Two approaches for induction and isolation of starch mutants in potato (*Solanum tuberosum* L.): random versus gene targeted mutagenesis

Twee benaderingen voor de inductie en isolatie van zetmeelmutanten in aardappel (*Solanum tuberosum* L.): ongerichte versus gen gerichte mutagenese

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STELLINGEN

1. Het concept om in een met joditum rood kleurende *amf* achtergrond een blauwkleurende variant te vinden in aardappel (*Solanum tuberosum* L.) is reëel toepasbaar. (Dit proefschrift)

2. Het gerichte mutatieonderzoek met behulp van *Ds* transposons uit maïs is een stuk willekeuriger dan verwacht aangezien het excisiegedrag erg moeilijk te voorspellen is. Dit wordt echter mede veroorzaakt door de gebrekkige kennis op moleculair niveau van transposons. (Dit proefschrift)

3. Bestudering van transpositie gedrag van transposons met een excisie marker leidt tot een onderschatting van de transpositie activiteit. (Enckevort et al., 2000. *Theor. Appl. Genet.* **101**: 503 – 510; dit proefschrift)

4. Partiële resistentie in gerst tegen dwergroest (*Puccinia hordei*) is deels gebaseerd op niet-waard resistentie (T.J.H. Hoogkamp et al., 1998. *Phytopathology* **88**: 856 - 861)

5. Goed plant materiaal is essentieel voor fundamenteel en strategisch wetenschappelijk onderzoek op het gebied van cultuurgewassen.

6. De interessantste bronnen van genetische variatie zijn omnummeren en vermenging!

7. Oude veredelingsmethoden zijn nog steeds goed toepasbaar, zeker wanneer men ze in een nieuw jasje steekt.

8. De officiële toestemming van de FIA voor toepassing van traction control in de Formule 1 zal weinig invloed hebben op het verloop van de races, omdat de meeste teams al jaren traction control gebruiken dat weggestopt is in de software van de auto's.

9. Invoering van de Euro leidt tot ongewenste prijsstijgingen niet alleen door afrondingsverschillen.

Stellingen behorend bij het proefschrift ' Two approaches for induction and isolation of starch mutants in potato (*Solanum tuberosum* L.): random versus gene targeted mutagenesis'.

Timo J.H. Hoogkamp
Wageningen, 8 oktober 2001.

TIMO J.H. HOOGKAMP

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Bibliographic abstract

In this thesis two approaches were used to induce structural mutations in potato starch biosynthesis genes in potato. First production of new monoploid *amf* genotypes through parthenogenesis made it possible to initiate mutation breeding for *amfae* double mutants. Two *amf* monoploids were selected which fulfilled most of the prerequisites. By inducing a mutation in one of the branching enzymes in an *amf*-mutant it is possible to select a double mutant having less branched amylopectin. This mutation can easily be identified by iodine staining. Amylose-free starch will stain red and less branched amylopectin will stain blue, like amylose containing starch. Mutations were induced by X-ray irradiation of leaf explants followed by adventitious shoot regeneration and microtuber induction or followed by several rounds of multiplication of axillary buds and microtuber induction. In both cases the starch of microtubers was stained with iodine to screen for aberrant types. In 56 tuber samples blue or otherwise abberant starch granules were found. With this kind of observations, the concept of mutation breeding for starch variants in monoploid potatoes is proven. A second way to induce structural mutations was the use of the *Ac (Activator)/Ds (Dissociation)* transposase system of maize in potato were the *Ds* transposon is activated by a transposase source. In this study the *Ds* element was linked to the GBSS gene, of which the phenotypic effect of deactivation is known i.e. red staining starch after iodine staining. The known *amf* mutation was used as a model system to gather more information about the transposition frequency of the *Ds* transposable elements in potato and to test the tagging of the wildtype GBSS gene. To activate the *Ds* element four *Ds* transposon containing plants were combined with the *Ac* transposase via cross combination or double transformation. Excision rates ranged from 14.8-48.4%. Three phenotypic starch mutants were found after screening by iodine staining of tuber cut surfaces. These amylose-free mutants were analyzed by *in vitro* tests, Southern blot hybridization and sequencing. Strong indications were found that inactivation of the GBSS gene was caused by a transposable element.

CONTENTS

General Introduction

Potato (*Solanum tuberosum* L.) originates from the Andes in South America, its domesticated form is a cross-fertilizing highly heterozygous autotetraploid (2n=4x=48) plant. Potato has one of the richest genetic resources of any cultivated plant. More than 200 *Solanum* species, which are often diploid, are recognized. By its capability to form tubers, vegetative propagation and multiplication is possible. After its introduction in Europe in the 16th century it has developed into one of the world's major tuber food crops. Also in the western part of Europe the growth of potatoes is economically very important. In the Netherlands an area of 182,600 ha potatoes were grown in 1999 which is the second biggest crop after maize. 42,000 ha of this area is used for the production of seed potatoes. Another 87,000 ha were grown for the production of potatoes for consumption (a.o. French fries and mashed potatoes). The growth of starch potatoes is mainly concentrated in the northeastern part of the Netherlands. In 1999 54,100 ha starch potatoes were grown for the production of starch which is approximately 18% of the fresh weight. After harvesting the potatoes the starch is obtained by industrial processing. The potato starch is used in many different applications because of its high molecular weight and its low amount of contaminants. For some of these applications it is necessary to change the starch properties by derivatization. A number of the chemicals used for derivatization of the starch are toxic or carcinogenic. During these processes a lot of water is used which leads to water pollution. Fortunately the increased knowledge about starch biosynthesis makes it possible to perform some steps of derivatization *in planta*. This might also lead to novel starches from which the starch industry can create new products and markets (Bruinenberg et al., 1995). The above mentioned aspects make it difficult but challenging and worthwhile to pursue the induction and isolation of new starch mutants in potato.

Starch biosynthesis

Starch biosynthesis in potato consists of the synthesis of amylopectin and amylose, the major starch components which represent respectively 77-82% and 18-23% of the starch present in amyloplasts. Amylopectin is a highly branched structure with besides linear chains of α(1,4) linked glucose residues multiple α(1,6) linked glucose residues with an average chain length of 20-30 units (Shannon and Garwood, 1984). Amylose is essentially a linear structure with α(1,4) linked glucose residues and a very few α(1,6) linked glucose residues with an average chain length of 100-10,000 units. Due to these molecular differences the amylose molecules can form helices with iodine which results in a dark blue color (Shannon and Garwood, 1984), amylopectin stains red.

Starch biosynthesis involves several enzymes. ADP-glucose pyrophosphorylase (AGPase; Figure 1) synthesizes ADP-glucose out of glucose-1-phosphate. A few starch synthase genes are responsible for elongation of α(1,4) glucan chains, the glucosyl part of an ADP-glucose molecule is linked to the non-reducing end of a glucan chain. Branching enzymes catalyze branching of linear chains (BE I and II) and some branches are trimmed by a de-branching enzyme (DBE, Ball et al., 1996) which leads to amylopectin. Amylose is believed to be synthesized by extension and cleavage from amylopectin (van der Wal et al., 1998; van der Wal, 2000) by GBSS I (Figure 1). The amylose/amylopectin ratio is one of the most important properties of the starch concerning gelatinization, retrogradation and viscosity. Small changes in starch composition or content may influence the properties of the starch considerably (Visser and Jacobsen, 1993; Bruinenberg et al., 1995). Apart from the practical point of view mutations in starch biosynthesis are an important tool to study the genetics of starch biosynthesis.

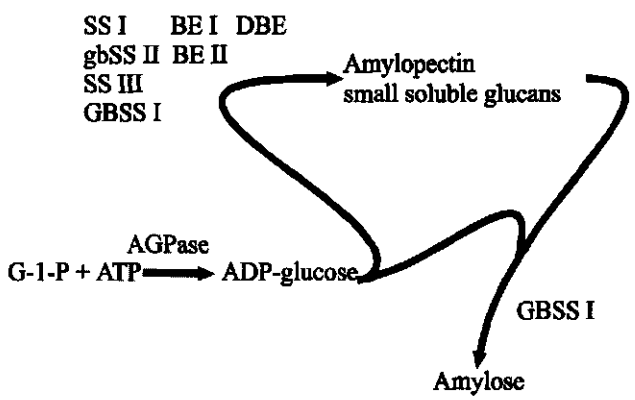

Figure 1. Schematic representation of the starch biosynthesis pathway (Bruinenberg et al., 1995 and van der Wal et al., 1998).

MODIFICATION OF STARCH BIOSYNTHESIS

In the past many researchers worked on the modification of starch biosynthesis in maize and pea. For almost all known enzymes in starch biosynthesis (structural) mutants are known in maize or pea (Kossmann and Lloyd, 2000). In potato until now only one structural mutant exists containing starch without amylose (*amf*), which was induced by using ionizing irradiation (Hovenkamp-Hermelink et al., 1987). Several genes of potato starch biosynthesis have been down regulated by the anti-sense approach. Inhibition of ADP-glucose pyrophosphorylase leads to sugar-storing tubers with effects on tuber formation and expression of tuber storage protein genes (Müller-Röber et al., 1992). For GBSS (Visser et al., 1991; Kuipers et al., 1994) this resulted in a minimization of amylose content leading to large changes in properties of the starch concerning gelatinization, retrogradation and viscosity. Down regulation of BE I in the *amf* mutant only resulted in small physico-chemical changes in starch (Flipse et al., 1996). Also the inhibition of BE II has almost no effect. The combined inhibition of BE I and II however results in an amylose content from 60% up to 75% (Schwall et al., 2000). Almost complete suppression of SS I in potato does not lead to any detectable changes in the starch, this is believed to be due to the fact that SS I only contributes a minor proportion (5–10%) of the total

starch synthase activity (Kossmann et al., 1999). The inhibition of the partial granule bound SS II (15% of activity) only had a small effect on starch metabolism (Edwards et al., 1995; Kossmann et al., 1999). Suppression of SS III (up to 80% of activity) has no effect what so ever on the starch in potato (Abel et al., 1996; Marshall et al., 1996). However the combination of SS II and SS III inhibition resulted in amylopectin with major changes in chain length (Edwards et al., 1999; Lloyd et al, 1999).

Besides knocking out endogenous genes also heterologous genes are introduced into potato to alter starch structure, composition or content. Introduction of bacterial genes of glycogen biosynthesis from *Escherichia coli* leads in case of glycogen synthase (*glgA*) to the production of a more highly branched starch. The amylose content is decreased and some chains within the amylopectin increase (Shewmaker et al, 1994). The introduction of glycogen branching enzyme (*glgB*) from *E. coli* in wildtype potato leads to more branched polymers where amylose is replaced by an intermediate between amylose and amylopectin (Kortstee et al., 1998). Also other genes are introduced into potato to produce for example cyclodextrins (Oakes et al., 1991) and fructans (van der Meer et al., 1994).

Salehuzzaman et al. (1993) were able to inhibit GBSS activity in potato by introduction of an anti-sense construct carrying cassava GBSS. So inhibition of endogenous genes might also inhibit similar genes from other species after transformation. Thus homologous genes from other species will also be inhibited by an anti-sense construct of a similar endogenous gene. Thus structural mutants in potato are an important tool to produce novel starch polymers because their mutant background will not influence genes which are introduced in these genotypes.

INDUCING MUTATIONS

Random mutagenesis

Before starting mutation experiments one has to find out the requirements for the basic plant material, which can be used to induce new starch mutants in potato. These mutants are expected to be recessive. An important tool to induce structural mutations is the use of X-ray irradiation on monoploid potatoes. To induce mutants ionizing irradiation as well as chemical mutagens were used in several plant species. In a study by Reddy (1991) mutagenic parameters based on chlorophyll mutation frequency in M_2 in single and combined treatments of gamma rays, ethylmethane sulphonate (EMS) and sodium azide in triticale, barley and wheat were determined. He found that in these three *Graminae* there is not much difference in efficiency between the individual and combined methods. In potato several mutants have been induced with reduced tuber glycoalkaloid content by using gamma irradiation (Love et al., 1996). In mutation induction experiments, most often use is made of X-rays and γ-rays (Broertjes and van Harten, 1988). When using adventitious shoots it is believed that these shoots originate from one cell (Broertjes and van Harten, 1988). However, the attempt of Jacobsen et al. (1990) to induce new mutations in the original amylose-free

(*amf*) mutant of potato failed. They were not able to select new structural mutants because their basic material proved to be chimeric for the *amf* trait (van der Leij et al., 1992). When using axillary buds as basic material for mutation experiments it is obvious that the derived vegetative generations are expected to be chimeric for the desired trait after mutagenic treatment (Ahloowalia, 1998).

Nomenclature

The nomenclature used in mutation breeding is confusing, therefore, the International Atomic Energy Agency (IAEA) has a standardized nomenclature for consecutive generations in a mutation experiment. The non-irradiated plant material is always referred to as M_0. In self-fertilizing crops the irradiated generation is called M_1 the second generative generation is called M_2 and so on. But for vegetatively propagated crops there are two possibilities to address the first vegetative generation after irradiation, vM_1 or M_1V_1 respectively. The second vegetative generation is referred to as vM_2 or M_1V_2 (van Harten, 1998). But when a generative step is made in the mutation process the first variant has to be changed in M_2. This should not, but does lead to confusion between M_2 and vM_2 whereas the other variant is simply changed in M_2V_1. Since potato is used in this thesis that can be propagated vegetative and generative the M_mV_n terminology is used where m is the number of cross generations and n the number of vegetative generations. Doing so it is clearer when a vegetative or a generative step is made (Donini et al., 1991).

Gene directed mutagenesis

McClintock (1948 and 1950) discovered the 'Ac-Ds (Activator-Dissociation) element family' in maize. There are two types of transposable elements, the first type consists of an autonomous element (like *Ac*) which can migrate independently through the genome. The second type is a non-autonomous element (like *Ds*) which can only transpose when another transposase source (like *Ac*) is present. The *Ac/Ds* family was successfully characterized and isolated (Fedoroff et al., 1983; Behrens et al., 1984; Müller-Neumann et al., 1984). All *Ac* and *Ds* transposable elements have almost the same (near identical) terminal inverted repeats (TIRs) of 11 bp. Families of other transposable elements like *Spm/dspm* (*En/I*) and *Mutator* in maize are discriminated by the length and composition of their inverted repeat termini (Bennetzen, 2000). For the *Ac/Ds* family, the non-autonomous *Ds* element contain deletions or are otherwise non-autonomous derivatives of the autonomous *Ac* element (Bennetzen, 2000). Transposition of the *Ac/Ds* family elements was studied (Dooner and Belachew, 1989) and they were first used to clone genes in maize (Fedoroff et al., 1984). Later it was shown that the *Ac/Ds* transposable elements could also transpose in other species like tobacco (Dooner et al., 1991), tomato (Yoder et al., 1988) and potato (Knapp et al., 1988). Instead of the autonomous *Ac* element as activator for the non-autonomous *Ds* element often use is made only of the transposase cDNA driven by several different promotors to enable transposition (Lassner et al., 1989; Rommens et al., 1992; Scofield et al., 1992; Long et al., 1993; Takumi et al, 1999). In potato a system was set up to induce structural mutations with the use of *Ac/Ds* transposable elements in potato (Pereira et al., 1992; El-Kharbotly et al., 1996). The transposition of

these *Ac/Ds* transposable elements in potato is proven to be somatic and thus leading to chimeric plants (Pereira et al., 1991; Enckevort et al., 2000).

CHIMERISM

Both random mutagenesis and gene directed mutagenesis will result in chimeric plants. So when doing structural mutation experiments in somatic tissue of potato it is very important to know how shoots and buds are organized to be able to minimize chimerism. Important is the number of cells involved in the formation of a shoot and the location of the histogenic layers L I, L II and L III (Broertjes and van Harten, 1988). It is generally found that axillary buds reproduce the layered pattern of the apical meristem (van Harten, 1998). For the propagation and multiplication of potato it is important to remind that generative tissue originates from L II and that tubers originate from L I and L III. The starch forming tissue within the potato tubers originates from only the L III layer (van Harten, 1998).

Organization of apical meristems

For mutation experiments involving apical meristems it is very important to know how those meristems develop. Especially when mutation frequencies are going to be calculated, the number of initial cells within the three histogenic layers has to be known. The general organization of all shoot apical meristems involves several zones that are located in different parts of the meristem (Figure 2).

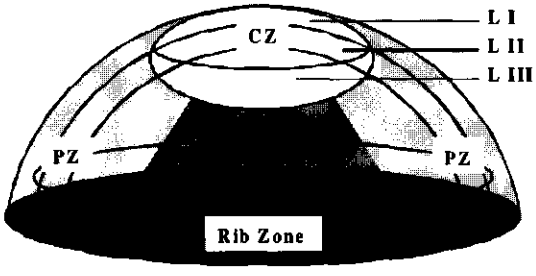

Figure 2. Diagram of the zones and layers within the shoot apical meristem. CZ = central zone, PZ = peripheral zone (Medford, 1992).

Most important are the so-called initial cells that are located in the central zone. The central zone is a small, oval-shaped, distally located group of cells that serve as the source of cells for other regions of the apical meristem and hence the shoot (Medford, 1992). After determining the central zone in the schematic representation of an apical meristem (Figure 3) the number of initial cells within the histogenic layers can be estimated (Broertjes and van Harten, 1988; Medford, 1992). Assuming that the cells per layer are ordered as a cylinder it is possible to use the formula pr^2 to estimate by calculation the number of cells involved.

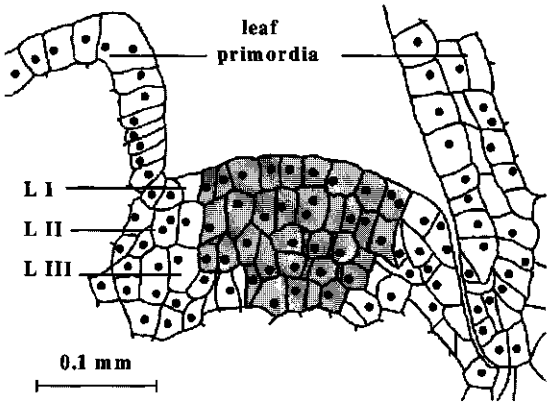

Figure 3. Schematic representation of the apical area showing the layered character of L I and L II, and the irregular character of the L III zone (modified from Broertjes and van Harten, 1988).

Surface: $\pi r^2 = \pi(0.5d)^2$, where d is the number of cells of a particular layer of the so-called initial ring involved in the formation of a new shoot, in a cross section of an apical meristem (Figure 3).

L I: $\pi(0.5*d)^2$	$= \pi(0.5*9)^2$	= 64 cells
L II: $\pi(0.5*d)^2$	$= \pi(0.5*8)^2$	= 50 cells
L III: multiple cell layers:		
- first layer $\pi(0.5*d)^2$	$= \pi(0.5*7)^2$	= 38 cells
- second layer $2\pi(0.5*d)^2$	$= \pi(0.5*4)^2$	= 13 cells
- third layer $3\pi(0.5*d)^2$	$= \pi(0.5*3)^2$	= 7 cells
L III total		= 58 cells

Types of chimerism

Randomly induced mutations as well as transposon insertion events are single cell events and, therefore, mutagenesis of multicellular organs will result in chimeric plants that carry one or multiple mutations in a part of the plant. A mutation in a shoot apical meristem will initially lead to a mericlinal chimera in which the mutated area is restricted to a part of one histogenic layer (D'Amato, 1997). Of course this type is unstable but in a vegetatively propagated crop like potato a few cycles of vegetative propagation might lead to a periclinal chimera. Periclinal chimeras are stable within one histogenic layer and this type of chimerism can be propagated vegetatively and can be undetected for a long time. This type of chimerism can be revealed when it is located in the L II and transferred to the next generation after a generative step. Also rearrangements of cell layers within a chimeric plant might result in revealing the mutation. There are three types of rearrangements, the first type is named perforation: cells of L II, L III or both penetrate the outer layer(s). The second type is called reduplication: cells of L I, L II or both penetrate the inner layer(s). The last type is referred to as transgression or translocation: cells within one layer are replaced laterally (van Harten, 1998; D'Amato 1997). The frequency of rearrangements in periclinal chimeras is rather low: thus the contribution of mutations in L I and L III transferred to the next generation is low (van Harten, 1998). Besides a generative step to stabilize periclinal chimeras and to obtain a solid mutant

(homohistont) periclinal chimeras can also be subjected to for example adventitious shoot formation or regeneration from certain tissue. This again will result in chimeras, but in some cases all three histogenic layers will contain the mutant phenotype (Tian and Marcotrigiano, 1993).

OUTLINE OF THE THESIS

The main objective of this thesis was to induce and select potato mutants that contain new structural mutations in the genes involved in the starch biosynthesis pathway resulting in an altered starch composition and/or content. Investigation of these mutants should lead to a better understanding of genes that act in starch biosynthesis. In this thesis two approaches are used to induce structural mutations in genes of starch biosynthesis. For the first approach of random mutagenesis new basic monoploid potato material was developed by prickle pollination of diploid *amf* clones. Several monoploids were produced and tested for *in vitro* properties like vigor, regeneration and tuberization capacity and also sensitivity to X-ray irradiation as is described in Chapter 2. Two monoploids were selected and used in two mutation induction protocols. The first protocol was the production of adventitious shoots followed by tuber induction. The second protocol was based on several rounds of multiplication in order to stabilize variant sectors. In Chapter 3 the outcome of these experiments is described. The second approach concerned the use of the maize *Ac/Ds* transposable elements system in potato. A model experiment was set up to test tagging of the wildtype GBSS gene. A few plants harboring a non-autonomous *Ds* element were selected based on the linkage of the *Ds* element to the GBSS gene. Before large-scale experiments could take place the excision rate of the *Ds* element under the influence of different transposase sources which activate the *Ds* element was determined. Chapter 4 gives an overview of the expected excision level when using maize transposons in potato in combination with different promotor driven transposase constructs. The actual use of these transposons for tagging of the GBSS gene in the model system is described in Chapter 5. And finally in Chapter 6, a general discussion of the research results for this study is presented together with a pathway for induction of mutations based on knowledge and properties of the gene(s) of interest. In addition alternative ways to induce and identify stable structural mutants in potato are discussed.

DEVELOPMENT OF AMYLOSE-FREE (*AMF*) MONOPLOID POTATOES AS NEW BASIC MATERIAL FOR MUTATION BREEDING *IN VITRO* USING X-RAY IRRADIATION[1]

T.J.H. HOOGKAMP, R.G.T. VAN DEN ENDE, E. JACOBSEN AND R.G.F. VISSER

ABSTRACT

For the induction and selection of double mutants with altered starch composition, a uniform (homohistont) amylose-free (*amf*) monoploid as basic plant is of vital importance. Therefore, new *amf* monoploids had to be developed via prickle pollination, and 26 monoploids were obtained which were screened *in vitro* for vigour, leaf size and for the percentage of monoploid leaf cells. The number of monoploid cells was underestimated at about 28%. Seven vigorous monoploids were tested *in vitro* for tuberization capacity, which varied from 0 to 100%, and for regeneration *in vitro*, which varied from 0 to 3.7 adventitious shoots per leaf explant. The tuberization capacity of the regenerated shoots varied from 0 to 48%. Two *amf* monoploids were selected which were sufficiently vigorous and tuberizing *in vitro*. A range for X-ray irradiation from 0 to 16 Gy was tested in order to balance the level of mutation induction and survival. The ideal range for survival and mutation induction appeared to be 4 to 8 Gy. This monoploid material is a new basis for the application of *in vitro* mutation breeding that should lead to the induction and selection of new starch mutants in potato.

INTRODUCTION

In maize, the recessive amylose extender (*ae*) mutation in combination with the amylose-free (*waxy*) mutation gives a new and interesting starch type which stains blue with iodine (Shannon and Garwood, 1984). On this basis, it might be possible to identify a similar double mutant in potato using monoploid amylose-free (*amf*) basic material. The existing *amf* mutation is based on a single point deletion and was induced by using X-ray irradiation on monoploid *in vitro* material (Hovenkamp-Hermelink et al., 1987; van der Leij et al., 1991). The idea of using the monoploid *amf* mutant as source for the selection of a revertant with loosely branched

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amylopectin was already suggested by Jacobsen et al. (1990). They selected a variant containing blue staining starch but it contained amylose and amylopectin. Apparently this selection was based on chimerism still present in the primary *amf* monoploid available (86.046), which they used as basic material (van der Leij et al., 1992). Therefore, for the induction of new mutations in functional genes in *amf* potato leading to blue staining starch, i.e. recessive mutations, new non-chimerical basic monoploid (2n=x=12) *amf* material (M_0) was needed in order to make mutations visible.

In the past for the production of monoploids of potato, anther cultures of diploid genotypes (androgenesis) as well as prickle pollination of diploid genotypes (gynogenesis) have been used (Breukelen et al., 1975; Irikura, 1975). Uijtewaal et al. (1987) produced monoploids in a frequency of up to one per ten berries using specific diploid genotypes with seed spot marker genes. They used selected diploid *Solanum phureja* genotypes as prickle pollinators on diploid *S. tuberosum* (Hermsen and Verdenius, 1973).

The ploidy level of monoploids is relatively stable when propagated in vitro, but spontaneous chromosome doubling may occur (Karp et al., 1984; Tempelaar et al., 1985). To monitor the ploidy level accurately, genotypes may be tested by flow-cytometry of leaf cells (Ramulu and Dijkhuis, 1986). By using this method, the percentage of monoploid cells in the leaves is under-estimated (Jacobsen et al., 1983). This is because a percentage of cells in G1 phase are 1C while the monoploid cells in G2-phase will be found in the 2C peak together with normal diploid cells in G1-phase.

Hovenkamp-Hermelink et al. (1987) used monoploid basic material for the isolation of a mutant using adventitious shoots to minimize the chance on chimerism with wildtype cells (Hovenkamp-Hermelink et al., 1988a). This method might be more appropriate for the induction of mutants without chimerism compared with the approach of repeated propagation of axillary buds (Donini and Sonnino, 1998). This latter method will normally result in chimeric (periclinal) mutants so that chimerism might cause problems when studying tuber properties during screening (Ahloowalia, 1998).

The aim of this study was to develop monoploid *amf* plant material, non-chimeric for the *amf*-trait which can be used in an *in vitro* protocol, similar to that of Hovenkamp-Hermelink et al. (1988a), for the induction of new potato starch mutants in an *amf* background. The newly produced monoploids were analyzed for a number of *in vitro* properties, like growth, somatic doubling in the leaves, regeneration capacity and tuberization.

MATERIALS AND METHODS

Plant material

Potential monoploid (2n = x = 12) amylose-free (*amf*) plant material was obtained by prickle pollination of seven diploid (2n = 2x = 24) *Solanum tuberosum* genotypes: 880004-2, 880004-3, 880004-6, 880004-9, 880004-11, 1029-31, 5002-18 and derivative KA91-895 (Jacobsen et al., 1991) with *S. phureja* pollinators IVP35, IVP48 and IVP101 (Hermsen and Verdenius, 1973). Two of these diploid genotypes were homozygous recessive *amfamf* (5002-18 and KA91-895) others were heterozygous *Amfamf*. Due to a homozygosity in the pollinators which causes seed embryo-spot and a nodal band on seedlings, hybrids can be easily identified. For the isolation of monoploids from these crosses, seeds without an embryo-spot (Hermsen and Verdenius, 1973) were selected and sown *in vitro*. After emergence the plantlets with 'nodal bands', controlled by the same genes as the embryo-spot marker, were removed (Uijtewaal et al., 1987). In non-hybrid seedlings without a nodal band, the ploidy level was estimated by counting the number of chloroplasts in the stomatal guard cells after iodine staining (Frandsen, 1968), and later determined by flow cytometry of leaf cells (Jacobsen et al., 1983).

Vegetative propagation was done by cutting. Shoot cultures were grown *in vitro* on a Murashige and Skoog (MS) medium (1962) supplemented with 30 g/l sucrose and 8 g/l agar. Conditions were 24°C, 16 h light and 40 $\mu molm^{-2}s^{-1}$ irradiance. After confirmation of the ploidy level, *in vitro* growth properties of the monoploids were determined based on vigour and leaf size of 32 shoots/monoploid in two series, on a scale from 0 (poor) to 4 (good). Vigour was scored as speed of growth in comparison with a diploid control. Only the genotypes with a score higher than one were subjected to repeated flow cytometric analyses. The genotypes with a score higher than two were included in further *in vitro* experiments because those with a score lower than two were almost impossible to maintain *in vitro*. The diploid genotype 5002-18 was used as a diploid control in all experiments.

In vitro *regeneration of adventitious shoots*

Selected monoploids were tested for their capacity to regenerate adventitious shoots which were produced according to Hovenkamp-Hermelink et al. (1988a). Leaves of sterile monoploid shoots were floated on a solution of 147 mg/l $CaCl_2 \cdot 2H_2O$ and 80 mg/l NH_4NO_3, supplemented with 10 mg/l BAP and 10 mg/l NAA. After one night (16 hrs), the leaves were cut into explants of 3-4 mm width and placed on a callus induction MS medium with 40 g/l mannitol, 10 g/l sucrose, 2.25 mg/l BAP, 0.0175 mg/l IAA and 8 g/l agar. After six days, explants were transferred to MS medium supplemented with 15 g/l sucrose, 2.25 mg/l BAP, 5 mg/l GA_3 and 8 g/l agar for regeneration. The explants were transferred to fresh regeneration medium every three weeks. Adventitious shoots were harvested and grown in containers on MS medium with 10 g/l

sucrose. When a shoot had formed more than five nodes, single nodes were used for *in vitro* tuber induction experiments.

In vitro *tuber induction*

Vegetatively propagated shoots and adventitious shoots were used for tuber induction. Single nodes were transferred to MS medium with 10 or 30 g/l sucrose in a container. Then after two to three weeks of growth, a liquid tuber induction (TI-A) MS medium was added on the solid medium. This liquid medium contained only 10 % of the original concentration of KNO_3 and NH_4NO_3. The final concentration of sucrose was 100 or 120 g/l (respectively TI-A1 and TI-A2) in the total volume supplemented with 500 mg/l ChloroCholine Chloride (CCC = $C_5H_{13}Cl_2N$; Sigma, Germany); TI-A2 was sometimes supplemented with 0.5 mg/l ABA (TI-A3) (Lillo, 1985; Hussey and Stacey, 1984).

Additionally, single nodes (axillary buds) were transferred to petri dishes containing tuber induction (TI-B) MS medium supplemented with 80 mg/l sucrose, 2.5 mg/l kinetine, 0.5 mg/l ABA and 8 g/l agar (Hussey and Stacey, 1984). The petri dishes and containers were sealed to prevent infection. Containers and dishes were placed in the dark at 18°C, where after five weeks the number of microtubers was counted. The starch composition (amylose containing or amylose-free) of the selected monoploid genotypes was determined by staining slices of microtubers with iodine (red = amylose-free, blue = amylose containing).

Irradiation

To find the right irradiation dose rate, single nodes were irradiated with X-rays at TNO-SCD (Arnhem, the Netherlands) using a Roentgen apparatus at 200 kV, 2 mA, no filter and at a distance of 80 cm; resulting in a dose rate (in air) measured in the centre of the field (40 cm diameter) of 1.5 Gy/min and a dose rate (in air) of 1.1 Gy/min measured 20 cm from the centre. Seven petridishes were placed in a circle at approximately 13 cm from the centre of the field. A series of 6 different doses, respectively 2, 4, 6, 8, 10 and 16 Gy was used to find a balance between mutation induction and survival. For each dose the growth and vigour of the shoots (M_1V_1) were scored.

RESULTS

Monoploid production

Prickle pollination of the selected diploid genotypes with *S. phureja* resulted in 480 berries from 24 parent-pollinator combinations. From the total batch of approximately 100,000 seeds, 995 were spotless. They were sown *in vitro* and 604 of them germinated. More than 75% of the seedlings were excluded from further studies because the presence of the nodal band indicated that they were hybrid and not potential monoploid. From the seedlings without nodal band

potential monoploids were selected by counting chloroplasts in the stomatal guard cells. However, this method was not reliable for identifying monoploids because, due to somatic doubling in the leaves, only a few could be identified and others remained arguable. Therefore, potential monoploids were evaluated by flow-cytometric analysis of leaf cells. Finally, among eight cross combinations (Table 1) 26 monoploids were obtained. The genotypes 880004-9 and 880004-11 were the best monoploid-producing parents. Half of them were derived from the combination 880004-9 * IVP 101 (Table 1). However only 10 of the 26 monoploids survived two years of propagation.

Table 1. Results of prickle pollination on diploid females with pollinators homozygous for seed spot and nodal band markers.

Female	GBSS[a] locus	Pollinator	Cross no.	# berries	# spotless seeds	# germinated seeds	Plants without nodal band		
							# died[b]	# 2x[c]	# x
880004-2	Amfamf	IVP 35	1014	15	38	24	1	1	3
880004-3	Amfamf	IVP 35	1017	23	18	6	0	0	1
880004-6	Amfamf	IVP 35	1020	5	21	5	1	0	1
880004-9	Amfamf	IVP 35	1023	2	12	8	1	0	1
880004-9	Amfamf	IVP 101	1022	34	177	112	21	10	13
880004-11	Amfamf	IVP 35	1026	8	37	5	0	0	1
880004-11	Amfamf	IVP 101	1025	3	52	35	2	21	5
KA91-895	amfamf	IVP 101	1041	12	19	14	0	1	1

[a] GBSS = gene for Granule Bound Starch Syntheses

[b] Plants which died before they could be analyzed for their ploidy level

[c] Ploidy level determined by flow cytometry, 2x= diploid (2n=2x=24), x=monoploid (2n=x=12), see Table 3.

Flow-cytometry was also used to determine the degree of somatic doubling in selected monoploids. The percentage of monoploid cells in leaves is an accurate but biased underestimate. The 1C peak is made up of cells in G1-phase and they are monoploid. Monoploid G2-phase cells with a 2C peak cannot be distinguished from diploid cells in G1-phase. The number of diploid cells, caused by somatic doubling, in the monoploids was estimated by the 4C/2C ratio in the diploids which were also progeny of the prickle pollinations (Table 2). From the results in Table 2 a formula was extracted: $\% x = 1C_x + \{2C_x - [4C_x /(4C_{2x}/2C_{2x})]\} = 1C_x * 1.287\%$.

Consequently, monoploid cells were multiplied by a factor of 1.287 leading to a less accurate but unbiased estimator for the monoploid level. From the flow-cytometric results the percentage of monoploid cells was calculated (Table 3). Originally, the 1C value varied between 32.7 and 64.6%, but after correction, the range became 42.1-83.1%. Some monoploids differed significantly, based on Duncan's multiple range test (Table 3). There was

no difference between the results of the multiple range tests performed for the original 1C values and the tests performed for the corrected ones.

Table 2. Estimation of the ploidy level of monoploids for cells in G2 phase in the 2C fraction.

Type	# cells analyzed	% of counts in each peak[a]			
		1C	2C	4C	Total
Diploids (2x)[b]	47566	0	71,08	28,92	100
Monoploids (x)	367005	46,11	42,14	11,76	100
Estimated % x in monoploids	-	46,11	13,25	-	59.36[c]

[a] Ploidy level determined by flow cytometry

[b] Average of all diploid offspring of the prickle pollinations

[c] **% x** = 46.11 + (42.14 - (11.76 / (28.92 / 71.08)= 46.11 * 1.287 = **59.36%**

Table 3. Vigour, leaf size and ploidy level of leaf cells of monoploids and a diploid control.

Genotype	Vigour[a]	Leaf size[a]	n[b]	% of counts in each peak[c]			%x[d]
				1C[d]	2C	4C	
5002-18 (2x)	4	4	2	0	86,1	13,9	0
1017M-15	2	1	1	34,1	49,4	16,5	43,9
1022M-47	3	3	10	51.5^{wx}	40,2	8,3	66.3^{wx}
1022M-54	4	4	12	34.9^{z}	44,9	20,1	45.0^{z}
1022M-66	3	3	11	45.1^{xy}	45,5	9,4	58.1^{xy}
1022M-76	3	2	9	53.0^{uvx}	39,2	7,8	68.2^{uvx}
1022M-118	4	4	13	52.6^{vw}	39,6	7,8	67.7^{vw}
1022M-132[e]	2	2	1	32,7	49	18,3	42,1
1022M-133	3	2	1	39,5	45,1	15,4	50,8
1022M-159	2	3	1	28	58	14	36
1023M-2	3	2	8	54.4^{uw}	37,9	7,7	70.0^{uw}
1025M-12[e]	2	2	2	47,3	38	14,8	60,8
1025M-17	2	1	4	64.6^{t}	33,2	2,2	83.1^{t}
1026M-6[e]	2	2	3	39.3^{yz}	44	16,8	50.5^{yz}

[a] Scored in a range from 0 – 4; 0 = poor, 4 = excellent, only genotypes with vigour > 1.

[b] n = number of times flow cytometry was performed.

[c] Ploidy level determined by flow cytometry.

[d] Percentages followed by the same letter do not differ significantly (p= 0·05, Duncan's multiple range test), only performed for n>2.

[e] Genotypes died during *in vitro* propagation.

In vitro *properties*

The monoploids were highly variable for vigour and leaf size. Two genotypes, 1022M-54 and 1022M-118, had the same vigour and leaf size as the diploid control (Table 3). During *in vitro* propagation, several monoploids were lost due to poor general growing properties. After two years ten monoploids were still alive *in vitro*, but they still differed in vigour and level of somatic doubling in the leaves. The percentage monoploid cells in the 1C peak after flow-cytometry based on all flow-cytometric measurements served as a parameter for the level of somatic doubling. Spearman's rank correlation was calculated between vigour and leaf size and the percentage corrected monoploid cells. The correlation coefficient of vigour and leaf size was 0.93 (α=0.01), of vigour and ploidy level -0.24 (α=0.05) and of leaf size and ploidy level -0.35 (α=0.01). This indicates a positive relationship between level of somatic doubling in the leaves and vigour and leaf size, and in particular a strong relationship between vigour and leaf size.

Seven monoploids, selected *in vitro* based on vigour (score 3 and higher), were tested for their micro-tuberization capacity. Tuber formation was measured as the number of tubers arising from shoots giving rise to at least one tuber, on vegetatively propagated plants (shoots) and single buds (nodes). On the solid TI-B medium only 1022M-118 formed tubers on 7 of 20 nodes, but when using the liquid TI-A media almost all monoploids formed tubers. Higher numbers of tubers were formed in the liquid media than on the solid medium. Among the three liquid media tested, TI-A1 was most effective. In six comparisons, shoots of monoploid material formed more tubers than the diploid control. For the liquid media, genotype 1022M-118 had a higher tuberization capacity than the diploid control (Table 4). There was no difference in size of microtubers between the monoploids and the diploid control.

The same monoploids were tested in the regeneration part of the mutation induction protocol to determine whether they were capable of regenerating adventitious shoots from leaf explants. After regeneration, four of seven monoploids gave adventitious shoots. The formation of callus was no guarantee for the formation of adventitious shoots. Some monoploids, like the vigorous 1022M-118, failed to regenerate shoots even when they formed callus (Table 5), showing that within the cross 1022 (Table1) there was clearly segregation for regeneration. Regeneration varied from 0 to 3.7 adventitious shoots per leaf explant. Two monoploids, 1022M-76 and 1022M-133 gave even more adventitious shoots than the diploid control. Flow-cytometry was used to determine ploidy level of regenerated shoots. Ten randomly selected early regenerated adventitious shoots were tested of which nine were doubled. From the diploid control a much lower fraction of adventitious shoots was doubled (two of 13).

Table 4. Tuberization capacity and starch composition assessment of seven selected monoploids and a diploid control.

Genotypes	Tuberization capacity								GBSS locus[b]
	TI-B (solid)a		TI-A1 (liquid)		TI-A2 (liquid)		TI-A3 (liquid)		
	# nodes[c]	# tuber forming nodes	# shoots	# tuber forming shoots	# shoots	# tuber forming shoots	# shoots	# tuber forming shoots	
1022M-47	15	0	32	5	32	3	40	0	Amf
1022M-54	16	0	136	40	93	10	158	11	*amf*
1022M-66	16	0	52	0	56	1	86	6	*amf*
1022M-76	16	0	38	24	24	8	44	3	Amf
1022M-118	20	7	56	45	64	47	40	36	*amf*
1022M-133	n.d.	n.d.	16	16	8	8	24	22	Amf
1023M-2	16	0	14	0	n.d.	n.d.	12	0	n.d.
5002-18 (2x)	32	22	64	37	40	35	72	50	*amf amf*

[a] See Materials and Methods for composition of the media.

[b] GBSS = gene for Granule Bound Starch Syntheses, amylose-free (*amf*) or amylose-containing (Amf).

[c] nodes = single buds, shoots = vegetatively propagated plants.

n.d. = not determined.

Table 5. Callus formation and regeneration capacity of seven monoploid genotypes and one diploid genotype.

Genotypes	# explants	% callus forming explants	Average # shoots/explant
1022M-47	109	100	0
1022M-54	738	100	0.26
1022M-66	304	94	0,01
1022M-76	476	100	3,73
1022M-118	323	100	0
1022M-133	117	95	2.80
1023M-2	104	52	0
5002-18 (2x)	316	100	1,3

Tuberization capacity of the available adventitious shoots was also tested just after they were taken from the regeneration medium on the three different liquid media. The diploid genotype formed more tubers than the monoploids. The tuberization capacity varied from 0 to 48%, indicating that tuberization is possible even directly after regeneration when there are still hormones present in the shoot. On the adventitious shoots, the liquid TI-A1 medium gave most tubers (Table 6).

Besides *in vitro* properties, the starch composition had to be determined because the background of the tubers originated from was sometimes heterozygous *Amfamf*. Induced

microtubers from the monoploids were stained with iodine and screened for starch type (Table 4). Of the seven monoploids, three were *amf*, the most vigorous ones were 1022M-54 and 1022M-118; the former showed regeneration and a sufficient level of tuberization and the latter did not regenerate adventitious shoots from leaf explants but had a high level of tuberization on vegetatively propagated shoots.

Table 6. Number of tuberizing adventitious shoots obtained directly after regeneration from leaf explants from three monoploids and a diploid control.

Genotypes	Tuberization capacity					
	TI-A1		TI-A2		TI-A3	
	# shoots	# tuber forming shoots	# shoots	# tuber forming shoots	# shoots	# tuber forming shoots
1022M-54 (*amf*)	56	14	43	9	66	13
1022M-76 (wt)	72	9	72	17	95	6
1022M-133 (wt)	23	11	25	11	16	0
5002-18 (2x, *amf*)	48	18	48	38	56	36

Irradiation

Irradiation experiments with single nodes of 5002-18 and 1022M-54 showed, first an increase in growth at low doses of X-rays and a negative growth effect at higher doses (Table 7). The monoploid was affected by irradiation at a lower dose rate (>4 Gy) than the diploid genotype (>10 Gy). These observations indicate that for mutation induction irradiation experiments at monoploid level can be practised in the range of 4-8 Gy.

Table 7. Number of internodes of M_1V_1 plants growing *in vitro* from irradiated single nodes with different doses of X-ray irradiation after two weeks.

	5002-18 (2x)		1022M-54 (x)	
Dose (Gy)	Average # internodes[a]	n	Average # internodes[a]	n
0	4.8^{x}	48	4.6^{w}	47
2	4.9^{x}	24	4.4^{w}	24
4	5.3^{w}	24	5.0^{w}	23
6	4.9^{x}	24	2.7^{x}	24
8	4.6^{xy}	24	3.0^{x}	24
10	4.3^{y}	24	0.9^{y}	24
16	3.2^{z}	24	0^{z}	24

[a] percentages followed by the same letter do not differ significantly (p= 0.05, Duncan's multiple range test)

DISCUSSION

Monoploid production

From literature, it is clear that the induction and selection of mutants is highly valuable, especially if biochemical pathway variants are needed. In a tetraploid vegetatively propagated crop like potato, the induction and selection of recessive mutants is difficult. Induction and isolation of the first amylose free (*amf*) mutant in potato was done by inducing mutations in a monoploid potato and selection with iodine for red staining starch (Hovenkamp-Hermelink et al., 1987).

To find new starch mutants this *amf* potato could serve as starting material for mutation induction experiments. Jacobsen et al. (1990) used the originally found *amf* mutant as starting material in their experiments designed to find mutants producing starches with altered branching. However, they apparently failed due to chimerism within their *amf* material (van der Leij et al., 1992). Therefore, new uniform (homohistont) monoploid *amf* material was produced via a sexual cycle using prickle pollination, as previously described by Uijtewaal et al. (1987).

In addition, a clear genotypic effect was found for the frequency of monoploids produced in specific crosses, with effects from both the maternal as well as the paternal side. The best pollinator in our experiments was IVP 101 (Table 1), which was also previously determined to be a good pollinator for dihaploid induction using tetraploids (Hutten et al., 1994). Another important observation was that new monoploids could be selected with high vigour and leaf size, which makes these monoploids good candidates for an *in vitro* mutation breeding protocol.

To ensure that we were dealing with monoploids a first screen was made based on determination of chloroplast number in stomatal guard cells. Some monoploids were identified, others were still arguable so it was clear that a more efficient way of determining ploidy levels was needed. Flow-cytometry of the leaf cells showed that the ploidy level of all monoploids was stable through all experiments. In the past, researchers found that monoploids spontaneously change in ploidy level during *in vitro* multiplication resulting in ploidy chimerism (Tempelaar et al., 1985; Karp et al., 1984). However, these authors based their assumptions on only one genotype, which showed some instability. Karp et al. (1984) stated that this might be explained by an unstable number of chromosomes within the basic material. Tempelaar et al. (1985) concluded that this instability causes doubling when monoploids are propagated *in vitro*. During *in vitro* propagation of more than 10 monoploids for more than two years, we could find no evidence for doubling of the monoploids. However, when leaves of monoploids were subjected to regeneration high frequencies of doubled plants were found. Regeneration of a

doubled somatic cell or chromosome doubling in cells during callus phase might be the cause. By using flow-cytometry, the level of somatic doubling in the leaves could be estimated more accurately by correction for the underestimated monoploid cells. A high frequency of monoploid cells is essential for the induction and regeneration of recessive mutations.

In vitro properties

Based on vigour and leaf size of the monoploids and the diploid control, it can be concluded that the ploidy level itself does not always determine these traits as stated by Hovenkamp-Hermelink et al. (1988a). Nevertheless, when we look at the monoploid offspring of a diploid, the level of somatic doubling in the leaves to some extent determines the leaf size and the vigour *in vitro* (Table 3). A higher 2C peak after flow-cytometry, however, might partly be caused by a higher mitotic activity in plants with good vigour.

The regeneration protocol of Hovenkamp-Hermelink et al. (1988a) was also suitable for some of the monoploids selected in these experiments. Six monoploids, progeny from cross 1022 (Table1), showed large variation for regeneration (Table 5). It can be concluded that between monoploids there is a large variation for regeneration capacity based on genetic differences.

For the induction of tubers, various *in vitro* systems are available. In the past, Hovenkamp-Hermelink et al. (1988a) developed a method in which single nodes were used for the induction of micro tubers on adventitious shoots of monoploids. Initially single nodes were used for the induction of tubers on solid medium (T1-B). However, this labour-intensive method gave poor results and only one monoploid tuberized. The system described by Lillo (1985), where whole plants of tetraploid material, were used for tuber induction in combination with liquid media (Table 4) gave excellent results, and up to 100% of vegetatively propagated shoots of some genotypes of the monoploids form tubers. A probable reason for the poor tuberization when using single nodes is the lack of a root system for efficient uptake of nutrients. Also the progressive exposure of roots to higher sucrose concentrations, by adding liquid sucrose media to the solid media up to 10-12% sucrose might be an advantage for tuberization rate on the liquid media (Lillo, 1985). When using adventitious shoots for tuberization there seems to be no problem concerning tuber formation because of a possible carry-over-effect of hormones (notably GA_3). Doubling of the monoploid cells during callus phase leading to homozygous diploid plant material might lead to a slightly lower level of tuberization for the adventitious shoots caused by inbreeding.

Results described here indicate that after the production of several monoploids a selection can be made for *in vitro* traits. For monoploids originating from one diploid, it can be concluded that some monoploids inherited more favorable genes than other monoploids with respect to *in vitro* properties. Some of the selected monoploids bearing an *amf* allele, such as 1022M-54

and 1022M-118, are suitable to be used in *in vitro* mutation protocols for the induction and isolation of new and interesting starch composition in tubers. In these selected monoploids, additional attention has to be paid to improve mutation and tuberization protocols. In Figure 2 a scheme is described that indicates the pathway in order to come to new starch mutants using *in vitro* techniques combined with irradiation.

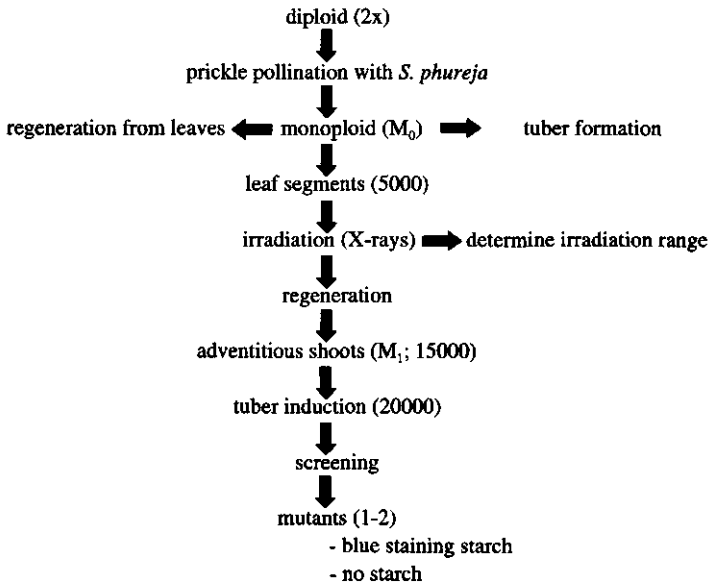

Figure 2. A schematic overview of the route which should lead to new mutants in potato.

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AN INDUCTION AND SELECTION PROCEDURE FOR ALTERED STARCH TYPES IN A MONOPLOID *AMF* POTATO (*SOLANUM TUBEROSUM* L.)

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ABSTRACT

After X-ray irradiation of amylose-free (*amf*) monoploid plant material, adventitious shoots and several generations of shoot multiplication from axillary buds were produced. 750 M_1 adventitious shoots and 14,000 M_1V_3 shoots were produced and subjected to tuberization. Screening of the starch of micro tubers was done at tuber as well as granule level. Iodine staining of cut surfaces of microtubers of all shoots made it possible to screen for aberrant starch phenotypes at macroscopic level. At cut surface level no aberrant starch phenotypes were observed. Additionally, based on the gelatinization profile determined by differential scanning calorimetry (DSC) at tuber level, three aberrant types were found. After propagation for a few generations none of these types found by DSC were recovered. Crude starch samples of tubers from 439 axillary buds in one experiment were all screened microscopically at granule level and classified in six different classes based on iodine staining pattern of which two classes had almost no blue staining. 56 tuber samples were found containing 1–4 aberrant staining starch granules. For the aberrant starch phenotypes only the first two most intensely blue staining classes of aberrant starch granules were reevaluated in later generations. In 19 out of 29 cases the granule phenotype could still be recovered in later generations. In one case 13 aberrant staining starch granules were found indicating an increase of the aberrant phenotype. These genotypes need to be propagated or regenerated to increase the mutant sector by decrease of chimerism to stabilize the aberrant staining type. In this study the concept of mutation breeding in monoploid *amf* potatoes to find altered blue staining granules is proven, however, more research is required to select solid mutants.

INTRODUCTION

In plants, starch consists of amylose and amylopectin. The amylose content in many species, including potato, is approximately 20-25%. In maize the amylose-free (*waxy*) mutation gives starch

which stains red with iodine (Shannon and Garwood, 1984). Later the recessive amylose extender (*ae*) starch mutant could be identified in the *waxy* background by blue staining of the starch because of loosely branching of the amylopectin. The amylose extender starch contains 45–60% loosely branched amylopectin molecules (Shannon and Garwood, 1984). To induce a recessively inherited mutant like the amylose extender in maize in an amylose-free (*amf*) potato background, monoploid (2n=x=12) basic material was developed as described in Chapter 2. This was based on the approach used by Jacobsen et al. (1990). They used the primary *amf* monoploid to find a revertant with loosely branched amylopectin. However, they failed because of the fact that the primary *amf* monoploid 86.046 was still chimeric for this trait (van der Leij et al, 1992).

Hovenkamp-Hermelink et al. (1987) used monoploid basic material for the isolation of the *amf* mutant using adventitious shoots to minimize the occurrence of chimerism with wildtype cells. This regeneration method might be more useful for the induction and selection of mutants without chimerism compared with the method of repeated propagation of axillary buds (Donini and Sonnino, 1998). After irradiation of the axillary buds micropropagation for four generations should be sufficient to produce a near solid mutant. It is expected that it will normally result in chimeric (periclinal) mutants and when studying tuber properties during screening, chimerism might cause problems (Ahloowalia, 1998). The major differences between using adventitious shoots and axillary buds are the regeneration step that is used for the adventitious shoots (Hovenkamp-Hermelink et al., 1988) and the initial number of plants to start with to reach the same number of irradiated plants in M_1.

To increase the mutation frequency physical and chemical mutagens are used (Novak, 1991). For the induction of mutations in potato in most cases ionizing irradiation like γ-ray, X-ray or fast neutrons are used (Kukimura, 1986). In the amylose-free (*amf*) potato a single point deletion was induced in monoploid material by using X-ray irradiation (Hovenkamp-Hermelink et al., 1987; van der Leij et al., 1991). In seed propagated crops mutations can be found after self-fertilization in the M_2 and M_3 generation which results in a non-chimeric genetically stable mutant (homohistont) (Donini and Sonnino, 1998). Since repeated self-fertilization in potato is difficult or even impossible, the use of monoploids is a prerequisite to come in one step to recessive mutants. The plant material developed in previous research (Chapter 2) is suitable to be used in the *in vitro* regeneration protocol used by Hovenkamp-Hermelink et al. (1987) and suitable to be used in an *in vitro* tuberization protocol (Chapter 2).

To be able to identify the putative mutants, Hovenkamp-Hermelink et al. (1987) first used spectrophotometric analysis at tuber level to determine the amylose percentage of starch of microtubers (Hovenkamp-Hermelink et al., 1988). Later on they were forced to use iodine staining of tuber cut surfaces as screening method due to the chimeric nature of the mutation. Another way to screen for aberrant types of starch at tuber level is the use of Differential Scanning Calorimetry

(DSC). Sanders et al. (1990) were able to discriminate starches from several mutant maize genotypes. Using DSC makes it possible to screen for mutations, which have an impact on the gelatinization profile. Together with the starch staining methods and screening at tuber level or at starch granule level this approach will result in a wide range of mutations which might be detected.

The aim of this study was to use previously produced monoploid plant material (Chapter 2) after using 4–10 Gy of X-ray irradiation as tested in previous experiments (Chapter 2) to produce mutants altered in starch biosynthesis. The protocol to produce adventitious shoots (Hovenkamp-Hermelink et al., 1988) which minimizes the chimerism was compared with the method of repeated propagation of axillary buds (Donini and Sonnino, 1998). Putative starch variants were expected to be identified by simple iodine staining or DSC profile of the starch. The chimeric variants found in this study might provide plant material which can be used to stabilize mutant sectors and obtain solid mutants.

MATERIALS AND METHODS

Plant material

Two amylose-free (*amf*) monoploid *Solanum tuberosum* genotypes 1022M-54 and 1022M-118, developed as described in Chapter 2, were used for mutation induction. Based on this research genotype 1022M-118 was only used for the approach of multiplication of axillary buds whereas 1022M-54 was also used for the production of adventitious shoots according to Hovenkamp-Hermelink et al. (1988) In both approaches a diploid control 5002-18 (Jacobsen et al., 1991) was included. Vegetative propagation of the genotypes was made by cutting, shoot cultures were grown *in vitro* on Murashige and Skoog (MS) medium (1962) supplemented with 30 g/l sucrose and 8 g/l agar. The temperature was 24°C, 16 h light, irradiance 40 $\mu molm^{-2}s^{-1}$.

Production of adventitious shoots

Of the monoploid genotype 1022M-54 and the diploid genotype 5002-18 adventitious shoots were produced in two series according to Hovenkamp-Hermelink et al. (1988). The leaves of sterile monoploid shoots were floated on a solution of 147 mg/l $CaCl_2 \cdot 2H_2O$ and 80 mg/l NH_4NO_3, supplemented with 10 mg/l BAP and 10 mg/l NAA. After one night floating (16 hrs), the leaves were cut into explants of 3-4 mm width and placed on a callus induction MS medium with 40 g/l mannitol, 10 g/l sucrose, 2.25 mg/l BAP, 0.0175 mg/l IAA and 8 g/l agar. At this point, the leaf segments were irradiated. After six days explants were transferred to MS medium supplemented with 15 g/l sucrose, 2.25 mg/l BAP, 5 mg/l GA_3 and 8 g/l agar for regeneration. The explants were transferred to fresh regeneration medium every three weeks. Adventitious shoots were harvested and grown in containers on MS medium with 10 or 30 g/l sucrose. When a shoot had formed more then five nodes, they were subjected to *in vitro* tuberization (Chapter 2).

Multiplication of axillary buds

Three-week-old shoots (M_0) of 1022M-54 and 1022M-118 were irradiated. After irradiation, single nodes (M_1) were transferred to fresh media. When a shoot (M_1V_1) arising from a single node had formed at least eight internodes, single nodes were harvested and placed on fresh MS medium. In addition, of each of the eight shoots the top was also transferred to fresh MS medium for tuber induction (Figure 1). Shoots (M_1V_2) arising from these nodes were again grown until they had formed at least four internodes, from each shoot four nodes were placed on fresh MS medium. In addition, again of each of the four shoots the top was transferred to fresh MS medium for tuber induction. In the first experiment this cycle was continued until M_1V_4 of which all plants were used for tuber induction (Figure 1). In the second experiment the cycle was continued until M_1V_3.

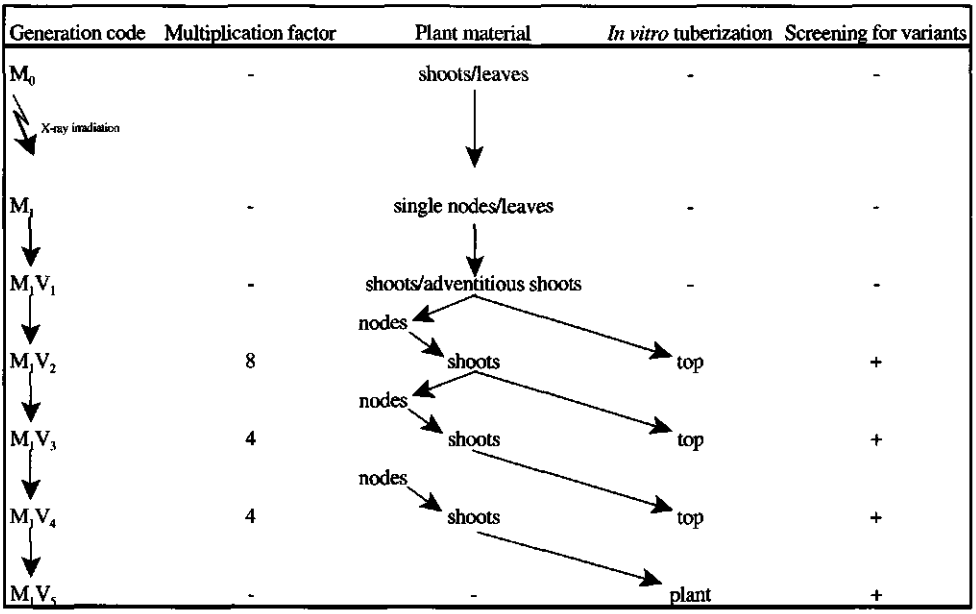

Figure 1. *In vitro* multiplication of shoots of monoploids when using X-ray irradiation (modified from: Donini and Sonnino, 1998)

Irradiation

Leaf segments on callus induction media of the adventitious shoot method and whole plants on MS30 of the axillary bud method were irradiated with X-rays at TNO-SCD (Arnhem, the Netherlands) using a roentgen apparatus at 200 kV, 2 mA, no filter at a distance of 80 cm. This resulted in a dose rate (in air) measured in the center of the field (40 cm diameter) of 1.5 Gy/min and a dose rate (in air) of 1.1 Gy/min measured 20 cm from the center. Seven Petri dishes or glass containers were placed in a circle at approximately 13 cm from the center of the field. Leaf segments were irradiated at 4, 6 and 8 Gy and whole plants at 6, 8 and 10 Gy based on a previous study (Chapter 2).

In vitro *tuberization*

Adventitious shoots that were harvested from the regeneration media were transferred to fresh media and subjected to tuberization as described in Chapter 2. In addition, the top of shoots from the axillary buds of different vegetative generations were also subjected to this tuberization protocol. Single nodes were transferred to MS medium with 10 or 30 g/l sucrose in a container. Then after two to three weeks, a liquid tuber induction MS medium was added to the solid medium. This liquid medium contained only 10% of the original concentration of KNO_3 and NH_4NO_3. The final concentration of sucrose was 120 g/l in the total volume supplemented with 500 mg/l ChloroCholine Chloride (CCC = $C_5H_{13}Cl_2N$; Sigma, Germany; Chapter 2). The containers were sealed to prevent infection and were placed in the dark at 18°C, where after five weeks microtubers were formed.

Screening of starch of in vitro *tubers*

The first screening of the microtubers was done at tuber level by standard iodine staining of tuber cuttings. Alternatively tubers were first treated with a cell wall degrading enzyme Rapidase LIQ$^+$ (Gist-brocades, Seclin, France) 1% in 0.2 M NaAc pH 4.0 overnight at 37°C and three times washed with mQ water. Then the starch from both methods was used for microscopy to screen for aberrant staining starch at granule level. Secondly a gelatinization profile at tuber level was made of the subsequent tubers or starch samples using Differential Scanning Calorimetry (DSC) to screen for possible mutations which are not visible after iodine staining. Thermal analysis was performed using a differential scanning calorimeter (Perkin-Elmer Pyris 1) equipped with a Neslab RTE-140 glyco-cooler. One half of a tuber with a diameter varying form 3 to 7 mm or a sample of 50 ml of the enzyme treated solution were placed in stainless steel sample pans (Perkin-Elmer B 050-5340). In case of the tubers Millipore water (20 ml) was added and the pan was sealed using the Perkin-Elmer Universal Crimper Press. The reference pan was empty. The heating rate was 10°C/min, from 40 to 100°C. Temperature and enthalpy were calibrated using indium (mp=156.6°C) and zinc (mp=419.5°C). For each endotherm the melting enthalpy ΔH (J/g) and the onset T_o (°C) were computed automatically using Pyris software. Due to the unknown weight of the tuber samples it was not possible to calculate enthalpies. After several measurements of wildtype and *amf* tuber material the T_{onset} showed to possess the smallest variation (not shown). So, the T_{onset} was taken as parameter for gelatinization profile and extremes and outliers were identified by using a boxplot test with T_{onset} as parameter. These were selected and subjected to further propagation and analysis. The combination of these screening methods was used to screen tubers coming from either the adventitious shoot method or the axillary bud method.

RESULTS

In all experiments production of adventitious shoots as well as multiplication of axillary buds was used to produce large irradiated populations.

Approach using adventitious shoots

Production of adventitious shoots

For the production of adventitious shoots initially leaves from 500 *in vitro* plants were used. From the monoploid genotype 1022M-54 and a diploid control 5002-18 respectively 750 and 1670 adventitious shoots were regenerated after irradiation and regeneration of leaf segments. The diploid 5002-18 started regeneration later, but was more efficient than the monoploid. For almost all levels of irradiation the regeneration capacity of 5002-18 was significantly higher (Duncan multiple range test, p=0.05) than the monoploid 1022M-54 in the two series (Table 1). Some of the shoots produced were not viable and were lost. The regeneration of adventitious shoots of the monoploid decreased with 87% when irradiated with 8 Gy compared with the non-irradiated explants. Even bigger was the decrease for the diploid genotype 5002-18 of 95%, but the regeneration of the diploid at lower irradiation levels was less reduced by irradiation.

Table 1. The average number of adventitious shoots after six weeks of regeneration of the monoploid (2n=x=12) 1022M-54 and diploid (2n=2x=24) 5002-18 treated with four different irradiation doses.

Genotype	Irradiation (Gy)	# leaf segments	Average # shoots/explant[a]
1022M-54	0	129	1.29[x]
1022M-54	4	1060	0.43[y]
1022M-54	6	430	0.15[z]
1022M-54	8	960	0.17[z]
5002-18	0	40	3.00[u]
5002-18	4	314	2.57[v]
5002-18	6	440	1.67[w]
5002-18	8	53	0.15[z]

[a] Values followed by a different letter differ significantly (p=0.05, Duncan's multiple range test, weight cases).

Tuberization

The adventitious shoots produced were subjected to tuberization. The variation in tuberization of the adventitious shoots was low, all the shoots from 1022M-54 from all irradiation levels had a tuberization rate of approximately 20%. Due to this low level of tuberization after one multiplication step several adventitious shoots got a retry for tuberization.

Screening

The origin of a few cells of the adventitious shoots made it possible to screen on tuber level for variants by using Differential Scanning Calorimetry (DSC) or iodine staining of tuber cut surfaces. Of all 557 tuber producing adventitious shoots one tuber each was used to determine the gelatinization

profile using DSC and the cut surface staining with iodine. The average T_{onset} was 75.7°C. Based on boxplot eight extremes and 17 outliers were selected for further testing. 21 samples had a T_{onset} <73.3°C and four samples a T_{onset} >78.2°C. Additionally three samples were selected on the basis of an aberrant gelatinization profile having an additional peak (Figure 2). No aberrant iodine staining tubers were identified. In the next vegetative generation the aberrant DSC profile types could not be recovered when screening starch of tubers with DSC.

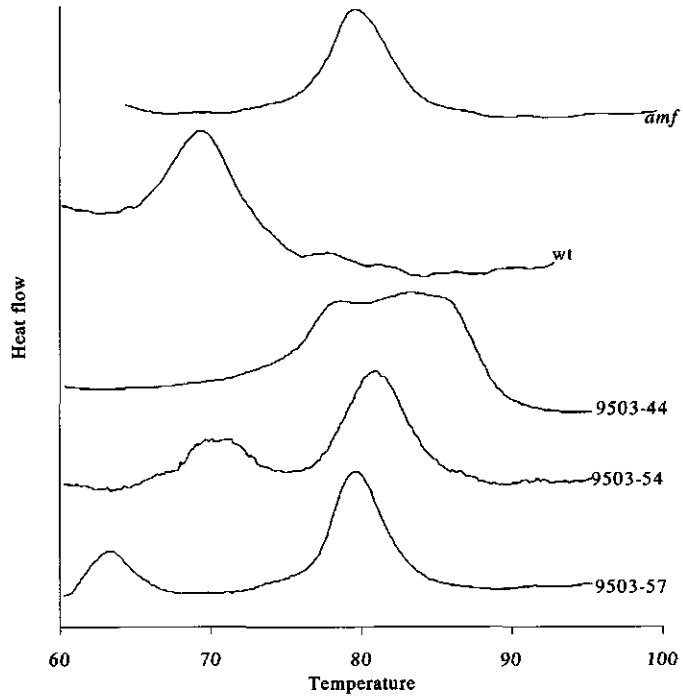

Figure 2. Differential scanning calorymetry (DSC) patterns of amylose free (*amf*), wildtype (wt) and three aberrant variant tubers from adventitious shoots (9503-44, 54 and 57).

Approach using axillary buds

Multiplication of axillary buds

For the axillary bud method initially 250 *in vitro* plants were irradiated at several doses in two experiments. Most of the axillary buds originated from the monoploid 1022M-118 which proved to be most suitable for this method based on growth and tuberization properties (Chapter 2). The first experiment using irradiated axillary buds was a randomized experiment and as a result of this the origin of vegetatively propagated shoots from the second generation on could not be noted. Therefore the second experiment was not randomized and all vegetative generations could be

traced back to one axillary bud. The monoploids 1022M-54 and 1022M-118 used, differed for survival when raising the irradiation dose. Irradiation had a more severe effect on the survival of 1022M-118 then on the survival of 1022M-54 (Table 2). But at higher irradiation levels also 1022M-54 had a lower survival. The diploid, however, did not show any loss of growth at higher irradiation levels. There were large differences in the results within one genotype as shown in the two experiments with 1022M-118 (Table 3).

Table 2. The number of irradiated nodes which gave rise to a shoot after three weeks in M_1V_1 for the monoploids (2n=x=12) 1022M-54 and 1022M-118, and diploid (2n=2x=24) 5002-18.

Irradiation (Gy)	1022M-54		1022M-118		5002-18 (diploid)	
	# nodes	# shoots M_1V_1	# nodes	# shoots M_1V_1	# nodes	# shoots M_1V_1
0	48	48	52	47	48	48
6	48	41	304	62	24	24
8	48	41	224	33	24	24
10	144	101	808	87	24	24

Table 3. The number of shoots after six weeks., in consecutive vegetative propagated generations of the irradiated shoots of monoploid 1022M-118 in two experiments.

Experiment	Irradiation (Gy)	M_1V_1 [a]		M_1V_2 [a]		M_1V_3 [a]	
		# nodes	# shoots (%)	# nodes	# shoots (%)	# nodes	# shoots (%)
I	0	52	47 (90.4)	-	-	-	-
	6	304	62 (20.4)	496	405 (81.7)	1336	685 (51.3)
	8	224	40 (17.9)	320	264 (82.5)	976	462 (47.3)
	10	808	124 (15.3)	992	706 (60.5)	2392	1599 (66.8)
II	8	472	320 (67.8)	2219	1588 (71.6)	10079	8660 (85.9)
	10	408	133 (32.6)	1201	803 (66.9)	4730	4140 (87.5)

[a] vegetative multiplication of the consecutive generations was done according to Figure 1.

Comparison of survival within series from M_1V_1 harvested at three consecutive time points shows that a low survival in the first generation is followed by a higher survival in the next generation and the other way around (Table 3; for example 8 Gy experiment I and II; Figure 3A.). An intermediate survival stays intermediate through generations. A detailed view of the shoots harvested from one single M_1V_1 is depicted in Figure 3B. Shoots harvested from the M_1V_1 at three consecutive time points are propagated separately in the M_1V_2 and M_1V_3 generations. It shows that the moment of growth of a shoot, harvested at three consecutive time points, of the M_1V_1 has an effect on the

vegetative propagation (survival) of these shoots in the M_1V_2 and M_1V_3 generations. In the M_1V_2 and M_1V_3 phenotypic changes were scored such as number of chlorophyll and growth mutants (Table 4). In general when the survival was higher the number of phenotypic mutations decreased. The correlation coefficient for percentage survival and percentage phenotypic changes was –0.697 when using Spearman rank correlation ($\alpha = 0.05$). From both experiments all shoots harvested from the M_1V_1 were multiplied for several generations.

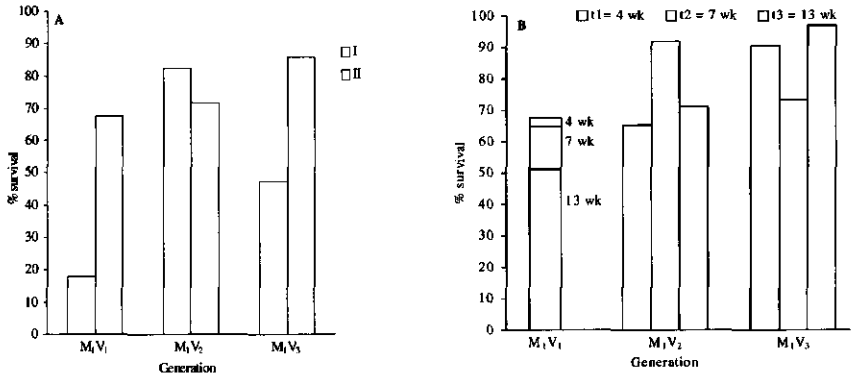

Figure 3. Survival rate during three vegetative generations **A.** of experiments I and II with treatment of 8 Gy and **B.** harvested at three consecutive time points (t1 = 4 weeks, t2 = 7 weeks, t3 = 13 weeks) from M_1V_1, propagated separately in the M_1V_2 and M_1V_3 generation from experiment II with treatment of 8 Gy.

Table 4. The number of phenotypic[a] variants in consecutive vegetative propagated generations of the monoploid 1022M-118 treated with different doses of irradiation.

Experiment	Irradiation (Gy)	M_1V_2		M_1V_3	
		# shoots	# variants (%)	# shoots	# variants (%)
I	0[b]	80	0 (0)	-	-
	6	459	2 (0.4)	694	57 (8.2)
	8	289	14 (4.8)	476	31 (6.5)
	10	758	21 (2.8)	1627	83 (5.1)
II	0[b]	40	0 (0)	-	-
	8	1588	51 (3.2)	8660	97 (1.1)
	10	803	24 (3.0)	4140	63 (1.5)

[a] phenotypic variants scored as number of chlorophyll and growth mutants

[b] normal propagated *in vitro* plants originating from one seedling which is vegetatively propagated for several generations

Tuberization

The top of M_1V_1 plants was used for tuberization. The shoots from axillary buds from 1022M-118 from all irradiation levels had a tuberization rate of approximately 97%, therefore in all the other experiments only 1022M-118 was used. There was no difference in tuberization between different irradiation doses.

Screening

The multicellular origin of vegetatively propagated shoots via axillary buds made it necessary to screen on starch granule level besides tuber level. Tubers that were induced on the shoots of tops of the 439 M_1V_1 shoots of the second experiment using axillary buds were used to make a crude starch sample. Based on the same criteria as described above no aberrant type was selected based on iodine staining of tuber cuttings. Another part of these 439 samples was used for microscopic screening at starch granule level. Microscopic screening of starch granule samples of the M_1V_1 tubers revealed several samples having 1–4 aberrant individual staining starch granules compared to the normal *amf* staining (Figure 4A).

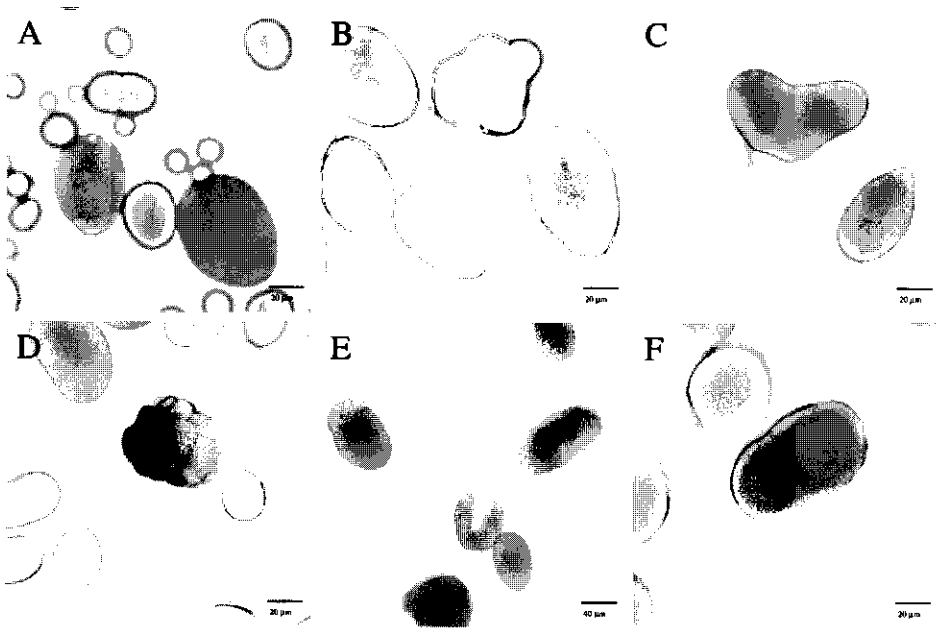

Figure 4. Starch granules stained with iodine. **A.** *amf* type. Starch granule shape variants found in the starch granule samples after 8 Gy **B–D.** and 10 Gy **E.** and **F.** of irradiation.

The aberrant granules were classified in six classes (Table 5) based on their blue staining. The last two classes (V and VI) had almost no blue staining and class VI was also found in the non-irradiated *amf*

control. The blue staining found in classes I–IV discolored after approximately 10 days. In one sample of the control a starch granule of class V was found. So based on the classes I–IV 56 tuber samples were found containing aberrant staining starch granules. Besides the aberrant staining starch granules also five aberrant shape starch granules were identified (Table 5, Figure 4 B–F).

Table 5. Six types of putative starch mutations microscopically scored in starch granules of tubers of the M_1V_1 of the monoploid 1022M-118 treated with two doses of irradiation (experiment II).

Irradiation (Gy)	n	Phenotype of aberrant starch granules						
		I	II	III	IV	V	VI[a]	Miscellaneous[b]
								
0	40	0	0	0	0	1	39	0
8	306	22	10	2	7	15	247	3
10	133	5	5	2	3	7	98	2

■ = blue staining with iodine

□ = red staining with iodine/discolor after 3 days

n = the number of starch granule samples scored

[a] this type of starch granules is detectable in all samples at magnification 400x.

[b] Miscellaneous are granule shape variants (Figure 4).

For the starch staining phenotypes only the first two bluest staining classes of aberrant starch granules were studied in crude starch samples of tubers of the M_1V_3 generation. For 8 Gy in 10 out of 19 and for 10 Gy in 9 out of 10 cases the phenotype could still be recovered in tubers of the M_1V_3 generation. In one case 13 aberrant staining starch granules were found indicating an increase of the aberrant phenotype. These genotypes need to be propagated or regenerated to increase the mutant sector by reduction of chimerism to stabilize the aberrant staining type. So some aberrant starch granule types were recovered, a few in a higher number than the original one. However, no tubers were found containing 50% or more of the aberrant starch granule types.

DISCUSSION

Approach using adventitious shoots

Production of adventitious shoots

The application of the adventitious shoot method using the material in this research proved to be very difficult. The previously developed plant material (Chapter 2) did not provide material similar to the monoploid 79.7322 used by Hovenkamp-Hermelink et al. (1987). Especially the regeneration capacity of the monoploid 1022M-54 was too low at higher levels of irradiation, which made it,

together with 20% tuberization capacity very difficult to produce 1000–3000 tubers needed to be able to find a variant (Hovenkamp-Hermelink et al., 1987). The difference in decrease of regeneration of adventitious shoots between the monoploid and diploid can be explained by the difference in ploidy level and the fact that more induced lethal mutations will be visible in the monoploids where no buffering by the other genome is possible.

Tuberization

The tuberization of the adventitious shoots of 1022M-54 was low compared to the tuberization of the shoots from axillary buds from 1022M-118. Hovenkamp-Hermelink et al. (1988) placed defoliated stem segments on high sucrose medium to induce tuberization of adventitious shoots of a monoploid. The same medium was tested in previous research on the monoploids used in this study, but gave low tuberization. The effect of irradiation on the tuberization of adventitious shoots was negligible.

Screening

The expected origin of a few cells of the adventitious shoots made it possible to screen on tuber level for variants by using Differential Scanning Calorimetry (DSC) or iodine staining of tuber cut surfaces. All aberrant types selected on the basis of DSC could not be recovered in subsequent generations. Apparently it was not possible to recover small chimeric sectors based on a few aberrant starch granules by screening with DSC. Therefore, DSC screening was not involved in the large second experiment using axillary buds. Hovenkamp-Hermelink et al. (1987) first also applied a fast method to determine changes in the amylose/amylopectin ratio in small amounts of tuber tissue of potato (Hovenkamp-Hermelink et al., 1998) and later on they were also forced to screen with the simple iodine staining of tuber cut surfaces. An extra barrier for the screening by DSC is the unfavorable frequency of finding variants and the costs of analysis per sample involved.

Approach using axillary buds

Multiplication of axillary buds

The variation in survival between consecutive generations can be explained by the fact that by irradiation also a lot of lethal mutations are induced which surface during multiplication. When a lot of plants die in the first vegetative generation the second generation has the advantage of a lower level of lethal mutations. This is true also the other way around. When a high number of plants survive in the first generation the second generation will have a low level of survival due to several lethal mutations which surface. There is a significant negative correlation between survival and relative number of phenotypic mutants. When a higher level of mutations is induced in the original axillary buds, after segregation of lethal mutations and phenotypic mutations, this will lead to lower survival and a higher number of phenotypic mutations in the remaining plants (Table 4). Although large differences were found between experiment I and II, this may not be explained by differences

in irradiation because the irradiation by X-rays was standardized. The differences found might be explained by differences in sensitivity of the M_0 plants to irradiation caused by growth of these plants in a different season.

Tuberization

The modified tuberization medium of Lillo (1985) gave 100% tuberization for the monoploid 1022M-118 (Chapter 2) and the irradiated axillary buds. Again the effect of irradiation on the tuberization of axillary buds was negligible.

Screening

The multicellular origin of vegetatively propagated shoots via axillary buds made it necessary to screen on starch granule level besides tuber level. Microscopic screening of the starch granules of subsequent tubers originating from M_1V_1 shoots revealed several different blue staining starch granules. Flipse et al. (1996) tried to inhibit the BEI-gene expression by sense and antisense transformation in an *amf* background and they found starch granules with a blue core with a maximum frequency of 305 per 1000 granules. A blue core is also found when the GBSS-gene expression is inhibited by antisense GBSS (Kuipers et al. 1994). In this study we found no starch granules having a blue core. The majority had a (small) red or discolored core with blue staining outer layers (class I – IV) or blue staining rings (class V and VI, Table 5). These blue-staining rings (class V and VI) seemed to be correlated with the growing rings of the starch granule. This was also found in the tubers of non-irradiated shoots. The blue staining of the growing rings was also observed by Kuipers et al. (1994). We conclude that this type of blue staining is not caused by irradiation. Although aberrant staining starch granules of the *amf* control were found in class V that is probably based on the natural mutation frequency we conclude that types V and VI are not aberrant. The starch granule types I-IV (Table 5) were never observed before in amylose-free potato starch. They are all different from wildtype or *amf* mutant. Most likely the variants found are similar to the *wxae* mutant of maize having loosely branched amylopectin (Shannon and Garwood, 1984) because the aberrant blue staining starch granules discolored after 10 days. Since during tuberization polyploidization takes place (Ramulu and Dijkhuis, 1986) it is to be expected that the variants found originate from mutation events due to irradiation. No examples of small cell clusters with altered starch were found in tuber slices.

56 axillary buds out of 439 showed aberrant staining starch granules presumably originating from one initial cell. So the mutation frequency for these types of aberrant starch granules is approximately $2,5 \times 10^{-3}$ based on the presence of 50 initial cells of an axillary bud (Broertjes and van Harten, 1988). For the isolation of the *amf* mutant in potato 3,000 minitubers were screened and three red-staining tubers were detected leading to a frequency of 10^{-3} (Hovenkamp-Hermelink et al., 1987). The mutation frequency in our study seems to be higher than the one found by Hovenkamp-

Hermelink et al. (1987), this might be explained by the differences in irradiation sensitivity of the genes of interest.

Donini and Sonnino (1998) suggest that screening for mutants should take place from M_1V_3 and up. Van Harten (1998) points out that it is not advisable to screen and select mutations already in the M_1V_1, as mutations may remain undetectable because of the small size of the mutated area. However, in this study we showed that it is possible to discriminate already 56 M_1V_1 families based on phenotypic changes of the starch of their tubers. Selection in M_1V_1 was also done by Love et al., (1996) who were able to select on glycoalkaloid content in leaves of the M_1V_1. This early selection can reduce the number of plants, which needs to be propagated through consecutive vegetative generations, considerably. Ahloowalia (1998) was able to select some solid potato variants with shallow eye, changed tuber shape, skin color and tuber size. He, however, used tetraploid potatoes that were irradiated with 20 Gy gamma irradiation. This is a much higher dose than used in this study, but we used monoploid potatoes of which the LD_{50} is much lower (Chapter 2). However only 19 of the 29 starch granule phenotypes of class I and II from tubers of M_1V_1 could be recovered in the M_1V_3. Only one variant was recovered having aberrant starch granules in an higher frequency in M_1V_3. Most likely the loss of aberrant types was caused by the chimeric nature of the initial shoot. Jacobsen et al. (1990) also encountered these problems involving chimerism. The selected M_1V_3 types form the basis for further research, but a problem is the limited number of cells that show the aberrant staining type in M_1V_3. Several rounds of multiplication will be necessary to stabilize the mutant sector to a periclinal chimera and finally a homohistont and thus to a stable mutant with the desired starch phenotype. An adventitious shoot regeneration step in between could speed up this proces. So when doing similar experiments in the future use should be made of the adventitious shoots method, or a similar method, were shoots originate from one or a few cells. This should result in at least 10,000 shoots of which after tuberization the starch can be screened for aberrant types at tuber or at starch granule level.

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GERMINAL TRANSMISSION AND SOMATIC EXCISION BEHAVIOR OF MAIZE *Ds* ELEMENTS IN POTATO USING THREE DIFFERENT TRANSPOSASE SOURCES

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ABSTRACT

Excision of the maize non-autonomous transposable element *Ds*, induced by *Ac* transposase and monitored by hygromycin resistance, was studied to determine the degree of transposition in potato. Four genotypes previously transformed with a *Ds* transposon containing T-DNA construct were combined with three different transposase sources by crosses or retransformation in order to induce excision of the *Ds* element. In two cases the transposase constructs consisted of the *Ac*-transposase cDNA driven by the 35S promotor or by the GBSS promotor. The other transposase source consisted of a natural, but stabilized *Ac* element. Based on hygromycin resistance the excision rate of the *Ds* element was calculated for the progeny of crosses between single transformants or out crosses of double transformants. In the progeny of double transformants besides early somatic excision in the seedlings also germinally transmitted excision of the *Ds* element occurred. Excision rates ranged from 14.8-48.4%. One (DsA16-458) of the four *Ds* carrying genotypes proved to harbor two independent loci of the *Ds* transposon and as a consequence a significantly higher excision rate was obtained. The stabilized *Ac* element proved to be the most effective transposase source in potato with an average excision rate of 38.3% for all four *Ds* carrying genotypes. Also a GBSS promotor driven transposase source was used which provided additional excision in the offspring of double transformants. The use of single transformants had the advantage of combining the transposase source and the *Ds* element in the same cross as excision is expected, but will only lead to unstable insertions. Outcrosses of double transformants are expected to result in both stable and unstable insertions. The stable insertions are found in *Ds* containing progeny seedlings missing the transposase source. However, as expected, fertility of the double transformants appeared to be much lower. Single transformants as well as double transformants are suitable to produce large populations of plants, containing both *Ds* element and transposase source. But a balance has to be found between a high level of excision, and stable insertions.

INTRODUCTION

Mutations are an important tool to study all kinds of metabolic pathways such as, for example, starch biosynthesis. Mutants with altered contents of amylose and amylopectin are known in several plant species and those with an increased amylose content have been identified in maize (Shannon and Garwood, 1984) and pea (Bogracheva et al., 1999). In potato only one starch mutant has been described containing amylose-free starch. This phenotype was obtained by X-ray mutagenesis of a monoploid potato plant (Hovenkamp-Hermelink et al. 1987). Another way to induce variants in potato is insertional mutagenesis with the use of transposable elements. For the induction of insertion mutants transposons were succesfully used in Arabidopsis, maize, petunia, tomato and rice. By this way of mutagenesis the gene involved can be identified by cloning the wildtype gene using the technique of transposon tagging (Fedoroff et al., 1984; Bancroft et al., 1993; Jones et al., 1994 and Chin et al., 1999). The use of transposable elements in potato for this purpose is more challenging due to the autotetraploid level, the vegetative propagation and the cross-pollinating nature of this crop.

Transposition of the maize transposable element *Ac* in potato has already been shown by Knapp et al. (1988). Since then, several mutation induction programs were set up for tagging of genes in potato using *Ac* or *Ds* elements. El-Kharbotly et al. (1996) for instance transformed potato plants with a *Ds* element with the goal to inactivate R-genes against *Phytophthora infestans*. Jacobs et al. (1995) mapped the location of 60 of these *Ds* element containing T-DNAs in diploid potato. This set of *Ds* carrying plants makes it possible to select genotypes carrying a *Ds* locus on a certain chromosome, where a gene of interest is located. Knapp et al. (1994) already stated that it is better to start with several plants each containing a different donor site for the *Ds* element instead to rely on one single *Ds* containing donor. If only one donor is used the excision from the respective donor site should be studied prior to a targeted tagging experiment. Pereira et al. (1991) used activation of the cloned GBSS gene, inactivated by insertion of the *Ac* element, as a marker in an amylose-free background for transposition to monitor excision of an *Ac* element.

In the present study a model system was developed to determine the transposition behavior of *Ds* elements in potato by possible inactivation of the gene for granule bound starch synthase (GBSS), which is located distally on the long arm of chromosome 8 (Jacobs et al. 1995). Because the phenotype of inactivation of GBSS in the starch composition is known, it is possible to visualize an insertion of a *Ds* element in the GBSS gene in a diploid potato background in which the allelic composition is heterozygous (Am*/amf*) for the *amf* mutation. Retransformation or introduction by crossing of transposase genes in *Ds* containing plants will lead to populations of plants with the desired genetic constitution in which the *Ds* element transposes infrequently also into the GBSS gene. A similar approach was earlier used for tagging of the *Cf-9* gene in tomato (Jones et al. 1994).

For the production of large populations of plants segregating for homozygous recessive insertion mutations in tomato, self-fertilization of *Ds* and transposase containing F_1 lines could be used to make the mutation detectable. Transposition of the *Ds* element is possible in such an F_1 parent (see Rommens et al. 1992; van der Biezen et al. 1996). This leads in an F_2 population, in which *Ds* and the transposase gene segregate, to stable and unstable insertions of the *Ds* element. The insertions of the *Ds* element in such a F_2 population originated from germinally transmitted excised *Ds* elements or from early somatic excision within the F_2 population. In tomato, the germinally transmitted and excised *Ds* elements in the F_2 population represented 10-50% of the total excisions (Rommens et al. 1993; Knapp et al. 1994).

In diploid potato repeated self-fertilization is not possible, because of self-incompatibility and inbreeding depression. Therefore, there are two approaches for the production of large populations of seedlings to provide excision of a *Ds* element with a transposase source. The first approach is a cross between two single transformants containing a *Ds* element and a transposase source, respectively (Figure 1A). The offspring contains plants with only a *Ds* element where no excision is possible and plants with both the *Ds* element and a transposase source present in which excision in early developmental stage is possible and the insertion is unstable (Knapp et al. 1994).

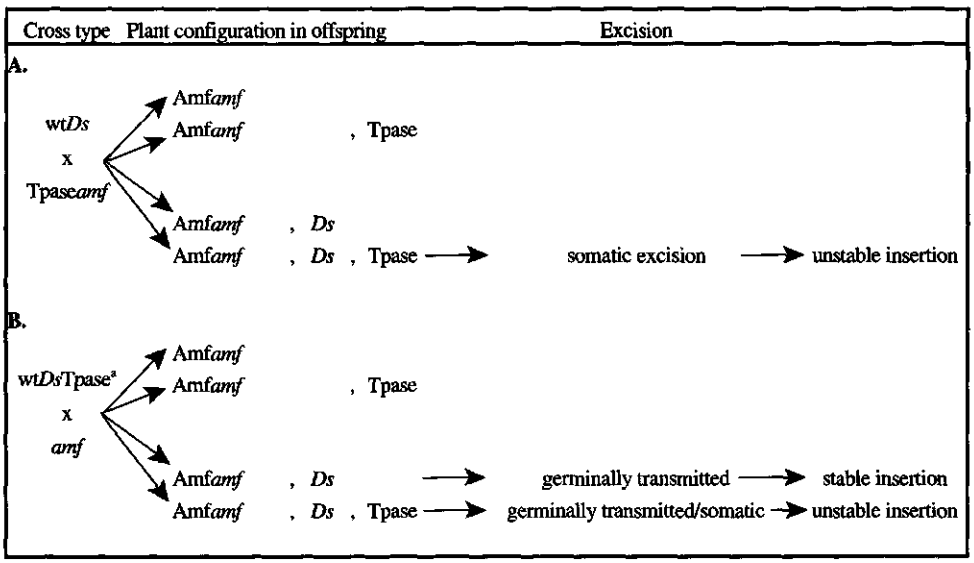

Figure 1. Crossing scheme for the activation of the *Ds* element by two approaches: **A.** single transformants and **B.** double transformants resulting in offspring with a specific plant configuration containing either stable or unstable excisions depending on the simultaneous presence or absence of the transposase gene and the *Ds* element. Tpase= transposase source. [a] excision is possible within the parent leading to chimerism.

The second approach is retransformation of *Ds* containing genotypes with a transposase source followed by an additional cross with a non-transgenic plant (Figure 1B.; Jones et al. 1994; Bancroft et al. 1993). After transposition the offspring contains useful plants with a stable transposed *Ds* element and also plants where both the *Ds* element and a transposase source are present in which the insertion is unstable. When going through meiosis germinally transmitted as well as somatic excision play a role in the same offspring as indicated above (see Rommens et al. 1993; Knapp et al. 1994; Stuurman et al. 1998).

Excision of particular *Ds* elements in potato has earlier been shown in a small population using one transposase source after retransformation (El-Kharbotly et al. 1996; Enckevort et al. 2000). The excision pattern of a *Ds* element in combination with several transposase construct sources has been studied earlier in tobacco (Rommens et al., 1992; Scofield et al. 1992). They used several promotors in combination with an *Ac* transposase cDNA or a stabilized *Ac* element and determined the excision activity in somatic tissue using different transposase sources in combination with a *Ds* element. Large differences, up to a factor 18 were found between excision rates for the different constructs.

Before performing large-scale experiments to find a *Ds* insertion in the GBSS gene, transposition behavior in diploid potato has to be known in more detail to estimate mutation frequencies for the gene of interest in the offspring. Additionally there might be differences in effect between introduction of a transposase source in *Ds* containing plants by double-transformation or crossing. Thus, the average transposition rate of a *Ds* element in combination with specific transposase sources needs to be determined by calculation of the number of plants having an excised *Ds* element. In this study, excision rates have been determined in two approaches using single and double transformants for three transposase sources in combination with the non-autonomous *Ds* element that has to be activated by an *Ac* transposase source in diploid potato.

MATERIALS AND METHODS

Plant material

From diploid (2n=2x=24) genotypes previously transformed with *Agrobaterium tumefaciens* strain GV3101 (pMP90RK) (Koncz and Schell 1986) containing the recombinant binary vector pHPT::*Ds*-Kan (HPT, hygromycin resistance; Kan, kanamycin resistance; Pereira et al. 1992; El-Kharbotly et al. 1996), a selection of four DsA16 genotypes (wildtype, AmfAmf) was made. These genotypes, DsA16-300, 95, 458 and 293 contained a *Ds* element in the vicinity of the gene for granule bound starch synthase (GBSS) at 9, 15, 22 and 41 cM respectively on chromosome 8 (Figure 2; Jacobs, 1995). Also two diploid amylose-free (*amf*) genotypes (4623-1 and 4625-14), that were offspring of the diploid *amf* mutant 1029-31 (Jacobsen et al. 1991), were used for transformation with transposase sources and as crossing parents.

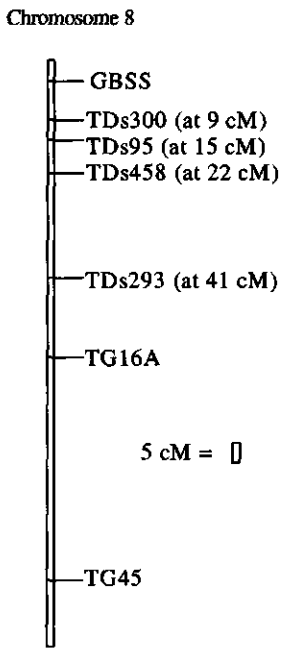

Figure 2. Schematic overview of chromosome 8 of potato with four *Ds* elements and their distance to GBSS, after Jacobs et al. (1995).

Constructs

To enable excision, three different transposase constructs were used in these experiments. Two constructs consisted of an *Ac* transposase gene (cDNA) under the control of the GBSS promotor: pGBSSAc::Mtx (GAMA) coded Gt, or under the control of the CaMV 35S promotor: p35S*Ac*::Mtx coded St (El-Kharbotly et al. 1996; Jacobs, 1995). Both constructs carried a methotrexate resistance gene as selectable marker. The construct SLJ10512 having an autonomous *Ac* element, which was stabilized by deletion of a specific border sequence (sAc), coded At from Scofield et al. (1992) was also used as transposase source. This construct carried a GUS gene for detection and a kanamycine gene as selection marker. A schematic overview of all constructs is given in Figure 3.

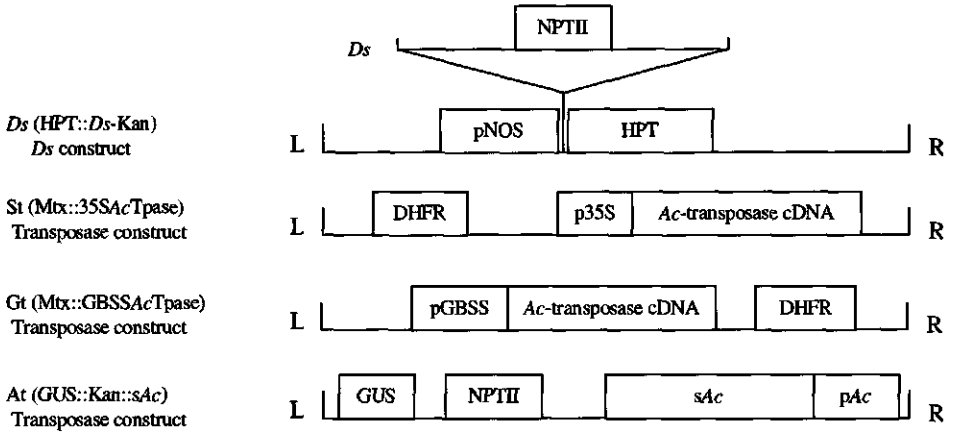

Figure 3. Schematic overview of constructs used in the experiments (*NPTII*=Kanamycin resistance, HPT=Hygromycin resistance, DHFR=Methotrexate resistance and GUS=ß-glucuronidase, pNOS=nopaline synthase promotor, p35S=califlower mosaic virus 35S promotor, pGBSS=granule bound starch synthase promotor, s*Ac*=stabilized *Ac* element under control of its own promotor=p*Ac*, L=left border, R=right border).

Transformation

Ds containing plants (DsA16-95, 293, 300 and 458), originating from genotype A16, which was originally selected for its transformation capability (El-Kharbotly et al. 1996), and the two diploid *amf* genotypes, were (re-) transformed with a transposase construct in order to induce *Ds* excision in either offspring of double or single transformants. Transformation was carried out as described by Visser (1991). But 2-3 days after inoculation, explants were transferred to MS medium (Murashige and Skoog 1962) containing 20 g/l sucrose, 200 mg/l cefotaxine, 200 mg/ml vancomycine, 100 μg/l methotrexate and 1 mg/l zeatine and then transferred to fresh medium every 3 weeks. From this medium shoots were isolated after 3 to 4 months and placed on MS medium containing 30 g/l sucrose and 100 μg/l methotrexate. Only transgenic shoots were able to root on this medium. Double transformants (*Ds*St+*Ds*Gt) and single transposase transformants (Gt, St and At) were produced to compare excision behavior of double transformants and single transformants in crosses. Of all transformants the ploidy level was tested, approximately 50% of the transformants was diploid. These diploid genotypes were selected for further experiments.

Crosses

For the induction of excision of the *Ds* element in the approach using single transformants, crosses were made between *Ds* containing and diploid genotypes with a transposase containing T-DNA (*Ds* x St, *Ds* x Gt and *Ds* x At). Additionally crosses were made for the second approach between double transformants and non-transgenic *amf* genotypes (*Ds*St x *amf* or *Ds*Gt x *amf*).

In vitro *tests for the presence of a transposase source and* Ds *element*

To monitor excision several *in vitro* tests have to be carried out. Representative samples of the seeds from the different crosses were sown *in vitro* on MS medium containing 20 g/l sucrose. In some cases 1 mg/l GA_3 was added to speed up germination. After four weeks the seedlings were investigated. For the Gt and St containing offspring a methotrexate resistance test was carried out as a screen for the presence of a transposase gene. Axillary buds of the seedlings were placed on MS medium supplemented with 150 μg/l methotrexate. For the At containing offspring a GUS assay was carried out using X-gluc according to Jefferson et al. (1987). When Gt and St transposase constructs were involved in crosses, it was possible to screen for the presence of *Ds* using a kanamycin resistance test. Thus, single axillary buds were placed on MS medium supplemented with 100 mg/l kanamycin. To monitor the assumed 1:1 segregation for the *Ds* element in all populations, a χ^2-test was carried out. To determine the excision of the *Ds* element, from all seedlings an axillary bud was placed on MS medium supplemented with 30 mg/l hygromycin.

DNA analyses

DNA was extracted from leaf material ground in liquid N_2 according to the CTAB protocol as described by Rogers and Bendich (1988). For determination of the presence of the *Ds* construct in

the transgenic clones, genomic DNA was digested with *Pst*I. The digested DNA was separated by electrophoresis and blotted onto Hybond N membranes (Amersham) under alkaline conditions as described by Sambrook et al. (1989). The membranes were hybridized, as described by Salehuzzaman et al. (1992), with a ^{32}P-ATP labeled 1.6 kb *Pst*I-*Kpn*I fragment of the vector pHPT::*Ds*-kan containing the HPT gene (Pereira et al. 1992).

RESULTS

Transformation

Several transformation experiments were carried out for the isolation of double transformants (*Ds*St and *Ds*Gt) or of plants only carrying a transposase gene (At, St and Gt).These transformants were the basis for the investigation of germinally transmitted and somatic *Ds* excision. No abnormalities were observed during transformation experiments other than the extreme aggressiveness of the *A. tumefaciens* strain carrying p35SAc::Mtx (St), which led to the isolation of only one single St transformant. Due to the high transformation capability of DsA16 genotypes the experiments for double transformants gave rise to respectively 31 (*Ds*St) and 50 (*Ds*Gt), diploid double transformants. Single transformant transformation experiments gave rise to 25 and 23 diploid transformants for respectively Gt and At transposase constructs after testing on methotrexate or kanamycin combined with a GUS assay, respectively. During further experiments, some (double) transformants were lost due to bad vigor. In the greenhouse the single transformants flowered much better than the double transformants of which some were excluded because of the absence of flowering. The bad flowering of the double transformants was the result of two transformation events and the use of the DsA16 genotype, which was originally selected for a high level of transformability but with a poor flowering capacity (El-Kharbotly et al. 1995).

In vitro *tests of offspring plants*

In order to induce excision of the *Ds* element followed by a possible insertion elsewhere in the genome, 1700 pollinations were made for both approaches i.e. single x single or double x *amf*. 400 pollinations resulted in 75 successful combinations between the four *Ds* containing genotypes and the diploid genotypes transformed with a transposase construct (Gt, St and At). On average three berries per combination were harvested and each berry contained more than 100 seeds. Additionally 1300 pollinations resulted in 75 successful combinations between double transformants (*Ds*St and *Ds*Gt) and the *amf* genotypes. On average also three berries per combination were harvested but here each berry contained only 10-50 seeds. The worst flowering plants were the double transformants containing a *Ds* element at 41 cM. The 150 combinations together resulted in more than 100,000 seeds of which approximately 80,000 were from crosses between single transformants. From all type-distance combinations 50–100 seeds were sown *in vitro* and 80-95 % of

the seeds germinated. In total 2010 plants of the various crossing populations were investigated (Table 1).

Table 1. Number of *in vitro* seedlings in the offspring tested for *Ds* excision in two types of crosses i.e. single x single and double x non-transgenic, using *Ds* sources with four distances to the GBSS gene in combination with three different transposase sources

	Basic *Ds* containing genotype (distance to GBSS gene in cM)				
Types of crosses	DsA16-300 (9)	DsA16-95 (15)	DsA16-458 (22)	DsA16-293 (41)	Total
wt*Ds* x *amf*At[a]	20	69	70	53	212
wt*Ds* x *amf*St[b]	n.p.	n.p.	247	243	490
wt*Ds* x *amf*Gt[c]	75	103	135	241	554
wt*Ds*St x *amf*[d]	205	37	371	7	620
wt*Ds*Gt x *amf*[d]	84	n.p.	41	9	134
Total	384	209	864	553	2010

[a] Single transformants: *Ds* wildtypes crossed with an *amf* genotype carrying the sAc construct

[b] Single transformants: *Ds* wildtypes crossed with an *amf* genotype carrying the p35SAc::Mtx construct

[c] Single transformants: *Ds* wildtypes crossed with an *amf* genotype carrying the pGBSSAc::Mtx construct

[d] Double transformants: wildtype genotypes which contain *Ds* and the p35SAc::Mtx or pGBSSAc::Mtx construct crossed with a non-transgenic *amf* genotype

n.p.: not performed

Per combination, the seedlings were tested for the presence of a transposase source and of the *Ds* element. No major abnormalities in growth behavior were observed during the *in vitro* methotrexate, kanamycin and hygromycin resistance tests of the 2010 plants of the crosses. Some representative results of single transformant and double transformant crosses are shown in Table 2. Two different types of calculations had to be used to determine *Ds* excision rate.

In case of the single transformants the segregation for transposase source together with the theoretically expected 1:1 segregation for the *Ds* element determined the number of plants containing the desired configuration. Together with the plants having an excised *Ds* element the excision rate could be calculated. For crosses involving the Gt or St transposase source the excision rate was calculated as follows. The number of plants displaying a hygromycin resistance in combination with a kanamycin resistance was divided by the number of plants carrying methotrexate resistance, which itself was first divided by two (based on the expected segregation of *Ds*) times 100%. For crosses involving the At transposase source the excision rate was calculated slightly different. The number of plants with hygromycin resistance in combination with GUS expression was divided by all plants showing GUS expression, which itself was first divided by two (based on the expected segregation of *Ds*) times 100%.

Table 2. Representative results for different types of offspring tested for methotrexate, kanamycin and hygromycin resistance *in vitro* and *β*-glucuronidase expression and their calculated excision rate.

	plants tested for transposase[a] and *Ds* element (kan)			# of plants with configuration				
		mtx	kan	mtx/kan/hyg			excision rate	
Cross examples	n	+/-	+/-[b]	+/+/+	+/+/-	-/+/+	calculation	%
Ds x Gt (single)	30	15/15	11/19	1	5	n.p.	1/(15/2) x 100%	13.33
DsSt x amf[c] (double)	38	18/20	21/17	2	4	1	(2+1)/21 x 100%	14.29
		GUS	kan	GUS/hyg				
		+/-	+/-[b]	+/+	+/-	-/+		
Ds x At (single)	20	5/15	n.a.	1	4	n.p.	1/(5/2) x 100%	40.00

mtx=methotrexate resistance, kan=kanamycin resistance, hyg=hygromycin resistance, GUS=expression of the *β*-glucuronidase gene.

[a] test for the presence of a transposase source carrying either methotrexate resistance gene or *β*-glucuronidase gene

[b] test for the presence of the *Ds* element carrying the kanamycin resistance gene

[c] excision of the *Ds* element is possible in the parent

n.p. not possible, no excision possible without transposase source.

n.a. not appropriate, transposase construct as well as *Ds* containing construct carry kanamycin resistance.

For the double transformants instead of the expected 1:1 segregation for the *Ds* element the real number of plants containing a *Ds* element was used in the calculation. This because somatic excision is possible within the basic double transformant which might lead to more independently segregating *Ds* elements with and without hygromycin resistance. Because somatic excision can be germinally transmitted to the offspring, excision of this nature might be found without the presence of a transposase source. The excision rate was calculated as the number of plants showing hygromycin resistance in combination with a kanamycin resistance divided by the number of plants carrying *Ds* times 100% (Table 2). The segregation pattern for the *Ds* element in all populations was tested using a $\chi^2_{1:1}(\alpha = 0.05)$. Based on this test the populations coming from one *Ds* genotype (DsA16-458, *Ds* at 22 cM from GBSS) had segregation ratios for *Ds* deviating from 1:1 but not from a 3:1, suggesting the presence of two independently inheriting *Ds* loci. Therefore, for analysis of excision rate the populations containing *Ds* at 22 cM from GBSS, in total 864 plants, were separated from the populations containing *Ds* at 9, 15 and 41 cM distance, in total 1146 plants, because of the two *Ds* loci present in the basic parent.

DNA analysis

Based on the segregation for kanamycin resistance the basic genotype DsA16-458 (22 cM) had two *Ds* loci. The Southern analysis was performed on the basic genotypes DsA16-458 (22cM), pollinator G254 and a few of the offspring plants (113-6, 16 and 17; Figure 4) to confirm the presence of two

Ds loci. The three bands present in DsA16-458 indicate a minimum presence of three copies of the construct. The three offspring plants clearly showed independent segregation of the band 2 and bands 1 and 3. This confirms the segregation of two different Ds loci within the basic genotype.

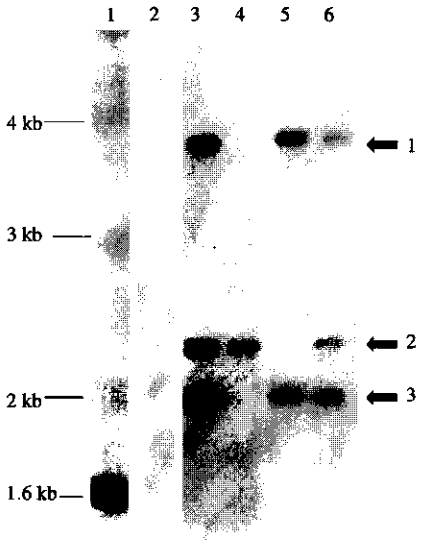

Figure 4. *Pst*I restriction of genomic DNA and hybridised with a 1.6 kb HPT *Pst*I-*Kpn* fragment of the construct pHPT::*Ds*-Kan to screen for plants containing the *Ds* construct in the parents G254 (lane 2) and DsA16-458 (lane 3), offspring 113Ds-6, 16 and 17 (lane 4,5 and 6) and the DNA marker (lane 1).

Excision

The expression of hygromycin resistance is an indication for excision of the *Ds* element which, in combination with the *in vitro* tests for presence of *Ds* and transposase, can be used to determine transposition. Depending on the approach, single transformants (*Ds* x St, Gt or At) versus double transformants were crossed (*Ds*St and *Ds*Gt x *amf*), the excision rate was calculated based on hygromycin resistance in *in vitro* tests as shown in Table 2. The variation of the excision rate within the five different crossing types was relatively low (Figure 5 and 6), between the five types the variation was rather high as shown in Table 3, Figure 5 and 6. For the four different distances of*Ds* to GBSS the average excision rate was calculated (weight cases by number of plants tested). These results are depicted in Figure 5A. In addition averages for crossing type were also calculated. The average excision rate of the 5 different types of crosses varied from 15.73% to 41.64% and they were all significantly different from each other based on Duncan's multiple range test ($\alpha = 0.05$) (Table 3, Figure 5B). The excision rate in the offspring of DsA16-458 (*Ds* at 22cM) was significantly higher than the rest based on Duncan's multiple range test ($\alpha = 0.05$). Based on this observation and the significant 3:1 segregation in particular populations mentioned before coming from DsA16-458 (22 cM), these excision rates appeared to be induced by two independent *Ds* loci and therefore they were separated from those of *Ds* parents at 9, 15 and 41 cM. These separated results are shown in Figure 6A and B. Based on the excision rates of 9, 15 and 41 cM the crossing types *Ds* x At and *Ds*Gt x *amf* had, based on Duncan's multiple range test ($\alpha = 0.05$), a significantly higher excision rate than the rest of the crossing types, 38.30 % and 35.13% respectively (Table 3, Figure 6A). For 22 cM only the excision rates from the crossing types *Ds* x At and *Ds* x St were significantly different from the rest due to large variation within the types of crosses or due to a small number of crosses, for example, for *Ds*Gt.

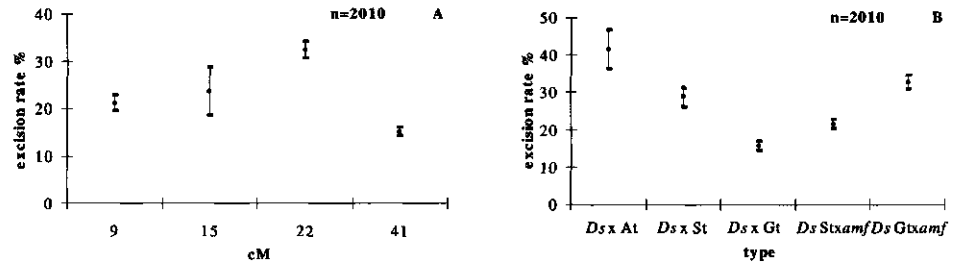

Figure 5. Excision rate with their 95% confidence interval for mean of the *Ds* element in the offspring of **A.** crosses for four different *Ds* loci located on chromosome 8. **B.** five different types of crosses having three different transposase sources.

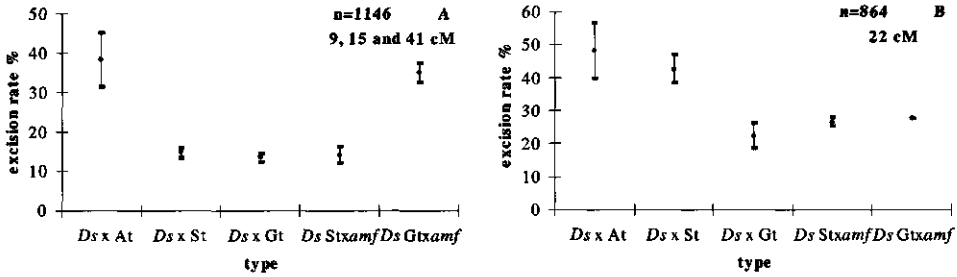

Figure 6. Excision rate with their 95% confidence interval for mean of the *Ds* element in the offspring of five different types of crosses having three different transposase sources for **A.** 9, 15 and 41 cM joined and **B.** 22 cM separate.

DISCUSSION

In vitro *tests*

Before doing large scale experiments for tagging of genes using *Ds* element containing parental clones in combination with several transposase sources it is important to get more insight into the transposition frequency, the percentage of plants containing a *Ds* element and a transposase source in combination with excision. The transposition of the non-autonomous *Ds* element can be tested in several ways. The *in vitro* tests were used to investigate the presence of *Ds* (kanamycin resistance), presence of transposase sources (GUS assay and methotrexate resistance) and excision leading to hygromycin resistance. The hygromycin resistance already proved in other potato experiments to be a reliable determinant for excision (Enckevort et al. 2000). In that study hygromycin resistance was tested in the range of 20-40 mg/l. It was found that on 30 mg/l hygromycin genotypes, still chimeric for excisions, could be scored as hygromycin resistant in specific genotypes, whereas on 40 mg/l only plants homogeneous for *Ds* excision were scored as resistant. Also in other studies the use of an antibiotics resistance gene as a marker for excision proved to be accurate (see Eisses et al. 1997;

Knapp et al. 1994). It has to be stressed that the excision rate of the *Ds* element measured by hygromycin resistance is a minimum rate. In the present experiments only the level of initial transposition was measured, as the excision of the *Ds* element from the donor site after which restoration of the hygromycin resistance is realized. However, in some cases the excision of*Ds* will not lead to sufficient restoration of the hygromycin resistance (Enckevort et al. 2000). Overall the*in vitro* resistance tests, used in our experiments, proved to be an excellent tool to determine the presence of *Ds*, transposase sources and excision, which resulted in reliable estimates for excision rate.

Table 3. Excision rate of the *Ds* element in the offspring of five different types of crosses with four different distances of the *Ds* element to the GBSS gene, with 22 cM separated.

	Excision rate (%)[a]		
Types of crosses[b]	9, 15 and 41 cM (n=1146)	22 cM (n=864)	total (n=2010)
wt*Ds* x *amf*At[c]	38.30[v]	48.42[v]	41.64[y]
wt*Ds* x *amf*St[d]	14.81[u]	42.66[v]	28.85[w]
wt*Ds* x *amf*Gt[e]	13.55[u]	22.49[u]	15.73[u]
wtDsSt x *amf*[f]	14.25[u]	26.63[u]	16.42[v]
wtDsGt x *amf*[f]	35.13[v]	27.59[u]	32.82[x]

[a] Values followed by a different letter differ significantly (p=0.05, Duncan's multiple range test, weight cases, each column separately).

[b] number of desired plants for the types for single and double transformants are shown in Figure 1 C and D.

[c] single transformants: *Ds* containing wildtypes crossed with an *amf* genotype carrying the sAc construct

[d] single transformants: *Ds* containing wildtypes crossed with an *amf* genotype carrying the p35SAc::Mtx construct

[e] single transformants: *Ds* containing wildtypes crossed with an *amf* genotype carrying the pGBSSAc::Mtx construct

[f] double transformants: wildtype genotypes which contain *Ds* and the p35SAc::Mtx or pGBSSAc::Mtx construct crossed with an *amf* genotype

From the research on potato of Wolters et al. (1998) it is known that transformation can lead to insertion of several copies of the T-DNA resulting in one ore more linkage groups within one transformant. The plants used in our experiments were originally selected for one linkage group. Thus when measuring excision, only the first jump of the *Ds* element that is excised from the T-DNA, which provides a restoration of the hygromycin resistance (Figure 2), is determined. But the excision of other *Ds* elements present in the same linkage group can not be determined because hygromycin resistance is already expressed. So in practice there will be an underestimation of the level of excision of all *Ds* elements within one plant. It is also important to remember that one *Ds* element can transpose several times so that the total frequency of transposition is probably higher.

Excision in crosses of single transformants and double transformants

When studying transposition by excision from a donor site it is very important to keep in mind that, in a plant containing a *Ds* element and a transposase source derived by crossing or double transformation, somatic excision may occur at different time points. This will result in small or larger chimeric sections of a plant containing excised *Ds* elements. In the present experiments the excision of a *Ds* element gave restoration of a hygromycin resistance and crosses of single transformants and double transformants were studied which provided excision of a *Ds* element. These excisions originated from somatic events during early development of the embryos after fertilization or in case of the double transformants from germinally transmitted events (Figure 1A and B).

Large populations were produced by crosses between single transformants and between double transformants and non-transgenic plants. The poor seed set in crosses with the double transformants was clearly a negative effect of the two transformation events and of the original A16. When crossing single transformants, one parent carrying the *Ds* element and the other one the transposase source, the excision will originate from early somatic events just after fertilization in the developing embryo. There are other differences in excision using offspring of single transformants or double transformants that have to be discussed. Besides unstable independent insertions in the offspring of single transformants because of the presence of transposase, the offspring of double transformants will also provide stable insertions without transposase and germinally transmitted insertions (Knapp et al. 1994).

Influence of transposase sources on excision

In the present study, the stabilized *Ac* transposase source showed the highest excision rate (38.3%). In case of the offspring of double transformants besides the somatic excision also germinally transferred excision, originating from chimeric sectors of the parents, was included (Figure 1B). In the offspring of double transformants the Gt transposase source (pGBSS*Ac*-transposase) had a higher excision rate (35.1%) than the St transposase source (p35S*Ac*transposase). Double transformants were also used by Stuurman et al. (1998) in tomato, a stabilized *Ac* transposase source was used and in the individual F_2 plants without a transposase source they found an overall germinally transferred excision frequency of 13.5%. Rommens et al. (1993) studied excision using a stabilized *Ac* transposase source in combination with a *Ds* element in tomato. In the F_2 they found that 23-87% of the excisions originated from germinally transferred excision events. This indicates that combining a *Ds* element and a transposase source by crossing will directly lead to excision in the F_1, but the insertion is expected to be lower in frequency and unstable due to the presence of a transposase source. So *Ds* containing plants should be combined with the stabilized *Ac* in a crossing or in a double transformant with additional crosses, to obtain the highest excision rate.

Excision originating from the parent where the *Ds* element and a transposase source were already combined was also found by Knapp et al. (1994). They investigated excision by restoration of a BAR resistance gene in an F_2 tomato population. 58% were independent *Ds* element excisions but 42% of them were found at the same position. The latter were probably due to only one early somatic excision in the F_1 parent. The other independent excisions were due to late somatic excisions in the F_1 parent or early excision in the F_2 individuals, which cannot be discriminated from each other. So in specific combinations of double transformants a relatively high level of germinally transmitted excision is expected leading to a high number of plants with the same excision which is not desirable when independent excisions have to be generated in offspring. Rommens et al. (1993) were able to generate a higher frequency of independent germinally transmitted excision events caused by late somatic transposition from the T-DNA.

Multiple loci within one basic genotype

Based on segregation of the *Ds* element and southern analysis it could be concluded that the genotype DsA16-458 with a *Ds* containing T-DNA insert at 22 cM from GBSS possessed two independently segregating *Ds* containing T-DNA loci. Therefore, these results were separated from those of 9, 15 and 41 cM (Table 3, Figure 6A and B). Due to this fact it might be expected that additional *Ds* elements, multiple copies or loci, in one plant can lead to more transpositions and thus to a bigger chance of tagging the gene of interest. Cooley et al. (1996) found this in tomato using somatic insertion at two single copy genes. In our case four crossing types of 22 cM had a significantly higher excision rate than the five crossing types of the rest, based on Duncan's multiple range test ($\alpha = 0.05$), as shown in figure 6B. For 22 cM the excision rates from the crossing types *Ds* x At and *Ds* x St were significantly higher. Between the others also differences might exist but due to variation within the individual types of crosses and due to a small number of crosses analyzed, no difference was found.

Differences in excision

The variation for excision between the *Ds* elements at the three distances (9, 15 and 41 cM) in the present study indicates that the efficiency of transposition depends on the genomic position of the Ds element, which was also suggested for tomato by Rommens et al. (1992). The variation within one type of crossing might also be explained by the position effect of the inserted transposase loci. The different transposase transcript levels might also influence the excision as earlier observed in maize and transgenic tobacco (Takumi et al. 1999). So, genomic position as well as transcript levels might have an effect on excision.

In literature several transposase constructs driven by tissue specific promotors have been used with variable results in different host species (see Honma et al. 1993; Scortecci et al. 1999). The transposition study of Scofield et al. (1992) was focused on excision patterns in cotyledons of

tobacco using streptomycin resistance as excision marker. They determined the expected transposition activity in somatic tissue using different transposase sources in combination with a *Ds* element. However, this may not directly be compared with excision behavior originating from early somatic excision within the embryo used in this research, in which the activity is different for the transposase genes under control of the constitutive 35S or the tissue specific GBSS promotor. Scofield et al. (1992) found a high excision pattern for the p35S-*Ac* transposase construct. This is in contradiction to the findings in the present study where the stabilized *Ac* transposase provided the highest excision rate, and the 35S-*Ac* transposase had a lower one.

Use of the tissue specific GBSS promotor in a transposase source

From figure 6A it can be deduced that the GBSS promoter driven transposase construct provided a higher excision rate when used in double transformants (Figure 1B). One could argue that the excision rate for the pGBSSActransposase construct with an additional meiosis is expected to be high due to a higher germinal excision transfer of the original double transformants. However, this is unlikely, because the excision rate of plants from the crossing type *Ds* x St and *Ds*St x *amf* were almost the same, indicating that the germinally transferred excision for this type is relatively low. This could mean that using a transposase gene combined with a promotor like the GBSS promotor, which is active in ovary and pollen (Visser, unpublished), in combination with a *Ds* element causes a much higher level of excision (real germinal excision) when applied in double transformants. This will result in more independent excision events and thus a higher chance of tagging the gene of interest.

Population size

When producing progeny of the selected plants for induction of transposition by crosses, transposon as well as transposase sources are segregating in the diploid offspring. When starting with 1000 seeds both non-autonomous *Ds* transposon and transposase source will independently segregate 1:1 in the offspring. When combined with the overall average excision rate (18.8%) this will lead to the number of useful plants in which a transposon has been excised leading to unstable or stable insertions of the *Ds* element (Figure 7A and B). Based on these useful plants it will be possible to calculate the insertion frequency into the GBSS gene. In case of using single transformants approximately 4.7% of the 1000 plants will have the desired configuration with excisions leading to unstable insertions. For the double transformants approximately 9.4% will be desired plants with excisions resulting in stable or unstable (precense of transposase source) insertions. In tomato the *Cf9* gene was tagged from a position located at a distance of 3 cM at a frequency of 1 in 1000 *Ds* transposition events (Jones et al., 1994). The tagging of multiple genes in petunia was done by using an autonomous *dTph1* element, insertion alleles for the various genes were found at a frequency of about 1 in 1000 plants (Koes et al., 1995). So to obtain at least one insertional GBSS mutant the

population size to start with should be at least 10,000 plants based on the 9.4% desired plants per 1000 offspring plants.

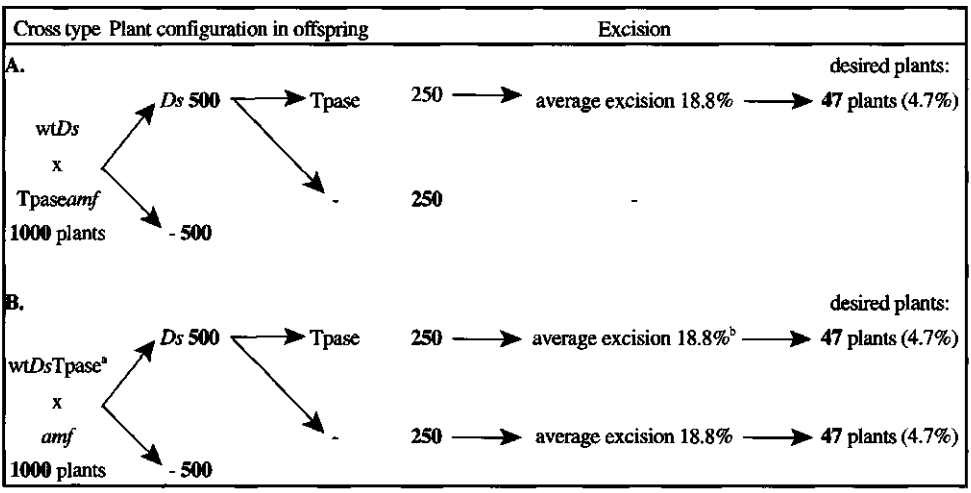

Figure 7. Scheme of crosses leading to the activation of the *Ds* element by two approaches using: **A.** single transformants and **B.** double transformants resulting in a specific plant configuration depending on segregation for *Ds* element and transposase source(s) and for both approaches the number of critical plants which have the desired configuration when used for transposon tagging, starting with 1000 plants. Tpase= transposase source,

[a]: excision is possible within the parent leading to chimeric behavior.

[b]: minimum excision generally higher due to new somatic excision events.

Conclusions

For the highest rate of excision in potato it seems to be important to use a heterologous system in which transposon and transposase source are used in their most natural way. Our experiments showed that in the approach using offspring of single transformants the genetically engineered transposase cDNA constructs exhibited lower excision rates than the stabilized *Ac* transposase under its own promotor (Table 2, Figure 5A). The *Ac* promotor seems to be active in all plant tissues and is expressed at a higher level in dividing cells (Fridlender et al., 1998). The excision results obtained in the present study for plants containing a *Ds* element linked to the GBSS gene may be generalized for the whole population of 60 *Ds* containing genotypes produced by El-Kharbotly et al. (1996). When a particular target gene is located on a particular chromosome the specific set of *Ds* containing plants of that chromosome can be selected and used for tagging of that gene. In our case plants containing a *Ds* element closely linked to GBSS are going to be used in the model system for the induction and selection of inserted *amf* mutants. Most suitable seem to be the cross parents in the group *Ds* x At with a high excision rate (Figure 5A and 6B). It is concluded that both approaches presented in this study are suitable for the production of populations in which *Ds* elements are

excised. But a choice has to be made whether the insertions leading to mutations have to be stable or that also unstable insertions will be useful.

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potato has already been shown by Knapp *et al.* (1988). Since then several programs were set up for the tagging of genes in potato.

To test the use of transposable elements in potato Kharbotly et al. (1996) transformed diploid potato with a *Ds* element derived from an *Ac* element with the goal to inactivate R-genes against *Phytophthora infestans*. Although shown to be mobile, there have been no reports until now of real targeted gene tagging by *Ac* or *Ds* transposable elements in potato. To test the *Ac/Ds* system in potato for tagging of genes in the starch biosynthetic pathway a model system was set up in this study for the tagging of the gene enconding granule bound starch synthase (GBSS) (Chapter 4). Because the phenotype of inactivation of GBSS is known, it is possible to visualize the effect of a mutation based on insertion of a *Ds* element in the GBSS gene in a diploid potato background heterozygous (*Amfamf*) for the *amf* mutation. The GBSS gene has already been used as an excision marker for the autonomous *Ac* transposable element (Pereira et al., 1991; Enckevort et al., 2000). To be able to implement the model system to other genes a PCR based insertional screening method might be suitable to identify inserted mutants when there is no known or easily observable phenotype. According to Cooley et al. (1996) even on the molecular level, it is relatively easy to show insertion in a known gene. Koes et al. (1995) screened large populations with a three-dimensional PCR-based selection approach to obtain mutant petunia plants in which a specific gene with known sequence was inactivated by a transposable element. In the present study the model system described in Chapter 4 was used to obtain inactivation of the gene for granule bound starch synthase (GBSS) by transposition of *Ds* elements in potato. Since plants have been selected of which the location of the *Ds* element inserts are linked to the GBSS gene, retransformation or introgression of these plants with transposase genes will lead to excision of these *Ds* elements as studied in Chapter 4, infrequently resulting in inactivation of the GBSS gene.

MATERIALS AND METHODS

Plant material

From diploid (2n=2x=24) genotypes previously transformed with the *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) (Koncz and Schell 1986) containing the recombinant binary vector pHPT::*Ds*-Kan (HPT, hygromycin resistance; Kan, kanamycin resistance; Pereira et al. 1992; El-Kharbotly et al. 1996), a selection of four DsA16 genotypes (wildtype, *AmfAmf*) was made. These genotypes, DsA16-300, 95, 458 and 293 contained a *Ds* element in the vicinity of the gene for granule bound starch synthase (GBSS) at 9, 15, 22 and 41 cM respectively on chromosome 8. From genotype DsA16-458 plants from the greenhouse as well as *in vitro* were used. Also the diploid *amf* genotypes with and without a transposase source described in Chapter 4 were used as pollinators. From the 100,000 seeds obtained from crosses, between single transformants or between double

transformants with non-transgenic *amf* genotypes, a batch of approximately 24,000 seeds was used in the present experiments.

Constructs

To enable transposition, four different transposase gene containing constructs were used in these experiments. One construct consisted of an *Ac* transposase gene (cDNA) under the control of the GBSS promotor: pGBSSAc::Mtx (GAMA) coded Gt, another one was under the control of the CaMV 35S promotor: p35SAc::Mtx coded St (El-Kharbotly et al., 1996; Jacobs, 1995). The constructs SLJ10512 (sAc) coded At and SLJ1111 (35S::Tpase) coded Bt, both from Scofield et al. (1992) were also used as transposase sources. Double transformants (*Ds*St +*Ds*Gt) and single transposase transformants (Gt, St, At and Bt), produced previously (Chapter 4), were used to be able to compare insertion behavior of double transformants and single transformants in crosses.

Ds selection in some populations

Seeds of all populations were sown in sowing pans in the greenhouse. In case of the crosses involving the Gt and St transposase source it was possible to select seedlings harboring the HPT::*Ds*-Kan construct by testing for kanamycin resistance. Four-week-old seedlings in the greenhouse were sprayed with 100 mg/l kanamycin according to Weide et al. (1998). After five to seven days chlorotic spots could be found on the negative control. These chlorotic spots indicate the absence of kanamycin resistance and, therefore, the absence of a *Ds* element.

Screening

The *Ds* element used in these experiments was derived from an *Ac* element cloned from the wx-m7 allele (Behrens et al., 1984) with an internal 1,6 kb *Hind*III deletion and a NPTII gene inserted in the *Xba*I site nearby (Pereira *et al.*, 1992). Four primers of the *Ds* element for the insertional PCR approach were taken of the *Ac* element from Cooley *et al.* (1996). Two additional primers for the *Ds* element and four primers for the GBSS gene were designed (see Table 1). Molecular screening for potential transposon insertion mutants was done by a 3-dimensional (3-D) PCR approach (Koes *et al.*, 1995). DNA was extracted from leaf material ground in liquid N_2 according to the CTAB protocol as described by Murray and Thompson (1980). By using PCR in combination with a GBSS and a *Ds* primer a product might be found indicating an insertion of the *Ds* element into the GBSS gene. By the 3-D approach a positive plant can easily be identified. Primers for the GBSS gene and the *Ds* element were tested for their accuracy on DNA from control plants (not shown). The PCR was completed with the following cycling conditions: hot-start at 94°C during 1 min, Taq polymerase was added at 80°C, followed by 40 cycles of 94°C for 1 min, 60°C for 2 min, and amplification at 72°C for 2 min, ending with a 10-min extension at 72°C. PCR products were separated on a 0.8% TBE agarose gel.

Because all diploid plants of the populations have an *Amfamf* composition for the GBSS gene, it is possible to visualize an insertion of a *Ds* element in the GBSS gene. So of all plants tubers were harvested and cut surfaces were stained with iodine. The default was blue staining tubers because of the presence of a wildtype GBSS gene. So red staining was found when a tuber had amylose-free starch due to a mutation of the GBSS gene.

Table 1. Primers of the *Ds* element, GBSS gene and pHPT::*Ds*-Kan T-DNA used for the PCR approach, empty donor site PCR and single point deletion PCR with their base composition. Location on the strands and the melting temperature.

Primer	Base composition	Strand	Melting temperature (°C)
Ds-1a[a]	5' TTTTCGTTTCCGTCCCGCAAGTTA 3'	+	70.0
Ds-1b[a]	5' GACCGTTACCGACCGTTTTCATCC 3'	+	74
Ds-1c	5' ATGAAAATGAAAACGGTAGAGGTA 3'	+	64.0
Ds-2a[a]	5' GTTTCCGTTTCCGTTTACCGTTTT 3'	-	68.0
Ds-2b[a]	5' CGTTTCCGTTCCGTTTTCGTTTTT 3'	-	68.0
Ds-2c	5' CTCAGTGGTTATGGATGGGAGTTG 3'	-	72.0
GBSS-1	5' GAGGGAGTTGGTTTAGTTTTTAGA 3'	+	66.0
GBSS-2	5' GCCGATTTTCTTTCTCTGACTTCC 3'	+	60.0
GBSS-3	5' CAAAGGAGGACGGAGCAAGAAACA 3'	-	72.0
GBSS-4	5' CAGGAATAGGCAAAATAAAGATGA 3'	-	64.0
GBSS-6	5' GGTTCCTGAGTCCTATCTGTGA 3'	-	66.0
GBSS-20	5' TTTTGATTCTCTTGCCTACTGT 3'	+	60.0
p1 NOS[b]	5' GCGCGTTCAAAAGTCGCCTA 3'	+	62.0
p2 HPT[b]	5' GTCAAGCACTTCCGGAATCG 3'	-	62.0

[a] from Cooley et al. (1996)

[b] from Enckevort et al. (2000).

In vitro *analysis*

When Gt and St transposase constructs were involved in putative insertional mutants, these were analyzed for the presence of *Ds* using a kanamycin resistance test, single axillary buds were placed on MS medium supplemented with 100 mg/l kanamycin. Also a methotrexate resistance test was carried out to confirm the presence of the transposase gene. Axillary buds of the seedlings were placed on MS medium supplemented with 150 μmg/l methotrexate. For the putative insertional mutants involving an At transposase source besides a kanamycin resistance test a GUS assay was carried out using X-gluc according to Jefferson et al. (1987). To determine the excision of the *Ds* element in the putative mutants, an axillary bud was placed on MS medium supplemented with 30 mg/l hygromycin.

Molecular analysis

Southern blot analysis was performed using *Hind*III or *Pst*I restricted genomic DNA. The digested DNA was separated by electrophoresis and blotted onto Hybond N membranes (Amersham) under alkaline conditions as described by Sambrook et al. (1989). The membranes were hybridized, as described by Salehuzzaman et al. (1992), with ^{32}P-ATP labeled probes. For determination of the presence of the *Ds* construct the membranes with *Hind*III digested genomic DNA were hybridized with a 1.7 kb *Bam*H1-*Hind*III fragment of the 5' end of the *Ds* element of the vector pNH19HRS-3 which was used for the construction of the pHPT::*Ds*-kan construct (Pereira et al. 1992). These membranes were also hybridized with the GBSS I cDNA probe pGB6 (Visser et al., 1991) to determine the allelelic composition of the phenotypic mutants and their parents according to van der Wal (2000).

The membranes with *Pst*I digested genomic DNA were hybridized with a 1.6 kb *Pst*I-*Kpn* fragment of the construct pHPT::*Ds*-kan (Figure 1), containing HPT, the 3' end of the construct, again to confirm the presence of the *Ds* construct. These membranes were also hybridized with a 3.7 kb *Pst*I fragment of the construct pHPT::*Ds*-kan, containing the 5' end of the *Ds* element together with the NOS promotor (Figure 1), to confirm the presence of the *Ds* element in original position in the transgenic clones called full donor sites (FDS) and to detect possible empty donor sites (EDS) containing a differently sized donor site after excision of the *Ds* element and to show reinsertion of the *Ds* element at various positions according to Enckevort et al. (2000).

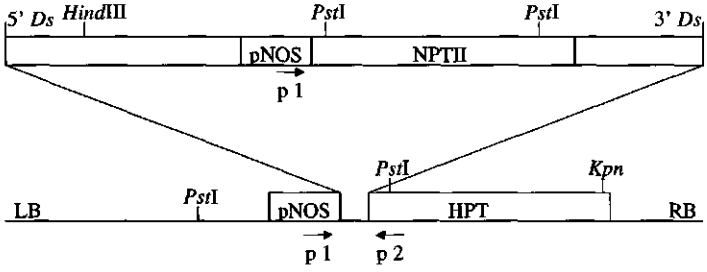

Figure 1. Schematic drawing of pHPT::*Ds*-Kan showing *Pst*I, *Hind*III and *Kpn* restriction sites and positions of the primers for the PCR full donor site (FDS) and for empty donor sites (EDS = 450 bp), p1 and p2. LB=left border, RB=right border, pNOS=nopaline synthase promotor, HPT=hygromycine phosphotranferase, NPTII=neomycin phosphotransferase.

Primers (p1 and p2, Table 1, Figure 1) previously designed by Enckevort et al. (2000) on pHPT::*Ds*-Kan together with PCR were also used to show FDS and/or EDS after excision of *Ds*. Primers were

also designed to reveal the allelic composition at base pair level to show whether both genomes contain the single point deletion of the *amf* gene at position 46–48 or not (van der Leij et al., 1991). Two primers (GBSS 1 and GBSS 6, Table 1) were used to amplify a specific fragment by PCR. These fragments of 624 bp were subcloned into a plasmid and sequenced using primer GBSS 20 (Table 1).

RESULTS

Populations

In order to make transposition of the *Ds* elements into the genes of interest possible, several hundred crosses were made between the *Ds*-containing genotypes and the genotypes transformed with transposase constructs or between double transformants and non-transgenic *amf* genotypes. Over 150 crosses were made with the four selected *Ds*-containing genotypes, which resulted in more than 100,000 seeds. A part of the populations was tested *in vitro* (Chapter 4). Of all populations available, categorized by distance of the *Ds* element to the GBSS gene and transposase source, seeds were sown ranging from 20 to 500 seeds per population depending on their availability (Table 2), resulting in a total of 22,056 seedlings. Based on the number of germinated seeds (Table 2), the excision rates and segregation (1:1) of the *Ds* element and transposase sources were described in Chapter 4. The number of seedlings having the desired genetic configuration, where excision of the *Ds* element is possible, was calculated being 2314 seedlings (Table 3). 13,586 seedlings from the crosses involving the St and Gt transposase sources were sprayed with 100 mg/l kanamycin to select for the presence of the HPT:*Ds*-Kan construct, which should result in the survival of approximately 50% based on the segregation of the *Ds* element. However, in some populations kanamycin resistance was not expressed, possibly due to co-suppresion and as a result of that complete populations were scored not resistant. As expected based on the findings in Chapter 4 the seedlings originating from plants carrying the *Ds* element at 22 cM sometimes showed a segregation for 2 loci of the *Ds* element. Finally 3700 kanamycin resistant plants were selected and transferred to 13 cm containers after subjecting the 13,586 seedlings to the kanamycin test. For the crosses involving At and Bt 8470 plants were transferred to 13 cm containers, so in total 12,170 plants were screened for a possible insertion of the *Ds* element into the GBSS gene.

Screening for Ds insertions molecularly and by iodine staining

From all plants three leaves were harvested and pooled in three dimensions. After PCR in some rare cases on the agarose gel a band was found between 1 and 4 kb, possibly indicating a insertion of a *Ds* element into the GBSS gene. But these bands were not found in all three samples of the 3D method, so identification of specific plants was not possible.

After screening of all the tubers of the 12,170 plants with iodine staining three phenotypic mutants were found having an *amf* type of starch of which one was chimeric (Table 4, Figure 2). The

phenotypic mutant 2154DsAt-228 was chimeric and its parents were 113Ds-16 and Ata7-4. The second phenotypic mutant 2147DsGt-139 was a result from the cross between 113Ds-6 and 3004Gt-16. The third and last phenotypic mutant 2101DsAt-310 resulted from the cross between 102Ds-3 and Ata7-1. There seems to be some relation between the number of plants tested per distance and the number of phenotypic mutants found for GBSS (Table 2). No relation was found between the number of populations involved and the number of phenotypic mutants.

Table 2. Number of populations and the number of seedlings in the offspring that germinated in the greenhouse for four different selected distances and the total number of plants used for tagging of the GBSS gene.

Cross type	Distance to GBSS gene (cM)								Total
	9		15		22		41		# plants
	# pop.	# plants	# pop.	# plants	# pop.	# plants	# pop.	# plants	
AmfAmf Ds x At *amfamf*	6	914	13	1593	15	2229[a]	9	2569	7305
AmfAmf Ds x Bt *amfamf*	1	138	1	222	4	705	n.p.	n.p.	1065
AmfAmf Ds x St *amfamf*	n.p.	n.p.	n.p.	n.p.	4	247	2	243	490
AmfAmf Ds x Gt *amfamf*	4	1860	6	896	5	631	8	1615[b]	5002
AmfAmf DsSt x amfamf	21	2991	5	426	24	2818	12	1084	7319
AmfAmf DsGt x amfamf	8	348	4	78	10	364	5	76	875
Total # plants		6251		3215		6994		5587	22056

n.p.: not performed

[a] two phenotypic mutants were found in this set of plants

[b] one phenotypic mutants was found in this set of plants

In vitro *analysis*

To screen for the presence of the *Ds* element the phenotypic mutant 2147DsGt-139 and its parents were subjected to a kanamycin resistance test. Also a methotrexate resistance test was carried out to confirm the presence of a transposase gene. Genotype 2147DsGt-139 did express kanamycin resistance indicating the presence of the *Ds* element from parent 113Ds-6, but did not express a methotrexate resistance connected with the transposase source of parent 3004Gt-16. For the other two phenotypic mutants and its parents involving an At transposase source besides a kanamycin resistance test a GUS assay was carried out. The chimeric phenotypic mutant 2154DsAt-228 did not have GUS expression not indicating the presence of the transposase source of parent Ata7-4, but a kanamycin resistance was present which might be expressed by the transposase source of parent Ata7-4 or the *Ds* element from parent 113Ds-16 or both. So the screening for GUS expression is more informative for transposase than the test for kanamycin resistance. But it is possible that a construct can be present, for example according to Southern blotting, without showing expression. Southern blotting will probably lead to an overestimation for the presence of full length constructs whereas the expression will lead to an underestimation. The last phenotypic mutant 2101DsAt-310

did not express a kanamycin resistance, but surprisingly neither did its parent 102Ds-3, which was earlier found to have a *Ds* element by PCR (not shown). According to the GUS expression it also does not contain the transposase source of parent Ata7-1. However after a hygromycin resistance test on all phenotypic mutants to determine the excision of the *Ds* element the latter phenotypic mutant expressed a hygromycin resistance which is an indication for excision of the *Ds* element.

Table 3. Calculation of the theoretical minimum number of plants having the *Ds* element and the transposase source combined, based on the desired configuration, segregation and excision rate[a] in the greenhouse for four different selected distances for tagging of the GBSS gene.

Cross type	Distance to GBSS gene (cM)								Total # plants
	9		15		22[b]		41		
	Excision rate	# plants	Excision rate	# plants	Excision rate	# plants	Excision rate	# plants	
AmfAmf Ds x At *amfamf*[c]	38.30	88	38.30	153	48.42	405	38.30	246	891
AmfAmf Ds x Bt *amfamf*[cd]	14.81	5	14.81	8	42.66	113	14.81	n.p.	126
AmfAmf Ds x St *amfamf*[c]	14.81	n.p.	14.81	n.p.	42.66	40	14.81	9	49
AmfAmf Ds x Gt *amfamf*[c]	13.55	63	13.55	30	22.49	53	13.55	55	201
AmfAmf DsSt x *amfamf*[e]	14.25	213	14.25	30	26.63	563	14.25	77	884
AmfAmf DsGt x *amfamf*[e]	35.13	61	35.13	14	27.59	75	35.13	13	163
Total		430		235		1248		400	2314

n.p.: not performed

[a] excision rates from Chapter 4

[b] offspring originating from crosses which involve two *Ds* loci and as a result higher excision rates (Chapter 4)

[c] number of plants having the desired configuration calculated based on the segregation for *Ds* and transposase source multiplied with the jump rate as deduced from Chapter 4.

[d] the Bt transposase construct was similar to the St construct, so in this case the jump rate of St was used.

[e] number of plants having the desired configuration calculated based on a 1:1 segregation for *Ds* multiplied with the jump rate.

DNA analysis

To confirm the results of the *in vitro* tests a Southern blot analysis was performed on the phenotypic mutants and their parents. A screen for the presence or absence of the *Ds* construct was done by hybridization of a 1.7 kb *Bam*H1-*Hind*III fragment of the 5' end of the *Ds* (Figure 3). The transposase source was also hybridized with this probe. The chimeric phenotypic mutant 2154DsAt-228 did contain the *Ds* construct from parent 113Ds-16, but did not contain the transposase source of parent Ata7-4 according to this hybridization experiment (Table 5). The same is true for the second phenotypic mutant 2147DsGt-139. It did contain the *Ds* construct from parent 113Ds-6 but did not contain the transposase source of parent 3004Gt-16. The third and last phenotypic mutant

2101DsAt-310 did not contain the *Ds* construct but neither did its parent 102Ds-3 although it did contain the transposase source of parent Ata7-1 according to this blot (Table 5).

Table 4. Three phenotypic mutants and their parents tested for antibiotic[a] resistance and GUS expression *in vitro*.

Genotype		Starch type	Presence of *Ds* and/or transposase	Presence transposase	Occurrence of excision
			kan^R	GUS	hyg^R
Parent 1	113Ds-16 (22 cM)	wt	+	-	-
Parent 2	Ata7-4	*amf*	+	+	-
Chimeric mutant[b]	2154DsAt-228	*amf*	+	-	-
	2154DsAt-228	wt	+	-	-
Parent 1	102Ds-3 (41 cM)	wt	-	-	-
Parent 2	Ata7-1	*amf*	+	+	-
Phenotypic mutant	2101DsAt-310	*amf*	-	-	+
			kan^R	mtx^R	hyg^R
Parent 1	113Ds-6 (22 cM)	wt	+	-	-
Parent 2	3004Gt-16	*amf*	-	+	-
Phenotypic mutant	2147DsGt-139	*amf*	+	-	-

[a] antibiotics test for kanamycin (kan^R), methotrexate (mtx^R) and hygromycin (hyg^R)

[b] genotype 2154DsAt-228 was chimeric for the mutant phenotype (see Figure 2.)

Hybridization with a GBSS I cDNA probe revealed the allelelic composition of the phenotypic mutants and their parents but in case of parent 102Ds-3 an allele (termed A5) was found which was not earlier described by van der Wal (2000) (Figure 4, Table 5). The phenotypic mutants 2154DsAt-228 and 2147DsGt-139 both had the allele composition A1A1. The phenotypic mutant 2101DsAt-310 had the allele composition A5A1. Also an insertion of a *Ds* element in the GBSS gene can be revealed by this hybridization. However, no aberrant bands were found for an insertion of a *Ds* element into the GBSS gene.

Another confirmation of the presence or absence of the *Ds* construct was done by hybridization with a 1.6 kb *PstI-Kpn* fragment containing HPT (Figure 5). This hybridization showed the presence of the donor sites containing the HPT gene (Figure 1). The segregation for the bands 1 and 3 and 2 from Chapter 4 was also clearly visible in these cross combinations. The chimeric phenotypic mutant 2154DsAt-228 did contain a *Ds* construct, as did its parent 113Ds-16 (Table 5). The same is true for the second phenotypic mutant 2147DsGt-139, which contained a *Ds* construct like its parent 113Ds-6. The third and last phenotypic mutant 2101DsAt-310 did not contain a *Ds* construct but neither did its parent 102Ds-3 (Table 5). Additionally a plant of genotype DsA16-458 together with some double transformants (DsGtG5-62, 78 and 103) of a DsA16-458 *in vitro* plant were also hybridized

with this HPT probe. The double transformants all showed the parental type (DsA16-458) of bands (Figure 5).

Figure 2. One out of the 11 tubers harvested from plant 2154DsAt-228 which indicates the chimeric nature of the phenotypic mutation (dark is blue staining, gray is red staining).

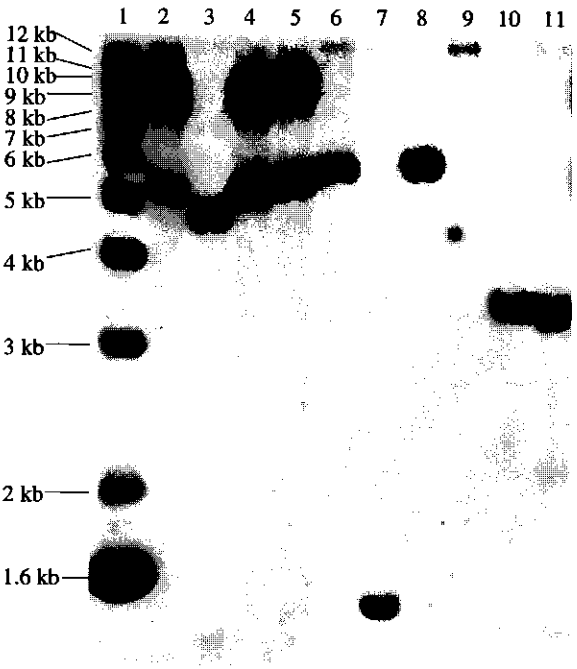

Figure 3. *Hind*III restriction of genomic DNA and hybridized with a 1.7 kb *Bam*H1-*Hind*III fragment of the 5' end of the *Ds* element of the vector pNH19HRS-3 which was used for the construction of the pHPT::*Ds*-kan construct to select for the presence of the *Ds* element and transposase source in *Ds* parent 113Ds-16 (lane 2), transposase parent Ata7-4 (lane 3), phenotypic mutant 2154DsAt-228 red staining tuber (lane 4), 2154DsAt-228 blue staining tuber (lane 5), *Ds* parent 113Ds-6 (lane 6), transposase parent 3004Gt-16 (lane 7), phenotypic mutant 2147DsGt-139 (lane 8), *Ds* parent 102Ds-3 (lane 9), transposase parent Ata7-1 (lane 10), phenotypic mutant 2101DsAt-310 (lane 11) and the DNA marker (lane 1).

To study the phenotypic mutants in more detail, hybridization with a 3.7 kb *Pst*I fragment of the construct pHPT::*Ds*-Kan was carried out to confirm the presence of full donor sites (FDS) containing

the *Ds* element in original position and to detect empty donor sites (EDS) containing a smaller sized donor site after excision of the *Ds* element. As a control several DsA16 plants of El-Kharbotly et al. (1996) were included in this hybridization. This hybridization could also show reinsertion of the *Ds* element at various positions. This blot confirmed the findings about the FDS of the previous blot (Figure 6, Table 5). The additionally tested cross parents DsA16-X and DsA16-458 showed a remarkable result. The DsA16-X plant clearly showed an EDS indicating an excised *Ds* element.

Table 5. Three phenotypic mutants and their parents tested by southern blot after *Hind*III digestion for their allelelic composition for GBSS, presence of the pHPT:: *Ds*-Kan construct, after *Pst*I digestion the presence of full donor site (FDS) and/or empty donor site (EDS) and the FDS and EDS tested by PCR.

Genotype		*Hind*III digestion			*Pst*I digestion			PCR		
		GBSS probe alleles	1.7 kb *Ds* probe 5' *Ds* construct[a]	Tpase	1.6 *Pst*I-*Kpn* HPT probe 3' *Ds* construct[a]	NOS probe FDS (4 kb)	NOS probe EDS (2.3 kb)	FDS (3.7 kb)	EDS (450 bp)	Tpase (1.1 kb)
Ancestor	A16	1:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Parent 1	113Ds-16 (22 cM)	1:1	+	-	+	+	-	n.d.	n.d.	n.d.
Parent 2	Ata7-4	1:1	-	+	-	-	-	n.d.	n.d.	n.d.
Chimeric mutant[b]	2154DsAt-228(*amf*)	1:1	+	-	+	+	-	+	-	+
	2154DsAt-228 (wt)	1:1	+	-	+	+	-	+	-	+
Parent 1	113Ds-6 (22 cM)	1:4	+	-	+	+	-	n.d.	n.d.	n.d.
Parent 2	3004Gt-16	1:1	-	+	-	-	-	n.d.	n.d.	n.d.
Phenotypic mutant	2147DsGt-139	1:1	+	-	+	+	-	+	-	-
Parent 1	102Ds-3 (41 cM)	4:5	-	-	-	-	-	n.d.	n.d.	n.d.
Parent 2	Ata7-1	1:1	-	+	-	-	-	n.d.	n.d.	n.d.
Phenotypic mutant	2101DsAt-310	5:1	-	+	-	-	-	-	-	+

n.d. = not determined

[a] the *Ds* construct consisted of the pHPT::*Ds*-Kan construct (Figure 1)

[b] the chimeric mutant is shown in Figure 2.

The gel electrophoreses of PCR products to show full and/or empty donor sites (EDS) with primers p1 and p2 are shown in Figure 7. The *amf* control showed some a-specific bands, which are also found in the phenotypic mutants. The chimeric phenotypic mutants 2154DsAt-228 and 2147DsGt-139 did contain a FDS but according to this PCR no EDS was found (Table 5). The same is true for the second phenotypic mutant, which contained a FDS like its parent 113Ds-6 but no EDS. The third and last phenotypic mutant 2101DsAt-310 did not contain a FDS but neither did its parent 102Ds-3, also no EDS were found in this phenotypic mutant according to this PCR (Table 5).

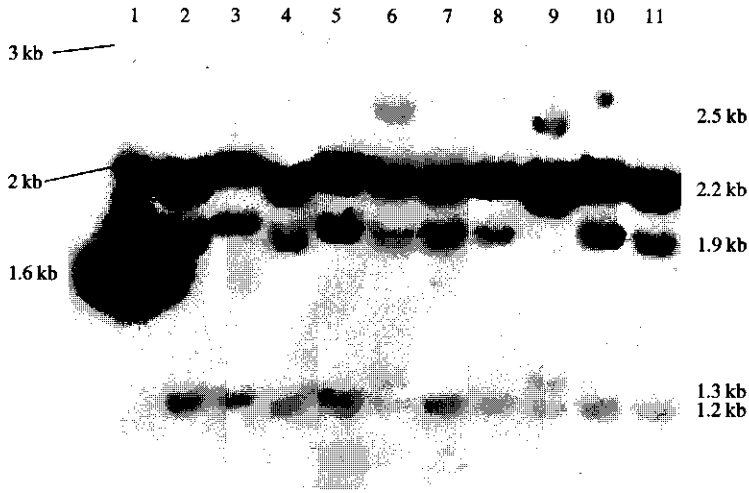

Figure 4. *Hind*III restriction of genomic DNA and hybridized with a GBSS I cDNA probe pGB6 (Visser et al., 1991) to determine the allele composition of *Ds* parent 113Ds-16 (lane 2), transposase parent Ata7-4 (lane 3), phenotypic mutant 2154DsAt-228 red staining tuber (lane 4), 2154DsAt-228 blue staining tuber (lane 5), *Ds* parent 113Ds-6 (lane 6), transposase parent 3004Gt-16 (lane 7), phenotypic mutant 2147DsGt-139 (lane 8), *Ds* parent 102Ds-3 (lane 9), transposase parent Ata7-1 (lane 10), phenotypic mutant 2101DsAt-310 (lane 11) and the DNA marker (lane 1).

Sequencing of approximately 30 DNA samples of each of the two red staining phenotypic mutants 2154DsAt-228 and 2147DsGt-139 containing the A1 allele showed that no wildtype allele could be amplified at the place of the original *amf* allele (Table 6). Also the blue staining control showed no straight 1:1 segregation indicating a preferential amplification of the *amf* allele. This could also be an indication that in both new *amf* alleles the fragment of 624 bp could not be amplified.

DISCUSSION

Populations

According to the results of Chapter 4 we were able to estimate the number of plants having the desired plant configuration for transposon tagging. Table 3 indicates that the chance of finding a mutant is the highest in populations having the *Ds* element at 22 cM which are crossed with the plants containing the At transposase construct or double transformed with the St transposase construct. However the phenotypic mutants found all resulted from crossing populations of single transformants. No red staining genotypes were found in case of offspring of the double transformants although they have the same number of excised plants. This might be explained by the high level of germinally transmitted excision of somatic excisions within the basic double transformant which leads to several plants in the offspring with the same excision and insertion

pattern (Enckevort et al., 2000; Chapter 4). Because of this, the number of individually different transposition events is probably much lower in the offspring of the double transformants than in the offspring of single-transformants and will, therefore, lead to a considerable overestimation of the number of desired plants. The number of populations does not seem to have any influence on the frequency (Table 2) of finding a phenotypic mutant which is to be expected because the essential genetic composition of all plants from a certain distance to GBSS is the same.

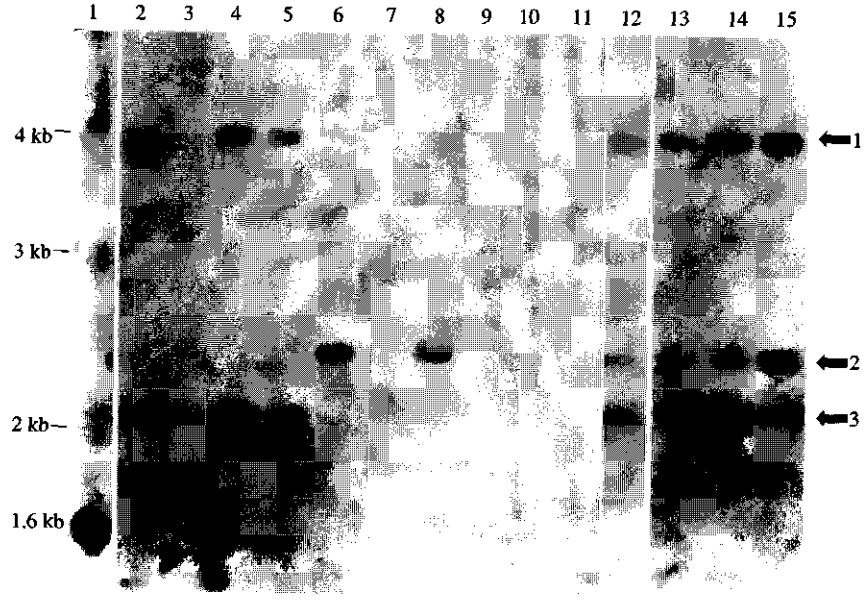

Figure 5. *Pst*I restriction of genomic DNA and hybridized with a 1.6 kb *Pst*I-*Kpn* fragment of the construct pHPT::*Ds*-kan, containing the 3' end of the construct: HPT, to confirm the presence of the pHPT::*Ds*-kan construct in *Ds* parent 113Ds-16 (lane 2), transposase parent Ata7-4 (lane 3), phenotypic mutant 2154DsAt-228 red staining tuber (lane 4), 2154DsAt-228 blue staining tuber (lane 5), *Ds* parent 113Ds-6 (lane 6), transposase parent 3004Gt-16 (lane 7), phenotypic mutant 2147DsGt-139 (lane 8), *Ds* parent 102Ds-3 (lane 9), transposase parent Ata7-1 (lane 10), phenotypic mutant 2101DsAt-310 (lane 11), cross parent DsA16-458 (lane 12), double transformants DsGtG5-62, 78 and 103 (lane 13,14 and 15) and the DNA marker (lane 1). The arrows indicate the two loci of the cross parent (first locus 1,3; second locus 2).

Screening

In the 3D-PCR screening some PCR products were found, however, it was not possible to identify potential mutants because these products were only found in one sample, which could not be repeated. Therefore, we were not able to point out one specific plant based on the 3D-approach. Enckevort et al. (2000) found that activation of the *Ds* element resulted in chimeric plants with small sectors of independent excision events. This might be an explanation for the finding of a PCR product in only one of the three samples, which is caused by chimerism for the insertion of the *Ds*

element in the GBSS gene. The discovery of the phenotypic chimeric mutant 2154DsAt-228 (Figure 2) by iodine staining supports this explanation. It is concluded that the 3D-PCR method in its present form was not suitable to screen for *Ds* insertions in the GBSS gene in our plant material.

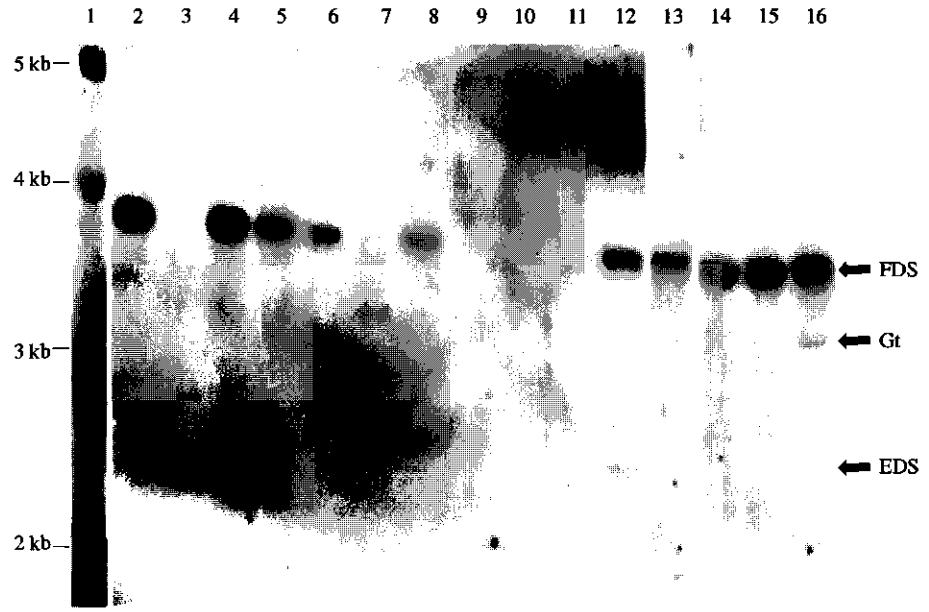

Figure 6. *Pst*I restriction of genomic DNA and hybridized with a 3.7 kb*Pst*I fragment of the construct pHPT::*Ds*-kan, containing the 5' end of the *Ds* element together with the NOS promotor (Figure 1.), to confirm the presence of the full donor sites (FDS = 3.7 kb), to possibly detect the empty donor site (EDS = 2.3) and GBSS*Ac* T-DNA (Gt) construct in *Ds* parent 113Ds-16 (lane 2), transposase parent Ata7-4 (lane 3), phenotypic mutant 2154DsAt-228 red staining tuber (lane 4), 2154DsAt-228 blue staining tuber (lane 5), *Ds* parent 113Ds-6 (lane 6), transposase parent 3004Gt-16 (lane 7), phenotypic mutant 2147DsGt-139 (lane 8), *Ds* parent 102Ds-3 (lane 9), transposase parent Ata7-1 (lane 10), phenotypic mutant 2101DsAt-310 (lane 11), cross parent DsA16-X (lane 12), parent DsA16-458 (lane 13), double transformants DsGtG5-62, 78 and 103 (lane 14–16) and the DNA marker (lane 1).

Table 6. Sequencing results of two phenotypic mutants.

Sequence		2154DsAt-228[a]		2147DsGt-139
		red staining	blue staining	
wt[b]	5'1...30-GTGTCAAGAAGCCAAACTTCAC... 3'	0[c]	12	0[c]
amf[b]	5'1...30-GTGTCAAGAAGCCAA CTTCAC... 3'	36[c]	21	32[c]

[a] phenotypic mutant 2154DsAt-228 was a chimeric mutant (Figure 2).

[b] positions and sequence according to van der Leij et al. (1991) starting at startcodon.

[c] significantly different from a 1: 1 segregation using $\chi^2_{1;1}(\alpha = 0.05)$.

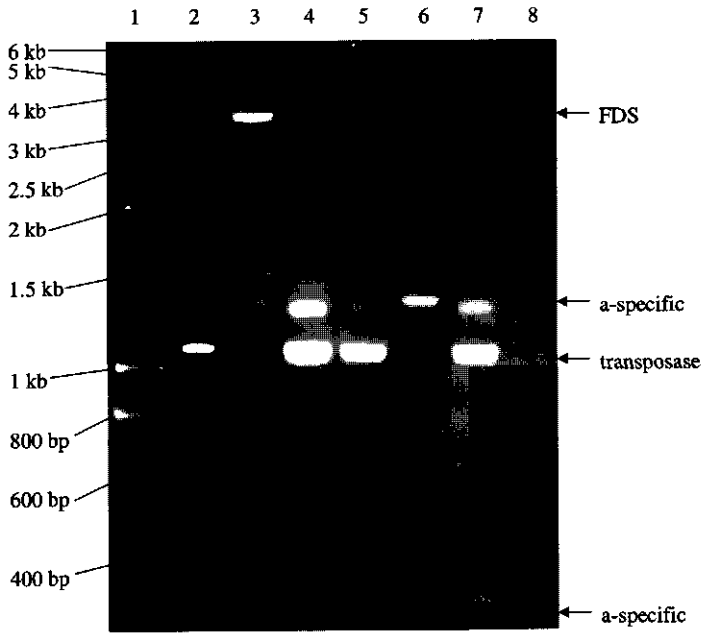

Figure 7. PCR using a pNOS and HPT primer (p1 and p2) to confirm the presence of the full donor sites (FDS = 3.7 kb), to possibly detect the empty donor site (EDS = 450 bp) and an At transposase construct, on a transposase carrying control Tm17-10 (lane 2) double transformant DsGtG5-62 (lane 3), phenotypic mutant 2154DsAt-228 red staining tuber (lane 4), 2154DsAt-228 blue staining tuber (lane 5), phenotypic mutant 2147DsGt-139 (lane 6), phenotypic mutant 2101DsAt-310 (lane 7), an *amf* genotype (lane 8) and the DNA marker (lane 1).

The occurrence of chimerism made it difficult to find a correlation between screening for *Ds* insertion in the leaves by PCR and the screening of the tubers for red staining starch (*amf* like) by staining tuber cut surfaces with iodine. Due to somatic transposition there might be differences in insertion into the gene of interest within one plant resulting in chimerism for the trait. The chimeric mutant found (Figure 1) shows evidence for this phenomenon. So in future research the genotype in the top of the plant found by PCR should be fixed by, for example, crossing or protoplast isolation followed by crossing to prevent loss of the mutated sector, which might not be present in the tubers.

In vitro *analysis*

Using *in vitro* tests for the genotypic characterization of the phenotypic mutants was difficult. It was not possible to discriminate between transposase source and *Ds* element in two phenotypic mutants and their parents due to the presence of a kanamycin resistance in both constructs (Chapter 4). Another problem was the use of GUS expression. When no GUS expression is found it can not directly be concluded that the construct, which contains the GUS gene, is not present. The

screening is purely based on the expression of GUS and will normally result in false-negatives. The same is true for kanamycin and hygromycin resistance. A *Ds* element might have excised from the donor site without resulting in functional restoration of the hygromycin resistance (Figure 1).

DNA analysis

Southern blot analysis allowed determination of the genotypic configuration of the phenotypic mutants and their parents. To confirm the presence of the *Ds* construct two hybridizations were carried out, one with a 1.7 kb *Bam*H1-*Hind*III fragment of the 5' end of the *Ds* construct and one with a 1.6 kb *Pst*I-*Kpn* fragment of the 3' end of the construct. There were no differences between these hybridizations, so the inserted constructs were still intact.

The Southern blot hybridization with the GBSS I cDNA probe showed the allelelic composition of the phenotypic mutants and their parents. There were no indications for an insertion of a *Ds* element in the GBSS gene because all alleles in the phenotypic mutants resembled the alleles that were found by van der Wal (2000). A new allele (termed A5) was found in the parent 102Ds-3, and in the mutant 2101DsAt-310, which was not described by van der Wal (2000). The A1A1 composition of the other two phenotypic mutants (2154DsAt-228 and 2147DsGt-139) might raise the question whether both A1 alleles are the original mutant *amf* allele or not. We conclude that this is not the case since the *Ds* element is present in both phenotypic mutants, which originated from the parents 113Ds-16 and 113Ds-6 respectively which were both *AmfAmf*. So even a rare recombination event will result in a gamete composition *DsAmf -Amf* in all cases. The *Ds* element will act as a marker for the presence of maternal gametes in the offspring. So it is evident that the phenotypic mutants 2154DsAt-228 and 2147DsGt-139 originate from another mutation than the original *amf* mutation, not enabling amplification of the GBSS specific PCR product using the two primers GBSS1 and GBSS6. Most likely as a result of the action of the *Ds* transposon or of a spontaneous mutation.

The Southern blot hybridization with a 3.7 kb *Pst*I fragment of the construct pHPT::*Ds*-kan, containing the 5' end of the *Ds* element together with the NOS promotor confirmed the presence of the *Ds* element in original position in the transgenic clones called full donor sites (FDS) which was already found by the hybridization with the 5' and 3' end of the *Ds* construct. With the latter probe also T-DNA loci conatining the Gt transposase gene could be revealed. In the phenotypic mutants no empty donor sites (EDS) were detected. However an EDS was found in the cross parent DsA16-X (Figure 6) which indicates endogenous transposase activity. To retest the phenotypic mutants for the presence of an empty donor site an EDS PCR was performed but this was not in full agreement with the Southern blots regarding the EDS. However the EDS PCR additionally gave indications for the presence of a transposase gene in the phenotypic mutant 2154DsAt-228.

The discovery of an empty donor site (EDS) within the cross parent DsA16-X, where no additional transposase gene is present, according to the Southern blots and *in vitro* tests, has enormous implications for the transposition of *Ds* elements in potato. From our results it can be deduced that transposition of a *Ds* element is possible without the presence of an additional transposase source. This indicates that there might be some endogenous transposase activity within the basic transformant DsA16-458. In potato the presence of endogenous transposon-like elements (Köster-Töpfer et al., 1990) or transposons, active (Pearce et al., 1996) or non-active (Oosumi et al., 1995) has been proven. These endogenous transposons might provide a transposase, which also recognizes the repeats of the introduced *Ds* element.

When there is transposition activity in a cross parent, this parent will act as a double transformant (Chapter 4). So germinal transmission of somatic transposition events will take place. This might be an explanation for the discovery of phenotypic mutants (2154DsAt-228 and 2147DsGt-139) where the *Ds* element is present but the heterologous transposase gene is absent. Double transformants were also used by Stuurman et al. (1998) in tomato. A stabilized *Ac* transposase source was used and in the individual F_2 plants without a transposase source they found an overall germinally transferred excision frequency of 13.5%.

Sequencing provided no additional evidence for a transposon acting as deactivating factor causing the phenotypic mutants. But it suggested the presence of two identical *amf* loci in the phenotypic mutants. Thus the mutation is caused by the *amf* allele or by another mutation in a gene unknown sofar to influence amylose content. One explanation is that some unknown recombination phenomenon resulted in two copies of the *amf* gene in the phenotypic mutants. However, it is unknown how two copies of the *amf* gene might end up in a diploid plant. A set of plants was derived from seed after pollination of a homozygous *AmfAmf* wildtype plant containing a *Ds* element as a marker with pollen of a homozygous *amf* mutant containing a transposase gene. Parthenogenesis can be excluded as explanation for this phenomenon because of the presence of the *Ds* element in two of the phenotypic mutants, which is maternally derived. The *Ds* element was found in two phenotypic mutants, which indicates the presence of a set of chromosomes from the homozygous wildtype parent. Another explanation might be partial deletion of the second (wt) allele blocking PCR amplification of the GBSS fragment. This will result in preferential amplification of the *amf* fragment only present in the phenotypic mutants. However, this explanation is not completely in agreement with the results of the Southern hybridizations. The results presented in this research indicate that the *Ds*-element is the mutating factor in these plants since such a high mutation frequency is not found in normal populations. For the true nature of the phenotypic mutants described additional research is needed.

Acknowledgements

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GENERAL DISCUSSION

ABSTRACT

The use of randomly induced mutagenesis and transposon tagging described in this research to obtain structural mutants, is compared. Also other potential ways to induce and identify stable structural mutants in potato are discussed together with a pathway for induction of mutations based on knowledge and properties of the gene(s) of interest. When performing mutation research one should first try to get as much background information as possible about the trait of interest. Based on this information it can be decided which mutation and selection procedure has to be applied. In this thesis two approaches of mutation breeding were used to induce structural mutations: 1. random mutagenesis using X-ray irradiation of monoploid potato material and 2. targeted gene tagging using *Ds* transposons in diploid potato of which the positions on the chromosomes are known. The trait of interest was alteration of starch composition using amylose-free (*amf*) starch as a model and inducing and selecting an amylose-extender like mutation in an *amf*-background by iodine staining.

BASIC PLANT MATERIAL

For both mutation induction and selection by transposon tagging or X-ray irradiation suitable plant material is needed. In case of recessive mutations the desired phenotype in potato could be screened in monoploid plant material after mutagenic treatment. The monoploid material produced in this study (Chapter 2) seemed to have all prerequisites for a successful mutation-breeding program. This monoploid plant material was used in two random mutation induction programs by irradiation of leaves followed by adventitious shoot induction and irradiation of whole plants followed by axillary bud propagation. However it had to be concluded that the monoploid material was not suitable enough for a successful large-scale mutation breeding program (Chapter 3). The most promising mutation breeding approach that has to be used in this respect was the adventitious shoot method which was also applied for isolation of the *amf* mutant in potato (Hovenkamp-Hermelink et al., 1987).

In case of transposon tagging new *amf* alleles were obtained. Three phenotypic mutants, with amylose-free starch were found in populations that resulted from crosses of two single transformants and not of crosses between double transformants and non-transformants (Chapter 5). It is obvious that the *Ds* containing plant material for transposon tagging must be easy to cross and to set seed.

However, the plant material used in this study, produced by El-Kharbotly et al. (1996), was male sterile and the female fertility was relatively low, but despite this disadvantage sufficient numbers of seed could be obtained. Generally it seemed that in this case the distance of the Ds element to the gene of interest for tagging was not highly important (Chapter 5). The crucial factor seemed to be that the tranposable element has to be located on the same chromosome arm as the target gene. However, our limited knowledge about the organization and activity of transposons within a chromosome might influence this view.

For both approaches the basic plant material is of vital importance. For the random mutagenesis approach the monoploid plants had to fulfill all requirements to be able to generate large plant populations after mutagenic treatment originating from one cell (Chapter 2). In case of transposon tagging fertile plants had to be available with several *Ds* elements on different locations of the target gene containing chromosome. For potato 50 to 60 well-characterized plants should be available which all have a *Ds* element on a known position of all chromosomes. Each chromosome arm should harbor four to five transposable elements on different positions. The desired plants should all have a single copy of the *Ds* element, of which preferably the excision pattern after combination with a transposase source is well known (Chapter 4) to predict the behavior of the *Ds* element. When a precise link is made in the future between the physical and genetic map of potato it might be possible to predict mutation frequencies when using transposons. Additionally transposase gene carrying plants should be available to induce transposition of the *Ds* element after crossing and large populations should be made. In spite of the fact that such a set of diploid *Ds*-containing plants existed the male sterility and not sufficiently female fertility does make the development of a set of Ds containing fertile plants necessary.

PROPERTIES OF THE TARGET GENE

When for the elimination of a certain trait a structural mutation is desired it is firstly important to gather information about this given trait. When the genetic background is unknown and mutations for this trait are desired than random mutagenesis might result in a mutant when it is possible to screen sufficient plants phenotypically for this alteration. Also when a trait is based on several genes it is still possible to use random mutagenesis. However, in a restricted number of cases it might be possible to use the transposon tagging approach in combination with phenotypic screening.

Screening for mutants

Direct screening for mutants in a random mutagenesis approach is a powerful method since direct identification of a recessive mutant is possible when using monoploid basic material (Chapter 3). This method has been successfully used in the past to isolate the *amf* mutant in potato (Hovenkamp-Hermelink et al., 1987). More difficult is the screening in a transposon tagging approach where an

indirect molecular screening should lead to the identification of insertional mutants (Chapter 5). In literature, this method did lead to tagging of particular genes. In our approach no proof was found for direct tagging of the *Amf* gene. This method was successfully (randomly) used in Arabidopsis (James et al., 1995) and petunia (Koes et al., 1995). However, the advantage of transposon tagging is the insertional mutagenesis of known genes, which do not have a clearly phenotypic effect. Another striking difference between both methods is that in case of random mutagenesis the mutation is irreversible, but in case of transposon tagging reversion might be possible.

MUTATION FREQUENCIES

In Chapter 1 it was shown that the starch containing tissue originates from approximately 50 initial cells. In Chapter 3 56 axillary buds out of 439 showed aberrant staining starch granules presumably originating from one initial cell. So the mutation frequency for these types of aberrant starch granules is approximately 2,5 x 10^{-3}. For the isolation of the first *amf* mutant in potato 3,000 microtubers were screened and three red-staining tubers were detected leading to a frequency of 10^{-3} (Hovenkamp-Hermelink et al., 1987). The mutation frequency in our study was higher than the one found by Hovenkamp-Hermelink et al. (1987), this might be explained by the differences in irradiation sensitivity of the genes of interest. The aberrant starch granules described in Chapter 3 might also be caused by mutations of different genes each with their own irradiation sensitivity.

The frequency of deactivation of the GBSS gene using transposons in Chapter 5 was about 4 x 10^{-4}. In tomato the *Cf9* gene was tagged from a position located at a distance of 3 cM, at a frequency of 10^{-3} *Ds* transposition events (Jones et al., 1994). The tagging of the FAE1 gene in Arabidopsis resulted from about 500-1000 transposition events of an *Ac* element at 22cM distance (James et al., 1995). The tagging of multiple genes in petunia was done by using an autonomous *dTph1* element, insertion alleles for the various genes were found at a frequency of about 10^{-3} plants (Koes et al., 1995). Higher frequencies are possible, for the tagging of the FEEBLY gene in tomato an additional selection was done for independent stable insertion of *Ds* and in doing so a mutation frequency of 1 in 150 was reached (van der Biezen et al., 1996). For the tagging of the DRL1 locus in Arabidopsis even a frequency of 1 in 50 was found by estimation of plants containing a transposed *Ds* element which were likely to represent primarily independent insertion events (Bancroft et al., 1993). So additional selection for independent stable insertions seems to raise the mutation frequency. Therefore, for transposon tagging in potato it is of great importance, to use selection markers such as NPTII and BAR which can be used in the greenhouse or a marker like GUS which can easily be used in material of greenhouse plants (Rommens et al., 1993; van der Biezen et al., 1996).

An alternative would be the activation of endogenous transposons. Endogenous transposons are, for example, present in the promotor region of an inactive patatin gene (Köster-Töpfer et al., 1990)

and in one of the introns of the potato starch phosphorylase gene (Camirand et al., 1990). These endogenous transposons might also be explored to induce mutations. Pearce et al. (1996) were able to activate the *Ty1-copia* group retrotransposons of potato during protoplast isolation analogous to the activation of transposable elements in tobacco.

Chapter 5 shows that it is possible to use *Ds* transposons for the direct targeting of the gene encoding GBSS. The three phenotypic mutants found are most probably the result of transposon insertion, but full proof has not been given. Another explanation for the observed GBSS mutation is normal spontaneous mutation of at least the mutation of the *amf5* GBSS allele in phenotypic mutant 2101DsAt-310.

CONCLUSIONS

Sufficient monoploid *amf* genotypes were found which could be used in an induction and selection procedure for *amfae* double mutants via the adventitious shoots method. The obtained blue staining starch granules (*amfae*) after irradiation of plant material indicated that the production of a double mutant is possible. Optimization of the adventitious shoot method followed by tuber induction are the next steps.

The obtained *amf* mutants are most likely caused by a transposon insertion followed by direct excision. The results clearly indicated that a cross performed between single transformants was most succesfull. The next step is to be able to detect potential mutants by PCR. Again optimization is very crucial here.

Transposition in potato using the *Ac/Ds* system is a real option. Depending on the basic plant material it is possible to obtain stable and unstable *Ds* insertion mutants. Their frequency is relatively high especially when an immobilized *Ac* element is used as transposase source.

In conclusion, it can be stated that for the start of mutation breeding experiments in potato it is important to use the right mutation breeding techniques based on knowledge of techniques and selection methods and properties of the gene(s) of interest. In Figure 1 a decision tree is depicted. Mutation breeding will eventually lead to an increase in genetic variation for the selected trait. In case of the potato starch biosynthetic pathway the additional genetic variation will lead to new natural starches which can be processed directly out of the tuber. This might open up the market for new and more applications and products which might be interesting for the potato starch industry.

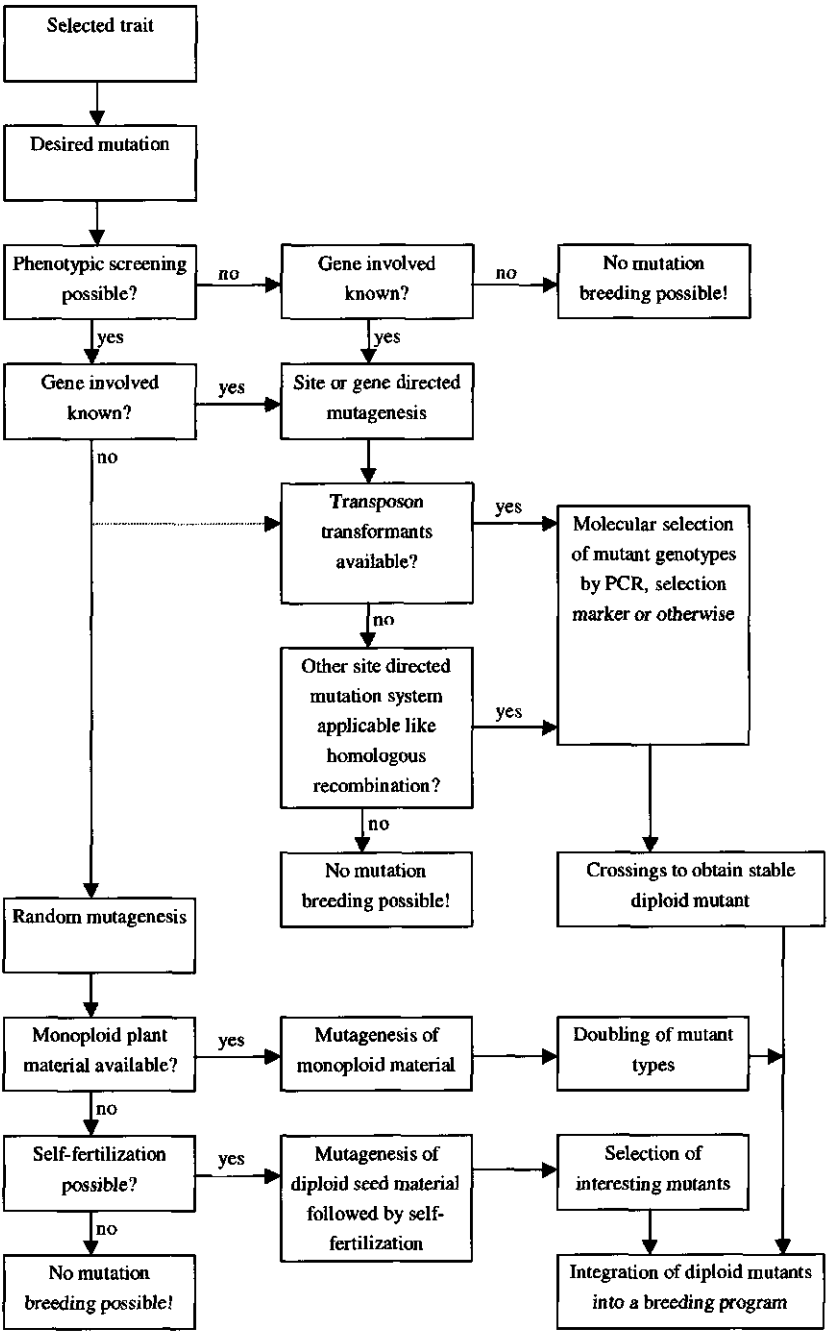

Figure 1. Decision tree for mutation breeding in potato based on knowledge and properties of the gene(s) of interest. (⋯➤ is possible but less favorable)

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SUMMARY

In all cultivated plants starch consists of a mixture of amylose and amylopectin. In a few of them like maize, rice, pea, wheat and potato, mutants are known having an altered starch composition and/or content. These new starch types are used for specific applications.

Through mutagenesis an amylose-free (*amf*) potato mutant was selected in 1986. To comply with the recessive nature of the trait and the tetraploid nature of the plant, conventional breeding for varieties will take 10-15 years. The wildtype *Amf* gene codes for granule bound starch synthase(GBSS) and was cloned in the late 80's. Transformation of potato clones with an antisense GBSS construct made it possible to generate amylose-free varieties in one step. This succes has negatively influcenced the interest in breeding research using the *amf*-mutant.
However, due to the social discussion about biotechnology and the absence of new transformants resulting in altered starch composition or content, there is nowadays an increased interest in breeding with the *amf*-mutant.

In this thesis two approaches were used to induce structural mutations in starch biosynthesis genes.

1. Production of new monoploid *amf* genotypes through parthenogenesis made it possible to initiate mutation breeding for *amfae* double mutants. Because of the expectation that the *ae* (amylose extender) mutation of the branching enzyme is recessive, monoploid material is essential. By inducing a mutation in one of the branching enzymes in an *amf*-mutant it is possible to select a double mutant having less branched amylopectin. This mutation can easily be identified by iodine staining. Amylose-free starch will stain red and less branched amylopectin will stain blue, like amylose containing starch. Mutations were induced by X-ray irradiation of leaf explants followed by adventitious shoot regeneration and microtuber induction or followed by several rounds of multiplication of axillary buds and microtuber induction. In both cases the starch of microtubers was stained with iodine to screen for aberrant types.
1. A second way to induce structural mutations was the use of the *Ac (Activator)/Ds (Dissociation)* transposase system of maize in potato were the *Ds* transposon is activated by a transposase source. In this study the *Ds* element was linked to the GBSS gene, of which the phenotypic effect of deactivation is known i.e. red staining starch after iodine staining. This known mutation was basis for the model study to determine the effectiveness of *Ds* transposition for mutation induction in potato.

Chapter 2 describes the research in which prickle pollination with *Solanum phureja* was used for the induction and selection of new solid monoploid amylose free potato plants. 26 monoploids were obtained and screened for several *in vitro* properties of importance for mutation breeding, such as vigor, leaf size, regeneration capacity, tuberization capacity and percentage somatic

doubling in the leaves. Finally, two *amf* monoploids were selected which fulfilled most of the prerequisites. In experiments with these two monoploids the ideal irradiation range for mutagenic treatment appeared to be 4 to 8 Gy of X-ray.

These monoploids were after mutagenic treatment tested for the phenotypic presence of the *amfae* mutation in two approaches. The first approach was adventitious shoot production followed by *in vitro* tuber induction. It was clear that the leaf explants of the irradiated plants had a regeneration frequency which was too low to find the desired mutation in a large scale experiment. The second approach was based on shoot multiplication for several generations in order to increase and stabilize mutant sectors. After X-ray irradiation of amylose-free (*amf*) monoploid plant material, 750 M_1 adventitious shoots were harvested. After two generations of shoot multiplication by axillary buds 14,000 M_1V_3 shoots were obtained. All these shoots were subjected to tuberization. Screening of the starch of these tubers was done at tuber level by iodine staining of cut surfaces to screen for aberrant starch phenotypes at macroscopic level. However, in this way, no aberrant starch phenotypes were observed. Additionally, based on the gelatinization profile determined by differential scanning calorimetry (DSC) at tuber level, three types with an altered profile were found. However, after shoot propagation for a few generations none of the types found by DSC were recovered (Chapter 3).

Iodine stained crude starch samples of tubers from 439 shoots were all individually tested microscopically for aberrant starch granules. These were classified in six different classes based on iodine staining pattern of individual starch granules from red to blue. In 56 tuber samples blue or otherwise abberant starch granules were found. Only tubers containing the most intensely blue staining starch granules were reevaluated in later generations. In 19 out of 29 cases the aberrant phenotype could still be recovered at starch granule level in later generations. In one case even 13 aberrant staining starch granules were found indicating an increase in the amount of aberrant granules. With this kind of observations, the concept of mutation breeding for starch variants in monoploid potatoes is proven. Further research is needed to stabilize and increase the mutant tissue induced (Chapter 3). The next step in order to obtain a *amfae* double mutant should be the irradiation of leaf tissue followed by adventitious shoot production and *in vitro* tuberization followed by selection of tubers having (partially) blue staining starch granules.

The second approach in this thesis was the application of the maize *Ac/Ds* transposable elements in potato. The known *amf* mutation was used as a model system to gather more information about the transposition frequency of the *Ds* transposable elements in potato and to test the tagging of the wildtype GBSS gene (Chapter 4 and 5). A few transformants harboring the non-autonomous *Ds* element were selected with linkage of the *Ds* element containing T-DNA insert to the GBSS gene on chromosome 8. Before large-scale experiments could be started excision of the maize non-autonomous transposable *Ds* element, induced by *Ac* transposase and visualized by hygromycin

resistance, had to be studied to determine the degree of transposition in potato (Chapter 4). Four *Ds* transposon containing plants were combined with the *Ac* transposase via cross combination or double transformation. To activate the *Ds* element three different transposase constructs were tested. Two transposase constructs consisted of the transposase cDNA isolated from the autonomous *Ac* element, driven by the 35S promotor or by the GBSS promotor. The third transposase source consisted of a natural, but stabilized *Ac* element. From the progeny of crosses between single transformants or crosses with double transformants the excision rate of the *Ds* element was calculated based on hygromycin resistance. Doing so 2010 plants were studied. Hygromycin resistance is expected when the *Ds* element has excised from the originally inserted construct. Excision rates ranged from 14.8-48.4%. In the progeny of double transformants besides early somatic excision in the seedlings also germinally transmitted excision of the *Ds* element occurred. The most effective transposase source in potato proved to be the stabilized *Ac* element with an average excision rate of 38.3%. Additional excision in the offspring of double transformants was provided by a GBSS promotor (which is also active in ovary and pollen) driven transposase source. Combining the transposase source and the *Ds* element by crossing has the advantage that excision is only to be expected in the offspring, however, it will most probably lead to unstable *Ds* insertions. Double transformants in contrast will result in both stable and unstable *Ds* insertions after crossing (Chapter 4). Based on these findings it was possible to pursue the inactivation of the GBSS gene in the model system.

Single transformants as well as double transformants were used to produce a population of over 22,000 plants. These plants contained both *Ds* element and transposase source for the possible inactivation of the gene for granule bound starch synthase (GBSS) by *Ds* insertions, which is located distally on the long arm of chromosome 8 (Chapter 5). Because the phenotypic effect of deactivation of GBSS is known, visualization of an insertion of a *Ds* element in the GBSS gene in the model system was possible. First an attempt was made to screen at molecular level for a potential insertion of the *Ds* element into the GBSS gene, but this gave no conclusive results. Three phenotypic starch mutants were found after screening of all 22,000 genotypes by iodine staining of tuber cut surfaces. These amylose-free mutants were analyzed by *in vitro* tests, Southern blot hybridization and sequencing. Strong indications were found that inactivation of the GBSS gene was caused by a transposable element, which might have inserted the GBSS gene and has excised again. Also indications were found for endogenous transposon activity in a crossing parent harboring only a *Ds* element (Chapter 5).

Based on promotor sequences 4 major groups of wildtype *Amf* alleles were found and encoded A1, A2, A3 and A4, respectively. Another wildtype allele, coded A5, was added as a result of the analysis of the plant material used in this study. The original *amf* mutation was derived from the wildtype A1 allele having a 1 bp deletion in the transit peptide. Two of the phenotypic mutants found were derived from the same wildtype A1 allele. However, the third mutant was derived

from the A5 wildtype allele. The next step will be further molecular characterization, of these new *amf* mutants, to find full proof for thepossible three new *amf* alleles.

In conclusion, it can be stated that new monoploid and solid *amf* genotypes were produced which can serve as basis for the induction and selection of *amfae* double mutants. The random mutagenesis appraoch using X-ray irradiation has shown to result in small sectors with blue staining strach granules (*amfae*). The most promising method to select such a mutant seems to be the the adventitious shoot method combined with *in vitro* tuber induction. The *Ds* insertional mutagenesis approach has shown that activation by *Ac* transposase of this element is possible. The model system concerning the *amf* mutation has shown that such an insertional mutation is most frequently found in the progeny of crosses of single transformants. This research indicates that this mutation induction system is functional in potato when an excellent phenotypic test is available like the iodine staining of starch.

SAMENVATTING

In alle cultuurgewassen komt reservezetmeel als een mengsel van amylose en amylopectine voor. In een aantal gewassen zoals maïs, rijst, erwt, tarwe en aardappel zijn zetmeelmutanten bekend waarbij alleen amylopectine of amylopectine met een verminderde vertakkingsgraad als zetmeel aanwezig is. Deze nieuwe zetmeeltypen worden voor specifieke toepassingen gebruikt.

Bij aardappel is via mutagenese in 1986 een amylose-vrije (*amf*) zetmeelmutant verkregen. Gezien het recessieve karakter van deze eigenschap en het tetraploide niveau van de plant duurt het in de conventionele veredeling 10-15 jaar voordat hiermee raswaardig materiaal verkregen wordt. Het wildtype *Amf* gen codeert voor korrelgebonden zetmeelsynthase en is eind 80'er jaren gekloneerd. Antisense constructen voor dit gen waren in staat bestaande rassen via transgenese in één stap amylose-vrij te maken. Dit succes heeft het veredelingsonderzoek met de *amf*-mutant op een laag pitje gezet. Door de huidige discussie over biotechnologie is het kweekwerk met de *amf*-mutant weer gestimuleerd. Het moleculaire werk met andere enzymen uit de zetmeelsynthese heeft bij aardappel via antisense genen geen andere successen met nieuwe zetmeelveranderingen gebracht. Dit uitblijven van nieuwe resultaten heeft bij aardappel de klassieke mutatieveredeling weer opnieuw onder de aandacht gebracht. In dit proefschrift wordt op deze benaderingswijze via verschillende invalshoeken ingegaan. Het onderzoek had twee doelstellingen:

1. door nieuwe monoploide *amf* planten via parthenogenese te isoleren werd de basis gelegd voor de mutatieveredeling van *amfae* dubbelmutanten. Omdat het hier bij *ae* (amylose extender) om een recessieve mutatie in één van de vertakkingsenzymen gaat is bij aardappel monoploid uitgangsmateriaal nodig. Door een mutatie te induceren in één van de vertakkingsenzymen bij de amylose-vrije mutant is het mogelijk om een dubbelmutant te verkrijgen met minder vertakt amylopectine. Deze mutatie is eenvoudig te scoren na jodiumkleuring van het zetmeel. Amylose-vrij zetmeel kleurt hiermee rood en amylose-vrij zetmeel, dat minder vertakkingen (*amfae*) bevat, kleurt weer blauw evenals amylose bevattend zetmeel. Mutaties werden geïnduceerd met behulp van Röntgenstraling gevolgd door adventiefscheutvorming en microknolinductie of gevolgd door cycli van okselknopvermeerdering en microknolinductie. In beide gevallen werd het zetmeel van de microknollen met jodium gekleurd en onderzocht.

2. een tweede manier om tot mutatieinductie te komen was met de hulp van insertie van het *Ds* transposon dat door transposase van het *Ac*-element geactiveerd wordt. Het *Ac (Activator)/Ds (Dissociation)* transposonsysteem is afkomstig van maïs. In dit geval is met een *Ds* element gewerkt dat gekoppeld was met het wildtype KGZ = *Amf allel*. Dit gen geeft na uitschakeling amylose-vrij zetmeel. Deze fenotypisch bekende mutatie is als modeleigenschap gebruikt om vast te stellen hoe effectief *Ds* transpositie voor mutatie-inductie in aardappel kan zijn.

In hoofdstuk 2 is het onderzoek beschreven dat na prikkelbestuiving met *S. phureja* tot de isolatie van 26 nieuwe monoploiden met amylose-vrij zetmeel heeft geleid. Deze planten zijn beoordeeld op een aantal *in vitro* eigenschappen zoals groeikracht, bladgrootte, regeneratievermogen, microknolvorming en percentage somatisch verdubbelde cellen in bladeren die allen van belang zijn om tot mutatie-inductie en selectie van de dubbelmutant te komen. Er werden uiteindelijk 2 *amf* monoploiden geselecteerd die het beste aan de basisvoorwaarden voldeden voor mutatieverdeling. De optimale dosisrange voor mutagene behandeling met Röntgenstraling bleek tussen 4 en 8 Gray te liggen.

De monoploiden werden na bestraling op twee manieren op het fenotypische optreden van de *amfae* mutatie getoetst. De eerste was via adventiefscheutvorming aan bladexplantaten gevolgd door *in vitro* knolinductie. Het was duidelijk dat bladexplantaten van bestraalde planten relatief een te lage regeneratiefrequentie hadden om op voldoende grote schaal naar de gewenste fenotypische mutant te kunnen zoeken. Bij de tweede methode werd gebruik gemaakt van vegetatieve vermeerdering van okselknoppen van bestraalde *in vitro* planten gedurende meerdere generaties. Op deze wijze werd aan aanwezige mutante sectoren de kans gegeven zich uit te breiden en te stabiliseren. Van de behandelde in vitro planten werden explantaten met 750 M_1 okselknoppen geoogst. Na twee maal vegetatieve vermeerdering werden 14.000 M_1V_3 scheuten geproduceerd. Al deze scheuten werden gebruikt voor de productie van microknollen. Het screenen van deze knollen op afwijkend zetmeel werd met jodium gedaan zonder dat dit een gewenste variant opleverde. Daarnaast werd zetmeel van de afzonderlijke knollen ook met behulp van differential scanning calorimetrie (DSC) getest waarbij het geleringsprofiel van het zetmeel zichtbaar wordt gemaakt. Hierbij werden drie afwijkende profielen ontdekt die na een aantal vegetatieve vermeerderingen van de bijbehorende plant niet terug gevonden werden (Hoofdstuk 3).

Jodium gekleurde zetmeelmonsters van knollen van 439 scheuten werden individueel microscopisch op afwijkingen getoetst. Deze afwijkende zetmeelkorrels werden in zes klassen ingedeeld variërend van rood naar blauw. Er werden 56 monsters gevonden waarin blauwkleurende of andere afwijkende korrels gevonden werden. De knollen met enkele zetmeelkorrels met de meest intense blauwkleuring werden na een aantal vegetatieve vermeerderingen nogmaals op deze afwijking gescreend. In 19 van de 29 gevallen werd het afwijkende type zetmeelkorrel teruggevonden hetgeen een indicatie is voor een toename van het weefsel aandeel met afwijkende korrels. Het concept van het gebruik van *amf* monoploiden voor het vinden van afwijkende zetmeelkorrels is hiermee bewezen. Verder onderzoek naar het stabiliseren van het mutante weefsel is gewenst (Hoofdstuk 3). De vervolgstap voor het verkrijgen van de dubbelmutant moet op basis van deze ervaring toch gezocht worden in de bladbestraling

in combinatie met adventiefscheutvorming en knolinductie gevolgd door selectie van knollen met (sectoren) blauw gekleurd zetmeel.

De tweede benaderingswijze die in dit onderzoek werd toegepast voor het verkrijgen van zetmeelmutanten was het gebruik van *Ac/Ds* transposons uit maïs in aardappel. De bekende *amf* mutatie werd hierbij als modelsysteem gebruikt om meer inzicht enerzijds in het transpositiegedrag van *Ds* transposons en anderzijds van de inactivatie van het korrelgebonden zetmeel (KGZ) synthase gen te krijgen (Hoofdstuk 4 en 5). Een aantal planten met daarin het *Ds* transposon werd geselecteerd op basis van koppeling van de plaats van het *Ds* transposon bevattende T-DNA met de plaats van het KGZ gen op chromosoom 8. Voordat er grootschalige experimenten konden worden opgezet moest het excisiegedrag van het *Ds* transposon worden bestudeerd. Dat was mogelijk doordat excisie van het *Ds* transposon zichtbaar kon worden gemaakt met behulp van herstel van het hygromycine resistentiegen. Op deze wijze zijn 2010 planten bestudeerd. Vier *Ds* transposon bevattende planten werden gebruikt bij kruisingen en dubbel transformatie experimenten. Hierbij zijn drie verschillende transposase constructen, om het *Ds* transposon te activeren, getest. Twee constructen bestonden uit het transposase cDNA in het ene geval gecombineerd met de 35S-promotor en in het andere geval met de KGZ-promotor. Het derde construct bestond uit een gestabiliseerd natuurlijk *Ac* element met het eigen transposase gen. Van de nakomelingschappen van alle kruisingen tussen twee enkelvoudige transformanten en de kruisingen van de dubbel transformanten met ongetransformeerde planten werd de excisiefrequentie berekend op basis van de verkregen hygromycine resistentie. Hygromycine resistentie treedt weer op als het geïnserteerde *Ds* transposon dat tussen de promotor en het structurele deel van het resistentiegen aanwezig is, weggesprongen is. De excisie frequentie varieerde van 14,8 tot 48,4 %. In de nakomelingschap van de dubbel transformanten vindt naast de vroege somatische excisie van het *Ds* element ook nog overdracht van excisie uit de vorige generatie plaats. Het gestabiliseerde natuurlijke *Ac* transposon was de meest effectieve transposase bron voor het activeren van het *Ds* transposon met een gemiddelde excisie-frequentie van 38,3 %. Additionele excisie werd in de nakomelingschap van dubbeltransformanten gevonden met een transposase dat door de KGZ-promotor werd aangestuurd die actief is in vruchtbeginsel en pollenkorrels. Het voordeel van kruisingen tussen twee enkelvoudige transformanten was, dat excisie alleen voorkomt in de nakomelingschap, maar deze zal wel uitsluitend resulteren in onstabiele insertiemutanten vanwege de aanwezigheid van de transposase bron. Dit in tegenstelling tot het gebruik van dubbeltransformanten die naast onstabiele ook stabiele insertie mutanten zal opleveren (Hoofdstuk 4). Op basis van deze bevindingen was het mogelijk om inactivatie van het KGZ-gen in het model systeem te onderzoeken.

Naast kruisingen van dubbeltransformanten werden kruisingen van enkelvoudige transformanten gebruikt om een populatie van meer dan 22.000 zaailingen te genereren die zowel het *Ds* transposon als een transposase bezaten om via insertie tot inactivatie van het KGZ- gen te komen.

Omdat het fenotypische effect van inactivatie van het KGZ-gen bekend is, was het eenvoudig om inactivatie van dit gen door het *Ds* transposon zichtbaar te maken. Allereerst werd onderzocht of het mogelijk was om via PCR een insertie van het *Ds* transposon in het KGZ-gen in een mengmonster van een groot aantal planten direct aan te tonen. Deze benadering gaf in dit materiaal geen eenduidige resultaten.

Via de klassieke weg werden in dit materiaal 3 nieuwe amylose-vrije zetmeelmutanten gevonden. Van de 22.000 zaailingen werd het snijvlak van de knollen met jodium gekleurd. Alle 3 mutanten werden in de nakomelingen van kruisingen tussen enkelvoudige transformanten gevonden en niet in kruisingen met dubbeltransformanten. Deze drie mutanten werden voorlopig gekarakteriseerd door middel van *in vitro* testen, Southern blotting en DNA sequentiebepalingen. Er zijn indicaties gevonden dat de inactivatie van het KGZ-gen werd veroorzaakt door het *Ds* transposon zelf dat mogelijk in het KGZ-gen en er weer uit gesprongen is. Tevens werden er indicaties gevonden voor eigen (endogene) excisieactiviteit (mogelijk door een endogene transposasebron) in een plant met alleen het *Ds* element (Hoofdstuk 5).

Van het wildtype KGZ-gen zijn op basis van promotor sequenties tot nu toe 4 allelen gevonden die aangeduid worden met A1, A2, A3 en A4. In het onderhavige materiaal van dit onderzoek is een nieuw A5 allel toegevoegd. De oorspronkelijke *amf* mutant is afgeleid van het wildtype A1 allel door een 1 bp deletie in het leaderpeptide. Twee van de nieuwe mutanten zijn van dit zelfde wildtype A1 allel afgeleid. De derde mutant is afkomstig van het wildtype A5 allel. Moleculaire karakterisatie is de volgende stap in het onderzoek om definitief vast te stellen dat het hier om 3 nieuwe mutante allelen gaat.

Concluderend kan gezegd worden dat het huidige onderzoek nieuwe stabiele *amf* monoploiden heeft voortgebracht die als basis kunnen dienen voor de inductie en selectie van *amfae* dubbelmutanten. Het mutatie-inductie onderzoek met Röntgenstralen heeft laten zien dat mutaties mogelijk zijn die met jodium in plaats van roodkleurend blauwkleurend zetmeel geven die bij *amfae* dubbelmutanten verwacht worden. De beste methode om zo'n dubbelmutant te verkrijgen is de adventiefscheutmethode in combinatie met microknolinductie. Het onderzoek met *Ds* insertiemutagenese heeft aangetoond dat dit element met *Ac* transposase in dit materiaal geactiveerd kan worden. In het modelonderzoek met de amf mutatie is naar voren gekomen dat de amf mutatie vooral in nakomelingschappen van de kruising tussen enkelvoudige transformanten optreedt. Het lijkt erop dat deze methode in modelsystemen bij aardappel werkt als er een goede fenotypische toets aanwezig is zoals hier via de zetmeelkleuring met jodium.

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CURRICULUM VITAE

Timothy Johan Herman Hoogkamp werd op 5 april 1971 geboren te Doetinchem. In 1989 behaalde hij het HAVO-diploma aan de Gemeentelijke Scholengemeenschap 'Doetinchem' (GSGD) in Doetinchem. Aansluitend begon hij met de studie aan de Chr. Agragrische Hogeschool (CAH) in Dronten waar hij in 1993 afstudeerde met als hoofdrichting Plantenteelt en differentiatie Bedrijfsmanagement. Een belangrijk onderdeel van deze studie was een stage bij het aardappelkweekbedrijf Hettema in Emmeloord. Vervolgens stroomde hij door in de reguliere studie Plantenveredeling aan de Landbouwuniversiteit Wageningen (LUW). De doctoraal studie omvatte een drietal afstudeervakken. Tijdens het eerste afstudeervak deed hij een literatuurstudie bij de vakgroep Agrarische Bedrijfseconomie naar de kosten en baten van bedrijfshygiënische maatregelen met betrekking tot rhizomanie in suikerbieten. Tijdens het tweede afstudeervak bij de toenmalige vakgroep Plantenveredeling deed hij onderzoek naar correlatie tussen niet-waard resistentie en partiële resistentie in gerst tegen dwergroest. Tijdens een derde afstudeervak, eveneens bij Plantenverdeling, werd er moleculair cytologisch onderzoek uitgevoerd aan Alstroemeria. In juni 1996 rondde hij zijn studie af en begon zijn aanstelling als Assistent in Opleiding bij wat nu heet het laboratorium voor Plantenveredeling van Wageningen Universiteit (WU). De resultaten van het promotie-onderzoek staan beschreven in dit proefschrift. Sinds 1 juli 2000 werkt hij als Hoofd Veredeling bij Koppe Veredeling B.V. te Ermelo.

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