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Isolation and characterization of potato-tomato somatic hybrids using an amylose-free potato mutant as parental genotype

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Summary. Using different genotypes of tomato and diploid potato, possessing alien selectable markers as well as endogenous markers, very high frequencies of protoplast fusion hybrids were obtained. One endogenous genetic marker, the amylose-free (*amf*) mutant of potato, was helpful not only for the confirmation of fusion products but also for the study of genetic complementation and the segregation of amylose-free starch in microspores. Cytological analysis of the fusion hybrids indicated that except for one which was hexaploid, all of them had a perfectly balanced chromosome number of allotetraploid constitution ($2n=4x=48$). Despite normal chromosome pairing and a diploid behaviour, the microspores in some of the fusion hybrids segregated for the recessive *amf*-locus. This anomalous segregation of a recessive character in these hybrids was shown not to be due to chromosome elimination or to the absence of the wild-type tomato *Amf* gene. Although all fusion hybrids were totally sterile, the hexaploid produced stainable pollen and berries with badly developed seeds. Embryo rescue has so far failed to produce backcross progeny.

Key words: *Solanum tuberosum* – *Lycopersicon esculentum* – Protoplast fusion – Amylose-free potato

Introduction

Distant hybrids that cannot be made by sexual methods can be obtained through somatic cell fusion. For example, while successful sexual hybridisation between potato and tomato has never been reported, such hybrids have been successfully produced through protoplast fusion (Melchers et al. 1978; Shepard et al. 1983).

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Based on morphological, biochemical and molecular analyses (Melchers 1978; Poulson et al. 1980; Schiller et al. 1982) Melchers (1978a, b, 1979, 1980, 1982, 1984) has pointed out some of the potential applications of protoplast fusion technology for crop improvement, as well as its limitations. In most of these applications an exchange (meiotic) of genetic material is required. The chromosome morphology, karyotypes and genetic maps (Gebhardt et al. 1991) of these two species are highly similar (Gottschalk and Peters 1955; Yeh and Peloquin 1965). Therefore, homeologous interchange of genetic material between them should be feasible. The transfer of genes between these species would be of value for broadening the genetic base of both these crops. Until now the sterility of this fusion hybrid has been the main bottleneck for a successful backcross program.

The methods used for the production of intergeneric fusion hybrids are becoming increasingly more efficient, partly because of the availability and introduction of selectable markers, such as resistance to kanamycin, hygromycin and herbicides, together with genes like B-glucuronidase (GUS). These markers are also useful in the backcross progenies of the fusion hybrids when they are linked to known chromosomes and genes. In addition to these marker genes of alien origin, there are a number of well known gene mutations within tomato and potato which are of basic as well as practical importance. One of these is the amylose-free (*amf*) mutant of potato which alters the starch composition through absence of the enzyme, granule-bound, starch synthase (GBSS; Jacobsen et al. 1989). This biochemical mutant affects the synthesis of amylose from starch and can be easily detected through the staining with iodine of any starch-containing tissue or cells such as tubers, stomatal guard cells of leaves, columella cells of root tips and microspores. Amylose-free cells stain red instead of blue as in the

wild-type. Because of this simple detection method, the amylose-free mutant is versatile in testing for genetic complementation, as well as for segregation in microspores and tubers in the fusion hybrids and their progenies.

With the long-term objective of introgressing alien genes and chromosomes across intergeneric and interspecific boundaries within the *Solanaceae* using newly available tools, we are applying both sexual and somatic cell-fusion methods. For this purpose, both alien and endogenous genetic markers are being used. Some of the results obtained in somatic fusion hybrids between the amylose-free mutant of potato and tomato form the subject of this article.

Materials and methods

Plant material

The potato fusion parents (*Solanum tuberosum*) and the earlier described amylose-free mutant diploids 87.1030/5 and 87.1029/31 (Jacobsen et al. 1989) were multiplied in vitro on MS30 medium (Murashige and Skoog 1962; MS with 30 g/l of sucrose). As the tomato (*Lycopersicon esculentum*) fusion parent, the albino mutant of cv Large Red Cherry (ALRC) (Hosticka and Hanson 1984) and selfed seeds of the kanamycin-resistant transformant ATW 4105 (Weide et al. 1989) were used. The albino character of the ALRC mutant was chloroplast encoded and its in-vitro multiplication was best on MS60. Recently, this albino mutant was successfully used in tomato-potato hybridisation experiments by Wolters et al. (1991). Kanamycin-resistant ATW 4105 shoots were selected from sterilized seeds germinated on MS20 + 100 mg/l of kanamycin and multiplied in vitro on the same medium. For protoplast isolation, potato shoots were grown in low glass jars containing 50 ml of MS10 and tomato shoots in high glass jars with 70 ml of culture medium. Transgenic parental plants and all fusion hybrids were grown according to Government safety rules in a temperature-controlled glasshouse. Plants were grafted on tomato root stocks for better growth and flower induction.

Protoplast fusion

In potato shoots grown on MS10 not much starch was accumulated in the leaf cells promoting regeneration of protoplasts. For the same reason tomato shoots were placed in the dark for 6–7 h in a refrigerator at 8 °C. The protoplasts were isolated according to Mattheij et al. (1991).

Protoplast fusion and culture

Protoplast fusion in poly-ethylene-glycol (PEG) was carried out according to Menczel et al. (1982). This protocol was modified by Wijbrandi et al. (1990) using 30% PEG MW 4000 instead of 40% PEG MW 6000. The PEG-treated protoplasts were cultured according to Wolters et al. (1991) until the second dilution. The second dilution was 14 days after PEG-treatment with TMD medium solidified with 0.2% agarose. One week later, the agarose-containing medium with the microcalli was transferred by pipette to solid-callus growth medium (MS11). The developed calli (2–5 mm) were cultured 6–7 days later on shoot-induction medium (MS12). After a few weeks the differentiating parts were transferred to shoot-elongation medium (MS13) and regenerated shoots were transferred to MS20-medium. The callus-growth, shoot-induction and shoot-elongation media were all according to Mattheij et al. (1991)

Cytological technique

Meiosis was studied in young anthers of the fusion hybrids and the diploid parents. Flower buds with suitable meiotic stages were fixed in a fresh solution of 3 parts of ethanol and 1 part propionic acid saturated with ferric acetate for a week or more. Fixed anthers were squashed in a drop of 2% acetocarmine following the routine procedure. All observations were made on well-stained temporary preparations and representative cells were photographed.

Morphological observations

Besides studies on the growth pattern of the fusion hybrids, four main types of morphological and phenotypic characters were taken into account. These were leaf and floral characteristics, tuberization, and pollen starch phenotype. The size of the leaves was measured from three representative samples. In all, five floral characteristics, viz., flower colour, shape of calyx and corolla, types of stamens and stigma extension, were analysed. Tuberization was estimated from mature plants grown in pots in the greenhouse. Pollen starch phenotype was determined by staining microspores with Lugol's solution. Isozyme electrophoresis was carried out according to Suurs et al. (1989).

Fertility, crossability and embryo rescue

The fertility of somatic hybrids was determined by two criteria: (1) pollen fertility based on stainability with lactophenol acid fuchsin and (2) female fertility based on crossability of fusion hybrids as seed parents with tetraploid pollinators. Pollen tube growth in the styles was monitored according to Martin (1959).

Starch composition determination

The amylose/amylopectin ratio in starch from tubers of greenhouse-grown potato plants and fusion hybrids was determined using the method described by Hovenkamp-Hermelink et al. (1988). Starch granules were isolated using the technique of Vos-Scheperkeuter et al. (1986).

Regeneration of protoplasts and detection of potential somatic hybrid calli

The potato clones 87.1029/31 and 87.1030/5 yielded about $4.3-5.0 \times 10^6$ protoplasts per gram of leaf tissue, whereas the tomato genotypes, ATW4105 and the ALRC mutant, yielded comparatively less – $1.4-1.9 \times 10^6$ protoplasts per gram of leaf tissue. Because tomato protoplasts regenerated much more slowly and only in restricted numbers as compared to potato protoplasts, this served as a criterion, in addition to other characteristics, for the selection of potential somatic hybrids (see later).

Screening of regenerants for potential hybrids

Potential hybrids were investigated for hybridity based on the following criteria: (1) growth in the presence of kanamycin, (2) morphological traits in vitro, (3) starch phenotypes determined from iodine staining and (4) the electrophoretic mobility of glutamate oxaloacetate transaminase (GOT: EC 2.6.1.1) isozymes. All regenrants of the S1 series were susceptible, while those of S2 were resistant, to kanamycin.

Results

Growth and morphological characteristics

Growth characteristics were analysed in some detail in 85 plants of both S1 and S2 series – 40 of S1 and 45 of S2

Table 1. Some flower characteristics of the tomato and potato parents used for somatic fusion

Characteristics	Tomato (ALRC + ATW 4105)	Potato (87.1029/31 + 87.1030/5)	Fusion hybrids S1 and S2 series
Flower colour	Yellow	White + purple	Yellowish white
Calyx	Deeply divided, regular	Less deeply divided, irregular	Deeply divided, regular
Corolla	Divided lobes (star)	Round	Star (S1), round (S2)
Stamens	United column, sterile tips	Single, fertile tips	United or single, fertile tips
Stigma extension	Non-protruded	Non-protruded + protruded	Protruded (S2), non-protruded (S1)

Table 2. Number of tuberizing and flowering fusion products of the S1 and S2 combinations producing some stainable pollen

Combination	Total	Number of fusion products		Stainable pollen
		Tuber set	Flowering	
S1	40	3	14	3[a]
S2	45	25	25	9
Total	85	28	39	12

[a] S1-61, 10–20% stainable pollen; all other genotypes $\leqslant 0.1\%$ or 0%; segregation of 140 blue and 16 red microspores after iodine staining

series. In plants grown in vitro, 13 of the S1 series but only two of the S2 series were relatively less vigorous than the parental genotypes. A month after transfer into soil in a greenhouse, however, there was a pronounced difference relative to in-vitro growth. In the S2 series six of the fusion hybrids showed retarded growth and 14 were more vigorous than the parents. No correlation was observed between hybrid genotypes with regard to a reduction of growth either in vitro or in vivo. In the S1 series, 25 of the fusion hybrids showed poor growth and four of them died before flowering. Among the S2 series, numbers 27, 29 and 36 were the most vigorous.

The leaves of the greenhouse-grown fusion hybrids displayed a large amount of variation. This was with respect to shape, size, arrangement, scarification and colour. Leaf arrangement was either pendulous or errect, the surface was smooth or irregular. The crenated leaf architecture of tomato was partially reflected in the somatic hybrids. Leaf colour varied from light green to dark green in both series.

In all, 39 hybrids produced flowers, almost all of them had uniformly yellowish white petals and the yellowish tinge was more evident just before and after opening of the flower. The purple anthocyanin pigmentation of 87.1029/31 was not clearly expressed in hybrids of the S1 series. In S2-12 and S2-23 the petals lacked a yellow tinge so that they looked almost pure white, comparable to the potato parent 87.1030/5. The lengths of the sepals exceeded those of the parents whereas petals were intermediate. The lengths of stamens were predominantly similar to those of the potato parent. The pistil length was heterotic, resembled the potato parent 87.1030/5 and protruded. Some of the floral characteristics that were investigated and their results are given in Table 1. The shape of the corolla was classified into only two types, viz., 'star' (more tomato-like) and 'round' (more potato-like). There was a slight difference between the S1 and S2 series with respect to corolla shape. In all S1 series of hybrids with the exception of S1-60 the corollas were of the star type, whereas a round type predominated in the S2 series of hybrids. Another clear difference between the potato and tomato parents was in the type and arrangement of anthers. In tomato the anthers were united into a column with the tips tapering, which are called 'sterile tips'. But in potato the anthers are independent from each other and their tips are normal. In the majority of fusion hybrids the anthers were loose and in some cases (Table 1) they appeared to be united into a column in the young flower bud. In these cases the mature flowers had loose anthers whose tips were slightly tapering but not sterile as in tomato.

Tuberization was observed in only three genotypes of the S1 series, whereas 25 of the S2 series tuberized, as shown in Table 2. Although stolon formation was observed in a few hybrids, in a majority of cases the tubers were produced so close to the main stem that no clear stolon part was detectable. The tubers of the fusion hybrids were strikingly similar to those of the parents and they appeared to be suitable for further propagation.

Ploidy level, meiotic behaviour and microspore phenotypes for amf character

Chromosome counts in the root tips of the fusion hybrids showed that all but one plant (S1-61) were tetraploid ($2n = 4x = 48$). This observation was also confirmed from chromosome counts in the pollen mother cells. Meiosis was investigated in the anthers of a sample of 20 flowering fusion hybrids (Table 3) with the following objectives: (1) to confirm their hybrid nature cytologically, (2) to

Table 3. Chromosome numbers, chromosome associations (average per cell) at metaphase-I and the types of sporads in the S1 and S2 series of fusion hybrids

Genotypes	Chromosome number (2n)	Number of cells studied	Chromosome associations	
			Bivalent	Univalent
S1-31	48	12	23.8	0.5
S1-42	48	15	23.4	1.1
S1-43[a]	48	14	22.6	2.4
S1-51	48	18	22.8	2.3
S1-56	48	12	23.6	0.8
S1-58	48	10	22.7	2.6
S1-60	72	nd	nd	nd
S2-3	48	15	22.3	3.5
S2-5	48	8	23.5	1.5
S2-12	48	13	22.2	3.5
S2-13	48	16	22.5	3.0
S2-27	48	11	23.1	1.8
S2-29	48	12	24.0	0.0
S2-31	48	18	23.4	1.2
S2-36	48	15	23.5	1.1
S2-41	48	17	23.1	1.8

[a] In three cells there were indications of multivalent formation

establish their chromosome number accurately, (3) to study the pairing behaviour and distribution of chromosomes and (4) to assess the quality of microspores and pollen grains.

Chromosome pairing was studied at prophase-I and metaphase-I stages. The hexaploid S1-61 (2n = 6x = 72) had a rather complicated meiosis, which was not analyzed in detail. A constant feature of all tetraploids was the formation of predominantly bivalents at prophase-I, pachytene and metaphase-I stages. Generally at pachytene one or a few bivalents were separated from the other chromosomes in the pollen mother cells and these could be identified. Because all 12 bivalents of tomato have distinct characteristics at the pachytene stage (Ramanna and Prakken 1967), advantage was taken of the pollen mother cells in which individual pachytene bivalents of tomato could be identified. Such an identification was helpful in confirming that all the 12 pairs of tomato chromosomes were intact in the fusion products. At metaphase-I, with one exception, there was autosyndetic pairing giving rise to exclusively bivalents (Table 3). Only in S1-43 was a low frequency of multivalents and univalents observed in addition to bivalents. Occasional univalent formation was also observed in other tetraploids but these univalents were due to precocious separation of bivalents rather than any lack of pairing.

The formation of bivalents in all tetraploids proved that they were undoubtedly fusion products and, as expected, behaved like allotetraploids. The hexaploid S1-61 formed several multivalents in addition to 12 or more bivalents and a low frequency of univalents.

A notable feature was that all plants had the perfectly balanced chromosome numbers of either a tetraploid (2n = 4x = 48) or a hexaploid (2n = 6x = 72). This means that, despite possessing chromosomes of alien genera, i.e., *Lycopersicon* and *Solanum*, there was no chromosome elimination. In spite of the formation of a high frequency of tetrads, however, there was only a low to a very low frequency of well-developed microspores and pollen grains in the anthers. Only S1-61 produced stainable pollen and up to 20% well-developed microspores. Two other hybrids of the S1-combination and nine of the S2 were highly male-sterile with very low frequencies of stainable pollen (< 0.1%). This means that the sterility of these plants could not be explained on the basis of chromosome pairing failure or of aneuploidy.

In six somatic hybrids starch-containing microspores were detected and analysed after iodine staining. In five of them the frequencies were very low but in the pollen-producing S1-61 it was fairly high. Segregation into blue and red starch containing microspores was found in all these hybrids. The frequency of red-staining microspores was relatively high in some cases (140 blue: 16 red in S1-61). The occurrence of red-staining microspores, which has a recessive inheritance, was unexpected in a typical allopolyploid.

Fertility

Pollen stainability was used as a criterion for male fertility. With the exception of S1-61, as mentioned earlier, all fusion hybrids proved to be highly male-sterile. Female fertility was determined by pollinating the styles of fusion hybrids with the viable pollen of 2x and 4x potato as well as tomato. To ensure that the pollinations were successful, pollen tube growth was monitored by staining the pollinated styles with aniline blue followed by fluorescent microscopy. The pollen tubes grew abundantly in the styles of fusion hybrids when pollinated with potato pollen whereas the tomato pollen was completely inhibited. Out of 83 pollinations with potato using 14 different fusion hybrids of the S1 series, not a single berry was set. However, S1-44 produced a spontaneous berry containing five deformed seeds, none of which germinated in vitro (Table 4). Over 1,000 pollinations using potato pollen were made on 25 fusion hybrids of the S2 series. In all, seven berries were obtained of which six were the products of crossing and one was probably spontaneous. Four berries form S2-29 possessed 135 relatively well-developed seeds. In-vitro germination of these unripe seeds (up to 20 and 34 days after pollination) was not successful.

Starch composition in tubers of fusion products

As expected, all harvested tubers synthesized amylose-containing starch which stained blue with iodine. This is

Table 4. Fusion products with berry and seed set spontaneously or after pollination with 4x potato

Fusion product	Berries	Number of			
		Seeds	Seeds/ berry	Germinating seeds	Pollinations
Spontaneous					
S1-44	1	5	5	0	0
S2-12	1	0	0	0	0
After pollination					
S2-5	2	4	2	0	5
S2-29	4	135	20.8	0	83

in accordance with the earlier described observations in other starch-containing tissues of these plants. Determination of starch composition in the parental *amf* clones resulted in the expected low percentage of amylose compared to over 21% in the wild-type cv. Bintje. The amylose percentages in tuber starch of the fusion hybrids was clearly intermediate varying between 8.1% and 13.7%.

Discussion

The first fusion hybrids produced by Melchers et al. (1978) had chromosome numbers ranging from 50 to 54, probably as a result of using cell suspensions as a protoplast source of potato in the fusion combination. In a later experiment, using leaf protoplasts, fusion hybrids with balanced chromosome numbers of 48 were found (Melchers 1980). Through somatic hybridization of tetraploid potato and tomato, Shepard et al. (1983) obtained near-hexaploid fusion hybrids which became unstable with respect to chromosome number during in-vitro culture.

The potato-tomato hybrids described here were stable allopolyploids after in-vitro multiplication. Almost all these hybrids were sterile despite being balanced allotetraploids derived from highly fertile parents. As has been pointed out by Melchers (1982) the isolation of fertile somatic hybrids might be possible when a greater number of different genotypes are hybridized and tested. The seed set of S1-29 and the pollen stainability of S2-61 are positive indications that functional pollen and egg cells do occur. In this new allopolyploid, fruit set with viable seeds has to be demonstrated before additional research can be carried out to detect and exploit its potential benefits.

The growth characteristics of the somatic hybrids were more potato-like as earlier indicated by the hybrids of Melchers et al. (1978) and Shepard et al. (1983). Tuber set depended on the combinations of genotypes used.

Starch composition of the somatic hybrids was investigated in all starch-containing tissues or complemented cells indicating that the GBSS-gene of tomato is also expressed in the tuber of the fusion hybrid. The observation that only 9–13% amylose is found in the tuber starch of fusion hybrids indicates that probably the tomato enzyme itself, and/or its regulation, is different and is not well integrated in the starch biosynthesis of the hybrid. Meiosis of the allotetraploid hybrids appeared to be normal and chromosomes showed clear indications of autosyndetic pairing. The microspores obtained are expected to be balanced in chromosome number containing one genome of potato and one of tomato and, therefore, always to be amylose containing (*Amf*/*amf*). Counts of S1-61 segregation for starch composition in the microspores showed that about 10% of them were red staining. An explanation for this phenomenon will be possible after successful back crosses with the *amf* potato and selection of amylose-free starch-containing offspring plants.

The availability of alien selectable markers and easily detectable markers together with endogenous markers, such as the *amf* gene and GOT, in the fusion hybrids is of obvious advantage. They are useful not only for detecting the fusion hybrids but also for monitoring their presence or absence in the subsequent sexual progeny, when these can be obtained. The advantage of an endogenous marker such as the *amf* gene is that its location in the genome is known, i.e., on linkage group 8 of potato (Gebhardt et al. 1989). Because the linkage groups of potato and tomato are nearly identical (Gebhardt et al. 1991), it is logical to infer that the wild-type *Amf*, corresponding to the potato *amf* locus, is also on chromosome 8 of tomato.

Pollination of the fusion products with potato and tomato resulted in some fruit set and even seed set with potato pollen. Within potato, AM 66.42 appeared to be an appropriate pollinator for selection of the best hybrid females. Unfortunately, the culture of 135 relatively unripe seeds was unsuccessful. Comparable negative results were described by Melchers (1984). In sexual interspecific crosses, such as *Lycopersicon esculentum* × *L. peruvianum*, incongruity barriers could be overcome by using embryo-callus techniques with a success rate of one regenerant out of 319 cultured seeds (Cap et al. 1991). Therefore, pollinations and embryo cultures will be made to obtain the offspring plants needed for introgression studies using *amf* as a marker, as well as for the creation of addition lines of potato with one or more extra tomato chromosomes for basic research, and for transferring agricultural traits from tomato into potato.

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