouse grown tubers (Table 2).

Table 2. The number of transgenic plants regenerated after *Agrobacterium tumefaciens* transformation, classified according to the inserted plasmid and the colour of te starch granules

	Colour			
Construct[a]	red	blue	no starch	total
pCVE 1.5 A	34	12	0	46
pCVE 1.5 B	16	7	0	23

[a] pCVE 1.5 A: 1.5 kb cDNA in antisense orientation;
pCVE 1.5 B: 1.5 kb cDNA in sense orientation

Hardly ever a blue core was found in large starch granules. A variation in starch granule colour was found between and within tubers. The change to blue colouring starch was not observed in the stomatal guard cells of the leaves. In two transformants, BV 2057 and BV 2060 the most prominent effect in starch colour change was found (Fig. 2). Not only more small starch granules had a blue core but also the average size of the starch granules with a blue core were larger than those of the other transformants (Table 3). These two transformants had a different starch granule size distribution as compared to the controls. Both transformants were used for further analysis. Of the 23 transformants with the pCVE 1.5 B construct, seven had a small blue core in the small starch granule fraction (Table 2). The pattern of starch colour throughout the tuber was comparable with that of the antisense cDNA transformants.

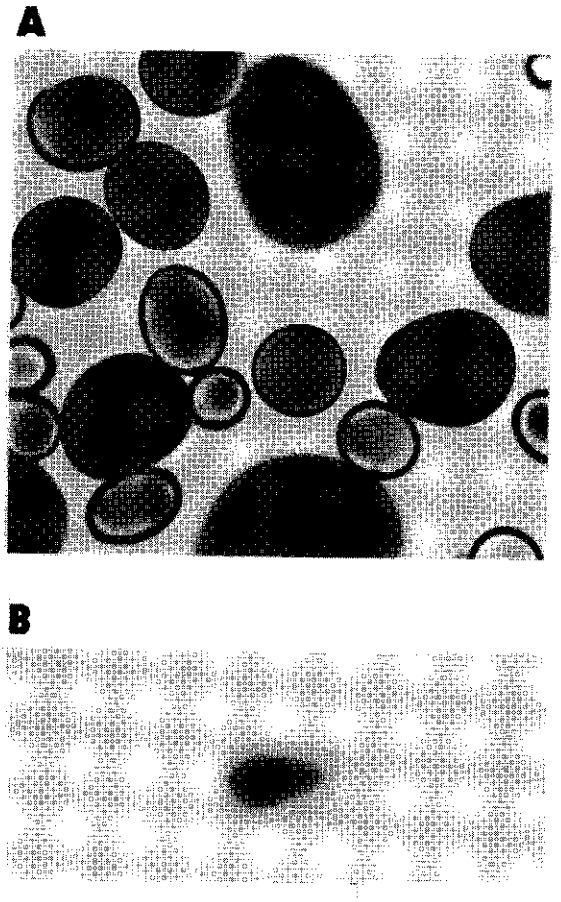

Fig. 2 A,B. Iodine staining of tuber starch granules.
A: Starch granules of clone BV 2057 some of them with a blue staining core and red staining outer layer
B: Starch granule of clone BV 2057 stained with a minimum amount of iodine.

Both, the size of the blue core and the number of small granules having a blue core, were low and comparable with that of the antisense BE transformants with small effect. No sense transformant with an effect as in BV 2057 or BV 2060 was found (Table 3). Two of the seven transformants with small blue cores in the starch granules were therefore selected for further analysis.

Table 3. The average granule size of the controls and the several antisense and sense transformants. The number of starch granules with a blue core (per thousand starch granules) and the size of the largest starch granule with a blue core found in antisense and sense transformants (ND = not determined; - no starch granules with a blue core present)

	starch granules with a blue core		
plant clone	average granule size in μm[a]	number per 1000[b]	maximum size[c]
Control			
1029-31	32.0	0	-
BC 222	ND	0	-
Antisense transformants			
BV 2011	ND	0	-
BV 2014	ND	92	-
BV 2057	40.6	305	32
BV 2060	37.8	237	21
Sense transformants			
BV 2120	ND	186	15
BV 2124	ND	0	-
BV 2134	ND	50	9
BV 2136	40.0	199	13

[a] The average starch granule diameter in μm;
[b] The number of starch granules with a blue core per 1000 starch granules present;
[c] The diameter in μm of the largest starch granule with a blue core observed

Expression of the gene encoding branching enzyme in transgenic potato plants

To determine the steady state level of mRNA for BE, total RNA was extracted from greenhouse grown tubers, blotted and hybridized with ^{32}P labelled full length cDNA for BE. In Fig. 3, the results are shown for the selected sense and antisense transformants and controls. Less mRNA for BE was detected in the selected transformants than in the transformed and untransformed controls. The best performing transformants BV 2057 and BV 2060 showed a very weak signal (Fig. 3) or sometimes even no detectable signal could be found (data not shown). The RNA transcribed from the inserted cDNA should have a size of 1.5 kb. Neither in the transformants with the sense, nor in those with the antisense construct, a signal was seen of 1.5 kb. This did not mean that the inserted cDNA wasn't transcribed, it

merely indicated that no detectable levels of RNA of 1.5 kb were present. The amount of BE protein present in the soluble and granule fraction was studied. It can clearly be seen from Fig. 3 that the sense (lane 5 and 6) and antisense (lane 1 and 2) transformants with a blue core in the small starch granules had no signal compared to the transformed and untransformed control with a clear signal (lane 3 and 4). The starch fraction was also screened for its BE content. Both controls (lane 3 and 4) showed a clear signal indicating that BE protein was present in the starch fraction of potato tubers. Most of the transformants, all of which have a small blue core in the small starch granules had a signal as well, indicating the presence of some BE in the starch fraction. The two antisense transformants BV 2057 and BV 2060 (lane 1 and 2) with the most prominent effect in starch colour gave no signal. Because the maximum amount of protein was loaded on the gel, we can conclude that no signal could be detected for the transformants BV 2057 and BV 2060. This indicates that the amount of BE was undetectably low, or that no BE was present in the granule fraction.

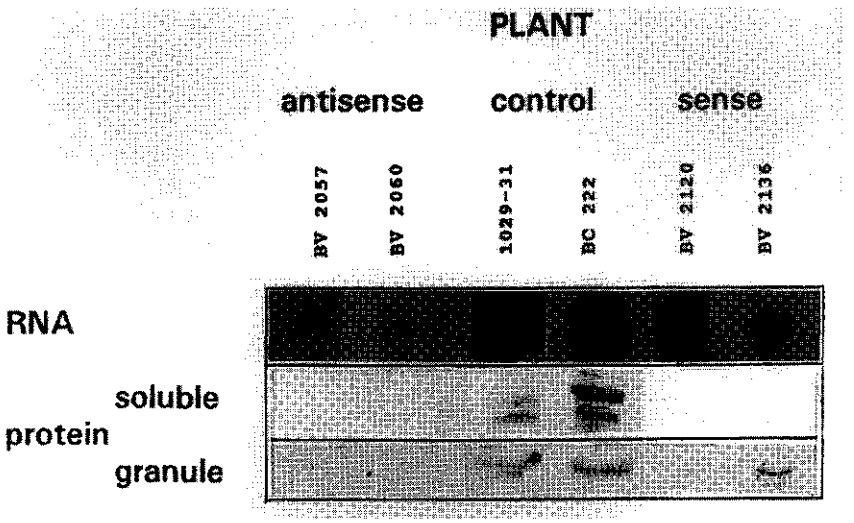

Fig. 3. The level of BE-mRNA and BE-protein in the soluble and granule fraction, in tubers of some transformants and their controls. BV 2057 and BV 2060: antisense 1.5 kb cDNA; 1029-31:
untransformed control; BC 222: control plasmid 8706; BV 2120 and BV 2136: sense 1.5 kb cDNA

Table 4. The branching degree, starch and sugar content of the *amf*-control 1029-31, transformed control BC 222 and four transformants with either the antisense or sense 1.5 kb cDNA inserted.

Plant clone	Branching degree[a]	Starch content[b]	Sucrose content[b]	Glucose content[b]	Fructose content[b]
Control					
1029-31	3.78 ± 0.45	125 ± 30	3.1 ± 0.5	1.0 ± 0.2	0.8 ± 0.3
BC 222	4.11 ± 0.22	140 ± 35	2.9 ± 0.6	0.8 ± 0.1	0.9 ± 0.2
Antisense transformants					
BV 2057	4.24 ± 0.28	118 ± 28	2.8 ± 0.6	1.1 ± 0.3	0.7 ± 0.2
BV 2060	4.65 ± 0.32	135 ± 27	3.3 ± 0.5	0.9 ± 0.2	1.1 ± 0.1
Sense transformants					
BV 2120	3.91 ± 0.35	152 ± 20	3.4 ± 0.7	0.7 ± 0.2	1.0 ± 0.4
BV 2136	3.84 ± 0.27	196 ± 40	2.5 ± 0.8	1.1 ± 0.3	0.6 ± 0.2

[a] the branching degree is given in DE;
[b] the starch and sugar conent is in mg/g fresh weight

Amylopectin characteristics of plants with an altered starch colour

The presence of a blue core in the starch granules suggests that amylose-like starch was present. The iodine binding ability of starch molecules depends on its branching degree. A change in iodine binding ability could be visualised by a shift in the λ_{max}. However, no significant difference in λ_{max} could be observed for the antisense transformants BV 2057 and BV 2060 (no data shown). Also no change in the branching degree could be found using the Luff-Schoorl procedure (Table 4). A reduced branching of the starch molecules is expected to result in larger starch chains. The chain length distribution was measured, but no changes compared to the controls were found (data not shown). This indicated that the lack of BE in the soluble fraction and/or granule fraction had no measurable influence on the branching degree of the starch. The branching degree of the glucose polymers in the soluble fraction (glucans) was also analyzed by scanning of the soluble fraction for its λ_{max}. The λ_{max} was variable for both the controls and the transformants, which made it difficult to visualize the effect of the introduced constructs. No differences in starch and sugar content were found (Table 4).

Physico-chemical properties of starch-water systems

Although no significant differences in the branching degree of starch was measured, other tests were made to determine whether a change in starch properties had occurred or not. It was known from other studies with starch from both potato and maize, that the rheological behaviour was influenced by the presence of amylose.

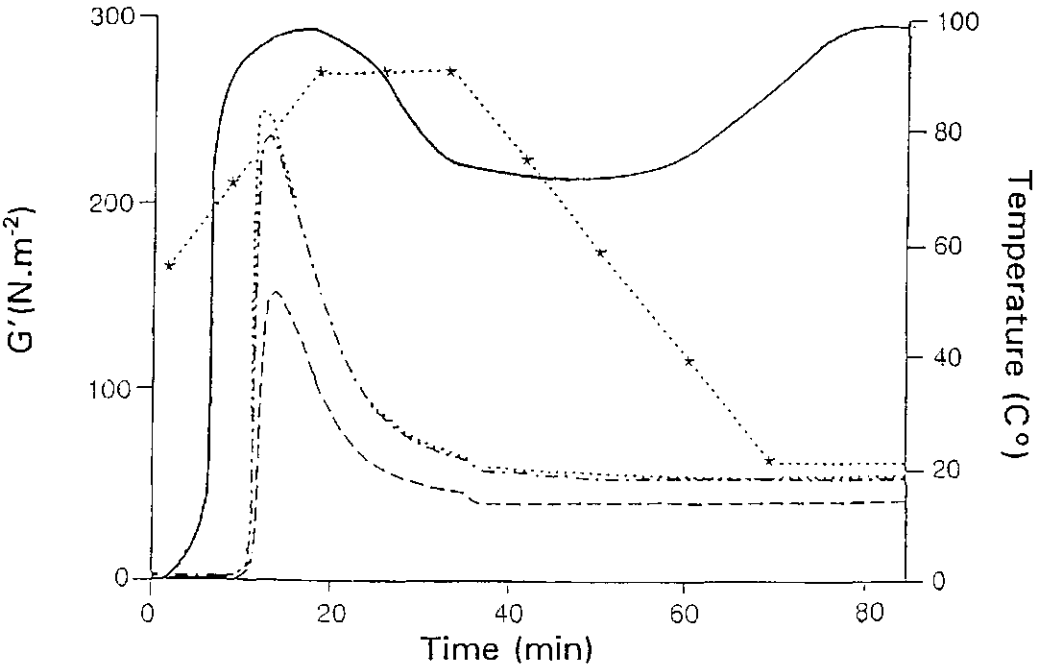

Fig. 4. Changes in the storage moduli of 5 wt. % potato starch suspensions during heating and cooling. Temperature against time (·⋆··); *amylose-free* control (— —); wild-type control (——); BV 2057 (— ·–); BV 2060 (- - -)

Changes in the storage moduli (*G'*) of heated 5 wt.% starch suspensions, measured during heating and cooling are shown in Fig. 4. It can be seen that the *G'* starts to increase strongly at approximately 70°C for both the *amf*-mutant and the antisense transformants, whereas the increase in *G'* starts at a lower temperature. This increase in moduli coincides with the first stages of crystallite melting, as determined with DSC (Fig. 5). At high temperatures the moduli decrease, and during cooling they remain at a constant level, for

both the *amf*-mutant and the antisense transformants. For the amylose containing starch the peak modulus was higher and decreased less during the time the starch system was at 90°C. During cooling the modulus increased. The moduli of the antisense transformants were higher than that of the *amf*-control, although the level of the wildtype wasn't reached.

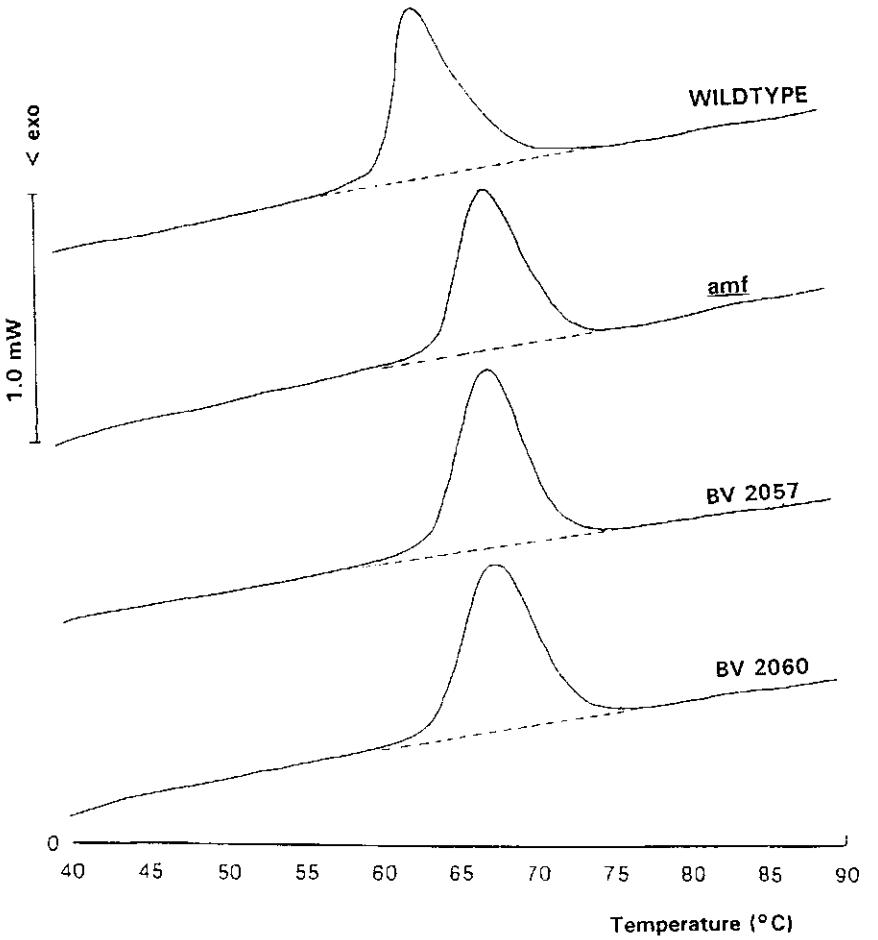

Fig. 5. Differential Scanning Calorimetry (DSC) thermograms of 20 wt.% starch suspensions of the antisense transformants BV 2057 and BV 2060, the *amf*-control 1029-31 and a wild-type

Discussion

The influence of introduced sense and antisense constructs of the cDNA for potato branching enzyme on the starch composition of the *amf*-mutant, could quickly be screened by using the *A. rhizogenes* transformation system. A few hairy roots regenerated after transformation with the antisense and sense orientated 1.5 kb cDNA had blue colouring starch in the columella cells of their roottips. The use of the *A. rhizogenes* transformation system as a first screening for the biological effects of the constructs was also applied by Kuipers *et al.* (1994).

The 1.5 kb antisense and sense BE cDNA constructs can inhibit the expression of the BE-gene

The starch granules in the tubers of some *A. tumefaciens* regenerants were partly blue. This blue colour was also found in the double mutant *aewx* of maize (Boyer *et al.*, 1976). This indicates that the *amf*-mutation is not epistatic over the reduced expression of the BE-gene as is the case in the double *duwx* mutant of maize, that lacks the BE IIa isoform and has a red starch colour when stained with iodine (Shannon and Garwood, 1984). Examination of the mRNA and protein level confirmed that this blue colour was formed due to suppression of the endogenous, only known potato BE gene. Plants with a blue core in the starch granules had less BE mRNA in the tubers as compared to the *amf*-mutant and no BE protein was detected in the soluble protein fraction of the tubers. It can therefore be concluded that the 1.5 kb antisense and sense cDNA of the BE are capable of suppressing the expression of its endogenous homologous sequence to a very large, maybe full, extent. Expression of other genes coding for chalcone synthase in petunia (van der Krol *et al.*, 1988), polygalacturonase in tomato (Smith *et al.*, 1988b) and Granule Bound Starch Synthase in potato (Visser *et al.*, 1991) have been reduced by introducing a part of the gene in antisense orientation. Suppressing the endogenous gene expression by introducing homologous sense genes has also been found for chalcone synthase in petunia (Napoli *et al.*, 1990; van der Krol *et al.*, 1990) and polygalacturonidase in tomato (Smith *et al.*, 1990).

The here presented research proved that the presence of BE protein is not only restricted to the soluble fraction of potato tubers. BE protein was also

found in the granule fraction of the *amf*-mutant, the transformed control and some of the transformants as it was found in the granule fraction of pea embryos (Denyer *et al.*, 1993). In the soluble fraction different bands are found within one lane. These bands indicate that different sizes of the BE protein are present. These different sizes of BE protein are always found in potato and are thought to be the result of protein processing (Blennow and Johansson, 1991). BE of only one size was found in the granule fraction, indicating that the protein is bound to the starch granule in a stable form.
The two antisense transformants with the largest amount of starch granules with a blue core, had no detectable amounts of BE protein linked to the starch granules. It seems that the reduction in BE protein is first noticeable in the soluble fraction. When the BE protein is more reduced no detectable amounts are found in both the soluble and starch fractions.
The reason why the 2.8 kb cDNA in sense or antisense orientation is incapable of suppressing the BE-gene expression is not known. This is especially so, because the 1.5 kb cDNA fragment is part of the 2.8 kb. Neither is the relationship clear between the reduced expression of the BE-gene and an increased size of the starch granules.

A reduction in the BE-gene expression has influence on the physicochemical properties of starch-water systems

Despite the change in starch colour, no differences could be found in the λ_{max}, branching degree and chain length of starch molecules. No differences in starch and sugar content were found as was reported for the BE mutants of maize and pea (Boyer *et al.*, 1976; Shannon and Garwood, 1984). However, when we looked at the mechanical properties at small deformations of 5 wt.% starch suspensions made from the transformants BV 2057 and BV 2060 and the *amf*-control we could observe a small difference (Fig. 4).
The increase in moduli was ascribed to swelling of the starch granules, which coincides with the first stages of crystalline melting in the granules (Keetels, 1995). The fact that the melting behaviour was similar for the antisense transformants and the *amf*-control (Fig. 4 and Fig. 5) would indicate that the stabilities of crystalline regions in the starch granules were not affected by the introduction of antisense cDNA for BE. The decrease in

modulus at high temperature might be caused by melting of the remaining crystallites and a slow breakdown of the amylopectin matrix (Keetels and van Vliet, 1994).
On the assumption that at high temperatures the systems were closely packed with swollen granules, the differences in moduli between the anti-sense transformants and the *amf*-control could be explained by differences in the stiffness of the swollen granules. The differences in stiffness might be induced by the transformation.

Starch colour

The location of the blue core in the starch granules suggests that amylose-like chains were only located in the centre of the starch granules. Although a blue colour was observed, no difference in branching degree of the starch was found. Starch granules with a small blue core and red outer layer were also observed by Kuipers *et al.* (1994) after inhibiting the expression of the GBSS-gene. They even found a small blue core in the starch granules of transformants in which the GBSS gene was inhibited, resulting in no measurable presence of amylose. This indicates that the staining technique with iodine was more sensitive for detecting starch chains with an altered branching degree than other techniques.
The growth of starch granules occurs via apposition, with the core being the starting point of growth (Badenhuizen and Dutton, 1956).
The fact that the blue starch was only present in the core, suggested that probably a second, but not yet known Branching Enzyme or another enzyme in the carbohydrate synthesis is responsible for catalysing the formation of branches in the later development of the starch granules. Kram (1995) showed that BE is present in the amyloplast stroma at the periphery of the starch granule. Therefore, it can also be inferred that the antisense RNA production is too low. When the surface of the starch granule is still very small, enough antisense RNA can be generated to capture the RNA produced by the endogenous gene for BE. When the starch granule has grown, not enough antisense RNA is produced to capture all the endogenous RNA, giving starch with more branching points that stains red with iodine. This does not explain why predominantly small starch granules have this blue core. We suggest that the formed loosely branched amylopectin is diffused

from the core into the rest of the starch granule during the further granule development resulting in a starch granule in which the amylose like chains are undetectale after staining with Lugol.
Even though the branching degree of the amylopectin molecules is not measurably decreased, this research shows that the only known BE isoform has influence on the starch properties in potato tubers. More investigations are necessary in order to understand the role of this BE isoform and the influence of other enzymes, if any, in the carbohydrate synthesis in potato.

Chapter 7

General discussion

Abstract

The *amf*-mutant as a model plant

In this study gene expression and gene silencing was examined of the two genes involved in starch biosynthesis. Both genes code for the last step enzymes in the pathway of amylose (Granule Bound Starch Synthase; GBSS) and amylopectin (Branching Enzyme; BE). Changes in expression of one or both genes were expected to influence the starch composition in transgenic plants. Much benefit was therefore obtained by using an *amylose-free* (*amf*) mutant of potato (Hovenkamp-Hermelink *et al.*, 1987) as a model plant. This monogenic and recessive *amf*-mutant lacks GBSS-activity and GBSS-protein in the starch granules due to a point deletion in the transit-peptide region of the gene coding for GBSS (van der Leij *et al.*, 1991b). The great advantage of this mutant was the easy procedure to distinguish the altered starch from wild-type starch. *Amylose-free* starch has a red colour when stained with iodine, whereas amylose containing starch has a blue colour (Hovenkamp-Hermelink *et al.*, 1987). This difference in iodine affinity is dependent on the length of the starch chains. The long chains of amylose can form an inclusion complex with iodine giving the blue colour. Screening by iodine staining was an easy and quick procedure and even a variation in gene-expression could be observed within tubers (Visser *et al.*, 1991; Kuipers *et al.*, 1991) as a mixed staining pattern. The staining of individual starch granules gave more detailed information about the level of GBSS gene expression (Kuipers *et al.*, 1994). Tubers with a relatively small decrease in GBSS gene-express-

ion had starch granules with a large blue core surrounded by a small red outer layer. With a further decrease in GBSS gene-expression the size of the blue core decreased until starch granules were formed with a very small blue core as frequently found in antisense GBSS plants with no measurable amylose content (Kuipers *et al.*, 1994). The mutant starch was not only present in tubers, but could also be found in the stomatal guard cells, the columella cells of roottips and in the microspores (Jacobsen *et al.*, 1989). This made examination of gene-expression throughout the plant possible. Segregation analysis after staining of microspores provided us with information about the number of wild-type GBSS-alleles in the plants of a gene-dosage population (Chapter 2). Microspore segregation analysis in combination with Southern hybridisation gave information about the minimum number of independently segregating active GBSS-inserts in the complemented *amf*-plants (Chapter 3).

There are gene-dosage and multi-allelic effects of the GBSS-gene on the GBSS-activity and amylose content

A gene-dosage population was obtained by crossing two genotypes that were duplex for the wild-type GBSS-allele; AmfAmfamfamf (Chapter 2). Nulliplex, simplex, duplex, triplex and quadruplex plants could be identified by monitoring the segregation of red and blue microspores. Triplex plants did not have solely blue staining microspores like quadruplex plants, but a small percentage of the microspores stained red due to double reduction (data not shown). In this case double reduction, as a result of quadrivalent formation during meiotic pairing, was expected because the GBSS-locus is positioned at the distal part of chromosome 8 (Gebhardt *et al.*, 1989; 1991). Triplex and quadruplex plants were placed in one group. A dosage effect of the wild-type GBSS-allele on both the GBSS-activity and amylose content was found. The dosage effect was linear for the GBSS-activity. This did, however, not account for a linear increase in the level of GBSS-protein in the starch granules as was the case in rice (Sano, 1984) and maize (Tsai, 1974). Already in simplex potato plants, wild-type levels of GBSS-protein could be found. Despite that, a dosage effect on amylose content was found although the effect was not linear. The presence of three or more GBSS-alleles did not

account for a further increase in amylose content compared to potato plants with two wild-type GBSS-alleles. The dosage effect on amylose content in the triploid endosperm of maize was also not linear (Boyer *et al.*, 1976). There was no difference between the presence of two and three wild-type alleles. In the endosperm of rice the dosage effect varied between crossings (Sano, 1984) when different wild-type alleles were present. For potato, plants within the simplex group appeared to have different amylose contents (Chapter 2). This indicates that different wild-type alleles are present in the simplex plants. In pilot studies, using PCR technique no different wild-type GBSS-alleles were detected at the molecular level (Chapter 2). Whether these differences in amylose content were mainly caused by variation in expression because of small differences between wild-type alleles (multi-allelism) or by differences in the genetic background or both remains unclear. The linear increase in GBSS-activity was not accompanied with a linear increase in GBSS protein. This indicates that less non-active GBSS protein is present in multiplex plants than in simplex plants. Despite the fact that there is not a detectable different amount of GBSS protein between simplex and duplex plants a significant difference in amylose content was measured.

The *amf*-mutation can be fully complemented with the wild-type GBSS-allele

A diploid *amf*-mutant was transformed with the GBSS-gene (Chapter 3). This led to diploid and tetraploid (mitotically doubled) transgenic plants with wild-type levels of GBSS-activity and amylose content and blue staining starch. A comparison of the results from microspore staining and Southern hybridisation indicated that in tetraploid plants the transgene was inserted after chromosome doubling. The complementation was much better than that of the nitrate reductase deficient mutant of *Nicotiana plumbagonifolia* where up to 6 % of the nitrate reductase (NR) activity of the wild-type was found in transgenic plants containing the NR transgenes (Vaucheret *et al.*, 1990). The minimum number of independently segregating active GBSS-inserts was estimated by genetic analysis after microspore staining. The fully complemented plants were back-crossed with the *amf*-mutant (Chapter 4). The level of complementation was investigated in sporophytic tuber cells and

the genetic constitution was determined by segregation analysis of gametophytic microspores of the sexual offspring. The expected segregation patterns (based on microspore staining with iodine of the original transformant) in the F1 offspring were confirmed by iodine staining of tuber starch. All expected genotypes in the F1 offspring with one or two inserts were observed by microspore segregation patterns of the individual F1 plants.
Microspore staining of fully complemented plants provided reliable information about the inheritance of the inserts avoiding time consuming crossing experiments.

Partial complementation of the *amf*-mutant is caused by co-suppression or unreliable expression of the transgenes

Not only fully complemented, but also transgenic plants with no or partial complementation were obtained (Chapters 3 and 5). Plants with no complementation had totally red staining starch granules after iodine staining. The partially complemented plants had blue, mixed and/or red staining tuber surfaces. The red and blue tubers of partially complemented plants could be distinguished from non, or fully complemented plants by staining the individual starch granules with iodine. They had no uniform colour, but had a blue staining core and a red outer layer. The size of the blue core depended on the level of restored GBSS-gene expression. The frequency of partial complementation was dependent on the ploidy level of the original plants. Partial complementation was more frequently found after transformation of diploid than of tetraploid *amf*-genotypes (Chapter 4). Beside that, the level of complementation was higher in microtubers of a partially complemented plant than in greenhouse or field grown tubers of the same transgenic plant. This is probably related to the difference in ploidy level between microtuber cells (2x or 4x) and greenhouse and field tuber cells (16 - 64x).
The position of the kanamycin resistance gene in relation to the GBSS-gene had no influence on the expression of the GBSS-transgene (Chapter 5) as was mentioned as a possible explanation by Grierson *et al.* (1991). The presence of an enhancer upstream the GBSS-gene lowered the number of transgenic *amf* plants with both partial or no complementation. This indicates that partial complementation is partly due to insufficient transgene

expression (Chapter 5).
Two transformants with partial complementation were investigated in more detail (Chapters 3 and 4). One of them (B1) was tetraploid with a duplex genotype for the GBSS-insert which had originated through mitotic doubling of transformed diploid cells. In the F1-offspring, after crossing with the *amf* potato, three phenotypic classes were found: *amf*, partially complemented and fully complemented. The latter two were not specifically related to a simplex or duplex situation. This indicates that in this case partial complementation was not dependent on the duplex situation. The level of transgene expression was thought to be influenced by the genetic background (Chapter 4). Another partially complemented plant (B10) was diploid and contained at least seven inserts. In the F1 offspring, after crossing with the *amf*-potato, the partly complemented phenotype was correlated with the presence of a block of five inserts. These five inserts were capable of inhibiting the expression (trans-inactivating) of the other inserts which were normally expressed. This could be observed in complemented F1 plants lacking this block of inserts (Chapter 4). Crossing this partially complemented plant with a wild-type showed that the block of transgenes was also capable of inhibiting the endogenous GBSS-genes. This leads to the assumption that partial complementation can also be caused by co-suppression besides the already mentioned phenomenon of reliable expression.

A reduced BE gene expression in an *amf* background.
The double mutant *aewx* of maize is defective for the GBSS enzyme and for one of the branching enzymes. It contains loosely branched amylopectin with long internal chains giving a blueish colour when stained with iodine (Boyer *et al.*, 1976). To obtain the same result in potato, the *amf*-mutant was transformed with sense and antisense cDNA's encoding Branching enzyme (BE) of potato (Chapter 6). The expression of the endogenous BE-gene was largely or fully inhibited as judged by the absence of both mRNA and protein of BE. The starch granules of some of such transgenic plants had a small blue core with a large red outer layer. This is one of the observations indicating that loosely branched amylopectin was formed in the core. The second observation is, that the physico-chemical properties of the starch

were changed in plants with reduced BE-gene expression. Here a higher peak viscosity was obtained in contrast to Kortstee *et al.* (1995) who found a lowered peak viscosity in an *amf*-mutant with increased branching degree due to introduction of the gene for BE of *E. coli*. Both observations indicate that the only presently known BE of potato catalyses branching in starch granules. It was not clear in this case whether the presence of a small blue core indicated that the inhibition was optimal during initiation of granule formation or not. The linear amylose in partially complemented plants, or plants with inhibited GBSS-gene expression - due to sense or antisense inhibition - was always present in the core of the starch granules. This might indicate that the empty space between the amylopectin molecules was filled, starting at the core of the starch granules. The fact that no measurable change in branching degree was found indicates that the gene expression is not sufficiently reduced or that other branching enzymes play a role. The BE investigated here is related to the BE I of maize. Guan and Preiss (1993) proposed that BE I forms a slightly branched molecule that is further branched by BE II. This suggests that a total reduction in potato BE-gene expression would only have a small influence on the branching degree, which was indeed observed. Furthermore, it supports the idea that another BE-gene, or gene which encodes for an enzyme with branching activity, must be present and active. The gene coding for this second BE can possibly be found by the application of mutagenesis in the *amf*-background. The fact that a blue core was formed in the starch granules, suggests that BE I is the only BE active in the granule core. Assuming that the activity of BE I is totally reduced throughout the granules suggests that BE II is also capable of branching linear chains and that an initial branching by BE I is not necessary.

An increase in amylose GBSS-activity does not lead to a higher level of amylose content than the wild-type

In the gene-dosage population a linear increase in GBSS-activity did not lead to a linear increase in amylose content (Chapter 2). The level of amylose reached a plateau. The introduction of additional sense genes coding for GBSS in the *amf*-mutant (Chapter 3) or in the wild-type potato (Chapter 5) which did account for an increased GBSS-activity above wild-type level did

also not lead to an increased amylose content above that of wild-types. This assumes that a maximum level of amylose is formed in wild-type starch granules. Above certain levels of amylose, the GBSS-activity is not the limiting factor for amylose formation. Probably the activity of other enzymes or the presence of intermediate products impose limitations for the production of amylose. It is questionable whether starch granules can possess more amylose or not. The starch granules in the duplex and triplex/quadruplex dosage groups were totally blue except for a few simplex plants in which a low percentage of the starch granules had a small red outer layer, indicating that the granules were not totally filled with amylose. According to Kuipers *et al.*, (1994), wild-type levels of amylose are present in the blue core of antisense inhibited wild-types with reduced amylose synthesis. The starch granule is formed due to a crystalline organization of the amylopectin molecules (Oostergetel and van Bruggen, 1989) and the empty spaces, with a restricted volume between these radially arranged amylopectin molecules, are filled with amylose to a maximum level (Jane *et al.*, 1992). This could mean that only a higher amylose content can be obtained in the starch granules when more space between the amylopectin molecules is available.
Rothacker and Effmert (1968) estimated a variability in amylose content in different potato cultivars. They found an amylose content up to 33% and a positive correlation between the parents and their progeny when the high level amylose plant was used in crossing programs. The GBSS-activity of those plants was not determined. We assume that the high amylose content was not correlated with a higher GBSS-activity but with an altered crystalline structure or size of the individual starch granules.

In this research the *amf*-mutant was successfully used for examining gene expression and gene silencing. It was possible to examine the expression of endogenous and introduced GBSS-genes by staining the starch with iodine. Variability in gene expression within and between tubers could be made visible by the easy and reliable iodine staining procedure. The starch composition could even be screened in the microspores. This made it possible to create a gene-dosage population for the GBSS-gene by simply staining the microspores with iodine and estimating the microspore segregation. Beside that the minimum number of independently segregating GBSS inserts in the *amf*-mutant could be estimated. The *amf*-mutant was further used to

examine inhibition of the BE-gene expression of potato. The blue core in starch granules lacking the BE indicates that loosely branched starch is present in the centre of the altered starch granules.

Summary

Gene-expression and gene-silencing were examined for Granule Bound Starch Synthase (GBSS) which catalyses the formation of amylose and Branching Enzyme (BE) which catalyses the formation of amylopectin. Changes in expression of both genes influenced the starch composition. To be able to monitor the effects and facilitate experiments an *amylose-free* (*amf*) potato mutant was used as a model plant. This plant is GBSS deficient. The great advantage of this mutant (*amf* = *gbss* = *a*) is the simple procedure to distinguish the altered starch phenotype from wild-type (Amf = GBSS = A) starch. *Amylose-free* starch has a red colour when stained with iodine, whereas amylose containing starch has a blue colour. Screening by iodine staining is an easy and quick procedure which can visualize the variation in gene expression in tubers. The staining of individual starch granules gives detailed information about GBSS gene expression. Furthermore, the expression of GBSS in the starch of microspores can be examined by staining with iodine enabling genetic analysis. The advantages of the *amf*-mutant were used in several types of investigation:

I) **Dosage effect.**

Plants, nulliplex (aaaa), simplex (Aaaa), duplex (AAaa), triplex (AAAa) and quadruplex (AAAA) for the wild-type

GBSS-allele could be identified by the segregation ratios of the microspores. Within the triplex plants a low frequency of microspores with red staining starch were formed as the result of double reduction during meiosis. In tubers, a dosage effect of the GBSS-allele on both GBSS-activity and amylose content was found. The dosage effect for the GBSS-activity was linear, but this did not account for a linear increase in GBSS-protein and amylose content. The presence of three or more GBSS-alleles did not lead to a further increase in amylose content compared to that in duplex plants. Within the simplex group of plants variation in amylose content was found indicating the existence of different wild-type alleles (multi-allelism).

II) **Complementation and co-suppression.**

A diploid *amf*-mutant was transformed with the genomic GBSS-gene. This led to diploid and tetraploid (mitotically doubled) transgenic plants

with wild-type levels of GBSS-activity and amylose content. The minimum number of independently segregating active GBSS-inserts was estimated by genetic analysis after microspore staining. The fully complemented plants were back-crossed with the *amf*-mutant of potato. The expected segregation ratios of complemented and *amf* F1 plants were found.

Not only fully, but also partially complemented primary transgenics were obtained. This partial complementation was more frequently found after transformation of diploid than of tetraploid *amf* genotypes. Besides that, the level of complementation was higher in microtubers than in greenhouse or field grown tubers of the same transgenic plants. This is probably due to the difference in ploidy level between cells in microtubers (2x - 4x) and greenhouse or field grown tubers (16x - 64x). The presence of an enhancer in front of the GBSS-gene lowered the number of plants with partial or no complementation. This indicates that partial complementation can be caused by an insufficient transgene expression.

In the offspring after back-crossing with *amf* of the partially complemented *amf*-plants B1 (tetraploid) and B10 (diploid), fully and partially complemented F1-plants were obtained. B1 was duplex for the GBSS transgene as a result of doubling of the genome after T-DNA insertion. The partial complementation in the F1 offspring was not correlated with the duplex situation. In the case of B10 the partial complementation was correlated with the presence of a block of 5 inserts. These 5 inserts were capable of inhibiting the expression of the other inserts with normal expression. Crossing of the partially complemented B10 with a wild-type showed that the block of transgenes was also capable of inhibiting the endogenous GBSS-gene. This indicates that partial complementation can also be caused by co-suppression besides the already mentioned low transgene expression.

Co-suppression was further obtained when the full size GBSS sequence or GBSS cDNA were introduced in the wild-type potato. Even the cloned *amf*-allele caused co-suppression. The orientation of the GBSS-gene towards the kanamycin resistance gene did not influence the frequency of transgenic wild-type plants with inhibited expression. The frequency of diploid wild-type plants showing sense inhibition (10 %)

was lower compared to the frequency using antisense inhibition (52 %) in earlier research. The frequency of plants with a complete inhibition was similar (3 %). This comparison is not made for tetraploid wild-types.

III) **Inhibition of branching enzyme gene expression.**

The *amf*-mutant was used to examine the inhibition of the branching enzyme (BE) of potato by sense and antisense cDNA sequences. A situation was created as in the *amylose-extender/waxy* (*aewx*) double mutant of maize. A mutation in both the GBSS-gene and one of the genes encoding BE led to the formation of loosely branched starch chains which stains blue instead of the red staining *waxy* starch. Introduction of the distal 1.5 kb cDNA for BE resulted in a few sense and antisense transgenic plants with a small blue core in the starch granules. This could indicate the presence of loosely branched amylopectin in the core of the starch granules. The expression of the endogenous BE-gene was largely or fully inhibited as judged by the absence of BE mRNA and protein. No differences in branching degree, amylose, starch and sucre content were found. However, the physico-chemical properties of this starch was altered compared to *amf*-starch.

In this study it is shown that the *amf*-mutant is successfully used as a modelplant to examine different aspects of gene expression. Insertion of the wild-type GBSS-gene in either an *amf* or wild-type showed that an increase in GBSS-activity higher than wild-type levels could be obtained, but that this did not account for a higher amylose content. This showed that the maximum level of amylose is already formed in these specific wild-type plants. Above a certain level of amylose formation, the GBSS-activity is not the determining factor. An explanation could be that the starch granules are totally filled with amylose and that no more amylose can be stored without altering the crystalline structure of the starch granules or the granule size.

Samenvatting

In dit proefschrift wordt de expressie bestudeerd van het gen voor Korrel Gebonden Zetmeelsynthase (KGZ) dat de vorming van amylose katalyseert en van het gen voor het vertakkingsenzym (VE) dat de vorming van amylopectine katalyseert. Een veranderde expressie van één of beide genen heeft direct invloed op de zetmeelsamenstelling. Om de effecten goed waar te kunnen nemen en de experimenten te vergemakkelijken werd als model plant de amylose vrije (*amf*) aardappelmutant gebruikt. Deze mutant (*amf* = kgz = a) bevat geen KGZ-eiwit in de zetmeelkorrels, waardoor daar geen amylose wordt gevormd. De *amf*-mutant heeft als voordeel dat het veranderde zetmeel met behulp van een simpele methode van dat van het wildtype (Amf = KGZ = A) zetmeel te onderscheiden is. Amylose vrij zetmeel heeft een rode kleur wanneer het met jodium gekleurd is, terwijl amylose bevattend zetmeel een blauwe kleur geeft. Ook de effecten van variatie in gen expressie kunnen hiermee zichtbaar gemaakt worden. Het kleuren van individuele zetmeelkorrels geeft gedetailleerde informatie over de verschillen in expressie niveaus van het KGZ-gen binnen de korrel. Verder kan de expressie van het KGZ-gen door middel van jodium kleuring in pollenkorrels worden bestudeerd, gevolgd door een genetische analyse. Van de genoemde voordelen van de *amf*-mutant werd in dit proefschrift gebruik gemaakt bij het onderzoek naar:

I) **Dosis effecten.**

Planten, die nulliplex (aaaa), simplex (Aaaa), duplex (AAaa), triplex (AAAa) en quadruplex (AAAA) voor het wildtype KGZ-allel zijn, konden aan de hand van de uitsplitsing van de pollenkorrels worden geïdentificeerd. De triplex planten bezaten een klein percentage rood kleurende pollenkorrels die ontstaan waren door dubbele reductie tijdens de meiose. In de knollen werd een dosis effect gevonden voor zowel de KGZ-activiteit als het amylose gehalte. Het dosis effect voor de KGZ-activiteit was lineair, maar dit resulteerde niet in een lineaire toename van de hoeveelheden KGZ-eiwit en amylose. De aanwezigheid van drie of meer wildtype KGZ-allelen leidde niet tot een toename in het amylose gehalte ten opzichte van de duplex planten. Binnen de groep van simplex planten werden duidelijke verschillen in amylose gehalte waargenomen. Dit impliceert het bestaan van meerdere wildtype allelen met een verschillende mate van effect (multi-allelie).

II) Complementatie en co-suppressie.

Een diploïde *amf*-mutant werd getransformeerd met een wildtype KGZ-gen. Dit leidde tot de vorming van diploïde en tetraploïde (mitotisch verdubbeld) transgene planten met een herstelde KGZ-activiteit en met een amylose gehalte gelijk aan dat van een wildtype. Het minimum aantal, onafhankelijk overervende en actieve KGZ-inserten werd door middel van een genetische analyse na het kleuren van de pollenkorrels met jodium bepaald. De volledig gecomplementeerde planten werden teruggekruisd met de *amf*-mutant. De verwachtte uitsplitsing van gecomplementeerde en *amf*-planten werd in de F1 generatie waargenomen.

Niet alleen volledig, maar ook partieel gecomplementeerde primaire transgenen werden verkregen. Deze partiële complementatie werd vaker gevonden na het transformeren van diploïde dan van tetraploïde *amf*-genotypen. Behalve dat, was het niveau van complementatie hoger in microknollen dan in knollen uit de kas of van het veld. Dit wordt mogelijk veroorzaakt door het verschil in ploïdie niveau van de cellen in de microknollen (2x-4x) en de cellen in de kas- of veldknollen (16x-64x). Expressie van ingebrachte genen zoals het KGZ-gen wordt na transformatie in diploïden schijnbaar vaker negatief beïnvloed in polyploïde knolcellen dan na transformatie in tetraploïden. De aanwezigheid van een enhancer voor het KGZ-gen verminderde het aantal planten met partiële of geen complementatie. Dit suggereert dat partiële complementatie het gevolg kan zijn van onvolledige gen expressie.

In de nakomelingschap van zowel de partieel gecomplementeerden B1 als B10, na kruising met een *amf*-genotype, werden volledig en partieel gecomplementeerde planten gevonden. De tetraploïde B1 was duplex voor het KGZ insert als gevolg van genoom verdubbeling na T-DNA insertie. De partiële complementatie in de nakomelingschap was niet gecorreleerd met het in duplex aanwezig zijn van de inserten. In het geval van B10 was de partiële complementatie gecorreleerd met de aanwezigheid van een blok van vijf insèrten. Deze vijf inserten waren in staat de expressie van de andere twee inserten te verminderen. Na het kruisen van deze diploïde B10 met een wildtype plant bleek het blok van vijf inserten ook in staat te zijn de expressie van het endogene KGZ-gen te verminderen. Dit suggereert dat partiële complementatie veroorzaakt

kan worden door co-suppressie, naast de al eerder genoemde onvolledige gen expressie.

Co-suppressie werd verder verkregen door zowel het volledige KGZ-gen als het KGZ-cDNA in wildtype aardappel in te brengen. Zelfs het gekloneerde *amf*-allel veroorzaakte in een lage frequentie co-suppressie. De oriëntatie van het KGZ-gen ten opzichte van het gen voor kanamycine resistentie had geen invloed op het percentage transgene wildtype planten met een verminderde KGZ-gen expressie. Het percentage planten met sense inhibitie (10 %) was lager dan het percentage planten met antisense inhibitie (52 %) dat in een eerdere studie was gevonden. Het percentage planten waarbij de afname in gen expressie volledig was, was echter gelijk (3 %). Niet onderzocht is de vraag of deze vergelijking ook geldt na transformatie van tetraploïde wildtypen.

III) Verlaging van de expressie van het vertakkingsenzym gen.

De *amf*-mutant werd ook gebruikt om een verlaging van de gen expressie voor het vertakkingsenzym (VE) door sense en antisense sequenties te bestuderen. Er werd op deze manier getracht een situatie te creëren die vergelijkbaar is met die in de dubbele mutant *amylose-extender/waxy* (*aewx*) van mais. Een mutatie in zowel het KGZ-gen als in het gen dat codeert voor één van de vertakkingsenzymen leidde tot de vorming van minder vertakte zetmeelketens die met jodium blauw kleurden. De introductie van het distale 1.5 kb cDNA voor het VE resulteerde in de vorming van enkele sense en antisense transformanten met een kleine blauwe kern in de zetmeelkorrel. Op basis van de afwezigheid van VE mRNA en VE eiwit werd geconcludeerd dat de expressie van het VE-gen in de knol grotendeels of volledig geremd was. Ondanks deze remming werden geen veranderingen gevonden in de vertakkingsgraad van het zetmeel en in het amylose-, zetmeel- en suikergehalte. De fysisch-chemische eigenschappen van dit zetmeel waren wél ten opzichte van het *amf*-zetmeel veranderd.

Dit promotie onderzoek laat zien dat de *amf*-mutant succesvol als model plant gebruikt kan worden om de expressie van het KGZ-gen en VE-gen te bestuderen. Door het wildtype KGZ-gen in een *amf* of wildtype achtergrond in te brengen kon een KGZ-activiteit worden verkregen die hoger was dan die

van het wildtype. Deze verhoogde activiteit leidde echter niet tot de vorming van meer amylose. Het maximum gehalte aan amylose is al aanwezig in de hier gebruikte wildtype planten. Dit betekent dat bij een bepaald amylose gehalte, de KGZ-activiteit niet meer de beperkende factor is. Een verklaring hiervoor zou kunnen zijn dat de zetmeelkorrels dan volledig met amylose zijn gevuld en dat geen extra amylose kan worden opgeslagen zonder de structuur of de grootte van de zetmeelkorrels te veranderen.

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Curriculum Vitae

Elise Flipse werd op 16 februari 1965 in Middelburg geboren. In 1983 behaalde ze haar diploma ongedeeld VWO aan de Christelijke Scholengemeenschap Walcheren te Middelburg. In datzelfde jaar ging ze Plantenveredeling studeren aan de Landbouwuniversiteit te Wageningen. Afstudeervakken deed ze in de plantenfysiology (knolinductie van aardappel) en de plantenveredeling (transformatie en regeneratie van aardappel). Het laatste afstudeervak werd op het voormalige instituut ITAL (nu CPRO) gedaan. In 1988 studeerde zij af waarna zij ging werken bij de vakgroep Plantenveredeling aan cytoplasmatische mannelijke steriliteit van Petunia. In 1990 stapte zij over naar het onderzoek aan aardappelzetmeel, waarvan de resultaten staan beschreven in dit proefschrift. Vanaf 3 oktober is Elise werkzaam bij het Scottish Crop Research Institute in Dundee, Schotland, waar zij onderzoek doet naar de expressie en vererving van verschillende ingebrachte genen in aardappel, en het effect van de inserten op de voedingswaarde van de aardappel knollen.

Nawoord

"Hè, hè, da was nie een bitje vé wark", maar zie hier het proefschrift is af en mijn koffers zijn gepakt. Echt een goed tijdstip dus om terug te blikken. Hoewel ik hier als student al was geweest, zette ik mijn eerste "werknemersstap" in mei 1988. Nu, vele stappen later is het proefschrift klaar. Gelukkig heb ik veel van deze stappen niet alleen hoeven zetten.

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Jacqueline Joosten, Nanda Huisman, José Kok, Frank van der Werff, Rob

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Anja: als de eerste twee starch sisters mochten we twee keer samen op stap. Tenminste, de eerste keer naar St. Andrews hadden we nog twee begeleiders bij ons, Evert en Herman (of waren wij de begeleiders ?). De tweede keer naar Santa Cruz durfden ze het aan ons alleen te laten gaan. Onze belevenissen en de aanvulling van ons vocabulair op deze trips zullen ons wel bijblijven, maar het lijkt me beter ze hier niet te vermelden. Ik vermeld alleen dat we alle lezingen netjes en serieus hebben gevolgd. Als starch pionier op de vakgroep zette jij dingen op waar ik gebruik van kon maken. Ook met betrekking tot de promotie en het proefschrift heb ik je heel wat kunnen vragen, bedankt.
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Anne: toen je op sollicitatiegesprek was geweest vroegen ze mij of ik wel met die Mej. Kortstee dacht te kunnen samenwerken, aangezien het een gezamenlijk project betrof en o.a. de analytische hulp moest worden ver-

deeld. Ik dacht toen dat het wel zou gaan. Volgens mij heb ik gelijk gekregen. Ik heb het in ieder geval leuk gevonden om samen met jou, maar toch apart binnen één project werkzaam te zijn. Je verhalen over salsa, de wodkajus club en de kledingruil beurs zal ik missen, maar misschien mail je nog eens?

Zonder enig vertier en afleiding was deze periode wel erg saai geweest. In de vrije uurtjes hebben velen op de één of andere manier een steentje hieraan bijgedragen; enkele personen wil ik daarvan met name noemen:
Marisca en Catrien: de "oudste vrienden", maar al het langste van elkaar gescheiden. Gelukkig weegt het eerste zwaarder. Irene en Eddy: Irene, onze wandeltochten waren uniek. We gaan nog een keer hè? Eddy: soms vergeet ik wel eens dat ik Irene al veel langer ken dan jou, het was een vriend erbij toen jij er met Irene vandoor ging. Henk: samen zijn we voor mij op computer strooptocht geweest. En je adviezen waren goed, proefschrift schrijven zou heel wat minder comfortabel zijn geweest. Vergeet niet dat je beloofde in Schotland te komen logeren. Martha en Peter: jullie hebben me al naar twee uithoeken van Nederland laten rijden om jullie op te zoeken. Weekends met een lekker wijntje, biertje, eten en veel gepraat. Ik pak jullie terug, ik zet de whisky in Schotland on the rocks.
En het thuisfront: het was mijn moeder die opperde of de Landbouw (toen nog) hogeschool niets voor mij was, ik hield namelijk wel van met laarzen door de modder lopen, en was al van kinds af aan van plan een rijke boerenzoon te trouwen. Van het eerste is het gekomen (zij het mondjesmaat), van het laatste niet echt. Mijn periode op de LUW, zowel tijdens de studie als het onderzoek is door mijn vader en moeder met interesse gevolgd. Zij weten meer over genen, aardappels en zetmeel dan de gemiddelde Nederlander, maar mijn kennis over viaducten, duikers en brugoverspanningen is ook niet te onderschatten. Sommigen van jullie zullen mijn kreet: " O, kijk eens wat een mooie overspanning" wel eens gehoord hebben. Het feit dat jullie (pappa en mamma) de flat hebben gekocht zodat ik hier in Wageningen een eigen plekje kreeg was geweldig. Ik heb er al die jaren van genoten en het spijt me hier te moeten vertrekken. De afstand in kilometers wordt groter, maar dat moet voor ons niet veel uitmaken. Jullie weten dat ik op zoek ben naar een appartement met een logeerkamer.
Gerald: voor jou zijn aardappels gewoon wat ze zijn: "lekker eten". Niks geen

gezeur over zetmeel, genen, onderzoek, artikelen, proefschrift, carrière, druk hebben ..... Doe maar gewoon: gezellig koffie drinken, lekker eten, mooie muziek, de juiste gezellige mensen om je heen, even smoezen voor het naar bed gaan, een glimlach van oor tot oor; wat is er nou belangrijker?

In de periode dat ik dit proefschrift schreef overleed mijn opa. Hij werd 90 jaar.
Ik was 9 toen opa en oma bij ons in de straat kwamen wonen. Ik liep dan ook vaak even bij hen binnen, om de laatste nieuwtjes van school te vertellen. Verhalen die door hen met grote aandacht werden gevolgd. Zoals vaak het geval is met een opa en oma werd ik (voornamelijk door oma) volgestopt met limonade en koekjes. Toen ik naar Wageningen verhuisde, bleef een bezoekje aan opa en oma een vast ritueel van een weekendje thuis, ook toen ze in het bejaardenhuis gingen wonen. De band met opa werd door de ziekte en het overlijden van oma alleen maar sterker. Ondanks zijn hoge leeftijd maakte hij zich druk over de Nederlandse maatschappij, het feit dat jonge mensen zo slecht aan het werk komen, de AOW maar ook de toestand in Rusland. Met veel aandacht bleef hij tot het eind mijn onderzoek volgen, hij was benieuwd of de lezing in Engeland goed was gegaan en of mijn computer het toch wel goed deed. Hij wilde weten waar ik naartoe ging op vakantie.
Op een bewonderingswaardige manier nam hij afscheid, toen hij wist dat hij niet meer beter zou worden.

Hij was een geweldige opa.

Elise