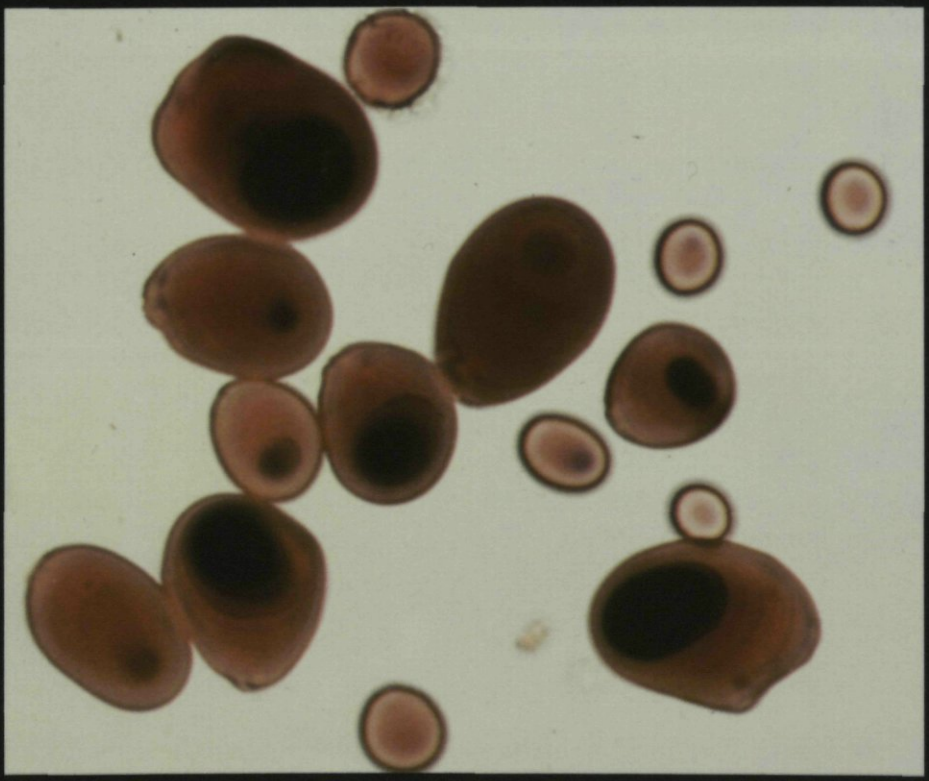
Antisense RNA mediated inhibition of granule-bound starch synthase gene expression in potato



Anja Kuipers

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Antisense RNA mediated inhibition of granule-bound starch synthase gene expression in potato

Proefschrift

ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op maandag 27 juni 1994
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

Stellingen

1. Reductie van de expressie van het GBSS gen in aardappel leidt in zetmeelkorrels van knollen tot de vorming van amylosevrij zetmeel wanneer de hoeveelheid GBSS eiwit door groei van de zetmeelkorrels is gedaald tot beneden een drempelwaarde.
(Dit proefschrift)

2. Introductie in aardappelplanten van antisense GBSS genen die de promoter van het endogene GBSS gen bevatten, leidt in de knol tot een stabielere remming van de expressie van het GBSS gen dan introductie van antisense GBSS genen die zijn voorzien van de CaMV-promoter.
(Dit proefschrift)

3. Toename van het geobserveerde antisense effect in knolweefsel tijdens de ontwikkeling van aardappelplanten, getransformeerd met een antisense GBSS gen, vindt zijn oorsprong in de specifieke wijze waarop de groei van de zetmeelkorrel plaatsvindt.
(Dit proefschrift)

4. Antisense RNA inhibitie van GBSS genexpressie in tetraploïde aardappelrassen is een goed alternatief voor een veredelingsprogramma op basis van de amylose-vrije aardappelmutant.
(Dit proefschrift)

5. Op grond van het histochemisch bepaalde expressiepatroon van een chimeer ß-glucuronidase gen voorzien van de promoter van het GBSS gen in wortels van transgene aardappelplanten dient het gekwantificeerde expressieniveau niet alleen te worden gerelateerd aan de hoeveelheid eiwit, maar ook aan de lengte van de geanalyseerde wortels.
(Visser et al. (1991), Plant Mol. Biol. 17: 691-699)

6. Er dient internationale consensus te worden bereikt omtrent de stijl van de referentielijst bij publicaties in wetenschappelijke tijdschriften.

7. De genomische *in situ* hybridisatie techniek kan worden beschouwd als een geslaagde cytogenetisch-moleculair genetische fusie.

8. De conclusie van sommige milieu- en ontwikkelingssamenwerkings-organisaties om een eenmalige bijdrage te beschouwen als de eerste van een nieuwe donateur werkt averechts.

9. De frequente vertraging van de stoptrein Leiden-Utrecht v.v. is onvoldoende structureel om op een dagelijkse basis tussen Vleuten en Rhenen te forenzen.

10. Bij een verdere uitbreiding van het aantal commerciële t.v.-zenders zal het mogelijk worden om zappend een avondvullend reclameblok samen te stellen.

Stellingen behorende bij het proefschrift getiteld 'Antisense RNA mediated inhibition of granule-bound starch synthase gene expression in potato' door Anja Kuipers.

Wageningen, 27 juni 1994.

Voorwoord

Nu al weer ruim vijf jaar geleden, op 1 februari 1989, begon ik mijn onderzoek bij de vakgroep Plantenveredeling. In de afgelopen jaren variëerden mijn bezigheden van moleculair biologisch werk tot de uitvoering en analyse van veldproeven. Door de ervaring, de kennis en de inzet van veel mensen waren de meest uiteenlopende werkzaamheden goed te combineren. Ik wil dan ook iedereen bedanken die heeft meegewerkt aan het antisense project. Een aantal mensen wil ik graag noemen.

Mijn promotoren, Dr. Ir. Evert Jacobsen en Dr. Ir. Will Feenstra, die in Groningen aan de basis stonden van het zetmeelproject, wil ik bedanken voor de plezierige begeleiding. Evert, meerdere malen heb je oplossingen gevonden voor het onderzoeksmedewerkersvraagstuk. Jouw voorwaarden scheppende activiteiten hebben door het hele project heen een belangrijke rol gespeeld. Will, bij het schrijven van de artikelen heb ik veel geleerd van de nauwgezette wijze waarop je de manuscripten hebt doorgenomen en met mij hebt besproken. Bedankt hiervoor. Mijn co-promotor, Dr. Richard Visser, heeft als directe begeleider een belangrijke rol in het project gespeeld. Richard, bedankt voor de samenwerking in de afgelopen vijf jaar, de organisatie van de verschillende veldproeven, en de snelheid waarmee je alle versies van de manuscripten hebt doorgenomen.

De leden van de begeleidingscommissie, Peter Bruinenberg, Paul Heeres, Jan van Loon en Jacob Eising, wil ik bedanken voor hun interesse in het verloop van het onderzoek. Hun enthousiasme over de veldproeven en de toepassingsmogelijkheden van de geselecteerde transformanten werkte aanstekelijk.

Bertus Meijer, Anneke-Thea Hertog-van 't Oever en Marja Schippers wil ik bedanken voor hun aandeel in het opzetten en uitvoeren van de vele transformatie experimenten, die de basis vormen van dit proefschrift. De honderden transformanten uit deze experimenten werden vervolgens in de (gaas-) kas uitstekend verzorgd door Jan Rijksen en Teus van den Brink. Wim Soppe heeft in het kader van zijn vervangende dienstplicht een groot deel van deze transformanten moleculair en biochemisch geanalyseerd, hetgeen resulteerde in een lovende oorkonde bij het afzwaaien. Voor de uitvoering van de veldproeven wil ik Herman Masselink, Frans Bakker en Bartho Stoffers graag bedanken. Ook wil ik graag Aat Vogelaar, Jouke Kardolus, Coert Engels, Ellen van Enckevort, Jeanette Vreem,

Kees van Beek en Remko Koeman bedanken, die in de vorm van een afstudeervak hebben meegewerkt aan de verschillende onderdelen van het project.

De meeste werkzaamheden zijn uitgevoerd op het moleculaire en het celbiologische lab. Iedereen die daar in de afgelopen periode heeft gewerkt wil ik bedanken voor de gezellige werksfeer, die onder andere tot uiting kwam in de grote aanvoer van taart. Van de collega's op het moleculaire lab hebben Luuk Suurs en Irma Straatman het nog het beste getroffen. Zij hebben een deel van de moleculaire en biochemische analyses aan de transformanten uitgevoerd. De *starch sisters* heb ik tot mijn spijt voornamelijk ingeschakeld voor de minder aangename klussen. Zij hebben een, overigens onmisbare, bijdrage geleverd aan het kleuren van vele honderden aardappelknollen, en het bereiden van (aangenaam ruikende) aardappelmonsters voor zetmeelanalyse. Met mijn collega-AIO's en OIO's heb ik in de afgelopen periode heel wat specifieke en minder specifieke AIO- cq. OIO-problemen besproken.

Aan het eind van dit voorwoord wil ik graag mijn ouders bedanken voor hun belangstelling tijdens mijn studie en mijn AIO-schap.

Maarten, al vond je het niet nodig om bedankt te worden in dit voorwoord, ik doe het bij deze toch.

Contents

General introduction

The formation of starch and its structural organization

Composition of starch

Starch is the major storage carbohydrate in most higher plants. According to the role in plant metabolism, two types of starch can be discerned. In chloroplasts, transitory starch is synthesized for the short time storage of photosynthetic products. Reserve starch is accumulated for long time storage in amyloplasts of tubers, roots and seeds (Shannon and Garwood, 1984). Starch is organized in granules that vary in size and shape, depending on plant source and developmental stage (French, 1984), and primarily consists of two polysaccharides: amylose and amylopectin. Minor components of starch granules are lipids, proteins and phosphorus, which together generally constitute approximately 1% of cereal starches and less than 0.2% of potato starch (Swinkels, 1985).

Amylose is an essentially linear, helical α(1,4) D-glucan polymer with very few α(1,6) glucosidic linkages and a molecular weight varying from 10^4 to 10^5. The presence of multiple α(1,6) glucosidic linkages in amylopectin, an α(1,4) α(1,6) glucan polymer, results in a highly branched structure with a molecular weight of up to 10^7. The average chain length can vary from 100 to 10,000 glucose units for amylose and from 20 to 30 units for amylopectin (French, 1984; Shannon and Garwood, 1984; Zobel, 1988). Due to the difference in structure, amylose and amylopectin have several distinctive characteristics, one of these being the affinity for iodine (Shannon and Garwood, 1984). The presence in starch of long linear amylose chains results in a blue colour with iodine, which results from complexation of iodine molecules and the helical amylose molecules. A red colour is observed after iodine staining of amylopectin.

Generally, amylose makes up 11 to 37% of the total reserve starch. In potato, 18 to 23% of the tuber starch consists of amylose (Shannon and Garwood, 1984). For several plant species, mutants have been described in which the amylose content deviates from that of the corresponding wildtype (Shannon and Garwood, 1984; Smith and Martin, 1993). In the *waxy* (or *amylose-free*, or *glutinous*) mutant, the formation of amylose is completely blocked, as has been described for *waxy* maize (Echt and Schwartz, 1981; Shure et al., 1983), barley, *Sorghum*, *Coix* (Shannon and Garwood, 1984) and amaranth (Sakamoto, 1982; Konishi et al., 1985), *glutinous* rice (Sano, 1984) and wheat (Kanzaki and Noda, 1988), and *amylose-free* potato (Hovenkamp-Hermelink et al., 1987). Starch of these mutants

exclusively contains amylopectin, which results in red-coloured starch granules after staining with iodine. Mutations resulting in an increase of the amylose content have also been described. *Amylose-extender* (or *amylose-1*, or *rugosus*) mutants have been identified for maize (Boyer and Preiss, 1981), rice (Okuno, 1985), barley and pea (Smith, 1988).

Biosynthesis of starch

Formation of ADP-glucose

As is depicted in Figure 1, the starch biosynthetic pathway consists of several enzymes, of which ADP-glucose pyrophosphorylase, starch synthases and branching enzyme are directly involved in the formation of amylose and amylopectin (reviewed by Preiss, 1991 and Smith and Martin, 1993). Prior to the synthesis of these two starch components, cytosolic sucrose is converted to hexose phosphates, which are transported to the amyloplast via a hexose translocator (Viola et al., 1991). Inside the amyloplasts, ADP-glucose pyrophosphorylase (AGPase) catalyses the conversion of glucose-1-phosphate into ADP-glucose, which is the preferred substrate for starch formation *in vivo*. The central role of AGPase in the biosynthesis of starch in potato tubers has been demonstrated by Stark et al. (1992) and Müller-Röber et al. (1992). Antisense inhibition of the expression of the gene encoding subunit B of AGPase resulted in the elimination of tuber starch and the accumulation of soluble sugars (Müller-Röber et al., 1992). The AGPase-catalysed conversion of glucose-1-phosphate into ADPglucose is the regulatory step in starch synthesis (Preiss, 1991). The enzyme is stimulated by 3-P-glyceraldehyde (corresponding with a high rate of photosynthesis), and inhibited by inorganic phosphate (corresponding with a low rate of photosynthesis).

Formation of amylose: granule-bound starch synthase

Amylose and amylopectin molecules are synthesized by the addition of ADP-glucose to the non-reducing end of the glucan chain. This reaction is catalysed by the starch synthases, of which two types can be discerned, namely granule-bound starch synthase (GBSS), which is tightly bound to the granule, and soluble starch synthases (SSS). GBSS as well as SSS are capable of elongating amylose and amylopectin molecules *in vitro* (Baba et al., 1987; MacDonald and Preiss, 1985; Ponstein, 1990). *In vivo*, GBSS is predominantly involved in the synthesis of amylose, which has been demonstrated by the absence of amylose in starch of

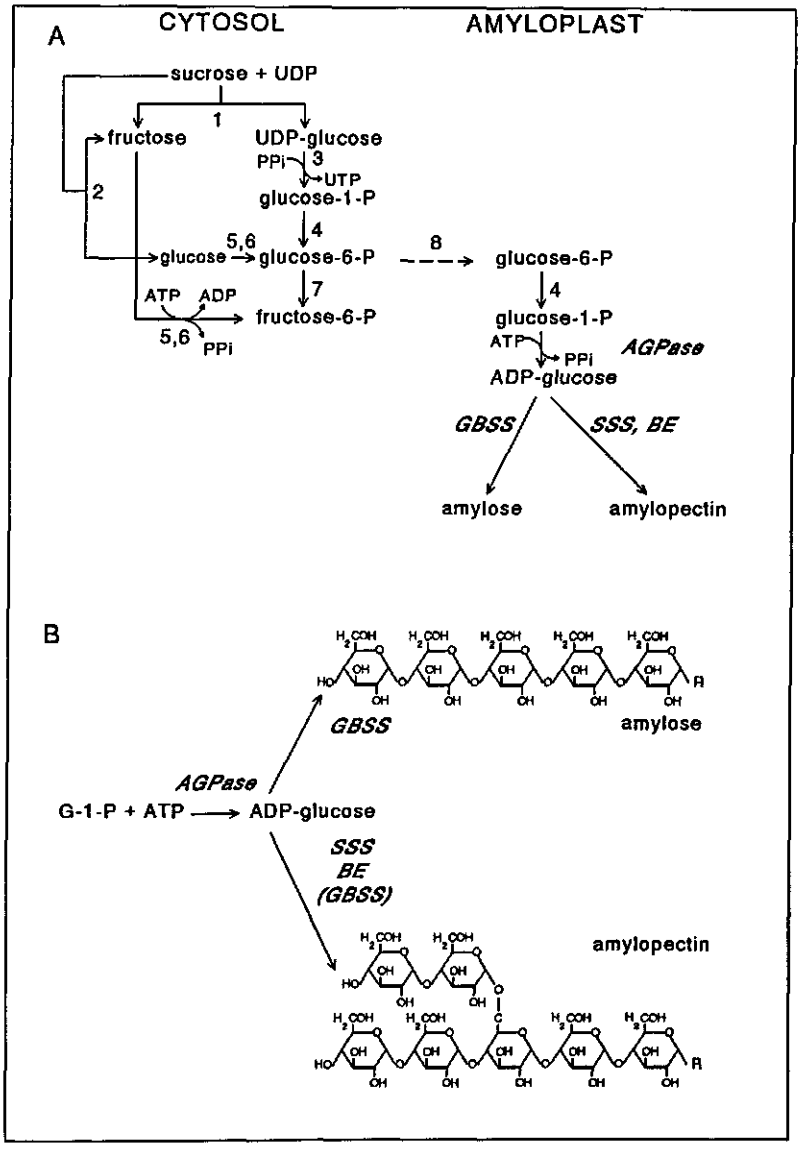

Figure 1: A. The starch biosynthetic pathway. B. The formation and structure of amylose and amylopectin.
AGPase, ADP-glucose pyrophosphorylase; BE, branching enzyme; G-1-P, glucose-1-phosphate; GBSS, granule-bound starch synthase; SSS, soluble starch synthase. All other enzymes are indicated by numbers: 1, sucrose synthase; 2, invertase; 3, UDP-glucose pyrophosphorylase; 4, phosphoglucomutase; 5, hexokinase; 6, hexose-6-phosphatase; 7, glucose-phosphate isomerase; 8, hexose translocator.

waxy mutants, that lack the GBSS protein and activity (Tsai, 1974; Echt and Schwartz, 1981; Sano et al., 1984; Konishi et al., 1985; Hovenkamp-Hermelink et al., 1987). In a recent study on a *waxy* mutant of *Chlamydomonas reinhardtii* it was proposed that GBSS is also involved in the synthesis *in vivo* of a fraction of amylopectin, since a structurally modified amylopectin was accumulated in this *waxy* mutant (Delrue et al., 1992).

Solubilization of granule-bound starch synthase from maize starch granules and separation by ion-exchange chromatography has revealed two isoforms: GBSS I (58-61 kDa) and GBSS II (93 kDa) (MacDonald and Preiss, 1985). The lack in *waxy* endosperm of the 60 kDa GBSS I protein (Echt and Schwartz, 1981) and the GBSS activity (Nelson and Rines, 1962; Nelson et al., 1978) has led to the identification of the *waxy*-locus of maize as the structural gene encoding GBSS I. Furthermore, the GBSS activity was found to vary linearly with the *Wx* gene dosage (Tsai, 1974), and the amount of 60 kDa GBSS I protein correlated with the *Wx* gene dosage (Echt and Schwartz, 1981). These data indicate that a single GBSS isoform is likely to be responsible for the formation of amylose. In pea, the identity between the GBSS enzyme activity and the GBSS (or Waxy) protein has been proven biochemically by immunoblotting (Sivak et al., 1993), although others doubt whether the GBSS enzyme and the Waxy protein are identical (Smith, 1990).

Several lines of evidence confirm the identity between the potato GBSS enzyme activity and the GBSS protein. Firstly, the GBSS activity in starch granules is severely reduced in the *amf*-mutant (Hovenkamp-Hermelink et al., 1987). This reduction is accompanied with the complete loss of the 60 kDa GBSS protein, and the formation of amylose-free starch in tubers, stomatal guard cells, columella cells of root caps, and microspores (Hovenkamp-Hermelink et al., 1987; Jacobsen et al., 1989). Considering these characteristics, the *amf*-mutant closely resembles the *waxy*-mutants of monocotyledonous plants.

Secondly, specific antibodies, raised against the 60 kDa potato GBSS protein, were found to cross react with the *waxy* proteins of maize and grain amaranth (Vos-Scheperkeuter et al., 1986). The amino acid sequence of the 60 kDa protein, that has been determined by Edman degradation (Ponstein, 1990) and deduced from the sequence of the isolated GBSS gene, showed a high degree of similarity with those of the *waxy* proteins of maize, rice and barley, and the *E.coli* glycogen synthase protein (van der Leij et al., 1991a).

Thirdly, the *amf* mutation has been identified by sequence analysis as a point deletion in the region encoding the GBSS transit peptide, which is involved in the routing and transport into the plastids (van der Leij et al., 1991a). The mutant *amf*

allele could be complemented by the introduction of the isolated wildtype GBSS gene into the *amf*-mutant via transformation with *Agrobacterium rhizogenes*, resulting in the complete restoration of the GBSS activity and the formation of amylose in all starch synthesizing tissues (van der Leij et al., 1991b).

Finally, antisense RNA mediated inhibition of GBSS gene expression resulted in a reduction of the GBSS activity in potato tuber starch of up to 100% (Visser et al., 1991a). Phenotypically, some of the transgenic plants expressing the antisense GBSS gene resembled the *amf*-mutant, as is shown by the absence of the 60 kDa GBSS protein, the GBSS activity and the lack of amylose in starch synthesizing tissues (Visser et al., 1991a).

Formation of amylopectin: soluble starch synthase and branching enzyme

Contrasting with granule-bound starch synthase, the soluble starch synthases are predominantly involved in the synthesis of amylopectin. The role of soluble starch synthase in the formation of amylopectin is further demonstrated in *Chlamydomonas st-3* mutants, that are defective for one (SSSII) of two isoforms of soluble starch synthase (Fontaine et al., 1993). These mutants displayed a reduced amount of starch and an increased amylose content, whereas the amylopectin fraction showed a shift from intermediate size chains, with a length of 8 to 50 units, to short chains (2-7 units) (Fontaine et al., 1993). For maize, two isoforms of soluble starch synthase have been described, SSS I (70 kDa) was found to be active in the absence of an exogenous primer, and hence is called the "unprimed" SSS (Pollock and Preiss, 1980; MacDonald and Preiss, 1985), whereas for the catalytic activity of SSS II (95 kDa) an α (1,4) glucan primer is needed (MacDonald and Preiss, 1985). For potato, two SSS isoforms (SSS I: 78 kDa, and SSS II: 85 kDa) have been described as well (Ponstein, 1990), although others reported a single SSS of either 90 kDa (Hawker et al., 1972) or 70 kDa (Baba et al., 1990).

The introduction of branch points in the amylopectin molecules is catalysed by the branching enzyme, which cleaves α (1,4) linked chains at 15 to 20 units from the non-reducing end. The cleaved fragment is then rejoined with the same or an adjacent molecule by the formation of an α (1,6) linkage (Smith and Martin, 1993). Multiple isoforms of the branching enzyme have been described for several plant species, including maize (BE I, BE IIa and BE IIb; Boyer and Preiss, 1981) and pea (BE I and BE II; Smith, 1988). The *amylose-extender* mutation of maize and the *rugosus* mutation of pea are known to interfere with branching activity, because of the absence of one of the isoforms of branching enzyme (Boyer and Preiss,

1981; Smith, 1988; Bhattacharyya et al., 1990). For potato, at least one isoform has been identified, which resembles BE I from maize (Vos-Scheperkeuter, 1989; Blennow and Johansson, 1991).

Localization of starch synthesizing enzymes

The possible role of the spatial organization of starch synthesizing enzymes inside the amyloplasts has been studied in potato tuber cells by immunogold labelling (Kim et al., 1989; Kram et al., 1993a). The regulatory enzyme AGPase was found to be located exclusively in the amyloplasts. Occasionally, the enzyme was observed along specific layers of the starch granules, which were supposed to reflect sites of active starch synthesis (Kim et al., 1989).

Kram et al. (1993a) demonstrated that branching enzyme is located at the interface of starch and stroma in the amyloplasts, indicating that branching occurs at the granule surface. With respect to GBSS, immuno electron microscopy indicated the presence of label throughout the starch (Kram et al., 1993b). However, a comparable amount of label was observed on amylose-free starch, although Western blot analysis has demonstrated the specificity of the GBSS antiserum (Kram et al., 1993b). The localization studies indicate that newly formed amylose might be spatially separated from branching enzyme, thus preventing the introduction of branch points in the amylose molecules.

Organization of starch granules

With respect to the initiation of starch granule formation limited information is available. The first step in granule formation may be the accumulation of a separate phase of amorphous starch. Crystallization of the amorphous starch at a certain moment might then provide the centre or hilum of the starch granule (French 1984). This centre may contain an amylose-lipid complex, which is occasionally visible as a blue-staining core in *waxy* starch granules of for example *Sorghum* (French, 1984), and might result from the specific binding and precipitation of amylose and lipoproteins, as has been reported for fungi (Varkey et al., 1985; Rutherford et al., 1986). Further growth of the starch granules occurs by apposition (Badenhuizen and Dutton, 1956), and has been suggested to occur mainly by the simultaneous elongation and crystallization of chains that are already incorporated into the starch granule structure (French, 1984; Smith and Martin, 1993).

Within native starch granules, concentric growth rings can be observed under light or electron microscopy. These rings represent alternating semi-crystalline and amorphous layers, and generally are between 120 and 400 nm in size (French, 1984). The semi-crystalline layers are known to consist of stacks of crystalline and amorphous lamellae, that originate from the organization of amylopectin molecules (Oostergetel and van Bruggen, 1989; Cameron and Donald, 1992; Imberty et al., 1991). A cluster model has been proposed for amylopectin of, for example, potato and maize starch. In this model, the crystalline lamellae represent the linear $\alpha(1,4)$ chains that are organized in double helices, whereas the amorphous layers consist of the $\alpha(1,6)$ branch points (Oostergetel and van Bruggen, 1989). The crystalline domains are organised in a continuous network of superhelices (Oostergetel and van Bruggen, 1993), and make up 40 to 50% of the granule (Gidley and Bociek, 1985). Optical diffraction analysis of electron micrographs of starch granule fragments showed the periodicity of the lamellar structure to vary from 9.2 nm for potato starch to 10.4 nm for barley starch (Oostergetel and van Bruggen, 1989). This periodicity was also measured by small angle X-ray diffraction and was found to be independent of the botanical source of the starch analysed (Oostergetel and van Bruggen, 1989; Jenkins et al., 1993). Both in wildtype and *waxy* maize a periodicity of 10.1 nm was found (Oostergetel and van Bruggen, 1989), which indicates that the lamellar structure of amylopectin is not affected in *waxy* maize.

Recently, cross-linking experiments with granular maize and potato starches have demonstrated that the amylose molecules are interspersed among the radially arranged amylopectin molecules (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). In the same study, it was demonstrated that the chain length of amylose molecules and amylopectin B-chains was larger at the core than at the periphery of the granules.

Potato development and starch formation

It is known that during plant growth changes occur in the amount and composition of starch that is deposited in the storage organs of various plant species (Geddes et al., 1965; French, 1984). During potato development, an increase of tuber size from 0-1 cm to 10-11 cm was found to be accompanied by an increase of the starch content from 5 to 18% of the tuber fresh weight, as well

as an increase of the average starch granule size (Geddes et al., 1965). Developmental changes of amylose contents have been reported for starch-containing tissues of several crops (French, 1984). In potato, the amylose content increased from 12.5 to 20% during tuber growth, and was found to be correlated with increasing granule size when granules from a single developmental stage were separated according to their size (Geddes et al., 1965). This is in accordance with the higher amylose content in starch from the outer layers of the granule in comparison with starch from the central part, as has been demonstrated by chemical gelatinization and fractionation of starch granules (Jane and Shen, 1993). Biochemical analysis demonstrated an increase during tuber development of the activity of several enzymes involved in starch biosynthesis (Hawker et al., 1979).

Inside the potato tuber, variations can occur in the distribution of the dry matter, of which starch constitutes 75%. The dry matter content is relatively low at the periphery of the tuber (14%), increases in the storage parenchyma associated with the phloem (25 to 28%), and decreases towards the centre of the tuber (18%) (Burton, 1966). Additionally, the dry matter content increases from the apical end to the stolon attachment end (Burton, 1966). Since these gradients reflect variation of cell maturity, it is expected that similar gradients occur for average granule size and amylose content.

Modification of the biosynthesis of starch

Strategies for the genetic modification of starch formation

The existence of mutants in which the quality or quantity of reserve starch in storage organs is altered, illustrates the potential for the genetic modification of the starch biosynthetic pathway. Several strategies can be followed to alter starch content and composition (Shewmaker and Stalker, 1992; Visser and Jacobsen, 1993). On the analogy of affecting the carbohydrate metabolism by mutations, the expression of specific genes can be inhibited by using antisense RNA technology, which is described below. In contrast with the recessive nature of mutations such as *wx* or *amf*, an antisense gene acts as a dominant suppressor gene, and hence can be directly applied in tetraploid potato cultivars. The application of the antisense technology in potato has been demonstrated for ADP-glucose pyrophosphorylase (Müller-Röber et al., 1992), granule-bound starch synthase (Visser et al., 1991a), and UDP-glucose pyrophosphorylase (Zrenner et al., 1993).

The overexpression of homologous genes by the introduction of additional copies of the gene of interest is often hampered by co-suppression, which results

in the inhibition of gene expression rather than overexpression (van der Krol et al., 1990a; Jorgensen et al., 1990; Napoli et al., 1990; Smith et al., 1990a; van der Leij, 1992). However, heterologous expression of, for example, bacterial genes that are driven by a plant promoter and contain a transit peptide for plastid targeting has been shown to affect carbohydrate metabolism. The expression of a bacterial ADP-glucose pyrophosphorylase gene (*E.coli* glgC) from a patatin promoter and a transit peptide from the small subunit (SSU) of Rubisco resulted in an increased starch content in the potato cultivar Russet Burbank (Shewmaker and Stalker, 1992). Cyclodextrins, which are cyclic oligosaccharides containing 6, 7 or 8 α (1,4) linked glucopyranose units, were produced in potato tubers that expressed a cyclodextrin glycosyl transferase gene from *Klebsiella pneumoniae* from the patatin promoter and the Rubisco SSU transit peptide (Oakes et al., 1991). Furthermore, the introduction of bacterial fructosyltransferase genes into the normally non fructan-storing potato plant was found to result in the accumulation of fructans in each tissue tested (van der Meer et al., 1994).

Besides the availability of characterised genes or cDNAs, storage organ specific promoters and, if needed for plastid targeting, transit peptide sequences, an effective system for plant transformation is essential for the successful modification of starch content and composition. For potato, which is amenable to *Agrobacterium*-mediated transformation, transgenic plants can be obtained from a range of varieties by cocultivation of *A.tumefaciens* (De Block, 1988; Ishida et al., 1989; Sheerman and Bevan, 1988; Stiekema et al., 1988; Visser et al., 1989a) or *A.rhizogenes* (Ooms et al., 1985; Visser et al., 1989b) with stem, leaf or tuber explants.

Antisense RNA

The antisense RNA technology is assumed to be based on the formation of a duplex between a target mRNA sequence and a complementary antisense RNA via base pairing. Gene expression can be inhibited by degradation of the RNA duplex, impaired nuclear processing of the target mRNA or inhibition of transport from the nucleus. The exact mechanism of antisense RNA mediated inhibition is not fully understood. Antisense RNA mediated regulation of gene expression occurs naturally in bacteria, as has been reviewed by Takayama and Inouye (1990).

Natural antisense RNAs have also been demonstrated for eukaryotes, although in most cases their function in the regulation of gene expression remains to be elucidated. Examples of natural eukaryotic antisense RNAs include *Xenopus*

fibroblast growth factor (Kimelman and Kirschner, 1989), mouse dihydrofolate reductase (Farnham et al., 1985), satellite RNA of cucumber mosaic virus (Rezaian and Symons, 1986), barley α-amylase (Rogers, 1988), and maize α-tubulin (Dolfini et al., 1993). The first report on the use of artificial antisense genes to inhibit eukaryotic gene expression came from Izant and Weintraub (1984). For plants, Ecker and Davis (1986) described a transient assay system in which carrot protoplasts were simultaneously electroporated with sense and antisense CAT genes. This resulted in the inhibition of CAT activity of up to 95% as compared to the control which only contained a sense CAT gene. Since these initial studies, the expression of a wide variety of genes has been effectively inhibited, either transiently, with *in vitro* transcribed antisense RNAs, or by the introduction of antisense genes via transformation (reviewed by van der Krol et al., 1988a; Watson and Grierson, 1993). Generally, antisense inhibition of gene expression results in a reduction of the mRNA steady state level, a reduced amount of protein and a reduced enzyme activity.

Application of the antisense RNA technology in plants

The feasibility of an increasing number of plant varieties for transformation and regeneration facilitates the stable introduction of antisense genes in the genome. This has resulted in the inhibition of the expression of a range of endogenous plant genes (e.g. van der Krol et al., 1988b; Elomaa et al., 1993; Shimada et al., 1993; Hemenway et al., 1988; Müller-Röber et al., 1992; Oeller et al., 1991), and reporter genes introduced by previous transformation (e.g. Cornelissen and Vandewiele, 1989; Delauney et al., 1988; Rothstein et al., 1987). A 90 to 100% decrease of the expression level of the target gene can be achieved with most antisense genes, although the extent of inhibition generally varies among transgenic clones that carry the same antisense gene (e.g. van der Krol et al., 1988b; Stockhaus et al., 1990; Sheehy et al., 1988; Visser et al., 1991a).

Most antisense genes consist of a full length cDNA of the target gene, cloned in reverse orientation behind the 35S CaMV promoter (e.g. van der Krol et al., 1988b; Elomaa et al., 1993; Hemenway et al., 1988; Müller-Röber et al., 1992). Occasionally, antisense gene constructs based on a 5′ or 3′ part of the cDNA have been reported to effectively inhibit the target gene expression (Smith et al., 1988; Bird et al., 1991; Hall et al., 1993; van der Krol et al., 1990b). Inhibition could also be achieved with antisense genes driven by an organ or tissue specific promoter (van der Krol et al., 1990b; van der Meer et al., 1992; Knutzon et al., 1992), or

antisense genes based on genomic intron-containing sequences in stead of cDNA sequences (Tieman et al., 1992; Shimada et al., 1993). The introduction of heterologous antisense genes, driven by the 35S CaMV promoter also resulted in the (partial) inhibition of the expression of the target gene (Temple et al., 1993; Oliver et al., 1993; Salehuzzaman et al., 1993).

The influence of construct composition on the effectiveness of antisense inhibition has been studied for the suppression of the chalconesynthase (CHS) gene in *Petunia* (van der Krol et al., 1990b) and the CAT enzyme activity in carrot protoplasts that transiently expressed sense and antisense CAT genes that were introduced simultaneously by co-electroporation (Bourque and Folk, 1992). Although relatively small numbers of transgenic clones have been evaluated, these studies indicate the occurrence of differences in the effectiveness of the constructs analysed. For petunia, inhibition of CHS gene expression, resulting in inhibited flower pigmentation, was achieved with antisense genes based on either the full length CHS cDNA or 3' cDNA fragments (van der Krol et al., 1990b). Antisense genes based on 5' fragments of the CHS cDNA did not affect flower pigmentation. Effective inhibition of flower pigmentation was achieved with antisense genes driven by the 35S CaMV- or the endogenous CHS promoter, which were found to be of comparable strength (van der Krol et al., 1990b). In carrot protoplasts, reduced CAT activity was observed after co-electroporation of a sense CAT gene and an antisense gene based on either a 5' fragment, a 3' fragment or the complete CAT gene (Bourque and Folk, 1992). The inhibition was highest after introduction of an antisense gene based on the 3' CAT gene fragment. At present, no information is available on the effectiveness of antisense genes based on genomic sequences in comparison with antisense genes based on the corresponding cDNA sequences.

In general, the introduction of an antisense gene results in transgenic clones in which the extent of inhibition of the target gene expression can vary from 0 to 100% (van der Krol et al., 1988b; Stockhaus et al., 1990; Sheehy et al., 1988; Visser et al., 1991a). This variability of inhibition among transgenic clones is likely to be due to position effects, that are assumed to be caused by influences of adjacent plant genomic DNA sequences or by the chromosomal structure at the integration site (Peach and Velten, 1991). Another factor that might affect the extent of antisense inhibition is the number of T-DNA insertions. Rodermel et al. (1988), who analysed the antisense inhibition of the ribulose bisphosphate carboxylase (Rubisco) enzyme level in five transgenic tobacco clones, have shown that the lowest Rubisco RNA and protein content were found in a clone that carried

at least four T-DNA copies. For tomato, selfings have been carried out with transgenic lines containing one T-DNA copy with an antisense polygalacturonase gene (Smith et al., 1990b) or an antisense gene from a cDNA clone involved in ethylene synthesis (Hamilton et al., 1990). Analysis of the progenies showed almost complete inhibition of gene expression in homozygous plants (2 T-DNA copies) and incomplete inhibition in hemizygous plants (1 T-DNA copy).

Stability of antisense inhibition

Several studies have demonstrated the stable inhibition of the target gene in subsequent generations of plants expressing an antisense gene. Clonal progeny of potato clones in which the AGPase B gene expression was inhibited showed a similar degree of inhibition as the primary transformants. Tubers from both generations were devoid of starch, and accumulated comparable amounts of sucrose and glucose (Müller-Röber et al., 1992). The sexual transmission of antisense genes has been studied by backcrossing of transgenic petunia clones (van der Krol et al., 1990b) and selfing of tomato (Hamilton et al., 1990; Smith et al., 1990b; Tieman et al., 1992). Progeny analysis has shown the stable inheritance, and thus the stable integration in the plant genome, of the introduced antisense gene.

With respect to the expression of antisense genes under varying environmental conditions a comparison has been made for field grown and greenhouse grown tomato plants expressing an antisense polygalacturonase (PG) gene (Kramer et al., 1990). This study has demonstrated that the 10 to 50% reduction of PG activity observed in greenhouse grown plants was maintained in the field.

Field analysis of transgenic plants

Genetic engineering, either by the expression of foreign genes or by the inhibition of endogenous gene expression, has been shown to be an important tool for the introduction or modification of specific traits. Regarding the applicability of genetic engineering in crop species, field trials are required to examine the stability of the expression of the introduced gene. In addition, field analyses have to be performed to evaluate the influence of the transformation and regeneration process and the presence and expression of the inserted gene on cultivar-related characteristics, particularly with respect to yield. To date, field trials have been carried out with transgenic plants of a wide variety of crops, including cotton, tomato, tobacco, soybean, potato rice, maize, sugar beet, rapeseed, cucumber,

alfalfa and flax (Knauf, 1991). Major targets are the introduction of *Bacillus thuringiensis* crystal protein genes (insect resistance; Delannay et al., 1989; Perlak et al., 1993), viral coat protein genes (virus resistance; Nelson et al., 1988; Jongedijk et al., 1992) and herbicide resistance genes (De Greef et al., 1989; D'Halluin et al., 1990; McHughen and Holm, 1991). Field trials have also been performed with tomato plants carrying an antisense polygalacturonase gene (Kramer et al., 1990). All experiments with transgenic potato plants described sofar have demonstrated that high levels of transgene expression can be achieved in field grown plants carrying chimeric genes encoding coat proteins of potato viruses X (PVX) or Y (PVY) (Kaniewski et al., 1990; Jongedijk et al., 1992) or the *Bacillus thuringiensis* cryIIIA protein (Perlak et al., 1993). High levels of resistance in the field can be achieved without significantly affecting tuber yield (Jongedijk et al., 1992). Furthermore, stable expression of the introduced genes has been observed throughout the growing season (Jongedijk et al., 1992) and across several clonal and sexual generations.

Scope of the thesis

In this study, the antisense RNA mediated inhibition of the expression of the gene encoding granule-bound starch synthase (GBSS) is analysed in transgenic potato plants. Earlier research on antisense inhibition of GBSS gene expression has led to the isolation of *Agrobacterium rhizogenes* transformed clones of a diploid potato genotype (Visser et al., 1991a). An initial evaluation of the extent of inhibition of GBSS gene expression in field grown tubers of four of these clones is described in Chapter 2. In the other chapters, several aspects of the antisense inhibition are discussed in more detail.

The influence of inhibition of GBSS gene expression on the synthesis of amylose and its deposition in starch granules is investigated in Chapter 3. The expression level of inserted (antisense) genes is known to be dependent on the composition of the gene construct and the genetic background at the insertion site. Chapter 4 describes the evaluation of the inhibitory effect of eleven antisense GBSS gene constructs that were introduced in a diploid potato genotype by *Agrobacterium tumefaciens* mediated transformation. The feasibility of the antisense GBSS system for application in potato cultivars, which primarily depends on the stability of the expression of the inserted antisense gene in field grown plants and the preservation of specific cultivar characteristics, is studied in Chapter 5. A general discussion is given in Chapter 6, followed by a summary.

Field evaluation of antisense RNA mediated inhibition of GBSS gene expression in potato

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Abstract

Granule-bound starch synthase (GBSS) catalyses the synthesis of amylose in starch granules. Analysis of antisense RNA mediated inhibition of GBSS gene expression in tubers from *in vitro* grown, greenhouse grown and field grown transgenic potato plants revealed complete and stable inhibition of GBSS gene expression in one clone. In three other transgenic genotypes partial and unstable inhibition was found. In these genotypes both GBSS activity and amylose content were remarkably reduced as compared with the non-transformed genotype. No relationship was found between the level of inhibition of GBSS gene expression and yield and dry matter content.

Introduction

It has been shown in a number of cases that an antisense gene can act as a dominant suppressor gene (Van der Krol et al., 1988b; Smith et al., 1988; Stockhaus et al., 1990). Introduction of an antisense gene in the plant genome leads to the transcription of antisense RNA which is complementary to the corresponding mRNA (Van der Krol et al., 1988a). It is assumed that in eukaryotic cells the hybridization of antisense RNA and mRNA inhibits the transport of RNA from the nucleus to the cytoplasm (Kim and Wold, 1985). When suppression of the expression of a certain gene is desired, introduction of an antisense gene may be less time-consuming than introduction of a recessive allele by mutagenic treatment, followed by homozygotisation and selection of the mutant phenotype. This holds especially true when the plant species concerned is cross-pollinating and tetraploid like the potato.

Visser et al. (1991a) used an antisense gene to suppress the expression of the gene for granule-bound starch synthase (GBSS), which catalyses the synthesis of amylose in amyloplasts. *Agrobacterium rhizogenes* mediated transformation was used to introduce the antisense GBSS gene construct pGB50 into the potato genome. Of the 16 transgenic clones that were obtained, *in vitro* plants and microtubers as well as greenhouse grown tubers were analysed. In tuber starch, the GBSS activity was inhibited to an extent of 70% to 100%. Complete suppression of GBSS activity correlated with the absence of GBSS protein as well as amylose, resulting in amylose-free tuber starch which stained entirely red with iodine (amylose containing starch stains blue). Partial suppression of GBSS activity was found to correlate with the partial reduction of GBSS protein level and amylose content. Tubers of clones belonging to this category contained either blue staining starch or a mixture of blue and red staining starch.

When the antisense technique is to be used in plant breeding, the stability of the suppression is of critical importance. In the present study, four transgenic clones were analysed more extensively with respect to antisense RNA mediated inhibition of GBSS gene expression under different physiological conditions. The analysis of the inhibition in tubers and other starch containing tissues of field grown, greenhouse grown and *in vitro* grown plants is presented.

Materials and methods

Plant material, in vitro conditions and microtuber induction

In vitro shoot cultures of the following *Solanum tuberosum* genotypes were used: PD007 (HH578;2n = 2x = 24); Ri007 (pBI121 transformed PD007; 2n = 2x = 24; Visser et al., 1989b); 1031-29 (F2 *amf*-mutant; Jacobsen et al.,1989); WA501, WA511, WA516, WA517 (pGB50 transformed PD007; 2n = 4x = 48; Visser et al., 1991a). The antisense gene of pGB50 consists of a full length GBSS cDNA, fused in reverse orientation between the Cauliflower Mosaic Virus 35S promoter and the nopaline synthase terminator. *Agrobacterium rhizogenes* mediated transformation had been used to integrate the gene into the plant genome (Visser et al., 1991a). Based on iodine staining of tuber starch the pGB50 transformed genotypes could be divided into three classes: I. All tubers contained red staining starch (WA501, WA511), II. All tubers contained blue staining starch at the stolon end and red staining starch at the apical end (WA517), III. All tubers contained blue staining starch (WA516). In these genotypes, which all have a decreased amylose content, one to three copies of the antisense GBSS gene are present (Visser et al., 1991a). Shoot cultures were grown on MS-medium (Murashige and Skoog,1962) with 30 g/l sucrose, at 21°C, 14h light (3200 lux). Microtubers were induced according to Hovenkamp-Hermelink et al. (1988a).

Field trial design

In August 1989 *in vitro* plants of the genotypes WA501, WA511, WA516, WA517 and Ri007, and greenhouse grown tubers of the genotypes PD007 and 1031-29 were planted in pots in the greenhouse. Plants were grown under standard greenhouse conditions. In April 1990 tubers were harvested. After two weeks the tubers were stained at the stolon end with Lugol's solution. One day later the tubers were planted in pots in the greenhouse and transferred to the field one month later. Some plants of each genotype were grown in the greenhouse. The field trial was set up according to security rules formulated by the government (Anon.,1990). The plant distance in the field was 40 cm, the ridges were 75 cm apart. The plot was surrounded with cv. Bintje. Flower buds were removed every 2-3 days. In September 1990 the plants were harvested. From each plant yield and dry matter content were determined. Dry matter content was determined by under water weighing.

Iodine staining of starch in different tissues

Iodine staining with Lugol's solution (I_2:KI) was used to visualize the presence of amylose in starch. A mixture of amylose and amylopectin stains blue whereas pure amylopectin stains red with iodine. Starch in stomatal guard cells of leaves was stained with Lugol/chloralhydrate (1:2) (Jacobsen et al., 1989) after extraction of chlorophyll from the leaves with 96% ethanol. Pollen starch was stained directly with Lugol/chloralhydrate (1:2). Starch in tubers was stained with Lugol/H_2O (1:1). Staining of tuber starch was carried out in different ways. All tubers of six plants per genotype were cut in 5 mm thick slices which were stained with Lugol/H_2O (1:1). All other tubers were stained at least at the stolon end. Tubers from genotypes with varying staining patterns (according to staining of sliced tubers) were stained at both ends.

Isolation of starch, determination of amylose content and GBSS activity

Starch was isolated from tubers pooled per plant from 3-9 plants per genotype by homogenizing them in extraction buffer (10 mM EDTA, 50 mM Tris pH 7.5, 1 mM DTT, 0.1% $Na_2S_2O_5$). The fibrous substance was washed several times with extraction buffer and placed at 4°C. After the starch granules had settled the supernatant was discarded. The starch granules were washed with extraction buffer, water and acetone respectively, and dried at 4°C. The amylose content was determined spectrophotometrically as described by Hovenkamp-Hermelink et al. (1988b). Using this method, amylose contents are slightly overestimated compared to amperometric titration. GBSS activity was measured as described by Vos-Scheperkeuter et al. (1986), and is expressed as pmol ADP-glucose incorporated per min. in 1 mg of starch. Both amylose content and GBSS activity were determined using 1-2 mg isolated starch per sample.

Results

Staining of starch in in vitro plants

The inhibitory effect of the introduced antisense gene was analysed phenotypically in *in vitro* plants of the genotypes WA501, WA511, WA516, WA517, PD007 and 1031-29. To this end, starch in stomatal guard cells, columella cells of root tips and microtubers was stained with iodine. Starch in these three cell types stained red in the *amf*-mutant 1031-29 and blue in the wildtype PD007. Starch in stomatal guard cells of leaves from the transgenic

clones, which was often difficult to stain, most often showed a blue colour. In WA501, WA511 and WA516 red staining starch granules could be found in up to 25% (WA501) of the stomatal guard cells. Leaves of WA517 only contained stomatal guard cells with blue staining starch. Root tips of plants from the genotypes WA501, WA511, WA516 and WA517, when stained three weeks after subculturing, mainly contained blue staining starch, but also red and intermediate staining starch could be observed. Intermediate staining root tips contained sectors with blue or red staining columella cells. One week after the first screening only root tips with blue staining starch were found. Microtuber starch of WA501 and 1031-29 always stained red. All microtubers obtained from WA511, WA516, WA517 and PD007 contained blue staining starch. For WA511 this observation differed from results of earlier experiments (R.G.F. Visser, unpublished data).

Iodine staining of greenhouse grown tubers

In vitro plants of the transgenic and control genotypes were multiplied and transferred to the greenhouse for tuber production. All tubers obtained were stained at the stolon end with iodine (Table 1). The genotypes PD007 and Ri007 produced tubers with blue staining starch, whereas tubers from 1031-29 always stained red. All tubers of WA501 and 85% of the WA511 tubers contained red staining starch. In some tubers from the genotypes WA516 and WA517 red staining starch was found (WA517: 5% red; WA516: 17% red).

After the iodine staining mentioned above, plants were grown in the greenhouse from three to five tubers of each genotype. The starch colour of these tubers was similar to that observed in previous staining experiments (Visser et al., 1991a; red for WA501, WA511, 1031-29; blue for WA516, WA517, PD007, Ri007). Furthermore, all blue staining WA511 tubers, all red staining WA517 tubers and eight out of fourteen red staining WA516 tubers were grown in the greenhouse. The tubers obtained from these plants were sliced and stained with iodine (Table 2). Besides red and blue staining tubers, mixed staining tubers were observed, in which red and blue staining sectors were present. Plants from red staining as well as blue staining tubers of WA511 mainly produced red staining tubers, occasionally in combination with some blue or mixed staining tubers. Both red and blue staining tubers from WA516 and WA517 gave rise to plants that produced a mixture of blue staining, red staining and mixed staining tubers.

Table 1: Iodine staining of tuber starch at the stolon end of tubers from *in vitro* plants that were grown to maturity in the greenhouse.

genotype	# red-staining tubers		# blue-staining tubers	
1031-29	46	(100%)	-	
PD007	-		57	(100%)
WA501	218	(100%)	-	
WA511	63	(85%)	11	(15%)
WA516	15	(17%)	74	(83%)
WA517	4	(5%)	71	(95%)

Table 2: Iodine staining of tuber starch throughout tubers of greenhouse grown plants. The plants were grown from previously stained tubers (colour mentioned under "parental starch colour" : r=red; b=blue).

genotype	parental starch colour	# plants	# tubers red	(%)	# tubers blue	(%)	# tubers mixed	(%)
1031-29	r	3	11	(100%)	-		-	
PD007	b	4	-		36	(100%)	-	
WA501	r	5	28	(100%)	-		-	
WA511	r	4	8	(53%)	-		7	(47%)
	b	10	34	(76%)	7	(15%)	4	(9%)
WA516	b	4	4	(19%)	8	(38%)	9	(43%)
	r	8	13	(24%)	18	(34%)	22	(42%)
WA517	b	4	3	(33%)	3	(33%)	3	(33%)
	r	4	4	(36%)	5	(46%)	2	(18%)

Yield characteristics of field grown plants

The field trial was set up with greenhouse grown tubers that were characterized phenotypically by iodine staining (Table 3). In order to characterize the transformants and to compare them with the non-transformed PD007, the average number of tubers per plant, the average yield per plant and the dry matter content (Figures 1A-B) were determined per genotype. Because the genetic background of 1031-29 differs from PD007 and the transformants, 1031-29 was not included in the comparison of average yield and dry matter content. For all genotypes high numbers of tubers per plant were found, although the transgenic clones produced less tubers per plant than PD007. The variation between and within genotypes was high. The average yield per plant for WA501 and WA516

was not significantly different from PD007, whereas WA511 and WA517 had a significantly lower yield (ANOVA; Figure 1A). Also for yield, a high level of variation was observed within and between genotypes (Table 3). The average tuber weight of WA501 and WA517 was significantly higher than of PD007 (ANOVA). With respect to dry matter content no significant differences were found for WA501 and WA516 as compared to PD007, whereas for WA511 and WA517 dry matter contents were significantly lower than for PD007 (ANOVA).

Table 3: Numbers and staining categories of parental tubers used for the field trial, total amount of field grown tubers harvested per genotype and average number of tubers per plant.

	parental tubers		harvested tubers		
genotype	# red	# blue	total number	average number per plant	(SD)
1031-29	40	-	1400	42	(31.0)
PD007	-	50	3465	77	(24.7)
WA501	200	-	7437	37	(19.3)
WA511	50	-	1200	26	(12.2)
WA516	6	70	3310	48	(17.4)
WA517	-	65	1500	26	(10.6)

Characterization of field grown tubers by iodine staining

The starch composition in field grown tubers was characterized phenotypically in order to evaluate the stability of the antisense RNA mediated inhibition. The tubers of six plants per genotype were sliced and stained with iodine. As expected, PD007 and Ri007 tubers contained blue staining starch. Tubers of 1031-29 stained red, as did tubers of the genotype WA501. From the six WA511 plants that were analysed only two produced red staining tubers. The other four showed variation for tuber starch colour. According to tuber starch colour four categories could be distinguished: red, blue, blue at the stolon end and red at the apical end (blue→red) and the reverse: red→blue. All plants of the genotypes WA516 and WA517 showed variation for tuber starch colour.

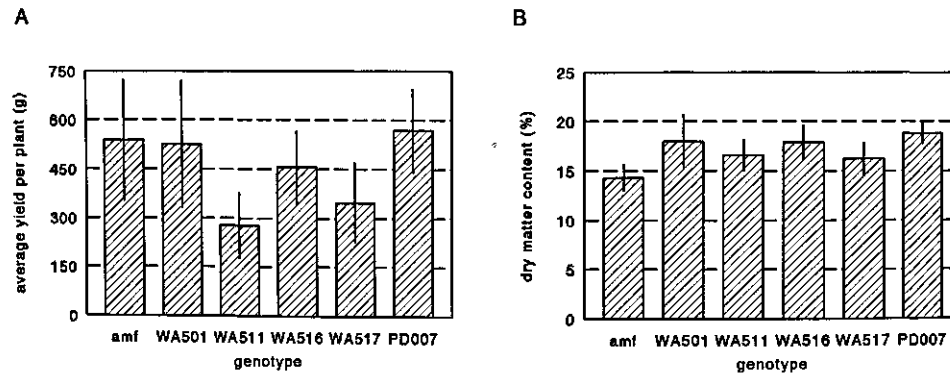

Figure 1: Average yield per plant (A) and dry matter content (B) of transformed and non-transformed potato genotypes. amf, *amf*-mutant 1031-29; PD007, non-transformed wildtype. Error bars represent standard deviations.

When compared to WA511 three extra categories could be discerned for WA517 tubers: blue and red throughout the tuber (blue/red), blue→blue/red and red→blue/red. Among WA516 tubers two additional categories were found: blue/red→blue and blue/red→red. In further staining analyses the tubers were classified using three colour categories: red, blue and mixed staining, the latter including all other categories mentioned for WA511, WA516 and WA517.

The tubers from all other field grown plants of the genotypes WA511, WA516 and WA517 were stained at both sides. The remaining tubers from field grown plants of the genotypes PD007, Ri007, 1031-29 and WA501, in which starch stained either blue or red, were stained at the stolon end only. All tubers obtained from these genotypes showed the expected blue or red colour with iodine. Within WA511, WA516 and WA517 variation was found with respect to the tuber colour categories (Figure 2: field). Among WA511 plants, tubers with only red staining starch were found in 14 out of 45 plants. The other plants produced red, blue and/or mixed staining tubers. None of the WA511 plants only produced blue staining tubers. The plants from WA516 originating from blue staining (WA516b; with one exception) or red staining (WA516r) greenhouse grown tubers and from WA517 produced a mixture of tubers with blue, red or mixed staining starch. One WA516b plant exclusively produced blue staining tubers.

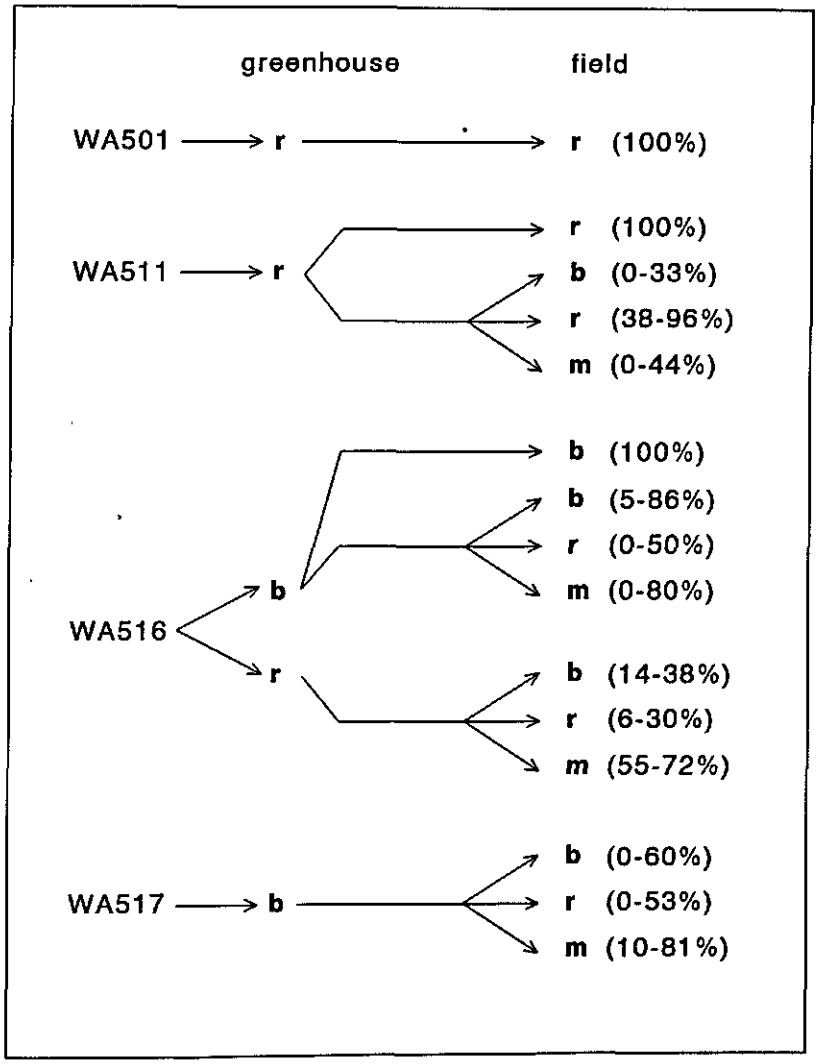

Figure 2: Schematic representation of staining characteristics of transformed genotypes. *In vitro* multiplicated plantlets were grown in the greenhouse for tuber production. The tubers were classified for starch composition by iodine staining and used for the field trial. Harvested field grown tubers were classified for starch composition as well. The range of occurrence of each category is indicated within parentheses. r, red staining tuber starch; b, blue staining tuber starch; m, mixed staining tuber starch.

The numbers of tubers per staining category were classified per genotype (Table 4), firstly according to the starch colour at the stolon end and secondly according to the starch colour throughout the tuber. A comparison of these two

classifications demonstrated that all tubers from WA511 with red staining starch at the stolon end, contained red staining starch throughout the tuber. This is in contrast with WA516 and WA517, where approximately two thirds of the red staining tubers were red troughout the tuber. Less than half of the blue staining tubers of WA511, WA516 and WA517 contained blue staining starch throughout the tuber. Staining of tubers only at the stolon end turned out to be insufficient for these genotypes, because a large number of tubers was found to contain mixed staining starch. Differences between WA516b and WA516r in relative amounts of tubers per staining category were only found when the starch colour throughout the tubers was determined. The differences in frequencies of tubers per staining category found between tubers from WA516b and WA516r plants (Table 4b) were neither significant for tubers from field grown plants, nor for tubers from greenhouse grown plants (ANOVA).

Table 4: Lugol staining of tuber starch in field grown tubers. The tuber starch colour is given for the stolon end of the tuber (A) and for stolon and apical end (B).

A. Stolon end.

	red		blue	
1031-29	895	(100%)	-	
PD007	-		3054	(100%)
WA501	6322	(100%)	-	
WA511	697	(83%)	140	(17%)
WA516b *	660	(26%)	1909	(74%)
WA516r **	77	(23%)	265	(77%)
WA517	290	(21%)	1128	(79%)

B. Stolon and apical end.

	red		blue		mixed	
1031-29	895	(100%)	-		-	
PD007	-		3054	(100%)	-	
WA501	6322	(100%)	-		-	
WA511	696	(83%)	69	(8%)	72	(9%)
WA516b *	480	(19%)	862	(33%)	1227	(48%)
WA516r **	48	(14%)	81	(24%)	213	(62%)
WA517	229	(16%)	508	(36%)	681	(48%)

* WA516b plants originate from blue staining parental tubers.
** WA516r plants originate from red staining parental tubers.

Staining of starch in microspores and stomatal guard cells

Leaf samples and flower buds (late bud stage) were collected several times during the plant growth period in the field and stored in 96% ethanol at 4°C. Starch in plastids of stomatal guard cells (±250 cells per sample) and in microspores (>1000 microspores per sample) was stained with iodine and examined microscopically. Often, microspores from flower buds could not be stained because of unfavourable developmental stage. Starch in microspores and stomatal guard cells of PD007 invariably stained blue, whereas in 1031-29 always red staining starch was found. Tuber starch of WA501 always turned out to be red, while starch in microspores stained blue. WA511, WA516 and WA517 also contained blue staining starch in microspores. In 1-3% of the stomatal guard cells of the genotypes WA501, WA511, WA516 and WA517 red staining starch was found.

Amylose content and GBSS activity in tuber starch

The amylose content and GBSS activity were determined in starch isolated from tubers that were pooled per plant and iodine stained previously (Fig.3). The values for amylose content and GBSS activity found for all transgenic genotypes were significantly lower than those found for the non-transformed PD007 (two-sided T-test, 5% level). Both amylose content and GBSS activity in WA501 starch were not significantly different from mutant starch. In WA511 starch, only the amylose content differed significantly from the mutant value. In starch from WA516 and WA517 both amylose content and GBSS activity were significantly higher than in mutant starch. A significant correlation was found between GBSS activity and amylose content (Spearman: ($r_s = 0.877$) > ($r_{26;0.2\%} = 0.586$)).

Discussion

The analysis of antisense RNA mediated inhibition of GBSS gene expression in potato plants is described. As was found previously in smaller scale experiments (Visser et al., 1991a), GBSS activity and amylose content were significantly reduced in the antisense GBSS transformed genotypes compared to non-transformed PD007. The most pronounced antisense effect was found in WA501. Both amylose content and GBSS activity of starch isolated from WA501 tubers equalled the values found for the *amf*-mutant 1031-29 (Fig.3) and the *wx*-mutant

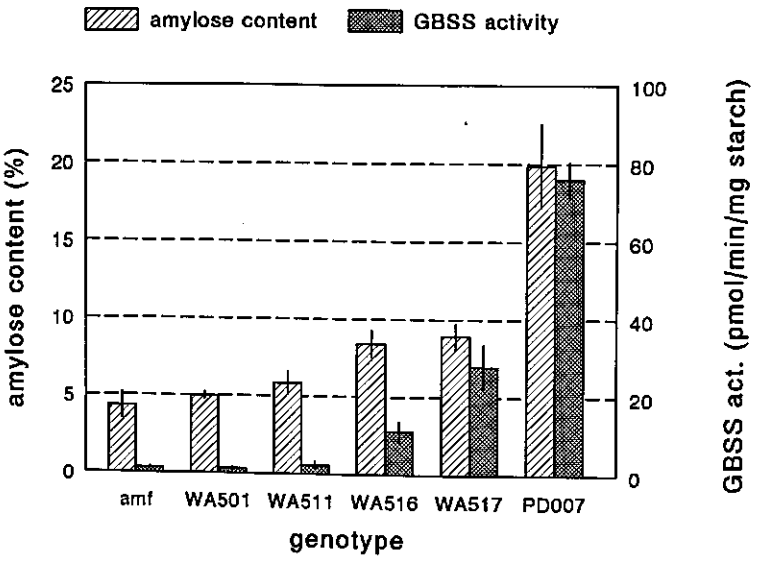

Figure 3: Amylose content and GBSS activity in siolated starch of transformed and non-transformed potato genotypes. Per genotype 3 to 9 starch samples were analysed. Each sample was analysed in triplicate. amf, *amf*-mutant 1031-29; PD007, non-transformed wildtype. Error bars indicate standard deviations.

of maize (Shannon and Garwood, 1984). No variation was found in starch colour between tubers produced by WA501 plants, which was also observed for field grown tubers from 1031-29 (Tables 2 and 4). Also microtubers from 1031-29 and WA501 always contained red staining starch. Therefore, it can be concluded that the introduced antisense GBSS gene led to complete suppression of GBSS gene expression in all tubers (field grown as well as greenhouse grown) and microtubers of WA501 plants. In contrast with the *amf*-mutant, in which the mutation is expressed in tubers and other starch containing tissues (Jacobsen et al., 1989), the inhibitory effect of the introduced antisense gene is only occasionally visible in stomatal guard cells and absent in microspores from WA501. This partial inhibition may be due to non-constitutive expression of the 35S CaMV promoter which was argued before (Visser et al., 1991a) and found in transgenic tobacco as well (Williamson et al., 1989).

Within WA511, WA516 and WA517, the occurence of different staining categories varied for individual plants. The variation found between as well as within the four transgenic genotypes might be caused by position effects: the

influence of plant DNA sequences flanking the inserted T-DNA on antisense gene expression (Smith et al., 1988). Variable antisense gene expression within individual plants was also found in petunia transformants (van der Krol et al., 1990c) where it was thought to be caused by changes in physiological conditions during flower development. When comparing the staining of starch in *in vitro* grown microtubers with greenhouse grown and field grown tubers from WA511, WA516 and WA517, physiological or environmental factors were found to affect the level of antisense RNA mediated inhibition of GBSS gene expression. Especially for WA511 the antisense effect is clearly expressed in soil grown tubers, whereas it is less pronounced in microtubers. This difference in antisense inhibition may be due to the absence (soil) or presence (*in vitro*) of sucrose at a high concentration, leading to a lower or higher GBSS gene expression, respectively. GBSS promoter studies have shown the sucrose inducibility of the GBSS gene expression (Visser et al., 1991b). Different ways of iodine staining were used for a rapid screening of antisense effects in large numbers of tubers. Staining of tubers at least at both sides turned out to be necessary for determining the stability of the antisense effect in transformants. The analysis of large numbers of tubers was shown to be essential for determining the stability of antisense RNA inhibition in individual transgenic clones, since one of the unstable transformants (WA511), was considered as stable in the smaller scale analysis (Visser et al., 1991a).

In two of the transformants used in this field trial yield and dry matter content were significantly reduced as compared to non-transformed potato which was shown before for *A.rhizogenes* transformed potato (Ooms et al., 1986). No relationship could be found between the level of inhibition of GBSS gene expression and decrease of yield or dry matter content, the latter being related to starch content. In fact, both yield and dry matter content found for WA501 (which showed the most pronounced antisense effect) were not significantly reduced as compared to PD007.

In our experiments GBSS gene expression in potato was effectively inhibited without a significant decrease in yield and dry matter content using *A.rhizogenes* mediated introduction of an antisense GBSS gene. The stability of antisense inhibition in WA501 and its variability in WA511, WA516 and WA517 will be studied in field trials for another year. In order to get more insight into the cause of the observed variability of antisense inhibition, a more detailed analysis of tubers and of the inserted T-DNA(s) will be carried out.

Formation and deposition of amylose in the potato tuber starch granule are affected by the reduction of granule-bound starch synthase gene expression

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Abstract

The synthesis of amylose in amyloplasts is catalysed by granule-bound starch synthase (GBSS). GBSS gene expression was inhibited via antisense RNA in *Agrobacterium rhizogenes*-transformed potato plants. Analysis of starch production and starch granule composition in transgenic tubers revealed that reduction of GBSS activity always resulted in a reduction of the production of amylose. Field experiments, performed over 2 years, showed that stable inhibition of GBSS gene expression can be obtained. Microscopic evaluation of iodine-stained starch granules was shown to be a sensitive system for qualitative and quantitative examination of amylose formation in starch granules of transgenic potato tubers. In plants showing inhibition of GBSS gene expression, the reduced amylose content in tuber starch was not a consequence of a lower amylose content throughout the entire starch granule. Starch granules of transgenic tubers were found to contain amylose, at a percentage similar to wild-type starch, in a core of varying size at the hilum of each granule. This indicates that reduced GBSS gene expression results in amylose formation in a restricted zone of the granules. The size of this zone is suggested to be dependent on the GBSS protein level. During development of the granules, the available GBSS protein is thought to become limiting, resulting in the formation of starch that lacks amylose. RNA gel blot analysis of tuber tissue showed that inhibition of GBSS gene expression resulted in a reduced GBSS mRNA level, but did not affect the expression level of other starch synthesizing enzymes. Antisense RNA could only be detected in leaf tissue of the transgenic plants.

Introduction

Starch, the major storage carbohydrate of higher plants, is synthesized for long-term storage in amyloplasts of, for example, potato tubers. In chloroplasts, starch is produced for the transient storage of photosynthetic products. The main components of starch are linear helical amylose (molecular weight between 10^4 and 10^5) and branched amylopectin (molecular weight up to 10^7), which are both glucose polymers. Reserve starch in potato tubers contains 18-23% amylose (Shannon and Garwood, 1984). The growth of starch granules occurs via apposition (Badenhuizen, 1963). The structure of starch granules is determined by the crystalline organization of the amylopectin molecules (Oostergetel and van Bruggen, 1989). From cross-linking experiments in potato and maize, it is known that amylose molecules are interspersed among the radially arranged amylopectin molecules (Jane et al., 1992). Transitory and reserve starch are synthesized from glucose by the same set of enzymes of which starch synthases and branching enzymes are key enzymes. Starch synthases catalyse the elongation of amylose and amylopectin by adding ADP-glucose to the non-reducing end of the polymers and can be divided into soluble starch synthases and granule-bound starch synthase (GBSS), the latter being involved in the biosynthesis of amylose (Robyt, 1984).

Recently, GBSS gene expression was shown to be inhibited after introduction of an antisense GBSS gene construct into the potato genome (Chapter 2; Visser et al., 1991a). The antisense RNA mediated inhibition was found to vary between and within the transgenic clones. In tuber starch of one of these clones, GBSS activity was inhibited up to 100%. Complete suppression of GBSS activity was shown to correlate with the absence of GBSS protein and amylose. Partially suppressed GBSS activity was accompanied by a reduction of GBSS protein level and amylose content.

Antisense gene expression, resulting in varying degrees of reduction of protein level and enzyme activity as observed in the transgenic clones mentioned above, has also been reported for other systems. In potato, inhibition of ADP-glucose-pyrophosphorylase (AGPase) expression resulted in a varying, but clearly reduced amount of protein and enzyme activity in eight out of 35 transformants (Müller-Röber et al., 1992). This led to the inhibition of starch synthesis in tubers and accumulation of soluble sugars. Antisense mediated inhibition of chalcone synthase (*CHS*) gene expression in petunia resulted in a reduction of CHS protein level varying for individual transformants (van der Krol et al., 1988b). Furthermore,

variation in flower phenotype was observed both between and within individual transformants (van der Krol et al., 1990c).

In this report, the varying antisense RNA-mediated inhibition of GBSS gene expression that was described before (Chapter 2) is further examined in field-grown potato plants. The influence of the introduction of the antisense GBSS gene on GBSS steady-state mRNA levels was studied in leaf and tuber tissue. Furthermore, the presence of GBSS antisense RNA was determined in these tissues and the effect of inhibition of GBSS gene expression on the expression levels of other starch synthesizing enzymes was determined in tuber tissue.

A model is presented to quantify the amylose content in starch using the iodine-staining pattern of starch granules from the transgenic plants, and the effect of reduced GBSS gene expression on the formation of amylose in starch granules is discussed.

Materials and methods

Plant material and field trial

The following potato (*Solanum tuberosum*) genotypes were used: PD007 (HH578;2n = 2x = 24); 1031-29 (F2 *amf*-mutant; 2n = 2x = 24; Jacobsen et al., 1989); WA501, WA511, WA516, and WA517 (pGB50 transformed PD007; 2n = 4x = 48; Visser et al., 1991a). In the pGB50-transformed genotypes, one to three copies of the antisense granule-bound starch synthase (GBSS) gene were present. This gene consists of a full-length GBSS cDNA, fused in reverse orientation between the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator, and was inserted into the plant genome using *Agrobacterium rhizogenes*-mediated transformation (Visser et al., 1989b; 1991a). *In vitro*-grown plants of the genotypes WA501, WA511, WA516, and WA517 and tubers of the genotypes PD007 and 1031-29 were transferred to the greenhouse for tuber production. Plants were grown under standard greenhouse conditions. Two weeks after harvesting the tubers were stained at the stolon end with Lugol's solution (1% Lugol's solution, I-KI [1:2, v/v]; Merck), and subsequently planted in pots in the greenhouse. Plants were transferred to the field 1 month later. The field trials were carried out according to governmental guidelines (Anonymous, 1990) as described before for experiments carried out in 1990 (Chapter 2). In 1991, the field trial was set up with tubers of all genotypes of which the tuber starch color had been determined the year before by means of iodine staining.

Iodine staining of starch

The staining of starch in several tissues was carried out using Lugol's solution (1% Lugol's solution, I-KI [1:2, v/v]; Merck). Starch in longitudinal tuber sections was stained with Lugol-H_2O (1:1). Starch isolated from individual tubers was suspended in water, stained with a few drops of Lugol's solution, and examined microscopically.

Isolation of starch

Starch was isolated from tubers that were iodine stained beforehand. Tubers were homogenized in extraction buffer (10 mM EDTA, 50 mM Tris, pH 7.5, 1 mM DTT, 0.1% $Na_2S_2O_5$). The fibrous substance was washed several times with extraction buffer and filtered subsequently. The filtrate was placed at 4°C. After the starch granules had settled, the supernatant was discarded. The starch granules were subsequently washed with extraction buffer, water, and acetone, and dried at 4°C.

Determination of amylose content and GBSS activity

The amylose content of individual tubers was determined spectrophotometrically in 1-2 mg isolated starch according to the method described by Hovenkamp-Hermelink et al. (1988b). Amperometric titration (Williams et al., 1970) was used for determination of the average amylose content per genotype. For this determination, total starch was isolated from 5 kg of potato tubers per genotype. Using spectrophotometric analysis, low amylose contents are overestimated in comparison with amperometric titration. The GBSS activity was determined in 1-2 mg isolated starch as described by Vos-Scheperkeuter et al. (1986), and is expressed as pmol ADP-glucose incorporated per min. in 1 mg of starch. The individual tuber starch samples were analysed in triplicate for amylose content and GBSS activity.

Measurements of blue-staining cores in starch granules

In iodine-stained starch granules, the radii of the blue staining core and the total granule were measured microscopically using an ocular micrometer. Due to the ovoid character of the granules both the long radius (R_{length}) and the short radius (R_{width}) were determined. The radii of the blue-staining core and the total granule were determined in more than 200 individual, randomly chosen starch granules. From the radii, the ratio (V_{blue}/V_{total}) between the volume of the blue staining core and the total granule volume was calculated:

$$V_{blue}/V_{total} = (R^2_{blue,width} \times R_{blue,length})/(R^2_{total,width} \times R_{total,length})$$

RNA analysis

RNA was extracted from leaves and individual iodine-stained tuber halves of each genotype. Tissue (1 to 2 g) was ground in liquid N_2 and mixed with 3 ml of extraction buffer (50 mM Tris, pH 9.0, 10 mM EDTA, 2% SDS) and 3 ml of phenol. The mixture was centrifuged (5 min at 1400*g*), the supernatant was extracted with 3 ml of phenol-chloroform (1:1), and the mixture was centrifuged again. Nucleic acids were precipitated with 3 ml of isopropanol and centrifuged (10 min at 9000*g*); the pellet was dissolved in 1 ml of H_2O. RNA was precipitated with 1 ml of 4 M LiCl for 3 h on ice and centrifuged (10 min at 9000*g*). The RNA pellet was dissolved in 0.5 ml of H_2O and precipitated with 50 μl of 3 M NaAc and 1 ml of ethanol. Following centrifugation the pellet was washed in 70% ethanol, dried, and resuspended in H_2O.

RNA gel blotting and hybridization were carried out using 30 μg of tuber RNA or 70 μg of leaf RNA per sample, as described by Sambrook et al. (1989) with some modifications. The membranes were hybridized with the following ^{32}P-ATP-labeled cDNA probes: a 1.3-kb EcoRI fragment of GBSS (Hergersberg, 1988; Visser et al., 1989c); a 1.5-kb EcoRI-HindIII fragment of sucrose synthase (Salanoubat and Belliard, 1987); a 1.7-kb EcoRI fragment of ADP-glucose pyrophosphorylase subunit S (AGPase S; Müller-Röber et al., 1990), a 2.8-kb SstI-HindIII fragment of branching enzyme (Kossmann et al., 1991); and a 2.3-kb EcoRI fragment of a potato 28S ribosomal RNA gene (Landsmann and Uhrig, 1985).

Strand-specific ^{32}P-UTP-labeled probes were transcribed from plasmids pT7T3α18GBc and pT7T3α19GBc. These plasmids were obtained by cloning the SpeI-BamHI GBSS cDNA into the XbaI and BamHI sites of pT7T3α18 and pT7T3α19 (Bethesda Research Laboratories). An antisense probe for detecting GBSS mRNA was obtained by restricting pT7T3α19GBc with BamHI and using T7 RNA polymerase for transcription. Sense probes for detecting antisense RNA were obtained either by restriction of pT7T3α19GBc with SalI followed by transcription with T3 RNA polymerase or by restriction of pT7T3α18GBc with SphI followed by T4 DNA polymerase treatment and transcription with T7 RNA polymerase. Probe DNA was removed from the filters by incubation 15 min in 2 mM Tris, pH 7.5, 0.1% SDS at 65°C; 30 sec in 0.4 M NaOH at 21°C, and 2 min in 0.2 M Tris, pH 7.5, 2x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate) at 21°C. The intensity of the RNA signals on the autoradiograms was scanned with a densitometer (Cybertech, Berlin).

Results

Phenotypic characterization of transgenic plants

The level of antisense RNA mediated inhibition of GBSS gene expression in tubers of the four transgenic potato clones was compared in two successive field trials. In 1990 the field trial was set up with tubers from greenhouse grown plants that were sprouted in pots in the greenhouse and subsequently transferred to the field. All harvested tubers were stained with iodine in order to evaluate the presence of amylose, as shown in Figure 1A (for a more detailed description see Chapter 2).

The 1991 field trial was set up with field-grown tubers obtained from the 1990 field trial. Tubers obtained from the 1991 field trial were phenotypically analysed in a random iodine staining test (Figure 1B). This analysis confirmed the results of the 1990 field trial concerning the total and stable inhibition of GBSS gene expression in tubers of WA501. However, in WA511, WA516 and WA517 the relative amount of mixed staining tubers was much higher than in 1990. Tuber staining for these genotypes ranged from almost red (amylose-free) via mixed staining (Figure 2A) to blue (amylose containing). Despite the shift toward a higher percentage of tubers with mixed staining starch, WA511 could be distinguished from WA516 and WA517 due to a higher percentage of red staining tubers in both years. Most tubers of the genotypes WA516 and WA517 were found to contain mixed staining starch. In both field trials, the phenotype of parental tubers of the genotypes WA516 and WA517 did not determine the ratio of red-, mixed-, and blue-staining tubers.

Distribution of amylose in isolated starch granules

To gain more insight into the nature of the different staining categories that were observed among tubers with reduced GBSS expression, iodine-stained starch granules from transgenic and control genotypes were examined microscopically. Completely blue-staining starch was found in wild-type starch granules, whereas starch granules of the amylose-free (*amf*) mutant of potato always were red, occasionally with a faint blue line at a growth ring. Remarkable staining patterns were observed in the starch granules of WA501, WA511, WA516, and WA517, which contained a blue-staining core around the hilum (at the center) of each starch granule, as shown in Figures 2B to 2D. Apparently, the formation of amylose in tubers with reduced GBSS expression is restricted to the hilum of the starch granules.

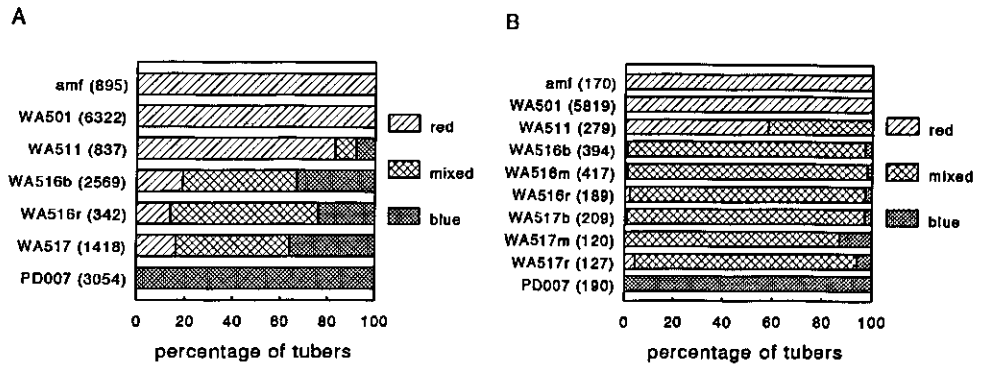

Figure 1: Comparison of the distribution of staining categories of tuber sections from field grown tubers. A. Tubers obtained from the field trial in 1990. B. Tubers obtained from the field trial in 1991.
amf, *amf*-mutant 1031-29; PD007, non-transformed wildtype; WA501 and WA511, plants grown from red parental tubers; WA517, WA516b and WA517b, plants grown from blue parental tubers; WA516m and WA517m, plants grown from mixed staining parental tubers; WA516r and WA517r, plants grown from red parental tubers. The number of harvested tubers per genotype is stated within parentheses.

In the transgenic genotypes, the blue core varied in size, but never filled the whole starch granule. At some of the inner red-staining growth rings, concentric blue lines could occur. In WA501 granules a small blue core was found at the hilum of each granule. The blue-staining core of starch granules from the other genotypes was larger in blue-staining tuber sections than in red-staining tuber sections. In individual cells, the size of the blue core, relative to the size of the complete granule, was found to be comparable for all amyloplasts. Starch granules with a blue core and a red outer part were observed in field grown and greenhouse grown tubers as well as in microtubers (data not shown).

According to the size of the blue core, three classes of starch granules were observed. They could be related to the macroscopically determined staining types of the tuber sections. The first class consisted of granules with a small blue core, corresponding with red tuber sections. The second class granules contained a medium-sized blue core, corresponding with mixed staining tuber sections, and the third class consisted of granules with a large blue core, which corresponded to blue tuber sections.

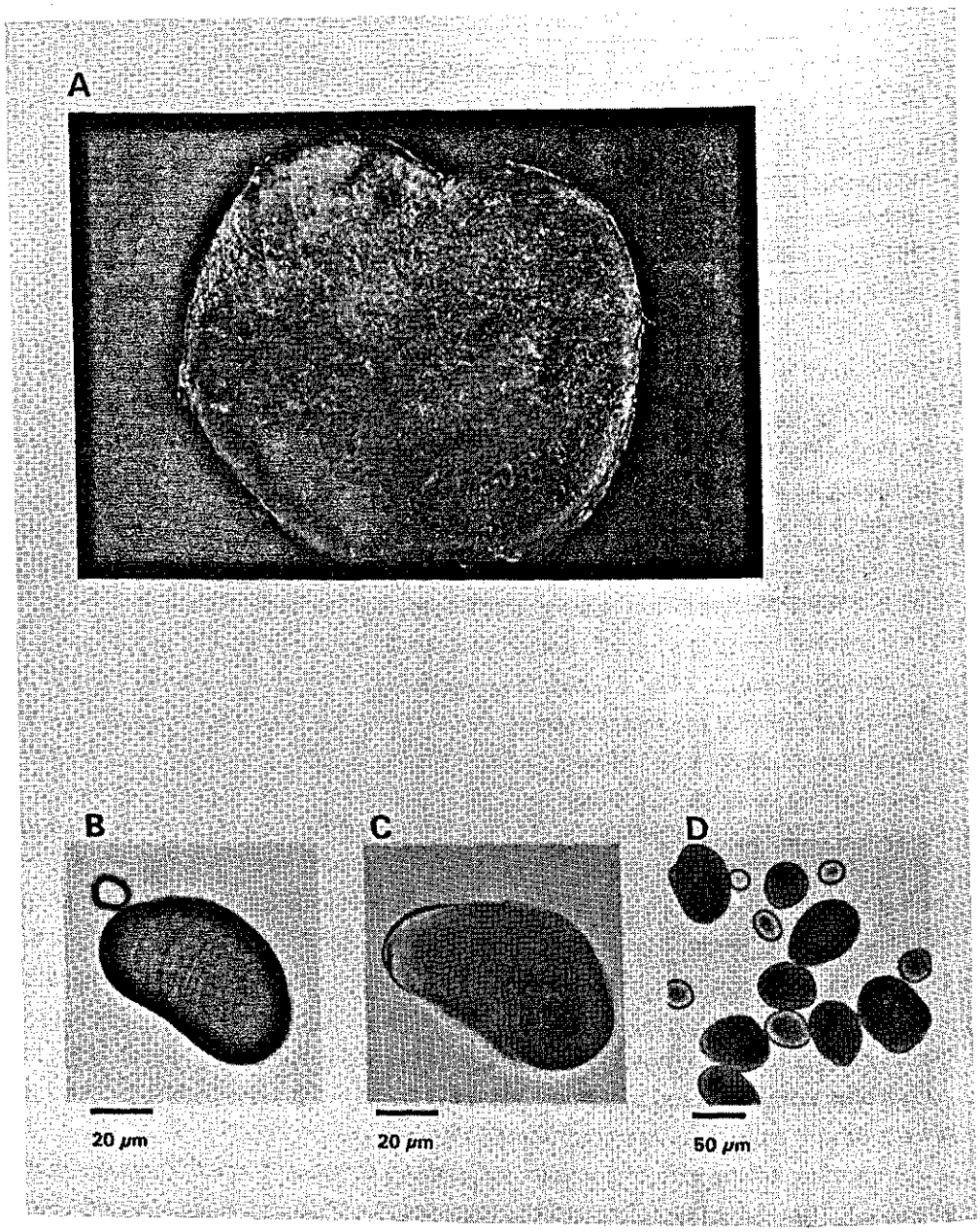

Figure 2: Iodine staining of tuber starch. A. Longitudinal section of a WA516 tuber with mixed staining starch. B. Blue staining starch granules of PD007. C. WA501 starch granules with blue staining core at the hilum and blue lines at the red staining growth rings. D. WA516 starch granules with blue staining cores of varying size. Dark zones represent blue staining starch. For full colour representation see Kuipers et al. (1994).

To quantify the size of blue cores in individual starch granules of each of these classes, we measured the short (R_{width}) and long (R_{length}) radii of the blue-staining core and the total granule using an ocular micrometer. Assuming an ovoid granule shape, the volume of the blue-staining core (V_{blue}) and the total granule (V_{total}) were calculated, and used for the calculation of the ratio (V_{blue}/V_{total}) between the volumes of the blue core and the total granule, as shown in Table 1. This ratio represents the relative size of the blue-staining core in starch granules of transgenic tubers.

Based on the relative occurrence of red-, mixed-, and blue-staining tubers (Figure 1A), which contain starch granules with small, medium, or large blue cores, respectively, the average volume ratio of the blue-staining core was determined for each of the transgenic genotypes. Table 2 shows that in the transgenic genotypes the average volume of the blue-staining cores is always less than 50% of the total volume of the starch granule. In the genotype WA501, which shows the most pronounced reduction of GBSS expression, 0.1% of the total starch volume contains blue-staining starch. The average volume ratios can be used to determine whether the blue cores represent the amylose that is present in the starch granules.

Table 1: Quantification of the size of the blue-staining core in starch granules from transgenic tubers obtained from the 1990 field trial.

observed size of blue core[a]	# granules analysed[b]	range V_{blue}/V_{total}[c]	average V_{blue}/V_{total}[c]
small(WA501)	42	0.3×10^{-4} - 0.5×10^{-2}	0.11×10^{-2}
small	50	0.2×10^{-3} - 0.04	0.80×10^{-2}
medium	54	0.04 - 0.70	0.25
large	58	0.70 - 0.95	0.85

[a] small(WA501), blue cores as present in starch granules from red staining tubers of WA501; small, medium, large, blue cores as present in starch granules from red-, mixed-, and blue-staining tubers, respectively, of the transgenic clones WA511, WA516, and WA517.

[b] Using an ocular micrometer, the short and long radii of the blue core ($R_{blue,width}$ and $R_{blue,length}$) and the total granule ($R_{total,width}$ and $R_{total,length}$) were measured.

[c] $V_{blue}/V_{total} = (R^2_{blue,width} \times R_{blue,length}) / (R^2_{total,width} \times R_{total,length})$

Table 2: Average volume ratio of the blue-staining core in starch granules from tubers of transgenic genotypes.

	classification of tubers according to size of blue core[a]				
genotype	%small(WA501)	%small	%medium	%large	$(V_{blue}/V_{total})_{genotype}$[b]
WA501	100	-	-	-	0.11×10^{-2}
WA511	-	83	9	8	0.97×10^{-2}
WA516b[c]	-	19	48	33	0.40
WA517	-	16	48	36	0.42

[a] Transgenic tubers obtained from the 1990 field trial were stained with iodine and classified according to the size of the blue core, which is related to the staining category of tuber sections as shown in Figure 1A.

[b] $(V_{blue}/V_{total})_{genotype} = [\%small \times (V_{blue}/V_{total})_{small} + \%medium \times (V_{blue}/V_{total})_{medium} + \%large \times (V_{blue}/V_{total})_{large}]/100\%$

[c] WA516b, plants grown from blue staining parental tubers.

Biochemical analysis of tuber starch

The amylose content and GBSS activity were determined in individual tubers of the transgenic genotypes, that were obtained from the 1990 field trial. Both amylose content and GBSS activity could be related to the amount of amylose-containing starch, as surveyed by iodine staining of these tubers, which is shown for WA517 in Figure 3. For WA511, WA516, and WA517, a higher relative radius of the amylose-containing blue core in iodine-stained starch granules was invariably associated with a higher amylose content and GBSS activity.

A larger scale analysis with respect to the amylose content was carried out on tuber samples of all genotypes from the 1990 field trial. Using amperometric titration, amylose could not be detected in starch from the *amf*-mutant, WA501, and WA511 in spite of the presence of blue-staining cores in starch granules of WA501 and WA511, as is shown in Table 3.

The determination of the amylose content and the average volume ratio of blue cores in starch granules is based on the formation of an iodine-starch complex. To evaluate the relationship between these two characteristics, a regression analysis was carried out for the average volume ratio and the amperometrically determined

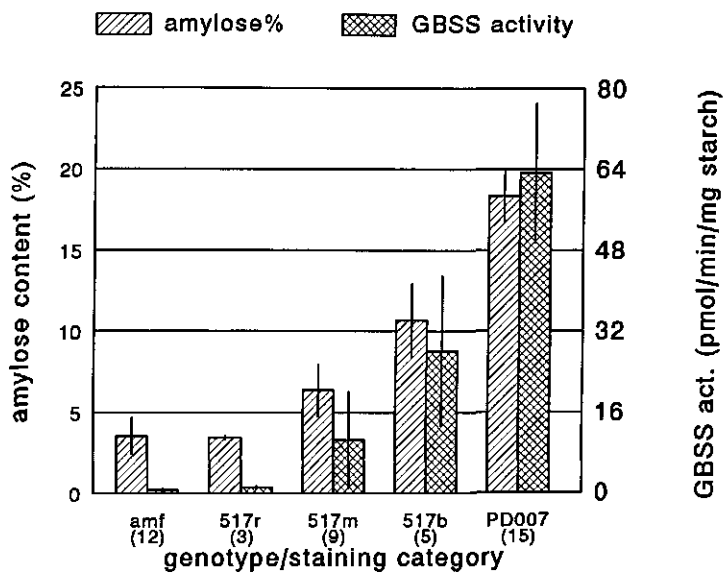

Figure 3: Amylose content and GBSS activity in tubers of WA517.
The amylose content (spectrophotometric method) and GBSS activity were determined in individual WA517 tubers of different staining categories that were obtained from the 1990 field trial. A comparison was made with the non-transformed wildtype and the *amf*-mutant. amf, *amf*-mutant 1031-29; PD007, non-transformed wildtype; r, red staining tuber starch; m, mixed staining tuber starch; b, blue staining tuber starch. Within parentheses are the numbers of tubers analysed per genotype or staining category. The error bars indicate standard deviations.

amylose contents of the transgenic and control genotypes, as shown in Figure 4. This resulted in the least squares regression curve $Y = 20 \times X^{1.5}$ with an R^2 value of 0.999. When expressed as a function of $(V_{blue}/V_{total})_{genotype}$ the equation is:

$$(\text{amylose \%})_{genotype} = (\text{amylose \%})_{PD007} \times [(V_{blue}/V_{total})_{genotype}]^{1.5}.$$

This relationship indicates that in tuber starch granules of the transgenic genotypes all amylose is located in the blue cores and that the amylose content of the blue cores is similar to that of wild-type starch, irrespective of the relative size of the blue cores. The nonlinearity of the relationship can be explained from the increase of the amylose content that is found during tuber development (Shannon

and Garwood, 1984). The equation enables calculation of the amylose content for transgenic genotypes based on the staining pattern of starch granules as shown in Table 3. A regression analysis was also performed for $(V_{blue}/V_{total})_{genotype}$ and the GBSS activity, which were shown to be correlated as well. Due to variation in GBSS activity measurements a reliable least squares regression curve could not be determined (data not shown).

Table 3: Amperometrically determined amylose content, and calculated amylose content based on $(V_{blue}/V_{total})_{genotype}$.

genotype	amylose content (%)[a]	calculated amylose content (%)[b]
WA501	0	0.0
WA511	0	0.6
WA516b[c]	5.0	5.0
WA517	5.4	5.5
PD007	20.0	20.0
1031-29	0	0

[a] Determined amperometrically in starch isolated from tubers pooled per genotype.

[b] Using $(V_{blue}/V_{total})_{genotype}$ and the amylose content determined for the nontransformed PD007, the expected amylose content could be calculated for the transgenic genotypes:
$(\text{amylose \%})_{genotype} = (\text{amylose \%})_{PD007} \times [(V_{blue}/V_{total})_{genotype}]^{1.5}$

[c] WA516b, plants grown from blue staining parental tubers.

RNA analysis

RNA was isolated from halves of individual tubers that were previously iodine stained and of which the other half was analysed for amylose content and GBSS activity. RNA gel blot hybridization with a ribosomal potato DNA fragment as a probe showed equal amounts of total RNA in each lane. Hybridization of tuber RNA using the GBSS mRNA specific single-stranded probe revealed a reduced amount of GBSS mRNA in all tubers from transgenic genotypes when compared with the wild-type, as shown in Figure 5A. Quantification of the autoradiograms showed a reduction of the GBSS mRNA steady state level varying from 34% to over 99%. However, there seemed to be no clear relation between the decrease of the GBSS mRNA level and the inhibition of GBSS activity or the reduction of the amylose content in individual tubers.

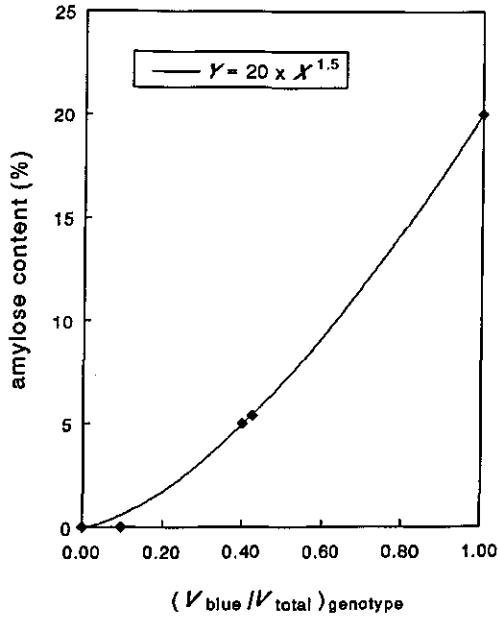

Figure 4: Amylose content of transgenic genotypes versus average volume ratio $(V_{blue}/V_{total})_{genotype}$ of blue cores in starch granules.
The amylose content was determined amperometrically (see Table 3). $(V_{blue}/V_{total})_{genotype}$ was determined as described in Table 2. Regression analysis was performed with the curve fit function of SlideWrite Plus 5.00 (Advanced Graphics Software, Inc., Carlsbad, CA).

Using strand-specific probes, it was not possible to detect antisense RNA in tubers. Therefore, RNA was isolated from leaves, in which the GBSS expression level is lower than in tubers, and hybridized with the GBSS cDNA fragment (Figure 5B) and with both strand-specific probes. This hybridization showed that leaf RNA of the transgenic genotypes contained reduced amounts of GBSS mRNA in addition to antisense RNA. The amount of antisense RNA appeared to be highest in WA501, the genotype with the most pronounced suppression of GBSS expression in tubers, and lowest in WA516 and WA517. This finding pointed toward a relationship between the amount of antisense RNA present in the leaves and the average level of antisense RNA mediated inhibition of GBSS gene expression in tubers of the four genotypes.

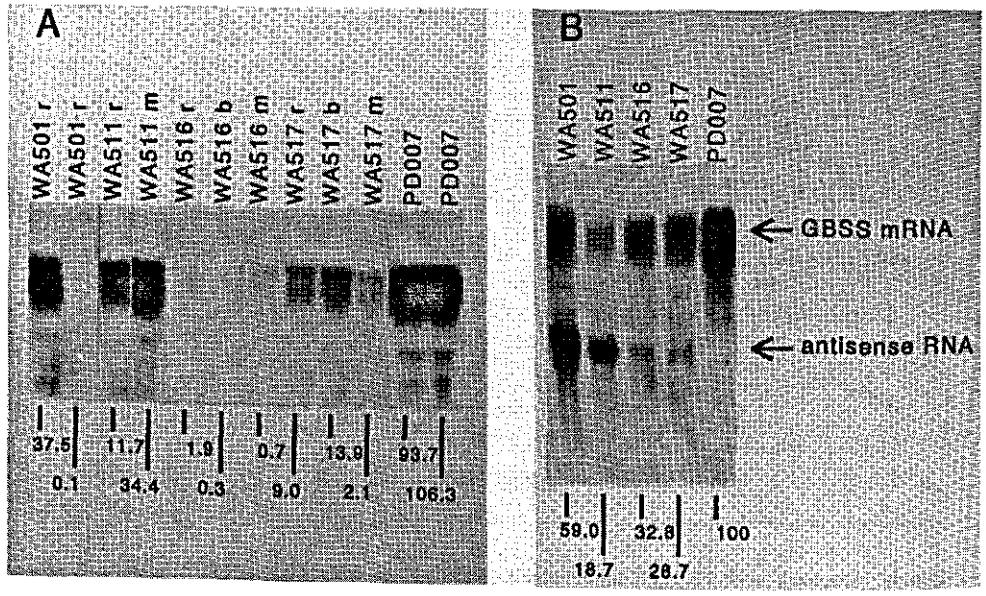

Figure 5: RNA gel blot analysis of tuber and leaf RNA of the transgenic clones.
A. Hybridization of RNA from individual tubers using a double-stranded GBSS cDNA probe. r, tuber with red staining starch; m, tuber with mixed staining starch; b, tuber with blue staining starch.
B. Hybridization of leaf RNA for GBSS mRNA and GBSS antisense RNA using a double-stranded GBSS cDNA probe. (GBSS mRNA and GBSS antisense RNA were detected individually using both strand-specific probes).
Below each lane, the GBSS mRNA steady state level is given as a percentage of the GBSS mRNA steady state level in the non-transformed PD007.

Because the antisense construct pGB50 is based on the full-length GBSS cDNA (approximately 2.4 kb), correct transcription of the antisense gene after insertion into the potato genome should result in an antisense RNA of about the same length as the GBSS mRNA. Our hybridization results showed an antisense RNA length of approximately 1.3 kb, which might be due to a premature stop of transcription.

To verify whether the antisense inhibition of GBSS gene expression affects the expression level of other genes involved in starch biosynthesis, RNA gel blot hybridizations were carried out using probes for several genes. Both sucrose synthase and ADP-glucose-pyrophosphorylase (AGPase) are involved in the

biosynthesis of ADP-glucose, the precursor for the biosynthesis of amylose and amylopectin. Sucrose synthase catalyses the cleavage of sucrose into fructose and UDP-glucose, which is converted into glucose-1-phosphate. AGPase subsequently converts glucose-1-phosphate into ADP-glucose. In this analysis, no significant alterations in the expression level of sucrose synthase and AGPase S, encoding one of the AGPase subunits, were observed in tubers with varying levels of inhibition of GBSS gene expression (data not shown). Also for branching enzyme, involved in the biosynthesis of amylopectin, mRNA steady state levels did not differ significantly from wild-type levels.

Discussion

Antisense inhibition in tubers: variability and subcellular origin

In this study, the effect of inhibition of GBSS gene expression on the production of amylose was analysed in tuber tissue and subcellularly in starch granules. The macroscopically determined staining patterns of tuber sections and the amylose content and GBSS activity showed that the stable and total suppression of GBSS gene expression, resulting in the production of nearly amylose-free starch in the transgenic clone WA501, was reproducible during two successive generations of field-grown tubers. Variable inhibition of GBSS gene expression among the genotypes WA511, WA516, and WA517 was observed in each of the tuber generations. In 1990 and 1991 a higher percentage of red staining tubers was found for WA511 compared with WA516 and WA517. For WA516 and WA517, the phenotype of parental tubers did not determine the classification of tubers into staining categories. Therefore, the variability among genotypes is supposed to be caused by position effects, which are known to affect the expression of inserted genes in transgenic plants (Dean et al., 1988).

Surprisingly, the variation in the degree of inhibition as observed in tuber tissue could be related to a distinctive staining pattern of starch granules that consisted of a blue core at the hilum surrounded by red growth rings. The staining pattern is an obvious distinction between the transgenic clone WA501 and the *amf*-mutant. This difference is due to the fact that the *amf*-mutation directly affects the GBSS gene expression in all starch synthesizing tissues, whereas the antisense RNA mediated inhibition is dependent on the relative expression levels of the GBSS gene and the antisense gene in each tissue. A small blue core at the hilum of

starch granules has been reported for *waxy* maize (Shannon and Garwood, 1984) and is thought to originate from the complexation of amylose and lipid at the initiation of granule formation (French, 1984).

Tuber starch granules with blue-staining cores of varying size were also observed after the inhibition of GBSS gene expression with an antisense GBSS gene driven by the promoter of the potato GBSS gene (A.G.J. Kuipers, unpublished results). In transgenic clones, the GBSS promoter driven antisense gene and the endogenous GBSS gene are likely to be expressed in a similar way, although position effects might modulate the expression level of the inserted gene. Therefore, the blue cores are supposed to originate from the reduced expression level of the GBSS gene rather than from a dramatic increase of the antisense effect during tuber development. This assumption is sustained by the observation of starch granules with an identical staining pattern after incomplete complementation of the *amf*-mutant with the potato GBSS gene (Flipse et al., 1994).

Because in individual cells the relative size of the blue core was found to be comparable for all amyloplasts, the available GBSS protein seems to be equally distributed to all amyloplasts in these cells. The relative volume of the blue staining core was shown to be related to the amylose content determined for starch synthesized in the transgenic genotypes (Figure 4, Table 3) and could also be related to the GBSS activity. Furthermore, the amylose content of the blue cores was shown to be similar to the amylose content of wild-type PD007 starch, irrespective of the relative size of the blue cores. This might point toward a GBSS protein threshold value for the production of a fixed percentage of amylose, resulting in the observed "all-or-nothing" pattern. Apparently, the amount of GBSS protein is sufficient for normal amylose production early in tuber development, whereas in later developmental stages the amount of GBSS protein seems to be below the threshold value or is entirely absent, which leads to the formation of amylose-free starch.

The decrease of the GBSS protein content between the core and the surface of the granule might be a consequence of a reduced amount of GBSS protein in transgenic plants in combination with the appositional mode of growth of starch granules (Badenhuizen and Dutton, 1956). Due to the increase in total granule surface during development, the amount of GBSS might be sufficient for the normal level of amylose production until a certain surface size is reached. From this point, which will depend on the level of inhibition of GBSS gene expression, the available GBSS protein may become limiting for adequate synthesis of amylose throughout the outer growth rings of the granule. This hypothesis is sustained by

the observation that starch granules of young transgenic microtubers are largely blue, and that the relative size of the blue core (V_{blue}/V_{total}) decreased with increasing microtuber age (A.G.J. Kuipers, unpublished results).

To determine the relationship between the staining pattern and the amylose content, the relative volume of the blue cores seems to be sufficient. This raises the question whether the blue lines that were frequently observed at the red growth rings surrounding the blue core represent amylose synthesized by GBSS. As the multiple concentric blue lines are specific for the transgenic antisense GBSS plants and differ from the single blue line that is only occasionally found at the periphery of starch granules of the *amf*-mutant, they probably contain a small amount of amylose due to the reduced GBSS gene expression. This granule zone might represent an intermediate between amylose-containing and amylose-free starch, and might indicate that the observed transition between amylose-containing and amylose-free starch is not abrupt. The occurrence of a zone with the alternate presence and absence of amylose resembles the specific labeling patterns that were observed in the immunocytochemical localization of ADP-glucose pyrophosphorylase (AGPase) in potato tuber cells (Kim et al., 1989). In this study, AGPase was suggested to be located within specific regions of the starch granule, which were supposed to reflect sites of active starch synthesis. Our findings seem to support this hypothesis.

RNA characteristics

The analysis of tuber and leaf RNA from the four *A. rhizogenes*-transformed genotypes showed an overall reduction of the GBSS mRNA steady-state level when compared with the nontransformed control. The absence in individual tubers of a relationship between the GBSS mRNA steady-state level and the extent of inhibition of GBSS gene expression contrasts with the presence in other antisense systems of a relation between the target mRNA level and the protein or enzyme level (van der Krol et al., 1988b; Stockhaus et al., 1990; Müller-Röber et al., 1992). Tuber RNA analysis has revealed fluctuations in the GBSS mRNA steady-state level in several nontransformed potato genotypes (A.G.J. Kuipers and R.G.F. Visser, unpublished results; Müller-Röber et al., 1992). These fluctuations are likely to occur in the transgenic genotypes as well. They might be the main cause of the lack of a relationship between the GBSS mRNA steady-state level and the extent of inhibition, since the amylose content, GBSS activity and amount of GBSS protein did correlate in several transgenic genotypes (Visser et al., 1991a; Chapter 2).

Although no antisense RNA was found in tuber tissue of the transgenic plants, we did find antisense RNA as well as GBSS mRNA in leaf tissue. This is likely to be due to the GBSS gene expression level, which is lower in leaves than in tubers (Visser et al., 1991b) and is comparable to what has been described for antisense RNA mediated inhibition of patatin gene expression in potato (Höfgen and Willmitzer, 1992). The presence of both antisense RNA and reduced amounts of GBSS mRNA in leaves can be explained from the CaMV-ß-glucuronidase (*GUS*) and GBSS-*GUS* expression patterns in leaves of transgenic potato plants (Visser et al., 1991b). Histochemical analysis of *GUS* activity showed CaMV-*GUS* expression throughout the leaf and GBSS-*GUS* expression in veins and stomatal guard cells. In the antisense transgenic plants, both GBSS mRNA and GBSS antisense RNA will be produced in veins and stomatal guard cells, resulting in a reduced GBSS mRNA steady state level as was found in tubers of these plants. The GBSS antisense RNA, which can be seen on the RNA gel blots, is likely to be produced in other parts of the leaf, where the GBSS gene is either poorly or not expressed.

The antisense RNA synthesized in plants carrying the pGB50 T-DNA was expected to be 2.4 kb, based on the GBSS cDNA sequence used for this construct. However, hybridization with a strand-specific probe showed an antisense RNA length of 1.3 kb. Screening of the GBSS cDNA sequence revealed three putative polyadenylation signals in the region between 1.2 and 1.4 kb downstream of the junction of the CaMV promoter and the reversed GBSS cDNA. This points toward a premature termination of transcription, which has also been reported for antisense genes for patatin (Höfgen and Willmitzer, 1992), polygalacturonase (Smith et al., 1988) and CHS (van der Krol et al., 1988b).

The specificity at the mRNA level of antisense RNA mediated inhibition of gene expression has been reported for several antisense systems (Rodermel et al., 1988; Stockhaus et al., 1990; van der Krol et al., 1990c), although antisense inhibition of one of the subunits of AGPase in potato was shown to affect the mRNA steady state levels of sucrose phosphate synthase and of storage protein genes (Müller-Röber et al., 1992). Because the inhibition of GBSS gene expression specifically down-regulates the biosynthesis of amylose, we conclude that antisense RNA-mediated inhibition of GBSS gene expression can be effectively applied for the suppression of the biosynthesis of amylose in potato. Furthermore, the correlation between the granule staining pattern and the amylose content (and to a lesser extent the GBSS activity), and the fact that antisense RNA mediated inhibition ranges from almost complete to no suppression of gene expression, make this system an asset for studying the production of amylose in starch granules.

Factors affecting the inhibition of granule-bound starch synthase gene expression in potato via antisense RNA

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Abstract

Starch is synthesized in potato tubers as a storage carbohydrate by several enzymes, of which granule-bound starch synthase (GBSS) catalyses the synthesis of amylose. Introduction of a cDNA based antisense GBSS gene into the potato genome is known to inhibit GBSS gene expression. In this study, eleven antisense constructs derived from the full length GBSS cDNA, the genomic GBSS coding region (gDNA) or fragments of each of these sequences were analysed with respect to their inhibitory effect. Introduction of full length gDNA constructs yielded a lower percentage of transgenic clones with complete inhibition than introduction of the full length cDNA constructs. This may be caused by a lower antisense RNA binding capacity due to the relatively low GC-content in intron-sequences present in the gDNA constructs. Complete inhibition of GBSS gene expression could also be achieved with a construct based on the 0.6 kb 3' end of the GBSS gDNA and with a construct based on a 0.7 kb internal fragment of the GBSS cDNA. For the full length cDNA and gDNA based constructs, a higher degree of inhibition of GBSS gene expression was found to be related with a higher T-DNA copy number. The presence of three or more inserted T-DNAs always resulted in inhibition of GBSS gene expression. Furthermore, the percentage of transgenic clones with inhibited GBSS gene expression was highest for constructs driven by the 35S CaMV promoter. Introduction of the GBSS promoter constructs, however, resulted in a higher percentage of clones with complete inhibition. Likewise, *A.rhizogenes* mediated transformation enhanced the antisense effect compared to *A.tumefaciens* mediated transformation, which is supposed to be due to the clustered insertion of the Ri-T-DNA and the vector T-DNA at the same genetic locus.

Introduction

The expression of specific endogenous genes can be inhibited via the expression of an integrated antisense gene in transgenic plants (van der Krol et al., 1988a; Watson and Grierson, 1993). With respect to the effectivity of antisense inhibition, little is known about the influence of the composition of the antisense gene construct. In most systems, effective inhibition was achieved with cDNA derived antisense RNAs covering either the complete target mRNA (Visser et al., 1991a; Müller-Röber et al., 1992; Höfgen and Willmitzer, 1992; Zrenner et al., 1993; Sheehy et al., 1988; Oeller et al., 1991), or a 5′ part of varying size (Bird et al., 1991; Smith et al., 1988; Rodermel et al., 1988). The chalcone synthase (CHS) gene expression in petunia was shown to be effectively inhibited by antisense genes containing the full length cDNA or a 3′ cDNA fragment. Contrasting with what has been reported for other systems, no inhibition was obtained with an antisense gene based on a 5′ CHS cDNA fragment (van der Krol et al., 1990b).

Whereas cDNA sequences are applied in most antisense gene constructs, genomic antisense genes are also suitable for effective reduction of gene expression. The expression of the tomato pectin methylesterase (PME) gene was shown to be almost completely inhibited after introduction of an antisense gene based on the nearly full length PME genomic DNA (Tieman et al., 1992). In rice, antisense inhibition of *waxy* gene expression using an internal genomic fragment of the rice *waxy* gene resulted in a reduction of the amylose content in grain starch (Shimada et al., 1993).

Most antisense gene constructs contain the 35S CaMV promoter. In petunia, complete inhibition of CHS gene expression could also be achieved by using a CHS promoter containing construct (van der Krol et al., 1990b). Anther specific inhibition of CHS gene expression was obtained with an antisense gene containing the 35S CaMV promoter with an anther box (van der Meer et al., 1992).

In potato the expression of the gene for granule-bound starch synthase (GBSS) could be effectively inhibited by the introduction of an antisense gene based on the full length GBSS cDNA and the 35S CaMV promoter (Visser et al., 1991a). This introduction resulted in the absence of GBSS protein and enzyme activity and the formation of amylose-free tuber starch. Among and within the transgenic clones varying levels of antisense inhibition were observed (Chapter 2). The level of inhibition of GBSS gene expression can easily and accurately be determined via iodine staining of starch granules (Chapter 3). Therefore, inhibition of the potato

GBSS gene is a suitable system for analysis of the antisense effect of different constructs.

In this study, a set of eleven GBSS antisense gene constructs was analysed with respect to the percentage of transgenic plants showing antisense inhibition and the level of this inhibition. Full length cDNA and genomic (gDNA) constructs were compared to determine the influence of the presence of intron sequences. The GBSS coding region contains twelve introns, which together comprise 37% of the genomic sequence (van der Leij et al., 1991a). Several partial genomic constructs were compared to identify GBSS sequences that are involved in antisense inhibition. Furthermore, a comparison was made between the 35S CaMV promoter and the GBSS promoter.

The antisense constructs were integrated into the potato genome by transformation with *A.rhizogenes* and *A.tumefaciens*. An important difference between the *A.tumefaciens* and *A.rhizogenes* strains used for transformation experiments is the presence in *A.rhizogenes* of the wildtype Ri T-DNA. Upon transformation the Ri T-DNA is introduced into the plant genome in addition to the binary T-DNA. It is known that the presence of the Ri T-DNA can affect the general plant metabolism, resulting in an Ri-phenotype (Visser et al., 1989d). The influence of this phenomenon on the antisense RNA mediated inhibition of GBSS gene expression was evaluated.

Materials and methods

Plant material and growth conditions

Two *Solanum tuberosum* genotypes were used for transformation. The diploid genotype 1024-2 originates from an F1 progeny of a cross between the *amf*-mutant 86.040 and a diploid wildtype clone (Jacobsen et al., 1989). 1024-2 Is heterozygous for the *amf*-character. The tetraploid genotype K892008 was obtained from KARNA, Valthermond, the Netherlands. *In vitro* shoot cultures were grown on MS-medium (Murashige and Skoog, 1962) with 30 g/l sucrose and 8 g/l agar (MS30; pH=5.8) at 21°C and 14h light (3200 lux). Two to four week old shoots were used for transformation. Microtuber induction was carried out as described by Hovenkamp-Hermelink et al. (1988a). *In vitro* plants of transgenic and control clones were transferred to the greenhouse and grown for tuber production under standard greenhouse conditions. Ploidy levels were determined according to Frandsen (1968).

Methods of cloning

Standard methods of molecular cloning were carried out according to Sambrook et al. (1989). For all DNA mediated transformations *Escherichia coli* strains JM83 (Vieira and Messing, 1982) and MH1 (Casadaban and Cohen, 1980) were used. Vectors with antisense genes were transferred to *Agrobacterium rhizogenes* strain LBA 1334 (Offringa et al., 1986) and to *A.tumefaciens* strain LBA4404 (Ooms et al., 1982) either by direct transformation (Höfgen and Willmitzer, 1988) or by triparental mating with *E.coli* HB101 (Boyer and Roulland-Dussoix, 1969) carrying the plasmid pRK2013 (Ditta et al., 1980).

Construction of antisense genes based on the GBSS-cDNA and the GBSS gene

Eleven antisense constructs were made based on GBSS cDNA and genomic sequences, the 35S CaMV promoter and the GBSS promoter (Figure 1). The construction of pGB50 has been described before (Visser et al., 1991a). The other constructs are based on pBI121 (Jefferson et al., 1987) and pPGB-1, which is derived from pBI121 by replacing the 35S CaMV promoter with the GBSS promoter (Visser et al., 1991b).

For the construction of pKGBA50, pGBA10 and pKGBA10 the GUS coding region of pBI121 and pPGB-1 was removed via digestion with SmaI and SstI followed by blunting of the SstI site with T4 DNA polymerase and religation. The resulting vectors pBI121S and pPGB-1S were digested with XbaI and BamHI. For the construction of pKGBA50 the 2.2kb BamHI-SpeI cDNA-fragment from pGB2 (Visser et al.,1991a) was ligated in reversed orientation into digested pPGB-1S. For the construction of pGBA10 and pKGBA10 the 4.2kb HindIII fragment containing the complete coding region of the GBSS gene (Visser et al., 1989c) was subcloned in pUC19 (=SUB10; Fig.1a). The 3.0kb BamHI-SpeI fragment of SUB10 was ligated in reversed orientation into digested pBI121S or pPGB-1S, respectively.

The partial genomic antisense constructs pGBA20, pKGBA20, pGBA30 and pKGBA30 are based on BamHI and SstI digested pBI121 and pPGB-1. The 1.8kb HindIII-NsiI fragment of the GBSS gene was subcloned in pMTL23 (Chambers et al., 1988) and isolated as an SstI-BamHI fragment (=SUB20; Fig.1a). This fragment was ligated in reversed orientation into pBI121 (=pGBA20) and pPGB-1 (=pKGBA20). The 1.4kb SstI-KpnI fragment of the GBSS gene was subcloned in pUC19 and isolated as an SstI-BamHI fragment (=SUB30; Fig.1a), which was ligated in reversed orientation into pBI121 (=pGBA30) and pPGB-1 (=pKGBA30). For the construction of pKGBA25 and pKGBA55, respectively, a 1.1kb fragment of the GBSS gene (positions 1552 to 2696; van der Leij et al., 1991a) or the

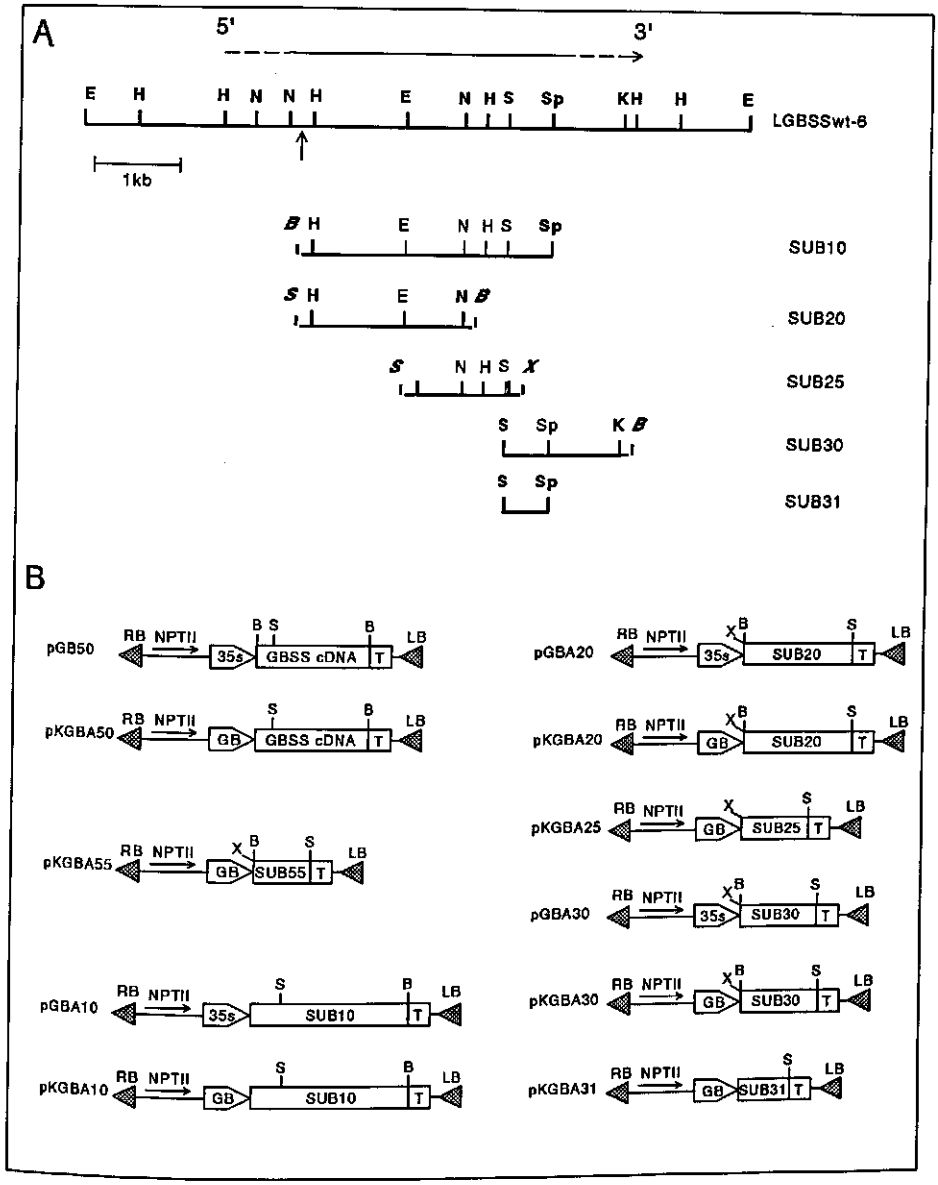

Figure 1: Construction of antisense genes based on the GBSS cDNA and the GBSS gene.
A. Origin of the GBSS gene fragments used for the genomic antisense constructs.
B. Composition of the constructs used in this study.
LGBSS-wt-6: full length genomic GBSS clone (Visser et al, 1989c). The arrow on top indicates the gene including the promoter region (5' dashed line) and the terminator region (3' dashed line). The small arrow indicates the position of the start codon.
B = BamHI; E = EcoRI; H = HindIII; N = NsiI; K = KpnI; Sm = SmaI; S = SstI; Sp = SpeI; X = XbaI (Italics indicate restriction sites in multiple cloning site). RB = right border; LB = left border; NPTII = neomycin phosphotransferase; 35S = 35S CaMV promoter; GB = GBSS promoter; T = nopalinesynthase terminator.

corresponding 0.7kb fragment of the GBSS cDNA were amplified via PCR using two 23-mer primers. Via the Sst-primer (GTTACACTGAGCTCATACTATGC) an SstI-site is introduced at the 5' end of the fragments, whereas the Xba-primer (CTGCAAGGCTCTAGACAACTGTT) introduced an XbaI-site at the 3' end of the fragments. The 1.8kb EcoRI-SpeI fragment of the GBSS gene and the 1.3kb EcoRI fragment of the GBSS cDNA were used as a template in individual PCR reactions. The PCR reaction mixture (100 μl) contained 250 ng of template DNA, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μm dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 1-2 U AmpliTaq (Perkin Elmer Cetus) and was covered with mineral oil. The amplification was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus) in 27 cycles of 1 min. 95°C, 2 min. 52°C and 3 min. 72°C. PCR products were isolated via the freeze-squeeze method (Tautz and Rentz, 1983), restricted with SstI and XbaI and ligated in reversed orientation into the XbaI-SstI digested pPGB-1. For the construction of pKGBA31 the 0.6kb SstI-SpeI fragment of the GBSS gene (=SUB31; Fig.1a) was directly ligated in reversed orientation into the XbaI-SstI digested pPGB-1.

Transformation of potato

Inocula of all *Agrobacterium* strains were prepared by culturing the bacteria overnight at 30°C in liquid LB-medium (Sambrook et al., 1989) with rifampicin (100 mg/l) and kanamycin (50 mg/l). *A.rhizogenes* strains were grown overnight at 30°C in liquid LB-medium (Sambrook et al., 1989) with rifampicin (100 mg/l), kanamycin (50 mg/l) and spectinomycin (200 mg/l). *A.rhizogenes* mediated transformations, isolation of kanamycin resistant hairy roots and shoot regeneration on hairy root segments were carried out according to Visser et al. (1989b). *A.tumefaciens* mediated transformations were carried out using internodal stem segments (Visser, 1991).

Iodine staining of starch

The staining of starch was carried out using Lugol (1% Lugol's solution, I-KI [1:2,v/v]; Merck). Tuber starch was stained at the surface of tuber slices with Lugol/ H_2O (1:1; v/v). Starch isolated from individual tubers was suspended in water, stained with a few drops of Lugol and examined microscopically.

Isolation of starch, determination of amylose content and GBSS activity

Starch was isolated from tubers as described before (Chapter 2). The tubers were iodine stained first, and then cut longitudinally. One half was used for the

isolation and analysis of starch, the other half for RNA isolation and analysis. The amylose content was determined spectrophotometrically in 1-2 mg isolated starch according to the method described by Hovenkamp-Hermelink et al. (1988b). Using spectrophotometric analysis, low amylose contents are overestimated in comparison with amperometric titration (Williams et al., 1970). The GBSS activity was determined in 1-2 mg isolated starch as described by Vos- Scheperkeuter et al. (1986), and is expressed as pmol ADP-glucose incorporated per min. in 1 mg of starch.

Determination of T-DNA copy number

DNA was isolated from 250 mg leaf tissue from greenhouse grown plants. The tissue was frozen in liquid nitrogen and ground with a cooled glass rod in a 2 ml Eppendorf tube. Upon grinding 0.75 ml extraction buffer (Dellaporta et al., 1983) and 0.5 ml phenol were added and mixed with the powder. After centrifugation for 10 minutes the supernatant was transferred to a new Eppendorf tube and the nucleic acids were precipitated with 0.75 ml isopropanol at -80°C for 15 minutes. Nucleic acids were pelleted by centrifugation for 5 minutes, dissolved in 0.3 ml 10mM Tris, 1mM EDTA, pH 8.0 (TE) with 0.1 μg/ml RNase, and incubated at 37°C for 30 minutes. After phenol/chloroform extraction the DNA was precipitated with 30μl 3M NaAc and 600μl 96% ethanol, spun down, washed with 80% ethanol, dried and dissolved in 50μl TE.

For determination of the minimum number of T-DNA insertions in the transgenic clones, isolated genomic DNA was digested with either SstI (transgenic clones with pGB50, pKGBA50, pGBA10 or pKGBA10) or BamHI (transgenic clones with pGBA20, pKGBA20, pGBA30 or pKGBA30). The digested DNA was separated by electrophoresis and blotted onto Hybond N membranes (Amersham) according to the manufacturers instructions. Hybridizations were carried out as described by Salehuzzaman et al. (1992). DNA-probes were labeled megaprime DNA labeling (Amersham). The 1.1kb EcoRI fragment of the GBSS cDNA (Visser et al., 1989c) was used as a probe for the detection of T-DNA insertions in transgenic clones with pGB50, pKGBA50, pGBA10, pKGBA10, pGBA20 or pKGBA20. The 1.4kb KpnI-SstI fragment of the GBSS gene (Visser et al., 1989c) was used as a probe for clones with pGBA30 or pKGBA30.

RNA analysis

RNA was extracted from leaves and individual tubers of transgenic and control clones as has been described before (Chapter 3). Northern blotting and

hybridization were carried out as described by Sambrook et al. (1989), using 30 μg of tuber RNA or 70 μg of leaf RNA per sample. The membranes were hybridized with a (^{32}P) ATP-labeled 2.4 kb EcoRI fragment containing the complete GBSS cDNA (Hergersberg, 1988; Visser et al., 1989c). Strand specific (^{32}P) UTP-labeled probes were transcribed from plasmids pT7T3α18GBc and pT7T3α19GBc as described before (Chapter 3).

Results

Biological effect of antisense constructs in hairy roots

The presence of starch in columella cells of roots enables the early screening of the biological effect of the antisense constructs in hairy roots obtained after *A.rhizogenes* mediated transformation. In a series of transformation experiments the four full length antisense GBSS constructs and the partial genomic constructs pGBA20, pKGBA20, pGBA30 and pKGBA30 (Figure 1) were introduced into the potato genome. Excised roots were screened for the presence of amylose and for kanamycin resistance in parallel experiments, because iodine staining of columella cells is most successful in young (approx. three week old) roots. With each of the constructs, approximately 40% to 50% of the roots were shown to be kanamycin resistant, and thus can be concluded to contain one or more copies of an antisense construct (Data not shown). Per construct 90 or more root tips, all representing independent transformation events, were screened for the presence of amylose. Transformation with *A.rhizogenes* LBA 1334 without an antisense construct resulted in blue (amylose containing) starch in columella cells of all hairy roots.

Iodine staining revealed three root tip phenotypes: blue (amylose containing), red (amylose free) and intermediate root tips (reduced amount of amylose). Intermediate root tips contained both blue and red staining columella cells. Depending on the construct used, complete inhibition of GBSS gene expression, resulting in red (amylose free) starch, was observed in 17 to 28% of the hairy roots, incomplete inhibition occurred in 22 to 31% of the hairy roots (Data not shown). A comparison of the percentages of kanamycin resistant roots and roots with completely or incompletely inhibited GBSS gene expression indicated that the introduction of an antisense construct generally results in inhibition of GBSS gene expression in columella cells of hairy roots. Because no significant differences were

observed between the individual constructs (Chi-square test; P=0.05; Data not shown), the effects of all constructs in tubers of *A.tumefaciens* transformed potato plants were analysed.

Antisense inhibition in transgenic potato plants

The GBSS antisense constructs (Figure 1) were analysed for their inhibitory capacity in potato tubers. *A.tumefaciens* mediated transformation was used to introduce the antisense constructs in the diploid genotype 1024-2. Per construct, at least 26 independent, kanamycin resistant shoots were isolated, from which microtubers and greenhouse grown tubers were obtained.

All transgenic clones were screened for inhibition of the GBSS gene expression by iodine staining of tuber starch. According to the staining pattern of the starch granule (Chapter 3), the transgenic clones were classified as indicated in Table 1. In general, iodine staining of greenhouse grown tubers resulted in a classification that was identical to that of microtubers of the same clones, irrespective of the construct that had been introduced. Upon the introduction of the full length cDNA

Table 1: Inhibition of GBSS gene expression assessed by iodine staining of (micro-) tuber starch from *A.tumefaciens* transformants of 1024-2 carrying different antisense constructs.

construct	# trans-formants	# transformants with inhibition: complete[a]		incomplete[a]		# transformants not inhibited[a]	
pGBA10	36	1	(3%)	23	(64%)	12	(33%)
pKGBA10	35	1	(3%)	17	(49%)	17	(49%)
pGBA20	36	0		1	(3%)	35	(97%)
pKGBA20	28	0		2	(7%)	26	(93%)
pKGBA25	49	0		6	(12%)	43	(88%)
pGBA30	87	0		0		87	(100%)
pKGBA30	71	0		1	(1%)	70	(99%)
pKGBA31	41	2	(5%)	21	(51%)	18	(44%)
pGB50	26	3	(12%)	21	(80%)	2	(8%)
pKGBA50	32	8	(25%)	14	(44%)	10	(31%)
pKGBA55	48	2	(4%)	2	(4%)	44	(92%)

The antisense constructs pGB50, pKGBA50 and pKGBA55 are based on the GBSS cDNA. The other constructs are based on the genomic coding region of the GBSS gene.
[a]: Complete inhibition: starch granules showing red staining starch with a small blue staining core after iodine staining. Incomplete inhibition: starch granules showing a medium sized or large blue staining core and a red staining outer part of the granule after iodine staining. No inhibition: starch granules showing blue staining starch after iodine staining.

or genomic constructs, the percentage of clones with complete inhibition varied from 3% to 25%, depending on the construct used. The percentage of clones with incomplete inhibition was found to vary from 44% to 80%. No inhibition was observed in 8% to 49% of the clones transformed with one of these constructs. Tuber starch from these transformants exclusively contained blue staining (amylose containing) starch, which was also found in non-transformed clones.

A.tumefaciens mediated introduction of the partial genomic constructs pGBA20, pKGBA20, pKGBA25, pGBA30, and pKGBA30 resulted in transgenic clones of which only a small percentage showed inhibition, which was nowhere complete. The introduction of pKGBA55, which contains a cDNA fragment corresponding to the genomic fragment present in pKGBA25, resulted in a comparable percentage of clones with inhibition as was found for pKGBA25, but in this case complete inhibition of GBSS gene expression was also observed. The ineffectiveness of the constructs pGBA30 and pKGBA30 was thought to be related to the genomic fragment used for these constructs, which contained the 3' non-coding end of the GBSS gene and a small part of a putative pseudo-gene (van der Leij et al., 1993) in addition to the 3'end of the GBSS coding region. The presence on the antisense strand of putative polyA-signals between the pseudo-gene and the GBSS fragment may prevent the GBSS sequence from being transcribed into antisense RNA. Therefore, the antisense vector pKGBA31 was constructed, in which the sequences not coding for GBSS were excluded. Integration of this antisense construct in the potato genome led to a comparable degree of inhibition of GBSS gene expression as was achieved with the full length genomic constructs.

Whereas the degree of inhibition of GBSS gene expression incited by eight of the GBSS antisense constructs differed only slightly in hairy roots, marked differences between the full length and partial GBSS antisense constructs were found in tubers of *A.tumefaciens* transformed plants. Therefore, the inhibitory effect of the GBSS antisense constructs was also analysed in tubers of *A.rhizogenes* transformed plants, regenerated from hairy roots. As can be seen in Table 2, the percentage of transgenic clones with inhibition was highest after introduction of the full length cDNA or genomic constructs. Although only small numbers of clones with the partial genomic constructs have been analysed, these constructs seemed to be less effective than the full length cDNA and genomic constructs. This is comparable to what was found for the *A.tumefaciens* transformed clones (Table 1).

Table 2: Inhibition of GBSS gene expression as surveyed by iodine staining of (micro-) tuber starch. The tubers were obtained from *A.rhizogenes* transformed plants of the genotypes 1024-2 and K892008, carrying different antisense constructs.

construct	# trans-formants	# transformants with inhibition: complete[a]		incomplete[a]		# transformants not inhibited[a]	
pGBA10	15	5	(33%)	5	(33%)	5	(33%)
pKGBA10	43	6	(14%)	5	(58%)	12	(28%)
pGBA20	3	0		1		2	
pKGBA20	6	0		2		4	
pGBA30	5	0		1		4	
pKGBA30	10	0		0		10	
pGB50	23	3	(13%)	16	(70%)	4	(17%)
pKGBA50	20	5	(25%)	13	(65%)	2	(10%)

The antisense constructs pGB50, pKGBA50 and pKGBA55 are based on the GBSS cDNA. The other constructs are based on the genomic coding region of the GBSS gene.
[a]: Complete inhibition: starch granules showing red staining starch with a small blue staining core after iodine staining. Incomplete inhibition: starch granules showing a medium sized or large blue staining core and a red staining outer part of the granule after iodine staining. No inhibition: starch granules showing blue staining starch after iodine staining.

Factors affecting the extent of antisense inhibition

A further evaluation of the data presented in Tables 1 and 2 indicated several factors that affected the degree of inhibition of GBSS gene expression. These factors were pairwise compared (Figure 2). *A.rhizogenes* mediated transfer of the antisense constructs was found to result more often in complete inhibition of GBSS gene expression than *A.tumefaciens* mediated transfer (Figure 2A), except for the constructs pGB50 and pKGBA50. Expression of these constructs resulted in a comparable level of complete inhibition in *A.tumefaciens* and *A.rhizogenes* transformed clones (Tables 1 and 2). The influence of the origin of the coding region and the promoter was analysed separately for *A.tumefaciens* and *A.rhizogenes* transformed clones, because of the difference in degree of inhibition between the two systems. Among the *A.tumefaciens* transformed clones introduction of the constructs pGB50 and pKGBA50 was found to result in a higher percentage of transgenic clones with complete inhibition than the constructs pGBA10 and pKGBA10 (Figure 2B). A comparable difference was observed between pKGBA55-transformed and pKGBA25-transformed clones (Figure 2C). A comparison of the 35S CaMV promoter and the GBSS promoter for *A.tumefaciens*-

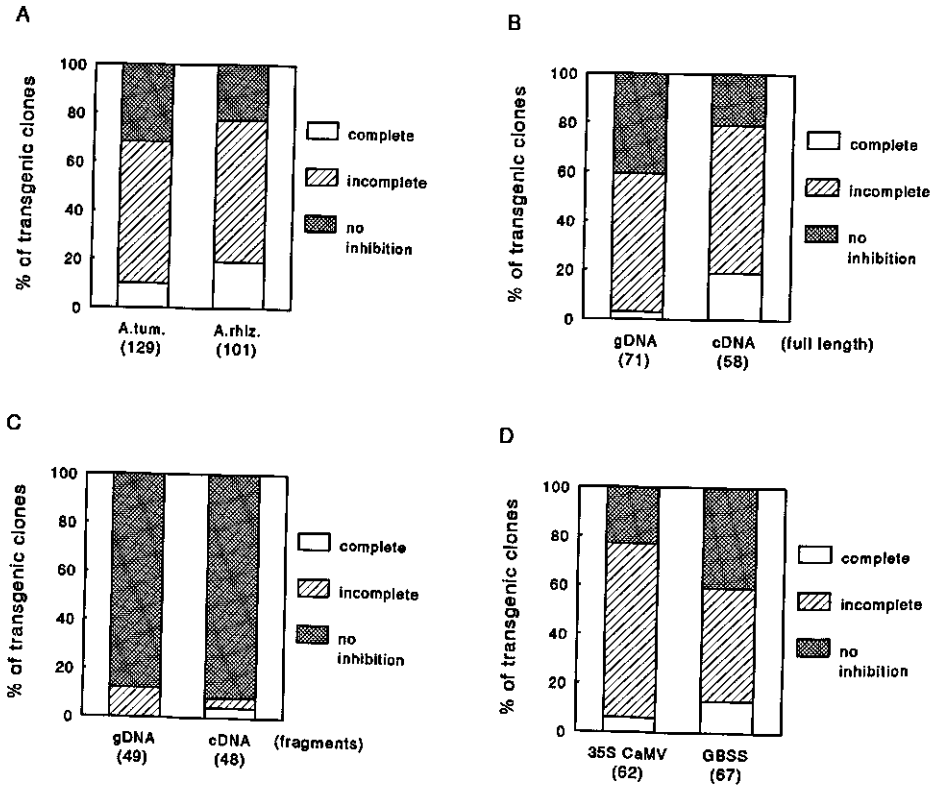

Figure 2: Evaluation of factors that influence the degree of inhibition of GBSS gene expression. A. Influence of transformation system. B-D. Influence of antisense construct composition: B. full length genomic GBSS DNA (pGBA10 and pKGBA10) versus full length GBSS cDNA (pGB50 and pKGBA50); C. internal fragment of genomic GBSS DNA (pKGBA25) versus corresponding fragment of GBSS cDNA (pKGBA55); D. 35S CaMV promoter (pGBA10 and pGB50) versus GBSS promoter (pKGBA10 and pKGBA50). A.tum, *A.tumefaciens* transformed clones; A.rhiz, *A.rhizogenes* transformed clones. Panels B-D represent data obtained for *A.tumefaciens*-transformed clones.

transformed clones (Figure 2D) showed that the percentage of transgenic clones with inhibition was highest for the constructs with the 35S CaMV promoter. However, in most of these clones the inhibition was incomplete. Introduction of the GBSS promoter constructs resulted in a higher percentage of transgenic clones with complete inhibition. The observed differences in the distribution of completely, incompletely and not inhibited clones were analysed in a chi-square test ($P = 0.05$). Significant differences were found for *A.tumefaciens* transformed clones between the full length GBSS cDNA and the genomic coding region of the GBSS gene, and

between the 35S CaMV promoter and the GBSS promoter. The inhibitory effect of the partial constructs pKGBA25 and pKGBA55 were not significantly different (Data not shown). The observed differences in degree of inhibition between the *A.tumefaciens* and the *A.rhizogenes* transformed clones were not significant either.

Amylose content and GBSS activity

Starch, isolated from greenhouse grown tubers, was analysed for amylose content and GBSS activity. For the 30 *A.tumefaciens* transformed clones and the 19 *A.rhizogenes* transformed clones that were analysed, the reduction of the amylose content always corresponded with the level of inhibition determined visually. The average amylose content and GBSS activity for *A.tumefaciens* and *A.rhizogenes* transformed clones with varying degrees of inhibition are shown in Table 3. In starch of clones showing complete inhibition the average amylose content and GBSS activity were comparable to amylose-free starch as present in the *amf*-mutant. As the variation in the degree of inhibition was rather high among clones classified as incompletely inhibited, the standard deviations for amylose content and GBSS activity were relatively high for this category.

Table 3: Amylose content and GBSS activity in starch isolated from tubers of *A.tumefaciens* and *A.rhizogenes* transformed clones with varying degrees of inhibition of GBSS gene expression.

type of clone	degree of inhibition[a]	amylose content(%)	(SD)	GBSS activity (pmol/min/mg starch)	(SD)
A.tumefaciens	complete	4.1	0.7	1.6	1.3
	incomplete	8.2	4.9	11.2	11.1
	no inhibition	19.3	1.4	45.3	10.4
A.rhizogenes	complete	5.2	0.4	n.d	
	incomplete	11.5	3.3	n.d	
	no inhibition	20.3	1.8	n.d	
non-transformed		20.9	1.6	53.8	8.4
amf-mutant		4.3	0.9	1.3	0.5

[a]: The degree of inhibition was determined by iodine staining (see Tables 1 and 2).
n.d: not determined.

T-DNA copy number

The minimum number of T-DNA inserts (T-DNA copy number) was determined in 132 *A.tumefaciens* transformed clones and 43 *A.rhizogenes* transformed clones. For *A.tumefaciens* transformed clones the T-DNA copy number varied from one to seven, the average being 1.9 inserts per clone. The T-DNA copy number for *A.rhizogenes* transformed clones ranged from one to eight, with an average of 3.0 inserts per clone. In Table 4 the T-DNA copy number is compared with the level of inhibition of the GBSS gene expression. For this comparison the results of the transgenic clones with the full length cDNA and genomic constructs were combined. Clones with partial constructs were not included in the analysis. It was shown that an increase in the average number of T-DNA inserts was related to a higher level of inhibition of GBSS gene expression. This relation was found to occur for *A.tumefaciens* and *A.rhizogenes* transformed clones and was also observed for the cDNA constructs and the genomic constructs when considered separately (Data not shown). For *A.tumefaciens* transformed clones 62% of the clones with 1 or 2 T-DNA inserts showed a certain degree of inhibition. For *A.rhizogenes* transformed clones antisense inhibition was found in 71% of these clones. In all clones with three or more T-DNA inserts the GBSS gene expression was inhibited. The degree of inhibition varied among these clones.

RNA analysis

RNA was isolated from tubers of clones carrying one of the antisense constructs and showing varying levels of GBSS gene expression. The isolated RNA was hybridized with the GBSS cDNA. In all *A.tumefaciens* transformed clones carrying the full length cDNA or genomic constructs the GBSS mRNA level was reduced compared to the non-transformed control (Figure 3A). Within the transgenic clones carrying either pKGBA10 or pKGBA50 the GBSS mRNA level seemed to be related to the level of inhibition of GBSS gene expression. In clones carrying the partial genomic constructs pGBA20, pKGBA20, pGBA30 or pKGBA30 which did not show antisense inhibition, the GBSS mRNA level was found to be comparable with the level found in non-transformed clones. A reduction of the GBSS mRNA steady state level was observed in clones with incomplete inhibition of GBSS gene expression (Figure 3B). Among the *A.rhizogenes* transformed clones the changes of the GBSS mRNA steady state levels were essentially the same as described for the *A.tumefaciens* transformed clones (Data not shown).

Some pGBA10, pKGBA10, pGB50 or pKGBA50 transformed clones with varying degrees of inhibition were analysed for the presence of antisense RNA in

Table 4: Comparison of T-DNA copy number and extent of inhibition of GBSS gene expression in tuber starch of transgenic plants obtained after transformation with *A.tumefaciens* (A) or *A.rhizogenes* (B), and average number of T-DNA inserts for complete, incomplete and not inhibited transformants. Combined results are presented for transformants carrying the constructs pGBA10, pKGBA10, pGB50 or pKGBA50.

A. *A.tumefaciens* transformed clones

# of T-DNA inserts	total number of transgenic clones	distribution according to extent of inhibition:		
		complete	incomplete	no inhibition
1	42	1	22	19
2	29	2	19	8
3	13	3	10	0
4	7	3	4	0
5	1	1	0	0
6	1	1	0	0
7	1	1	0	0
average T-DNA copy number		3.7	1.9	1.3

B. *A.rhizogenes* transformed clones

# of T-DNA inserts	total number of transgenic clones	distribution according to extent of inhibition:		
		complete	incomplete	no inhibition
1	8	0	6	2
2	6	3	1	2
3	13	3	10	0
4	4	1	3	0
5	2	0	2	0
6	1	0	1	0
8	1	1	0	0
average T-DNA copy number		3.4	2.9	1.5

leaf tissue by hybridization with the GBSS cDNA probe (Figure 3C). In addition to the 2.4 kb GBSS mRNA, leaf RNA of pGB50 transformed clones contained a band of approx. 1.2 kb. This band has been identified before as GBSS antisense RNA via hybridization of leaf RNA with a strand specific probe (Chapter 3). In leaf RNA of pKGBA50 transformed clones the 1.2 kb antisense RNA signal is absent, which can be explained from the lower expression level in leaf tissue of the GBSS promoter compared to the 35S CaMV promoter. Extra bands varying in size from 0.8 kb to 1.8 kb were found in addition to the GBSS mRNA in leaf RNA from the pGBA10 transformed clones. These bands were absent in pKGBA10 transformed clones. On the analogy of the results obtained for pGB50 and pKGBA50 transformed clones, they were supposed to represent antisense RNA. The size of the additional RNA bands was reduced as compared to the expected size of 3.0kb, based on the GBSS fragment present in pGBA10. This difference can be explained from a premature stop of transcription due to putative polyadenylation signals at 0.8kb, 1.3kb and 1.8kb downstream of the junction of the 35S CaMV promoter and the reversed GBSS sequence. Leaf RNA of all analysed clones transformed with the partial genomic constructs only contained GBSS mRNA (Data not shown).

Discussion

In this study, a comparison of eleven GBSS antisense constructs showed that inhibition of GBSS gene expression can be achieved with each of the constructs. However, differences were found to exist with respect to the percentage of clones with antisense inhibition, and the ratio of clones with completely and incompletely inhibited GBSS gene expression. The extent of inhibition was also found to differ between *A.tumefaciens* and *A.rhizogenes* transformed clones. The variation of antisense inhibition found for each of the constructs may be caused in part by position effects, which has been observed for antisense inhibition of tomato polygalacturonase gene expression (Smith et al., 1990b) and, in general, after the introduction of transgenes into various plant species (Peach and Velten, 1991). As all antisense gene constructs were based on the same vector (pBI121) and high numbers of transgenic clones were analysed per construct, the variation due to position effects is supposed to be similar for all constructs. The same holds true for the variation in the T-DNA copy number of each of the constructs. Therefore, significant differences in inhibitory capacity can be attributed to the composition of individual antisense genes.

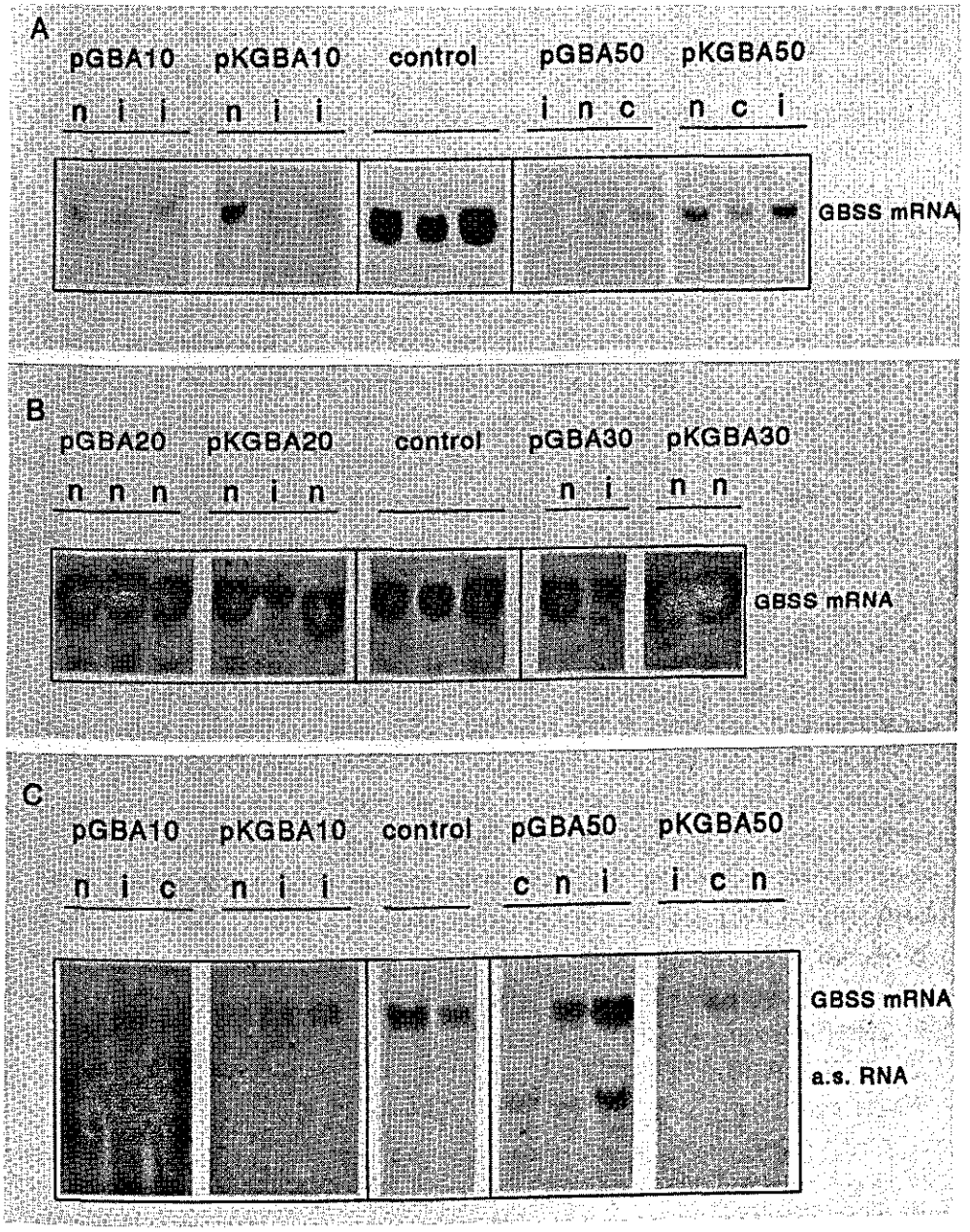

Figure 3: Northern hybridization of tuber RNA with a 2.4 kb GBSS cDNA probe.
A. Tuber RNA of clones transformed with the antisense constructs pGBA10, pKGBA10, pGB50 or pKGBA50. B. Tuber RNA of clones transformed with the antisense constructs pGBA20, pKGBA20, pGBA30 or pKGBA30. C. Leaf RNA of clones transformed with the antisense constructs pGBA10, pKGBA10, pGB50 or pKGBA50.
c = complete inhibition of GBSS gene expression in tuber tissue; i = incomplete inhibition of GBSS gene expression in tuber tissue; n = no inhibition of GBSS gene expression in tuber tissue.

Effect of construct composition on antisense inhibition: cDNA versus genomic DNA

The GBSS sequence origin was shown to be an important factor determining the effectivity of antisense inhibition. The full length GBSS cDNA (pGB50, pKGBA50) and genomic DNA (pGBA10, pKGBA10) constructs were all found to be capable of complete inhibition of GBSS gene expression, but it was shown that the antisense GBSS cDNA constructs resulted in complete inhibition of GBSS gene expression in a higher percentage of transgenic potato clones (Tables 1 and 2). This was also observed for the partial cDNA construct pKGBA55 as compared to the corresponding partial genomic construct pKGBA25. The total percentage of clones with inhibited GBSS gene expression was shown to be higher for the antisense GBSS cDNA constructs than for the genomic DNA constructs (Figure 2B), which sustains the assumed role of RNA-RNA interactions in antisense inhibition (van der Krol et al., 1988a; Höfgen and Willmitzer, 1992). The presence of intron sequences in the genomic constructs might attribute to the observed differences in antisense inhibition as compared to the cDNA constructs. The full length GBSS gene contains twelve introns (van der Leij et al., 1991a), four of which are also present in the gene fragment used for pKGBA25. These introns can not be processed when present in antisense orientation. The duplex formed with the antisense RNA based on the genomic GBSS DNA, is therefore expected to be most stable with the non-processed GBSS pre-mRNA, whereas the duplex formed with the GBSS cDNA based antisense RNA will be most stable with mature GBSS mRNA. This suggests that RNA duplex formation takes place both before and after RNA processing, but is most effective with mature GBSS mRNA. The differences in stability of the duplexes can be explained by a difference in the GC-content, which is 42.7% for exon (cDNA) sequences and 33.5% for intron sequences. In contrast with the relatively stable duplex involving the cDNA-based antisense RNA, the genomic antisense RNA will form a duplex with alternating stretches of higher and lower stability. In this way, the presence of intron sequences with a low GC-content might hamper the effectivity of antisense inhibition of gene expression.

Effect of construct composition on antisense inhibition: full length versus partial genomic DNA

In *A.tumefaciens* transformed clones the degree of inhibition of GBSS gene expression was found to vary for the genomic GBSS antisense constructs. However, similar frequencies of complete and incomplete inhibition could be achieved with pGBA10, pKGBA10 and pKGBA31 (comprising 0.6kb of the 3' end of the GBSS coding region and containing one intron sequence). This indicates that

the size of the antisense RNA does not affect the effectiveness of inhibition. Furthermore, it demonstrates that the GBSS fragment used in pKGBA31, or at least part of it, is essential for the inhibition of GBSS gene expression, as the inhibitory effect of pGBA20, pKGBA20 and pKGBA25 was much lower.

For pGBA30 and pKGBA30, the low inhibitory effect may be caused by a premature stop of transcription. The genomic fragment used for these constructs contains a 3' non-GBSS sequence which comprises a part of a putative pseudogene (van der Leij et al., 1993) in addition to the GBSS fragment that is also present in pKGBA31. Sequence analysis of the reversed fragment present in pGBA30 and pKGBA30 has revealed the presence of putative poly-adenylation signals between the pseudogene and the GBSS fragment. A premature stop of transcription does not necessarily result in the absence of antisense inhibition, as has been described for pGB50 (Chapter 3) and several other antisense genes (Smith et al., 1988; van der Krol et al., 1988b; Höfgen and Willmitzer, 1992), but in the case of pGBA30 and pKGBA30 the resulting antisense RNA might lack sequences that are complementary to the GBSS mRNA.

The varying inhibitory effect of the partial genomic antisense constructs points towards a function of certain regions of the gene for antisense inhibition.Effective inhibition with antisense genes based on a 3' fragment of the target gene has also been reported for petunia chalcone synthase (van der Krol et al., 1988b). However, 3' fragments are not always essential, as effective antisense inhibition has also been reported for antisense genes based on 5' fragments (Rodermel et al., 1988; Smith et al., 1988; Bird et al., 1991) and internal fragments (Sandler et al., 1988) of the target gene. This might indicate that certain sequence characteristics are involved in the process of antisense inhibition. Sequence analysis showed the presence of a few imperfect inverted repeats at the 5' end of the antisense RNA which corresponds to the 3' non-translated end of the GBSS mRNA. This part is common for pGBA10, pKGBA10, pKGBA31, pGB50 and pKGBA50. The inverted repeats might enhance the stability of these antisense RNAs by the formation of stem-loop structures. Furthermore, the formation of a heteroduplex between these antisense RNAs and the 3' untranslated region of the GBSS mRNA might prevent the formation of stabilizing stem-loop structures in the GBSS mRNA thus enhancing the antisense effect. For the human transferrin receptor a stem-loop in the 3' untranslated region has been reported to be required in the regulation of mRNA stability (Müllner and Kühn, 1988). As little is known about the influence of secundary structure on plant mRNA stability, further research is needed to elucidate the possible role of the inverted repeats at the 5' end of the GBSS

antisense RNA.

Effect of the T-DNA copy number on the level of antisense inhibition

As in most antisense systems described sofar only few transgenic clones have been analysed, little is known about the effect of T-DNA copy number on antisense inhibition. Rodermel et al. (1988), who analysed the antisense inhibition of the ribulose bisphosphate carboxylase (Rubisco) enzyme level in five transgenic tobacco clones, have shown that the lowest Rubisco RNA and protein content were found in the clone which carried at least four T-DNA copies. For tomato, selfings have been carried out with transgenic lines containing one T-DNA copy with an antisense polygalacturonase gene (Smith et al., 1990b) or an antisense gene from a cDNA clone involved in ethylene synthesis (Hamilton et al., 1990). Analysis of the progenies showed almost complete inhibition of gene expression in homozygous plants (2 T-DNA copies) and incomplete inhibition in hemizygous plants (1 T-DNA copy).

Our results clearly demonstrate the existence of a relationship between T-DNA copy number and antisense effect among *A.rhizogenes* and *A.tumefaciens* transformed clones carrying a full length cDNA or genomic construct. It was shown that an increase of the T-DNA copy number coincided with an increased percentage of transgenic clones with complete or incomplete inhibition of GBSS gene expression. The presence of three T-DNA copies always resulted in antisense inhibition. Therefore, it is concluded that a higher average T-DNA copy number increased the chance for complete inhibition of GBSS gene expression. The organization of multiple antisense GBSS gene copies in repeats does not seem to play an important role in the effectiveness of inhibition (A.G.J. Kuipers, unpublished results). However, since only small numbers of transgenic clones were screened for the presence of transgene repeats, their possible involvement in the inhibition of GBSS gene expression can not fully be excluded. The enhancing effect of multiple T-DNA copies might be caused by a combination of a higher chance for a positive position effect, and an additive effect of multiple insertions on the extent of antisense inhibition.

Effect of the transformation system on the level of antisense inhibition

The antisense effect in tubers of transgenic plants turned out to be slightly more pronounced in *A.rhizogenes* transformed clones than in *A.tumefaciens* transformed clones, except for pGB50 and pKGBA50. The higher average T-DNA

copy number in *A.rhizogenes* transformed clones is likely to attribute to this difference, because of the observed relation between the T-DNA copy number and the extent of antisense inhibition (Table 4). Another important factor underlying the observed differences between *A.tumefaciens* and *A.rhizogenes* transformed plants may be the fact that *A.rhizogenes* mediated transformation results in co-transfer of the vector T-DNA, which carries the antisense gene, and the Ri-T-DNA (Hamill et al., 1987). Introduction of the NPTII gene with *A.rhizogenes* LBA1334 resulted in a higher level of kanamycin resistance in potato plants than transformation with *A.tumefaciens*, even without selection for this trait (Visser et al., 1989a; 1989d). The clustered insertion of the Ri-T-DNA and the vector T-DNA at the same genetic locus (Visser et al., 1989d; Visser, unpublished results) might enhance the expression of the vector T-DNA gene.

Field evaluation of transgenic potato plants expressing an antisense granule-bound starch synthase gene: increase of the antisense effect in starch granules during tuber development

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Abstract

Antisense GBSS genes, based on the full length GBSS cDNA driven by the 35S CaMV promoter or the potato GBSS promoter, were introduced into the genome of a tetraploid potato cultivar by *Agrobacterium tumefaciens* mediated transformation. Expression of each of these genes resulted in the complete inhibition of GBSS gene expression, and thus in the production of amylose-free tuber starch, in mature field grown plants originating from rooted *in vitro* plantlets of 4 out of 66 transgenic clones. Clones in which the GBSS gene expression was incompletely inhibited showed a developmental increase of the antisense effect in iodine stained starch granules. This increase is likely to be due to the increase of starch granule size during development and the specific distribution pattern of starch components in granules of clones with reduced GBSS activity. Expression of the antisense GBSS gene from the GBSS promoter resulted in a higher stability of inhibition in tubers of field grown plants as compared to expression from the 35S CaMV promoter. Field analysis of the transgenic clones indicated that inhibition of GBSS gene expression could be achieved without significantly affecting the starch and sugar content of transgenic tubers, the expression level of other genes involved in starch and tuber metabolism and agronomic characteristics such as yield and dry matter content.

Introduction

The antisense technology (van der Krol et al., 1988a; Watson and Grierson, 1993) has proven to be a suitable approach for the modification of several agronomically important characteristics. In tomato for example, increased resistance to mechanical damage and retarded ripening were achieved via antisense RNA mediated inhibition of the expression of ripening-related genes encoding enzymes involved in cell wall degradation (polygalacturonase) or ethylene synthesis (e.g. ACC synthase), respectively (Gray et al., 1992; Hamilton et al., 1990; Oeller et al., 1991; Smith et al., 1988).

Essential requirements for the agricultural application of antisense technology are the stable expression of the inserted gene under field conditions, and the preservation of intrinsic cultivar properties. Stable expression of inserted genes under field conditions can be achieved in successive clonal generations of transgenic plants, as was found for inhibition of GBSS gene expression in potato tubers (Chapter 3). Jongedijk et al. (1992) have shown that in several transgenic clones of two potato cultivars the inserted PVX coat protein gene is expressed throughout the growing season. With respect to the field performance of transgenic plants it is known that, although plant morphology and yield can be affected by the transformation and regeneration process, transgenic clones can be obtained in which the inserted transgene is expressed without affecting cultivar characteristics (Dale and McPartlan, 1992; Jongedijk et al., 1992; Kramer et al., 1990).

In this study, we describe the inhibition of GBSS gene expression in a tetraploid potato cultivar which is grown for the production of starch. After *A.tumefaciens* mediated introduction of antisense genes based on the full length GBSS cDNA and driven by the 35S CaMV or the GBSS promoter, transgenic clones with varying degrees of inhibition of GBSS gene expression were isolated and characterized. The stability of the expression of each of the antisense genes in individual transgenic clones was analysed during tuber development in the field and in mature field grown tubers. Furthermore, the clones were analysed with respect to the effect of the introduced antisense GBSS genes on the expression of other genes, and the preservation of cultivar characteristics.

Materials and methods

Plant material, transformation and growth conditions

In vitro shoot cultures of the tetraploid *Solanum tuberosum* cultivars Astarte and K892002 (KARNA, Valthermond, the Netherlands) were grown on MS-medium (Murashige and Skoog, 1962) with 30 g/l sucrose and 8 g/l agar (MS30; pH = 5.8) at 21 °C and 14 h light (3200 lux). Two to four week old K892002-shoots were used for *Agrobacterium tumefaciens* mediated transformation (Visser, 1991). Via transformation the antisense GBSS gene constructs pGB50 and pKGBA50 were integrated into the potato genome. The construction of pGB50 has been described before (Visser et al., 1991a). pKGBA50 is based on pPGB-1, which is derived from pBI121 (Jefferson et al., 1987) by replacing the 35S CaMV promoter with the GBSS promoter (Visser et al., 1991b). The GUS coding region of pPGB-1 was removed via digestion with SmaI and SstI followed by blunting of the SstI site with T4 DNA polymerase and religation. The resulting vector pPGB-1S was digested with XbaI and BamHI. For the construction of pKGBA50 the 2.2kb BamHI-SpeI fragment from pGB2 (Visser et al., 1991a) was ligated in reversed orientation into digested pPGB-1S. *In vitro* shoots of transgenic and control clones were used for microtuber induction (Hovenkamp-Hermelink et al., 1988a) and were transferred to the greenhouse.

Field trial design

The field trial was set up with 23 transgenic clones, derived from K892002, with varying levels of inhibition of GBSS gene expression as determined in microtubers. Ten of these clones contained the antisense vector pGB50, the other 13 clones contained pKGBA50. Per clone 20 plants were used, except for the clones tB50-17, tB50-20, tBK50-8, tBK50-10 and tBK50-64, of which 12 to 18 plants were used. As a control, 20 plants of the non-transformed cultivars K892002 and Astarte were included. *In vitro* plants of all clones were transferred to the greenhouse in May 1992 and planted in the field one month later. The field trial was carried out according to governmental guidelines (Anonymous, 1993). The plot was set up in two replicates and surrounded with the cultivar Lady Rosetta. The plant distance in the field was 50 cm and the ridges were 75 cm apart. Flower buds were removed every two to three days. Tuber samples were taken at six week intervals. In July and August two to four plants per clone were harvested. The matured tubers of the remaining plants were harvested in

September, two weeks after the foliage was killed. Yield and dry matter content were determined on mature tubers of each clone. The dry matter content was determined by under water weighing.

Iodine staining of starch

Tuber starch was stained at the surface of tuber slices with Lugol-H_2O (1:1, v/v; Lugol: 1% Lugol's solution, I-KI [1:2, v/v]; Merck). Starch isolated from (micro-) tubers was suspended in water, stained with a few drops of Lugol and examined microscopically. In iodine-stained starch granules, the longitudinal radii of the blue staining core and the total granule were measured microscopically using an ocular micrometer (Chapter 3).

Isolation of starch, determination of amylose content and GBSS activity

Tuber starch was isolated as described (Chapter 2). Samples were taken from two tubers of each of the three harvests of eight representative transgenic clones and the two controls. The amylose content was determined spectrophotometrically in 1-2 mg isolated starch as described by Hovenkamp-Hermelink et al. (1988b). Using the spectrophotometric method, low amylose contents are overestimated in comparison with amperometric titration (Williams et al., 1970). GBSS activity was determined in 1-2 mg isolated starch as described by Vos-Scheperkeuter et al. (1986), and is expressed as pmol ADP-glucose incorporated per min. in 1 mg of starch. The distribution of starch granule size was determined in 1 g of isolated starch with a Multisizer and used for the calculation of the volume-weighted mean granule size.

Determination of starch and sugar content

Tuber tissue samples of 150-180 mg were taken from two tubers of each of the three harvests of eight transgenic clones and the two controls. These samples were frozen in liquid nitrogen, homogenized in Eppendorf tubes, extracted in 500 μl 80% ethanol at 70°C for one hour and centrifuged for 10 minutes at 9000 g. The supernatants were transferred to new Eppendorf tubes. The starch content was analysed in the pellets of the samples using the Boehringer test-combination for starch (Boehringer, Mannheim). Solubilization of the pellets and determination of starch were carried out according to the manufacturer's recommendations. The supernatants were used for the determination of sucrose, glucose and fructose using the Boehringer test combination for sucrose, D-glucose and D-fructose. The test was carried out basically as described by the manufacturer, but was adjusted

for absorption measurement in a microplate reader (BioRad 3550-UV) at 340 nm (Viola and Davies, 1992).

Protein electrophoresis and immunoblotting

Protein samples were prepared by boiling 20 mg starch for 10 seconds 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 placed on ice. Per sample 15 µl was analysed on 10% polyacrylamide gels (Laemmli, 1970) that were subsequently stained for protein with $AgNO_3$ according to Slisz and van Frank (1985). Immunoblotting was carried out as described by Hovenkamp-Hermelink et al. (1987) using antiserum raised against potato GBSS (Vos Scheperkeuter et al., 1986). Alkaline phosphatase was used as a second antibody and the antigens were detected by incubating the filters in the dark in 100 ml AF-buffer (100 mM Tris.HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) with 200 µl NBT (4-nitro blue tetrazolium chloride; 75 mg/ml in dimethylformamide) and 200 µl BCIP (5-bromo-4-chloro-3-indolyl-phosphate; 50 mg/ml in H_2O). The reaction was stopped by incubation in AF-buffer.

Determination of T-DNA copy number

The T-DNA copy number was determined in leaf tissue from greenhouse grown plants as described in Chapter 4.

RNA analysis

RNA was extracted from samples of tubers that had also been used for determination of the amylose content and the GBSS activity, using the procedure described in Chapter 3. Northern blotting and hybridisation were carried out as described by Sambrook et al. (Sambrook et al., 1989), using 40 µg of tuber RNA or 80 µg of leaf RNA per sample. The RNA gel blots were hybridized with the following ^{32}P-ATP-labeled cDNA probes: granule-bound starch synthase (GBSS; (Visser et al., 1989c), ADP-glucose pyrophosphorylase (Müller-Röber et al., 1990), branching enzyme (Kossmann et al., 1991) and patatin (Rosahl et al., 1986).

Results

Isolation and characterization of transgenic clones

Via *Agrobacterium tumefaciens* mediated transformation the antisense constructs pGB50 and pKGBA50 (see Chapter 4, Figure 1), both based on the full length GBSS cDNA, were integrated into the genome of the tetraploid potato cultivar K892002. Sixty-six individual kanamycin-resistant clones were isolated and screened for the degree of inhibition of GBSS gene expression in microtubers by iodine staining of starch granules. Introduction of each of the constructs resulted in red-staining starch granules with a blue-staining central core of varying size. The size of the blue-staining core has been demonstrated before to be related to the amylose content of tuber starch (Chapter 3). A small blue core correlated with complete antisense inhibition, whereas incomplete inhibition resulted in a large blue core. In completely blue staining starch granules, the expression of the GBSS gene was not inhibited. For both constructs, approximately 50% of the transgenic clones showed either complete or incomplete inhibition of GBSS gene expression. In ten of these clones complete inhibition of GBSS gene expression was observed.

Based on the degree of inhibition of GBSS gene expression in microtubers as surveyed by iodine staining, a series of clones was chosen for further analysis. These clones were grown in the greenhouse for the determination of the T-DNA copy number and the amylose content. The minimum number of T-DNA inserts in these clones varied from one to four (Table 1). For both constructs, the average T-DNA copy number was found to be highest in transgenic clones with complete inhibition and lowest in transformants without inhibition of GBSS gene expression. At least two T-DNA copies seemed to be necessary for complete inhibition. This indicates a relationship between the T-DNA copy number and the degree of inhibition of GBSS gene expression, which has also been demonstrated for transgenic clones derived from a diploid potato genotype (Chapter 4).

The amylose content of starch from greenhouse grown tubers of the pKGBA50-transformed clones and most of the pGB50-transformed clones was found to correspond with the degree of inhibition as determined by iodine staining of microtuber starch granules (Table 1).

Inhibition of GBSS gene expression in field grown plants

With respect to the feasibility of antisense RNA mediated inhibition of GBSS gene expression for the production of amylose-free potato starch, the extent and the stability of the antisense effect were analysed in field grown plants originating

from rooted *in vitro* plantlets of the selected transgenic clones. For some of the clones the antisense effect in mature field grown tubers was different from that in microtubers (Table 1). In most of the pGB50-transformed clones, the GBSS gene expression was found to be incompletely inhibited in mature tubers, whereas either complete or no inhibition of GBSS gene expression was observed in microtubers.

Table 1: Analysis of transgenic clones derived from the potato cultivar K892002.

clone	degree of inhibition[a]: microtubers	mature field tubers	copy number	amylose content (%)
tB50-1	n	i	1	13.3
tB50-5	n	i	2	4.4
tB50-7	c	i	4	3.9
tB50-8	c	i	3	4.2
tB50-17	n	i	2	8.3
tB50-20	n	i	2	n.d
tB50-22	c	i	4	4.8
tB50-35	n	n *	1	17.6
tB50-42	c	i	2	n.d
tB50-46	c	c *	2	5.9
tBK50-3	n	n *	n.d	n.d
tBK50-8	n	n *	1	n.d
tBK50-10	i	i *	1	20.7
tBK50-12	n	n *	2	20.7
tBK50-13	c	c *	2	4.4
tBK50-29	c	i	2	3.8
tBK50-33	c	c *	3	4.4
tBK50-34	i	i *	1	8.9
tBK50-39	c	i	4	5.2
tBK50-48	n	n *	1	n.d
tBK50-50	n	n *	1	20.1
tBK50-64	i	i *	1	n.d
tBK50-66	c	c *	3	4.3
K892002	n	n *	0	21.6

The clones were isolated after transformation with pGB50 (code: tB50-n) or pKGBA50 (code: tBK50-n), and characterised for degree of inhibition of GBSS gene expression in microtubers and mature field grown tubers. The T-DNA copy number and the amylose content were determined in leaves and tubers, respectively, of greenhouse grown plants. Asterisks indicate a similar extent of inhibition in microtubers and mature field grown tubers. n.d: not determined.
[a]: The transgenic clones were classified according to the staining pattern of starch granules. c = complete inhibition: starch granules showing red staining starch with a small blue staining core after iodine staining. i = incomplete inhibition: starch granules showing a medium sized or large blue staining core and a red staining outer part of the granule after iodine staining. n = no inhibition: starch granules showing blue staining starch after iodine staining.

Expression of the antisense gene from the GBSS promoter in pKGBA50-transformed clones generally resulted in a similar antisense effect in mature field grown tubers as in microtubers. Therefore, expression of the antisense gene from the GBSS promoter seems to have a stabilizing effect on the extent of inhibition of GBSS gene expression under varying physiological conditions. The expression of the GBSS gene was completely inhibited in mature tubers of one pGB50-transformed clone (tB50-46) and of three pKGBA50-transformed clones (tBK50-13, tBK50-33 and tBK50-66). Complete inhibition was also observed in microtubers of these four clones.

To monitor the occurrence of changes in antisense inhibition during tuber development, plants of each clone were harvested at six week intervals. As can be seen in Table 2, the antisense effect as surveyed by iodine staining of starch granules increased during development for pGB50- as well as for pKGBA50-transformed clones. According to the observed antisense effect in tubers from each of the harvests, four types of clones could be discerned (Figure 1). Most of the pKGBA50-transformed clones were classified as inhibition group A (complete inhibition in second and third harvest tubers) or D (no inhibition), whereas most pGB50-transformed clones and the remaining pKGBA50-transformed clones, which appeared to carry one T-DNA copy, were classified as inhibition groups B or C (incomplete inhibition). The developmental increase of the antisense effect in group B and C clones was found to be significant (Chi-square test ($P<0.05$); Data not shown).

The observed increase of the antisense effect in tuber starch was further evaluated by measuring the mean granule size and the size of the blue core in starch granules of developing and mature tubers. As is shown in Figure 2, the mean granule size increased during development. The size of the blue core did not increase or increased to a lesser extent, thus resulting in a decrease of the ratio between the volume of the blue core and the totel granule volume (V_{blue}/V_{total}) as has been described before (Chapter 3). The mean granule size of starch granules of first, second and third harvest tubers was found to significantly increase during development (ANOVA; data not shown). Since no significant differences were observed among the analysed clones, the inhibition of GBSS gene expression was concluded not to affect the mean granule size.

Table 2. Inhibition of GBSS gene expression in tubers harvested at six week intervals.

clone	First harvest (6 weeks after planting) degree of inhibition[a]:				Second harvest (12 weeks after planting) degree of inhibition[a]:				Third harvest (18 weeks after planting) degree of inhibition[a]:			
	# tubers	c (%)	i (%)	n (%)	# tubers	c (%)	i (%)	n (%)	# tubers	c (%)	i (%)	n (%)
tB50-46	44	2	91	7	61	25	75	0	50	100	0	0
tB50-8	42	17	67	17	63	70	24	6	50	86	14	0
tB50-7	53	2	53	45	63	33	49	17	50	44	56	0
tB50-20	4	0	0	100	12	0	50	50	50	28	66	6
tB50-22	24	0	21	79	40	0	70	30	50	28	66	6
tB50-42	29	0	59	41	38	5	63	32	50	24	68	8
tB50-17	5	0	20	80	23	0	13	87	45	2	51	47
tB50-5	29	0	0	100	50	0	2	98	50	0	12	88
tB50-1	9	0	0	100	23	0	4	96	50	0	6	94
tB50-35	28	0	0	100	55	0	0	100	50	0	0	100
tBK50-33	37	100	0	0	49	100	0	0	50	100	0	0
tBK50-13	22	86	14	0	60	100	0	0	50	100	0	0
tBK50-66	13	85	15	0	29	97	3	0	50	100	0	0
tBK50-39	22	14	86	0	54	76	24	0	50	98	2	0
tBK50-29	25	36	60	4	42	79	19	2	50	96	4	0
tBK50-64	6	0	83	17	37	0	30	70	50	30	66	4
tBK50-10	5	0	80	20	19	0	37	63	50	18	78	4
tBK50-34	8	0	0	100	39	0	31	69	50	16	76	8
tBK50-3	8	0	0	100	25	0	0	100	50	0	0	100
tBK50-12	26	0	0	100	59	0	0	100	50	0	0	100
tBK50-48	9	0	0	100	13	0	0	100	50	0	0	100
tBK50-50	32	0	0	100	42	0	0	100	50	0	0	100
K892002	45	0	0	100	47	0	0	100	20	0	0	100
Astarte	19	0	0	100	37	0	0	100	35	0	0	100

The extent of inhibition was assessed by iodine staining of tuber starch of clones transformed with pGB50 (code: tB50-n) or pKGBA50 (tBK50-n). For each clone the percentage of tubers per category was determined. The clones were ranged according to the extent of inhibition in mature tubers.
[a]: The degree of inhibition was determined via iodine staining of tuber slices and verified in a random microscopic check of iodine stained starch granules.
c = complete inhibition; i = incomplete inhibition; n = no inhibition of GBSS gene expression.

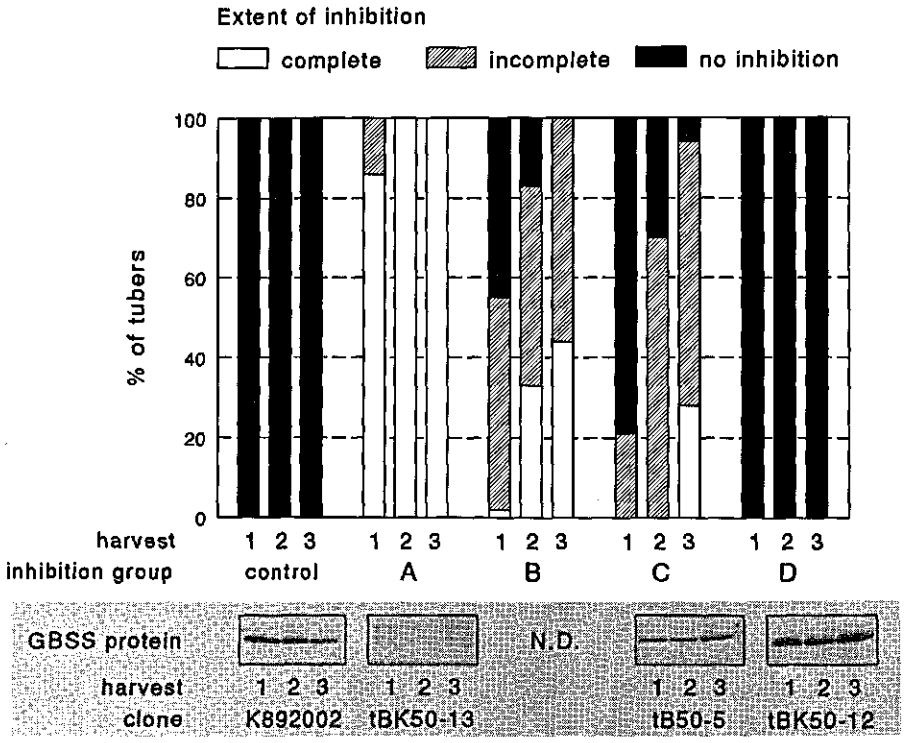

Figure 1: Extent of antisense inhibition during tuber development and immunoblot analysis of tuber starch samples.
Inhibition group A: tBK50-13, tBK50-33 and tBK50-66. Group B: tB50-7, tB50-8, tB50-46, tBK50-29 and tBK50-39. Group C: tB50-1, tB50-5, tB50-17, tB50-20, tB50-22, tB50-42, tBK50-10, tBK50-34 and tBK50-64. Group D: tB50-35, tBK50-3, tBK50-8, tBK50-12, tBK50-48 and tBK50-50. The immunoblot analysis was performed with clones representative for each inhibition group. Per sample, 15μl of a boiled mixture of 20 mg starch and 120 μl of sample buffer was analysed on a 10% polyacrylamide gel with antiserum raised against the potato GBSS protein.
1, six week old first harvest tubers. 2, twelve week old second harvest tubers. 3, mature, eighteen week old third harvest tubers.

Biochemical analysis

The phenotypical analysis of tubers from the transgenic clones showed the effect of the inhibition of GBSS gene expression to be determined in part by the developmental stage of the tuber. Starch, isolated from tuber samples of clones of each of the inhibition groups shown in Figure 1, was analysed for amylose content, GBSS activity and the relative amount of GBSS protein. As is shown in Table 3, the amylose contents and GBSS activities were found to be consistent with the results obtained by iodine staining. For group A clones, the amylose contents and GBSS activities were comparable to those of the amylose-free potato mutant

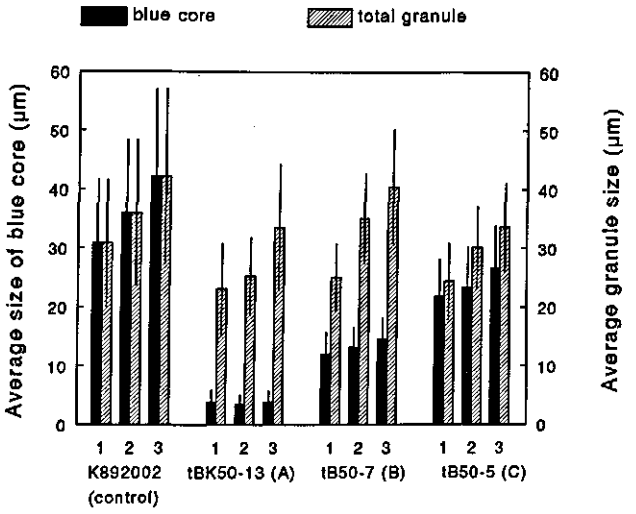

Figure 2: Mean granule size and size of blue-staining central core of individual starch granules. Starch granules were isolated from developing and mature tubers of transgenic and control (K892002) clones.
1, Starch from first harvest tubers. 2, Starch from second harvest tubers. 3, Starch from third harvest tubers. For each clone the inhibition group is indicated within parentheses. Error bars indicate standard deviations.

(amylose content 4.3%; GBSS activity 1.3%; (Chapter 2)). The amylose contents and GBSS activities of group B clones were slightly higher than those of group A clones. Both amylose content and GBSS activity of tBK50-12 (inhibition group D) were comparable to the non-transformed control. For group C clones, intermediate values between B and D were found. The increase of the antisense effect in starch granules during tuber development, which was observed for most clones after iodine staining, was also expressed in the amylose content of group C clones, although for some of the clones the standard deviation was relatively high.

GBSS protein analyses were carried out on tuber starch samples of the transgenic clones tB50-5, tBK50-12 and tBK50-13. From silver stained gels (Data not shown) and immunoblotting with antiserum raised against potato GBSS (Figure 1) it can be seen that GBSS protein was present in starch of tB50-5 and tBK50-12. In tB50-5 starch, the amount of GBSS protein was reduced compared to the non-transformed control. The GBSS protein was lacking in starch of tBK50-13, in which the GBSS gene expression is completely inhibited. The amount of GBSS protein in tuber starch samples from the three harvests did not vary for the transgenic clones, nor for the non-transformed control (Figure 1).

Table 3: Amylose content and GBSS activity of tuber starch.

inhibition group	analysed clones	harvest	amylose content (%)[a]	GBSS activity[a] (pmol/min/mg starch)
A	tBK50-13, tBK50-33	1	3.5 (0.3)	n.d.
		2	5.4 (0.8)	1.2 (0.4)
		3	5.4 (0.8)	1.1 (0.5)
B	tB50-7, tB50-46	1	5.7 (1.9)	n.d.
		2	5.5 (0.6)	3.2 (3.2)
		3	5.2 (1.0)	3.9 (2.8)
C	tB50-5, tBK50-34	1	15.2 (0.8)	n.d.
		2	12.4 (7.1)	54.5 (44.3)
		3	9.3 (2.5)	41.8 (32.2)
D	tBK50-12	1	19.8 (0.2)	n.d.
		2	20.1 (2.4)	90.1 (28.3)
		3	20.1 (0.7)	139.2 (32.0)
control	K892002	1	19.6 (0.2)	n.d.
		2	20.2 (1.3)	104.8 (39.9)
		3	18.8 (1.0)	93.0 (11.3)

Average values were determined for first, second and third harvest tubers of transgenic clones belonging to each of the four inhibition groups. n.d.: not determined.
[a]: Between parentheses: standard deviation.

The starch and soluble sugar content of mature tubers of transgenic clones did not significantly differ from the non-transformed K892002 (ANOVA; data not shown), although a large degree of variation was found among the individual clones (Figure 3). The absence of significant differences indicates that the inhibition of GBSS gene expression did not affect the starch and soluble sugar content in mature tubers of these clones. During tuber development in the field, the average starch content was found to increase significantly (data not shown). The glucose and fructose contents of mature tubers of the clones tB50-5 and tBK50-34 were found to be considerably higher than those of the other transgenics and of K892002.

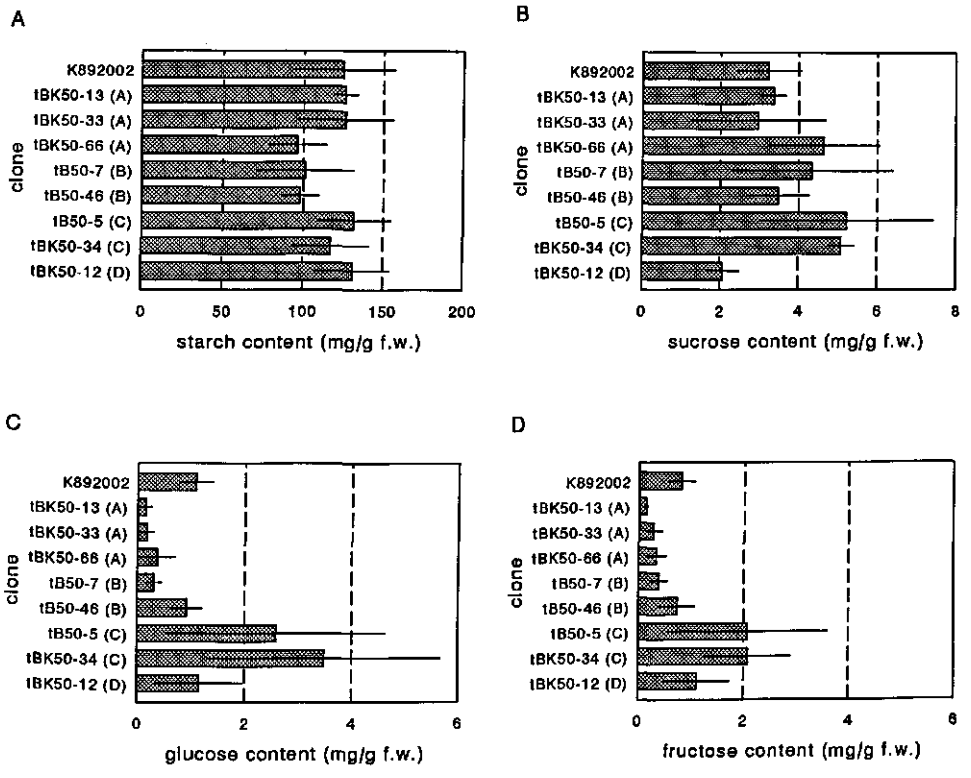

Figure 3: Starch and soluble sugar content of tissue samples of mature tubers of transgenic and control (K892002) clones. A. Starch content. B. Sucrose content. C. Glucose content. D. Fructose content.
For each clone the inhibition group is indicated within parentheses. The starch and soluble sugar contents are expressed in mg per g fresh weight. Error bars indicate standard deviations.

RNA analysis

GBSS mRNA steady state levels were determined in samples from tubers of K892002, Astarte and eight transgenic clones. As illustrated in Figure 4, this analysis showed that in all transformants the GBSS mRNA steady state level was reduced as compared to the control. In general, the degree of inhibition of GBSS gene expression and the GBSS mRNA steady state level were found to be inversely related. Third harvest tubers from all transformants contained less GBSS mRNA than first and second harvest tubers. Since this difference was also observed for K892002 and Astarte, it was thought to be caused by maturation of the tubers.

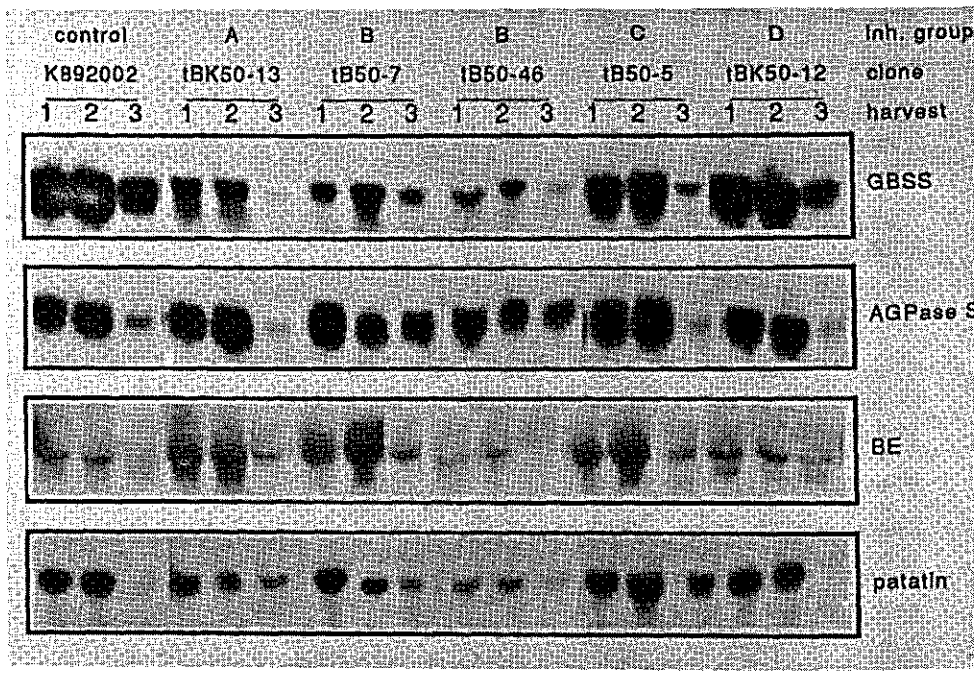

Figure 4: Northern blot analysis of RNA from developing and mature tubers of transgenic and control clones. Hybridizations were carried out with ^{32}P-ATP-labeled probes for GBSS, ADP-glucose pyrophosphorylase subunit S (AGPase S), branching enzyme (BE) and patatin.
1, RNA from first harvest tubers. 2, RNA from second harvest tubers. 3, RNA from third harvest tubers. inh. group = inhibition group.

In order to test this hypothesis, hybridization with probes for one of the subunits of ADP-glucose-pyrophosphorylase (AGPase S), branching enzyme (BE) and patatin were performed (Figure 4). For all clones, the mRNA levels of these genes were found to be reduced in third harvest tubers as compared to first and second harvest tubers of K892002, tBK50-13, tBK50-12 and tB50-5, although variation of the mRNA steady state levels occasionally occurred in first and second harvest tubers. The fact that the mRNA steady state levels of GBSS, AGPase S, BE and patatin in transgenic and control clones showed a decrease in most of the third harvest tubers, suggests that the reduced GBSS mRNA level indeed is a consequence of a maturation-related decrease of the starch or tuber metabolism.

Plant morphology, yield and dry matter content

With respect to the application of the antisense technology for modification of the potato tuber starch composition, possible effects of somaclonal variation or the expression of the transgene on plant morphology and the agronomic characteristics yield and dry matter content were evaluated. The overall plant morphology (plant height, leaf size, plant growth) of all clones was similar to the non-transformed K892002, except for tBK50-8, which showed severely retarded growth. Because *in vitro* grown plantlets were used, a detailed analysis of UPOV morphological characteristics (Anonymous, 1986) could not be performed during this field trial.

Yield and dry matter content were determined for mature tubers. As can be seen in Table 4, considerable variation for yield was found among the transgenic clones. This is likely to be caused by the *in vitro* origin of the plant material used for the field trial. The possible effect of plant origin was less pronounced for dry matter content. Statistical analysis (ANOVA; data not shown) indicated that both yield and dry matter content of the transgenic clones did not differ significantly from the non-transformed control.

Table 4: Yield and dry matter content of mature tubers of transgenic and control clones.

	transgenic clones		K892002 (control)	
	average	SD	average	SD
yield per plant (g)	792	119	898	17
dry matter content (%)	21.6	0.8	21.5	0.1

Discussion

Stability of inhibition

The introduction of an antisense GBSS gene in a tetraploid potato cultivar yielded a series of transgenic clones with varying degrees of inhibition of GBSS gene expression (Table 1). A high degree of inhibition was invariably associated with the absence of the GBSS protein (Figure 1), whereas the GBSS activity and the amylose content (Table 3) were comparable to mutant values (Chapter 2). Expression of the antisense GBSS gene did not significantly affect starch and

soluble sugar contents (Figure 3), although fluctuations were observed for the sucrose, glucose and fructose contents among the transgenic clones and among tubers of the same genotype. Similar fluctuations of the sucrose and glucose contents have been observed between and within individual non-transformed potato tubers (Merlo et al., 1993).

With respect to the extent of inhibition of GBSS gene expression and its stability in individual clones, a comparison was made between *in vitro* grown microtubers and field grown tubers of pGB50-and pKGBA50-transformed clones. For most pKGBA50-transformed clones, the extent of inhibition in microtubers was similar to that in field grown tubers. Most of the pGB50-transformed clones showed incomplete inhibition in field grown tubers, which contrasted with the degree of inhibition observed in microtubers (Table 1). This demonstrates that the antisense inhibition was most stable after introduction of the antisense GBSS gene driven by the GBSS promoter, which is likely to be due to the higher expression level in starch synthesizing tissues of the GBSS promoter as compared to the 35S CaMV promoter, as has been shown in GUS fusion experiments (Visser et al., 1991b). Besides the difference in promoter strength, the nature of the GBSS promoter might also attribute to the enhanced stability of the inhibition. Because of the presence of the potato GBSS promoter in the antisense gene of pKGBA50, the expression level of this gene is thought to be regulated in a similar way as the endogenous GBSS gene. This could explain the relatively limited influence of physiological and developmental factors on the extent of inhibition in pKGBA50-transformed clones.

Antisense effect in starch granules during tuber development

For field grown tubers of transgenic clones with incompletely inhibited GBSS gene expression, the antisense effect was found to significantly increase during development (Figure 1, Table 2). This increase, which was observed in iodine stained tuber starch as well as for the amylose content, was found for both antisense constructs. A similar increase was found during microtuber development (Unpublished data). The observed increase of the antisense effect is not likely to be due to a pronounced increase of the expression of the introduced antisense GBSS gene, since the GBSS protein level (Figure 1) and the GBSS activity (Table 3), that were reduced in tuber samples of each of the three harvests, did not significantly decrease during development. Furthermore, the decrease of the GBSS mRNA level in third harvest tubers did not result from the inhibition of GBSS gene expression, but was demonstrated to result from a maturation-related decrease of

the starch or tuber metabolism (Figure 4).

Based on previous studies on the deposition of reduced amounts of amylose in starch granules (Chapter 3), it is suggested that the observed increase is caused by the specific way of starch granule development. From earlier research (Chapter 3), it is known that a reduction of the GBSS gene expression is accompanied by the presence in starch granules of red-staining (amylose-free) starch and a blue-staining central core, in which the amylose content is comparable to that of wildtype starch. The size of the blue core was found to be related to the amylose content as determined in isolated tuber starch (Chapter 3). During tuber development, the starch content (Figure 3) and the average total granule size were found to increase, whereas the size of the blue-staining cores increased only slightly (Figure 2). Based on the growth of starch granules, which occurs by apposition (Badenhuizen and Dutton, 1956), it was suggested (Chapter 3) that once the transition from amylose-containing to amylose-free starch has been made, the synthesis of amylose-free starch will continue until the development of the granule is completed. Due to the increase of the red-staining amylose-free zone, the ratio between the size of the blue-staining core and the total granule size, as well as the average amylose content in isolated starch will decrease, thus resulting in an increase of the antisense effect in starch granules with respect to these characters. This hypothesis is confirmed by the present study.

Feasibility for application

In this study we describe the application of the antisense RNA technology for the modification of the composition of potato tuber starch in a cultivar which is currently being used for starch production. Inhibition of the expression of the gene encoding granule-bound starch synthase was found to result in complete inhibition of the formation of amylose in mature field grown tubers of four transgenic clones. The specificity of the antisense RNA mediated inhibition was demonstrated by the absence of significant changes in starch content (Figure 3), starch granule size (Figure 2) and steady state levels of several genes involved in starch and tuber metabolism (Figure 4) as compared to the non-transformed control. Field evaluation of the transgenic clones derived from the potato cultivar K892002 demonstrated that in this stage of investigation complete inhibition of GBSS gene expression could be achieved without affecting agronomic traits such as yield and dry matter content. At present, the expression of transgenes at a desirable level combined with the preservation of cultivar characteristics has been reported for potato (Dale and McPartlan, 1992; Jongedijk et al., 1992; Perlak et al., 1993) and several other

crops, e.g. tomato (Kramer et al., 1990; Nelson et al., 1988), flax (McHughen and Holm, 1991) and canola (Arnoldo et al., 1992). The reduction of GBSS gene expression was analysed in a field trial based on in vitro grown plants, as has also been described for the field analysis of insect resistant potato plants expressing a *Bacillus thuringiensis* crystal protein gene (Perlak et al., 1993). The *in vitro* origin might explain the high degree of variation among the clones that was observed for tuber yield (Table 4). However, increased variability for agronomic and morphological characteristics has also been described for field trials based on plants from (field grown) seed tubers (Dale and McPartlan, 1992; Jongedijk et al., 1992). To further evaluate the variability for plant morphology, yield and dry matter content in transgenic clones expressing the antisense GBSS gene, multilocation field trials will be carried out that are based on seed tubers of these clones.

The antisense RNA mediated inhibition of GBSS gene expression was shown to enable the synthesis of amylose-free starch in potato tubers. When compared to the use of the amylose-free (*amf*) potato mutant (Hovenkamp-Hermelink et al., 1987), the antisense technology has several advantages. Since the recessive *amf*-mutation has been induced in a monohaploid potato clone, the isolated mutant had to be introduced into a breeding program to develop a cultivar in which the *amf*-character will be combined with appropriate agronomic traits (Jacobsen et al., 1991). As demonstrated in this study for K892002, the dominant character of the inserted antisense GBSS gene(s) allows the application of antisense technology in existing cultivars, which will reduce the period of time required to develop amylose-free potato cultivars.

Aspects of antisense RNA mediated inhibition of GBSS gene expression in potato: overview and conclusions

Stability and variability of antisense inhibition

Initial studies have demonstrated that the extent of inhibition of GBSS activity in potato tuber starch ranged from 70% to 100% upon the *A.rhizogenes*-mediated introduction of an antisense GBSS gene based on the full length GBSS cDNA and driven by the 35S CaMV promoter (Visser et al., 1991a). The complete suppression of GBSS activity in one of the clones was shown to correlate with the absence of GBSS protein and amylose, and was maintained in tubers of three clonal generations of potato plants (Chapters 2 and 3). An extensive analysis of the complete inhibition of GBSS gene expression was performed with three *A.tumefaciens*-transformed tetraploid clones which carried an antisense GBSS gene consisting of the potato GBSS promoter and the full length GBSS cDNA (Chapter 5). The starch that is formed in tubers of these clones was comparable to starch produced in the amylose-free (*amf*) potato mutant (Hovenkamp-Hermelink et al., 1987; Jacobsen et al., 1989) regarding the absence of amylose, GBSS activity and GBSS protein. The analysis of clones with completely inhibited GBSS gene expression demonstrated that complete inhibition occurred in microtubers, greenhouse grown tubers and field grown tubers (Chapter 2) and remained stable throughout tuber development in the field (Chapter 5). This led to the conclusion that complete inhibition is not influenced by physiological fluctuations.

Incomplete inhibition of GBSS gene expression was accompanied by a reduction of the GBSS protein level, the GBSS activity and the amylose content (Visser et al., 1991a). In transgenic clones with incompletely inhibited GBSS gene expression, the extent of antisense inhibition was found to vary between and within individual clones (Chapter 2). The observed variation of inhibition between transgenic clones is thought to be caused by the effect of genomic sequences adjacent to the T-DNA insertion site that might modulate the expression level of the inserted gene (position effect; Peach and Velten, 1991). The assumed genetic origin of the variation between clones might explain that, for most of the incompletely inhibited clones, the extent of inhibition is similar in microtubers, greenhouse grown tubers and field grown tubers.

Analysis of tuber starch isolated from clones with incomplete antisense inhibition through iodine staining clearly demonstrated the variability of the antisense inhibition within individual transgenic clones (Chapter 2). Variable antisense gene expression within transgenics was also found in *Petunia* (van der Krol et al., 1990c), where it was thought to be caused by changes in physiological conditions during flower development. For potato, large fluctuations have been reported to occur in the growth rate of tubers on a single plant (Engels and Marschner, 1985). Since the starch content as well as the amylose content and the starch granule size are known to increase during tuber growth (Geddes et al., 1965), individual tubers represent different stages of the developmental process. Furthermore, gradients for tuber growth and carbohydrate metabolism are known to occur inside individual tubers along the longitudinal axis (Burton, 1966; Merlo et al., 1993), and from the periphery of the tuber inwards (Burton, 1966). The influence of developmental factors on the antisense effect in tuber starch was clearly demonstrated in Chapter 5. For clones with incompletely inhibited GBSS gene expression, the observed antisense effect was found to increase during tuber development in the field. A similar increase was observed during development of microtubers of comparable transgenic clones. It is therefore argued that the variation in the net antisense effect in tubers with incomplete inhibition of GBSS gene expression is caused by developmental factors related to tuber growth rather than fluctuations of the expression level of the inserted antisense gene.

Effect on the formation and deposition of amylose

The suppression of the GBSS gene expression in transgenic clones resulted in the formation of starch with a reduced amount of amylose. The amylose formed was found to be confined to a core of varying size at the hilum of each granule (Chapter 3, Figures 2C and 2D). The size of the blue central core was found to be closely related to the amylose content as determined in isolated tuber starch. Since the occurrence of blue cores was independent from the antisense gene used, the specific staining pattern is most likely to originate from the reduced expression level of the endogenous GBSS gene and the mode of deposition of amylose in starch granules. This hypothesis is sustained by the observation of starch granules with an identical staining pattern after incomplete complementation of the *amf*-mutant with the potato GBSS gene (Flipse et al., 1994).

Based on the appositional mode of growth of starch granules (Badenhuizen and Dutton, 1956), this implies that early in starch granule development the reduced

amount of GBSS protein is adequate for the formation of starch with a normal amylose content. Due to the increase in total granule surface during development, the reduced amount of GBSS protein might become insufficient for the normal level of amylose formation from a certain surface size onwards. This point was suggested to represent a GBSS protein threshold value (Chapter 3). The moment at which the threshold value is reached, will depend on the extent of inhibition of GBSS gene expression in each individual transgenic clone. Once the transition from amylose-containing to amylose-free starch is made, the formation of amylose-free starch will continue throughout the further development of the starch granules. Thus, the amount of amylose-free starch will increase, whereas the size of the amylose-containing core does not, or only slightly, increase (Chapter 5, Figure 3). The resulting decrease of the relative size of the blue core can explain the increase of the antisense effect during tuber development that was observed for all clones with incompletely inhibited GBSS gene expression that were included in the field trial of 1992 (Chapter 5). It is therefore concluded that the observed increase originated from the specific deposition of the reduced amount of amylose inside starch granules during tuber growth, and not from a change in the extent of antisense inhibition of the expression af the GBSS gene.

With respect to the biosynthesis of starch, the specific effect of the inhibition of GBSS gene expression on the formation and deposition of amylose is additional proof for the occurrence of separate biosynthetic pathways for amylose and amylopectin (Tsai, 1974; Echt and Schwartz, 1981; Konishi et al., 1985; Hovenkamp-Hermelink et al., 1987; Visser et al., 1991a). In addition, the biosynthesis of amylose in starch granules seems to be strictly regulated. The presence in the blue cores of starch with an amylose content which is equal to that of the non-transformed control, demonstrates that a limited reduction of the GBSS protein level early in granule development does not affect the formation of amylose. This indicates that tuber starch granules from non-transformed clones probably contain an excess of GBSS protein, and might imply that the amylose content in tuber starch is regulated at a fixed percentage.

The cessation of the formation of amylose as soon as the GBSS protein level drops below the GBSS protein threshold value can not be fully explained by the gradual decrease of the GBSS protein level due to the increase of the total granule surface during development. A decrease of the ratio between GBSS and other enzymes involved in starch synthesis, due to the reduced amount of GBSS protein, might affect the availability of substrate for the various enzymes, thus adding to the transition from amylose-containing to amylose-free starch.

Composition of the antisense GBSS gene and effectiveness of inhibition

Regarding the effectiveness of antisense inhibition of gene expression, several factors were analysed that might influence the effectiveness of inhibition incited by the introduced antisense gene. The tight relationship between the iodine staining pattern of starch granules and the amylose content (i.e. the extent of inhibition of GBSS gene expression; Chapter 3) allowed a comparison of eleven antisense GBSS gene constructs in at least 30 *A.tumefaciens* or *A.rhizogenes* transformed clones per construct. A comparison of cDNA- and genomic DNA based antisense genes demonstrated that complete inhibition of GBSS gene expression was achieved most often after introduction of the full length cDNA based constructs pGB50 and pKGBA50, followed by the constructs pGBA10 and pKGBA10 that are based on the full length genomic coding region of the GBSS gene. This difference in effectiveness, which was also observed for antisense GBSS genes based on corresponding internal cDNA and genomic fragments, is suggested to originate from the presence of introns in the genomic sequences. The relatively low GC-content of the intron sequences (Chapter 4) might reduce the binding capacity of antisense RNA transcribed from antisense genes based on the genomic DNA as compared to cDNA based antisense genes. The similar inhibitory effect of the full length genomic constructs and the partial genomic construct pKGBA31, based on the 0.6 kb 3' end of the GBSS gene, demonstrate that the GBSS sequence present in pKGBA31 (or at least part of it) is required for the suppression of GBSS gene expression. The involvement of this sequence in antisense inhibition is supported by the low inhibitory effect of the partial genomic constructs that lack the fragment present in pKGBA31.

Expression of antisense GBSS genes from the GBSS promoter resulted in a higher percentage of clones with complete inhibition than expression from the 35S CaMV promoter (Chapter 4). This difference was especially clear during development of field grown tubers (Chapter 5), and may be explained from the three to tenfold higher expression level in tuber tissue of the potato GBSS promoter as compared to the 35S CaMV promoter (Visser et al., 1991b). The more pronounced inhibition of GBSS gene expression in the case of a GBSS promoter driven antisense gene suggests that the occurrence of antisense inhibition is promoter-independent, whereas the effectiveness of inhibition is influenced by the promoter used.

In addition to the above mentioned factors regarding construct composition, a positive correlation was observed between the number of inserted T-DNA copies (antisense genes) and the extent of inhibition of GBSS gene expression. This copy

number effect may be due to a combination of a higher chance for a positive position effect and an additive effect of the presence of multiple T-DNA copies in these clones. The possible role of the additive effect can be elucidated by the analysis of progeny plants in which the T-DNA copies are segregated. Taken together, the combined data demonstrate that the chance for complete and stable inhibition of GBSS gene expression throughout tuber development is highest upon the introduction of three or more copies of the antisense gene based on the full length GBSS cDNA and driven by the GBSS promoter (Chapters 4 and 5).

With respect to the mechanism of antisense inhibition, the observed differences between the various constructs suggest the involvement of RNA-RNA interactions, although genomic interactions between the target gene and the antisense gene, which have been suggested to be involved in sense inhibition or co-suppression (Jorgensen et al., 1990) can not be excluded. From the higher inhibitory effect of the full length cDNA-based antisense constructs as compared to the full length genomic constructs, hypothetical RNA-DNA interactions (van der Krol et al., 1990b) are not likely to be involved in the inhibitory process.

Application of the antisense RNA technology in tetraploid potato cultivars

As is shown in Chapter 5, complete inhibition of GBSS gene expression was achieved in mature tubers of four transgenic clones derived from the tetraploid cultivar K892002. Starch from these clones was devoid of amylose and lacked the GBSS protein and activity. Except for the presence of small blue staining central cores at the hilum of each starch granule, the four clones were comparable to the *amf*-mutant of potato (Hovenkamp-Hermelink et al., 1987; Jacobsen et al., 1989) regarding the starch characteristics determined. A second similarity between the clones with completely inhibited GBSS gene expression and the *amf*-mutant was the maintenance of the suppression in several tuber generations. The stability of complete suppression in field grown tubers of K892002-derived clones (Chapter 5) was confirmed in a second, larger scale field trial, performed in 1993 (unpublished data). Stable inhibition in field grown tubers was demonstrated before for three tuber generations of the transgenic clone WA501 which originated from a diploid genotype (Chapters 2 and 3). Complete inhibition of GBSS gene expression in mature field grown tubers did not significantly affect the starch and sugar contents, nor the expression level of other genes involved in starch and tuber metabolism (Chapter 5). Agronomic characteristics such as yield and dry matter content were not affected either, although it has to be taken into account that the

field trial of 1992 was performed with rooted *in vitro* plantlets.

The applicability of the antisense RNA technology in other potato cultivars will depend on the transformation efficiency of individual cultivars. Since complete inhibition of GBSS gene expression occurs in approximately 10% of the transgenic clones, high numbers of transgenic clones are required to enable the selection of clones in which the GBSS gene expression is completely inhibited without affecting specific cultivar characteristics such as yield, resistances and starch content. In an experiment hitherto not reported in this thesis, antisense GBSS genes were introduced into several potato cultivars by *A. tumefaciens*-mediated transformation. A comparison of 16 cultivars demonstrated considerable differences with respect to the transformation efficiency, that was found to vary from almost 100% of shoot-producing stem explants in three of the cultivars, to less than 5% for most other cultivars. This indicates the necessity of evaluating the transformation efficiency of potato cultivars prior to the application of the antisense RNA technology.

Introduction of the antisense GBSS gene based on the full length GBSS cDNA into the three cultivars that were amenable to *A. tumefaciens*-mediated transformation demonstrated that variation occurred between these cultivars regarding the percentage of clones with complete inhibition of GBSS gene expression (Unpublished data). As compared with data obtained for transgenic clones derived from the diploid potato genotype 1024-2 (Chapter 4), introduction of the antisense gene into the tetraploid cultivars resulted in a lower percentage of clones with complete inhibition. The lower extent of antisense inhibition observed in transgenic clones of the tetraploid cultivars might be caused by the presence of four GBSS-encoding Amf-alleles, whereas the diploid 1024-2 contains only one active Amf-allele. Further research is needed to investigate whether all four Amf-alleles are (equally) active in non-transformed tetraploid genotypes, and whether this is affected by the expression of antisense GBSS genes.

In conclusion, the complete and stable inhibition of GBSS gene expression that is achieved in transgenic cultivar-derived clones, without significantly affecting specific cultivar characteristics, offers good perspectives for the production in potato tubers of starch that is devoid of amylose. Three of the transgenic clones with amylose-free tuber starch were submitted to the Board for Plant Breeder's Rights for registration. These clones will be extensively analysed in field trials to evaluate whether yield losses have to be taken into consideration. However, potential yield losses may be compensated for by a higher economic value of the starch produced by the transgenic clones, due to the improved starch quality. With

respect to plant breeding, transformation of potato cultivars with antisense GBSS genes can be applied in two ways, namely for the direct selection of a new variety which produces amylose-free starch, and, secondly, for the selection of transgenic clones with complete and stable inhibition of GBSS gene expression that can be used as breeding parents. The second approach will have to be applied when serious yield losses are connected with the altered trait and/or the transgenic nature of the selected potato clones. In these cases, introduction of the antisense trait into a different genetic background through breeding may compensate for the negative yield effects. As compared to the recessive *amf*-mutant, the introduction of transgenic clones into a breeding program is more efficient, because of the dominant inheritance of the antisense genes.

Summary

Potato starch and its derivatives are widely used in several fields of application. The manufacturing of most products requires the modification of native starch with respect to, for example, viscosity and physical stability. In addition to the currently used physical, chemical and biochemical derivatization and gelatinization of extracted starch, modification of the starch biosynthetic pathway *in planta* is regarded as a valuable approach for altering the quality and quantity of potato starch.

This thesis describes the application of antisense RNA technology for the modification of the composition of potato tuber starch. Starch consists of amylose and amylopectin, and is predominantly synthesized in amyloplasts of tubers and seeds, where it is deposited in starch granules. Several enzymes are involved in the biosynthesis of starch, of which granule-bound starch synthase (GBSS) catalyses the formation of amylose. Generally, starch contains 20% of amylose and 80% of amylopectin. However, mutants are known in which the ratio between amylose and amylopectin has changed. An example is the amylose-free (*amf*) potato mutant, of which the starch exclusively contains amylopectin due to a recessive mutation in the GBSS-gene. The suppression of the expression of specific genes can also be achieved by means of antisense inhibition, which is supposed to be based on the formation of an RNA-duplex between the target mRNA and the antisense RNA, thus blocking the transport of the mRNA from the nucleus.

To inhibit the GBSS gene expression, several antisense genes were introduced into the potato genome via *Agrobacterium*-mediated transformation. Among transgenic clones in which inhibition of GBSS gene expression was observed, two types could be discerned:

A. Transgenic clones with completely inhibited GBSS gene expression. These clones lacked the GBSS protein, GBSS activity and amylose. The complete inhibition was demonstrated in microtubers, greenhouse grown tubers and field grown tubers, and was maintained in several tuber generations. The composition of tuber starch from these clones and from the *amf*-mutant was similar.

B. Transgenic clones with incompletely inhibited GBSS gene expression. In these clones the GBSS protein content, GBSS activity and amylose content were reduced as compared to non-transformed clones. With respect to the extent of inhibition, variation was observed between transgenic clones which was

supposed to result from position effects. Variation was also observed between tubers of individual plants and within individual tubers. This type of variation was suggested to be caused by factors related to tuber development.

Microscopic analysis of iodine stained tuber starch granules from transgenic clones showed that the reduced amount of amylose was not equally distributed throughout the entire granule, but was confined to the central core of the granule. Iodine-staining of starch granules yielded a blue core, in which the amylose content equalled that of wildtype starch, and a red, amylose-free, outer part. The size of the blue core was shown to be positively related to the amylose content, as determined in starch isolated from the transgenic clones. This finding led to the hypothesis that at a certain point in starch granule development the available amount of GBSS protein will become insufficient for the formation of starch with a normal amylose content. From this point onwards, amylose-free starch will be produced throughout further starch granule development.

With respect to the effectiveness of antisense inhibition, the influence of the composition of the antisense gene on the percentage of transgenic clones with complete or incomplete inhibition was studied. To this end, eleven antisense genes were constructed, based on the full length GBSS cDNA, the genomic GBSS coding region, and some cDNA- and genomic fragments. Furthermore, a comparison was made between the 35S CaMV promoter and the potato GBSS promoter. The extent of inhibition of GBSS gene expression was studied in at least 30 transgenic clones per antisense gene construct. In addition to construct-related differences, the number of T-DNA insertions was found to be positively related to the extent of antisense inhibition. It was concluded that the chance for complete inhibition of GBSS gene expression was highest in transgenic clones with three or more copies of the antisense gene based on the full length GBSS cDNA driven by the GBSS promoter.

In order to evaluate the applicability of the antisense GBSS system, two antisense genes based on the full length GBSS cDNA and driven by the GBSS- or the 35S CaMV promoter were introduced into several potato cultivars via transformation with *A.tumefaciens*. The trangenic clones of one of the cultivars were analysed for the extent of antisense inhibition and its stability in a field trial based on *in vitro* plantlets. Complete inhibiton of GBSS gene expression, resulting in the formation of amylose-free starch, was demonstrated in mature tubers of four of these clones. Other characteristics, such as the total starch- and sugar content, the total yield and the dry matter content, did not deviate significantly as compared

to the non-transformed cultivar. This led to the conclusion that antisense inhibition has good perspectives for the formation of amylose-free starch in potato tubers. In 1993, three of the transgenic clones with amylose-free tuber starch that are described in this thesis, were submitted to the Board for Plant Breeder's Rights for registration.

Samenvatting

Aardappelzetmeel en zetmeelderivaten worden op grote schaal toegepast in zeer uiteenlopende produkten. Tijdens de fabricage van veel van deze produkten wordt het oorspronkelijke zetmeel vaak gemodificeerd, door bijvoorbeeld de viscositeit of de stabiliteit te veranderen. Voor modificatie worden doorgaans fysische, chemische en biochemische processen gebruikt. In dit proefschrift wordt de mogelijkheid beschreven om door toepassing van biotechnologische technieken de zetmeelsamenstelling te modificeren tijdens de vorming van het zetmeel in de aardappel.

Zetmeel bestaat uit amylose en amylopectine, en wordt voornamelijk gevormd in knollen en zaden waar het wordt opgeslagen in de vorm van zetmeelkorrels. Bij de vorming van zetmeel zijn verschillende enzymen betrokken. Van deze enzymen is het korrelgebonden zetmeelsynthase (granule-bound starch synthase; GBSS) betrokken bij de vorming van amylose. Normaal gesproken bestaat zetmeel voor ± 20% uit amylose en voor ± 80% uit amylopectine. Er zijn echter mutanten bekend waarin de verhouding tussen amylose en amylopectine is veranderd door een mutatie in een van de genen die coderen voor de bovenstaande enzymen. Een voorbeeld hiervan is de amylosevrije mutant van de aardappel (*amf*), waarvan het zetmeel alleen amylopectine bevat door een recessieve mutatie in het GBSS-gen.

Uitschakeling of remming van de expressie van specifieke genen is ook mogelijk door toepassing van antisense inhibitie. Hierbij wordt het cDNA van het te remmen gen of het gen zelf geïsoleerd en gebruikt om een antisense gen te maken. In het antisense gen is het cDNA of het geïsoleerde gen achterstevoren (in antisense oriëntatie) achter een promoter geplaatst. Het antisense gen wordt vervolgens door middel van transformatie ingebracht in een bestaand aardappelras, waar de niet-coderende streng van het antisense gen wordt afgelezen, hetgeen leidt tot de vorming van antisense RNA. Antisense inhibitie vindt waarschijnlijk plaats door de vorming van een RNA-duplex, bestaande uit het mRNA van het te remmen gen en het antisense RNA, waardoor het transport van het mRNA uit de kern, en daardoor de vorming van het corresponderende enzym, wordt verhinderd.

In deze studie werd de expressie van het GBSS-gen in aardappel geremd door antisense inhibitie. Door middel van transformatie met *Agrobacterium* werden verschillende antisense genen ingebracht in de aardappel. Onder de transgene planten waarin de remming van het GBSS-gen werd aangetoond konden twee typen worden onderscheiden:

A. Transformanten met volledige remming van het GBSS-gen. In deze planten kon geen GBSS eiwit, geen GBSS activiteit en geen amylose worden aangetoond. De volledige remming werd aangetoond in micro-, kas-, en veldknollen en bleef gehandhaafd in meerdere generaties van vegetatief vermeerderde knollen. Het zetmeel dat wordt gevormd in deze transformanten lijkt zeer sterk op dat van de *amf*-mutant.
B. Transformanten met onvolledige remming van het GBSS-gen. Deze planten hebben een verminderde hoeveelheid GBSS eiwit, een lagere GBSS activiteit en een lager amylosegehalte. Tussen klonen van deze groep werd variatie van het niveau van remming gevonden. Deze variatie is waarschijnlijk een gevolg van de invloed van het DNA van de plant dat het ingebrachte gen flankeert. Ook werd variatie gevonden tussen knollen van individuele planten en binnen individuele knollen. Deze variatie wordt mogelijk veroorzaakt door factoren die de ontwikkeling van aardappelknollen beïnvloeden.

De microscopische analyse van met jodium gekleurde zetmeelkorrels uit knollen van de transgene planten toonde aan dat de verminderde hoeveelheid amylose niet gelijkmatig was verdeeld door de hele korrel. Met jodium kleurde de amylose-bevattende kern blauw en het overige deel van de korrel, dat geen amylose bleek te bevatten, rood. De grootte van de blauwe kern bleek gerelateerd te zijn aan de hoeveelheid amylose: hoe groter de blauwe kern, des te meer amylose. Dit gegeven leidde tot de hypothese dat tijdens de ontwikkeling van de zetmeelkorrel de beschikbare hoeveelheid GBSS eiwit op een gegeven moment ontoereikend wordt voor de vorming van zetmeel met een normaal amylosegehalte. Voorbij dit punt wordt amylose-vrij zetmeel gevormd gedurende de verdere ontwikkeling van de zetmeelkorrel.

Met betrekking tot de effectiviteit van de antisense inhibitie werd de invloed van de samenstelling van het antisense gen op het percentage transformanten met volledige of onvolledige remming van he GBSS-gen bestudeerd. Hiervoor werden elf verschillende antisense genen gemaakt, gebaseerd op het volledige GBSS cDNA, het volledige coderende deel van het GBSS-gen en enkele fragmenten van het cDNA en het GBSS-gen. Ook werd een vergelijking gemaakt tussen de veel gebruikte 35S CaMV promoter en de eigen promoter van het GBSS-gen. De mate van remming van de expressie van het GBSS-gen werd bestudeerd in tenminste 30 transformanten per antisense gen. Naast verschillen tussen de genen werd gevonden dat de antisense remming sterker was naarmate er meer kopieën van een antisense gen in de transformant aanwezig waren. Op basis van de verkregen resultaten kon worden geconcludeerd dat de kans op volledige remming van de

GBSS genexpressie het hoogst was in transformanten met drie of meer kopieën van het antisense gen gebaseerd op het volledige GBSS cDNA en de GBSS-promoter.

De toepassingsmogelijkheden van het beschreven systeem werden bestudeerd door twee antisense genen gebaseerd op het GBSS cDNA en de GBSS- of de 35S CaMV-promoter via transformatie met *A.tumefaciens* in te brengen in enkele zetmeelrassen. De transformanten van een van deze rassen werden geanalyseerd in een veldproef op basis van *in vitro* planten voor wat betreft de mate van remming en de stabiliteit. Volledige remming van de GBSS genexpressie werd aangetoond in volgroeide knollen van vier van de transformanten. Het zetmeel van deze transformanten bevatte geen amylose. Andere eigenschappen, zoals het totale zetmeel- en suikergehalte, de opbrengst en het droge stof gehalte waren niet significant veranderd ten opzichte van het niet-getransformeerde zetmeelras. Dit leidt tot de conclusie dat antisense inhibitie goede perspectieven biedt voor de vorming van amylosevrij zetmeel in aardappelknollen. In 1993 werden drie van de in dit proefschrift beschreven transgene klonen die amylosevrij zetmeel produceren bij de Raad voor het Kwekersrecht aangemeld voor registratie.

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Curriculum vitae

Anja Kuipers werd geboren op 28 juni 1964 te Groningen. In 1982 behaalde zij het VWO-diploma aan het Nienoordcollege te Leek. Na een jaar te hebben doorgebracht op de PABO te Groningen, begon zij in 1983 aan de studie Biologie aan de Rijksuniversiteit te Groningen. De doctoraalfase omvatte onderwerpen bij de werkgroep Cel- en Plantengenetica van de vakgroep Genetica en de werkgroep Moleculaire Biologie van Planten van de vakgroep Biologie van Planten. In januari 1989 werd de biologiestudie afgerond, waarna zij werd aangesteld als assistent in opleiding bij de vakgroep Plantenveredeling van de Landbouwuniversiteit te Wageningen. De resultaten van het onderzoek, dat werd uitgevoerd van februari 1989 tot februari 1993, zijn beschreven in dit proefschrift. Vanaf 1 april 1994 is zij werkzaam als postdoc bij de vakgroepen Plantenveredeling en Erfelijkheidsleer van de Landbouwuniversiteit Wageningen.