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Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.)

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Summary. An amylose-free potato mutant was isolated after screening 12,000 minitubers. These minitubers had been induced on stem segments of adventitious shoots, which had been regenerated on leaf explants of a monoploid potato clone after Röntgen-irradiation. The mutant character is also expressed in subterranean tubers and in microspores. Starch granules from the mutant showed a strongly reduced activity of the granule bound starch synthase and loss of the major 60 kd protein from the starch granules.

Key words: *Solanum tuberosum* – Mutant – Starch composition – Granule-bound starch synthase – amylose-free

Introduction

Two types of starch occur in plants: assimilatory starch is formed in the chloroplasts in green tissues as the result of photosynthesis. At night this starch is broken down into sugars, which are transported to other organs. Reserve starch is synthesized from the transported sugar-molecules. It accumulates in amyloplasts in specific storage organs, such as endosperm or perisperm in seeds, and in vegetative organs such as potato tubers (Shannon and Garwood 1984).

Normally, the major constituents of starch are amylose and amylopectin, which have different properties with respect to water solubility and iodine binding capacity. The amylose content in most reserve starches is approximately 20%–25% (Shannon and Garwood 1984). However, in many plant species mutants are known with altered contents of amylose and amylopectin. In maize (*Zea mays* L.) mutants exist containing starch almost without amylose (*waxy* mutants). Such mutants are also known in other plant species such as rice, barley, sorghum and amaranth (*Amaranthus hypochondriacus* L.). Mutants with an increased amylose content were found in maize, e.g., *dull* and *amylose extender* (Okuno and Sakaguchi 1984; Shannon and Garwood 1984). In all these cases starch in endosperm or perisperm was involved. To date, no mutant plant has been found in which the reserve starch in a vegetative organ, such as the potato tuber, is affected. From the work of de Nettancourt and Dijstra (1969) we know that mutation leading to amylose-free starch can occur in a *Solanum* species. These authors observed waxy-like microspores in developing buds of *S. verrucosum.* The spontaneous mutation frequency was extremely low: among 2.5 million microspores analysed, two showed the characteristics of amylose-free starch. Hovenkamp-Hermelink et al. (1987a) found occasional cells in minitubers of a monoploid clone of *S. tuberosum* showing reddish-brown starch granules when exposed to an I_2-KI solution. This indicated the presence of amylose-free starch in these cells. In order to obtain a solid plant mutant we carried out an experiment in which potato tubers, obtained on X-rayed plant material, were screened for an altered starch composition.

Waxy alleles in maize and other plant species proved to be recessive to the wildtype allele. Therefore, in the search for a potato mutant we used a monoploid clone ($2n = x = 12$) as starting material. Adventitious shoots formed on leaf explants of potato can be readily used for the isolation of mutants. According to Broertjes and van Harten (1985) such shoots arise mainly from single cells, since the large majority of the mutant plants which are obtained do not show chimerism. Large scale

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adventitious shoot regeneration on a suitable monoploid clone was developed by Hovenkamp-Hermelink et al. (1987a). Minitubers were induced on stem segments of these shoots in vitro. These minitubers with a starch composition similar to normal tubers (Hovenkamp-Hermelink et al. 1987b) were used for mutant selection. In waxy mutants of maize the activity of granule bound starch synthase was absent or largely diminished. This enzyme is reponsible for amylose synthesis in endosperm and pollen. Potato tubers also contain such an enzyme (Frydman 1963; Tsai and Kuo 1980). The isolation and characterization of the enzyme from potato has recently been described by Vos-Scheperkeuter et al. (1986). Therefore, when a potato mutant lacking amylose is found it can be characterized with respect to this enzyme.

Materials and methods

Plant material and mutagenic treatment

The androgenetically obtained monoploid *Solanum tuberosum* clone AM 79.7322 (kindly supplied by Prof. G. Wenzel, Institut für Genetik, Grünbach, West Germany) was used throughout. This genotype has relatively large leaves and firm stems when grown in vitro (i.e. characters required for easy induction of adventitious shoots and minitubers).

Shoot cultures were grown in vitro on a Murashige and Skoog medium (1962) supplemented with 1% sucrose and 0.8% agar. Temperature was 21 °C, 14 h light, light intensity 3200 lux. Vegetative propagation was by cutting. Under these circumstances the ploidy level of the monoploid plants is fairly stable (Tempelaar et al. 1985). Adventitious shoots and minitubers were produced according to Hovenkamp-Hermelink et al. (1987a). The leaves were X-irradiated just after floating using an ENRAF-Röntgen apparatus at 90 kV and 2 mA; no additional filter, HVL 1.2 mm Al at 78 kV, dose 8.5 Gy and dose rate 1.9 Gy/min. The maximum dose allowing moderate regeneration was 8.5 Gy. This dose was used in all experiments.

Ploidy level of adventitious shoots was determined by chromosome counts using the squash technique (Pijnacker et al. 1984). Chromosome doubling was performed by means of adventitious shoot formation (Hermsen et al. 1981; Hovenkamp-Hermelink et al. 1987a).

Isolation of variants

The amylose percentage in minituber starch was determined spectrofotometrically according to Hovenkamp-Hermelink et al. (1987b). During part of the investigation one minituber from an adventitious shoot was taken for starch analysis, and the remaining tubers grown from the same shoot were used for plant propagation. After finding indications of chimerism in shoots, a part of each analysed tuber was saved for propagation.

A portion of the tubers was pre-screened by visual inspection. The cut surface of minitubers, from which part had been removed, was exposed to iodine vapor produced by a diluted (1:3 v/v) Lugol's solution (I_2-KI, Merck), heated to 30 °C. Minitubers staining abnormally were analysed spectrofotometrically for determination of the amylose content.

Characterization of starch

In tubers. For the chromatographic analysis of starch the method of Lustinec et al. (1983) was used in a shortened form. Minitubers were extracted with 45% (w/v) perchloric acid. The cooled extract was neutralized with NaOH. Two volumes of 96% ethanol were added to part of the neutralized solution, and the resulting precipitate was filtered onto a strip of glass fiber paper (2.5 × 10 cm) which was placed between the two parts of a Millipore filter apparatus. After washing with a small volume of 96% ethanol the strip of glass fiber paper was dried and the chromatogram was developed with dimethylsulfoxide (DMSO) and stained by placing it on a piece of glass fiber paper which had been soaked in Lugol's solution.

In microspores. Microspores of young, unopened flowers were stained with Lugol and made translucent with a solution of oil cloves, xylol and chloralhydrat (1:1:1 w/w). The color of the starch granules was established microscopically.

Isolation of enzymatically active starch granules and assay conditions

For isolation of starch granules 12 minitubers were incubated separately with 4 ml 50 mM Na-citrate buffer, pH 4.8 containing 0.1% (w/v) pectolyase, 1.5% (w/v) cellulase, 0.6 M mannitol, and 25 mM $CaCl_2$ for 36 h at 30 °C, with gentle shaking. The protoplasts formed were collected by centrifugation (10 min, 5,000 × g), and lysed by resuspension in 50 mM Tris-HCl buffer pH 7.5 containing 5 mM $MgCl_2$, 1 mM EDTA, 100 mM KCl, and 30% (w/v) sucrose at 4 °C. The pellet was resuspended in the latter buffer (8 ml), and layered upon a 25 ml 75% (w/v) sucrose cushion in the same buffer (Shure et al. 1983). Starch granules were re-collected by centrifugation (10 min, 10,000 × g) and washed three times with acetone at −20 °C. The white powders were dried and stored at −20 °C until further use. The activity of the granule bound starch synthase (GBSS) was measured as described by Vos-Scheperkeuter et al. (1986), in the presence and absence of 10 mM parachloromercuribenzoate, a potent inhibitor of the enzyme, to correct for non-enzymatic absorption of label.

Gel-electrophoresis and immunoblotting

Proteins were extracted from the various starches as described by Vos-Scheperkeuter et al. (1986) and analysed on 10% SDS polyacrylamide gels, according to Laemmli (1970). Gels were stained for protein with $AgNO_3$ according to Wray et al. (1981) or immunoblotted.

For immunoblotting, proteins were transferred electrophoretically to nitrocellulose filters (Schleicher and Schuell 0.45 μm). The transfer was performed in 25 mM Tris-HCl buffer, containing 150 mM glycine and 20% (v/v) methanol, pH approximately 8.3, for 2 h at 0.35–0.40 A. Proteins bound to the nitrocellulose filter were analysed immunochemically as follows: the filter was washed 8 times with 100 ml icecold TTBS [20 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl and 0.05% (v/v) Tween-20] and antigens were detected by incubation with antiserum, diluted 1:250 in 100 ml TTBS, raised against the GBSS isolated from potato (Vos-Scheperkeuter et al. 1986). After incubation at room temperature for 16 hours, the filter was washed 8 times with icecold TTBS, and incubated with protein A-horseradish peroxidase conjugate, diluted 1:10,000 in 100 ml TTBS. The filter was then washed 8 times with 100 ml icecold TTBS and twice with 100 ml TBS (TTBS without Tween-20), at room temperature. Antigens were visualized according to the Bio-Rad instruction manual. All steps were performed with gentle shaking.

Results

Isolation of variants

About 9,000 minitubers obtained from 3,600 adventitious shoots were analysed spectrofotometrically for starch composition. Among them, two were found with clearly reduced amylose contents (approximately 5%). Shoots were raised from sister tubers of the two aberrant ones (i.e. tubers obtained from the same adventitious shoot). On these shoots a second generation of minitubers was induced. However, analysis of these tubers showed the presence of completely normal starch. The most likely explanation of this phenomenon is chimerism of the original adventitious shoots. Therefore, after this finding only a part of a tuber was used for starch analysis, and shoots were raised on the remaining part.

In order to speed up the screening, but at the same time sacrificing the possibility to isolate variants with an increased amylose content, a further 3,000 minitubers were pre-screened with the iodine vapor method before spectrophotometric analysis. It was assumed that amylose-free tubers would show up by a distinct color, as did imbibed kernels of waxy maize. Three red-staining tubers were detected: two stained completely reddish-brown (86.039 and 86.040) (Fig. 1), while the cut surface of the third tuber (86.041) was partly reddish-brown, partly blue. When analysed spectrophotometrically, 86.039 and 86.040 showed an absorption ratio (A618/A550) of 0.70, indicating the virtual absence of amylose (Hovenkamp-Hermelink et al. 1987b). A starch extract, stained with I_2-KI, showed maximal absorption at 550 nm, as does pure amylopectin (Hovenkamp-Hermelink et al. 1987b). Since the three aberrant tubers were found in a lot arising from only five to seven adventitious shoots, they all probably came from one chimera shoot. Progeny tubers induced on plants which had been raised on tubers 86.039 and 86.040 also contained amylose-free starch. The plants grown from 86.039 and 86.040 were still monoploid. Shoots were raised on tuber 86.040 and further propagated by shoot culture and minitubers. The resulting variant clone was denoted amf (1×). A diploid (2×) and a tetraploid (4×) form were obtained by adventitious shoot regeneration on leaf explants of the monoploid. Minitubers induced on these shoots were likewise amylose-free. When transferred to a temperature controlled glasshouse and grown in soil, amf-plants were as vital as wildtype. Their tubers also had amylose-free starch. Clone AM79.7322 (2×) flowered well but had low male and female fertility (Uiitewaal et al. 1987). Plants of amf (2×) showed the same characteristics, which renders sexual analysis cumbersome, but not impossible. The microspores of wildtype AM79.7322 (2×) contained blue staining starch granules, whereas the microspores of amf (2×) contained brown staining starch granules.

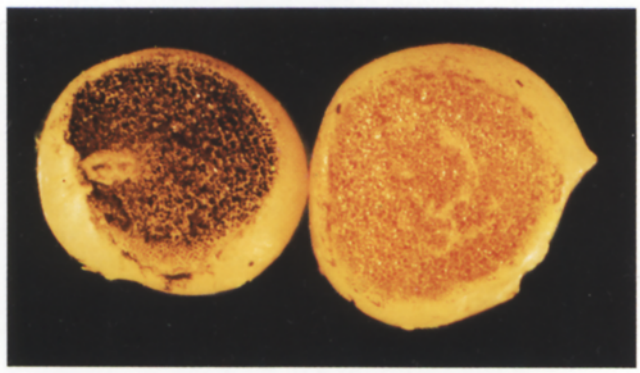

Fig. 1. The cut surface of minitubers after exposure to iodine vapor. The variant 86.040 (*right*) is amylose-free and stains reddish-brown in contrast to the dark blue staining wildtype AM79.7322 (*left*)

Biochemical analysis of the variant

Starch was extracted from minitubers and analysed chromatographically on glass fiber paper. After staining with I_2-KI solution, extract of clone AM79.7322 showed two spots: amylose, with dark blue color and moving with the solvent front, and amylopectin, reddish brown and on the loading spot (Fig. 2). An extract of amf tubers only showed the latter spot, confirming the absence of amylose.

The results of the SDS-polyacrylamide gel electrophoresis and immunoblotting are shown in Fig. 3. It is apparent that the major protein band from wildtype potato starch is missing in amf. Immunoblotting experiments with antibodies raised against the GBSS from potato confirmed the absence of this protein.

The activity of GBSS in wildtype and amylose-free potato starch was measured in duplicate. Wildtype starch showed an incorporation rate of 65.6 pmol/min/mg starch, using ADPG as a substrate, whereas amylose-free starch showed an incorporation rate of 2.5 pmol/min/mg starch. This result indicates that the activity of starch synthase in variant starch granules is only 4% of that in wildtype starch granules.

Discussion

In this paper we describe the isolation of an amylose-free potato variant. Biochemical analysis of the starch granules of this variant revealed two major characteristics: (1) starch granules lack the major protein of 60 kd which is present in wildtype starch granules and has

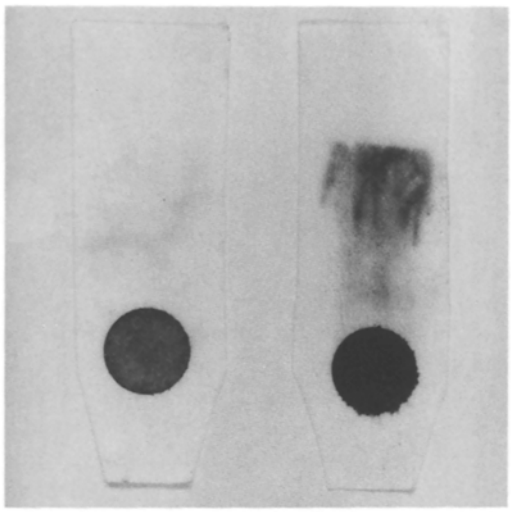

Fig. 2. Chromatographic analysis of starch extracted from minitubers of wildtype AM79.7322 and mutant amf. The mutant is amylose-free (*one spot*) and the wildtype contains amylopectin and amylose (*two spots*)

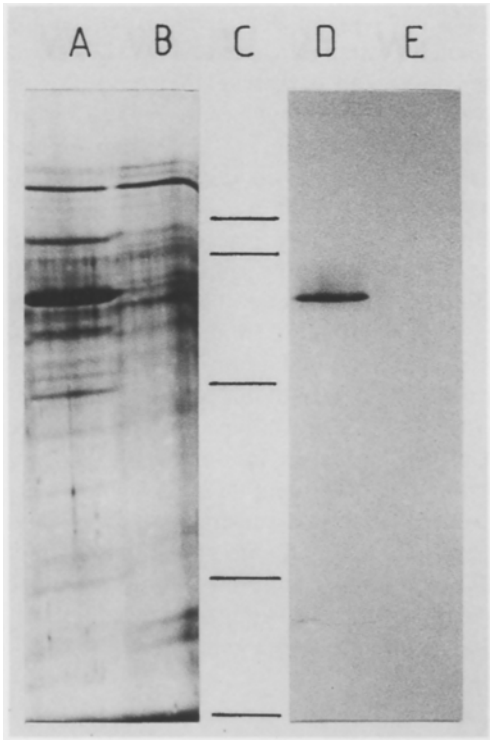

Fig. 3. Protein patterns of wildtype (*A*) and amylose-free (*B*) potato starches and immunoblots of the protein patterns tested with antibodies raised against the granule bound starch synthase (wildtype *D*, amylose-free *E*). Starch granules were (3 mg) extracted with denaturation buffer at 100 °C, and analysed by SDS gel-electrophoresis. *C* is a reference line. The lines are corresponding with the molecular weights of ovotransferrin (78 kd), albumin (68 kd), ovalbumin (45 kd), carbonic anhydrase (30 kd), myoglobin (front) (17 kd)

been identified previously as GBSS (Vos-Scheperkeuter et al. 1986). This conclusion is based on SDS-gels after both protein staining and immunological detection. (2) starch granules have a strongly reduced activity of GBSS as compared to wildtype starch granules (4% rest activity).

Based on these results we conclude that our amylose-free variant of potato is characterized by the absence of GBSS and is, therefore, analogous to waxy maize (Nelson et al. 1978). This conclusion, and the observation that the aberrant phenotype is stable upon vegetative propagation both in vitro and in soil, strongly point to a genetic basis for the absence of amylose in our potato variant. Moreover, the aberrant character is expressed in microspores, indicating that it passes through meiosis. This strengthens the conclusion that the amylose-free variant is a mutant. Definite proof will await the results of a genetic analysis.

Since only one mutant was found, no dependable estimation of the mutant frequency can be made. Moreover, the chimerism of adventitious shoots might point to a multicellular origin of these structures, rendering calculation of the number of mutants per cell even more difficult.

Evidence for chimerism in the shoots obtained from leaves of clone AM79.7322 not only came from the analysis of minitubers, but also from a number of chimeric chlorophyll variants (data not shown) and regenerated shoots with mixoploid chromosome numbers (Hovenkamp-Hermelink et al. 1987a). The suggestion that almost all adventitious shoots are derived from one cell (Broertjes and van Harten 1985) does not hold true for our material. Norris et al. (1983) also found indications for a multicellular origin of adventitious shoots.

Thus far, mutants with an altered composition of reserve starch were restricted to plants in which this starch is accumulated in the endosperm (maize, barley, rice) or in the perisperm (amaranth) of seeds. Our results show that in a plant in which reserve starch is accumulated in a vegetative organ, a mutation can lead to a type of starch in which amylose is absent. In view of the similarity between the enzyme involved in the synthesis of amylose both in a grain type reserve starch (in maize) and in tuber starch (in potato), this finding is not surprising.

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