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Vigorous growth of fusion products allows highly efficient selection of interspecific potato somatic hybrids: molecular proofs

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Summary An early identification of fusion products was based on the presumed vigorous growth of hybrid calluses after fusion between *Solanum brevidens* and *S. tuberosum* leaf protoplasts. The *S. brevidens* protoplasts were unable to form multicellular colonies under the applied culture conditions. Three size groups of calluses were separated and analyzed at two different early phases of culture period. "Squash blot" hybridization with a *S. brevidens* specific repetitive DNA probe showed that the group of the largest calluses consisted of putative somatic hybrids with a frequency of 80-100% in three independent experiments. Furthermore, approximately 80-95% of the middle sized calluses and 33-90% of the smallest ones were shown to be hybrid. The unexpectedly high percentage of fusion products, even in the case of the smallest calluses, may result from the suppression of the development of parental potato colonies in cultures with mixed cell population. Till this time 120 independent colonies selected as putative hybrids have been regenerated into plants. All of them exhibited hybrid phenotype, and their hybrid origin was proved by cytological and restriction fragment length polymorphism analyses.

Key words: hybrid vigor - potato - *Solanum* - somatic hybrids - species-specific repetitive DNA

Abbreviations: BA: N^6-benzyladenine; NAA: α-naphthaleneacetic acid; RFLP: restriction fragment length polymorphism; UV: ultraviolet

Introduction

The mass fusion of protoplasts can result in a heterogeneous cell population consisting of unfused parental cells and products of homo- and heterofusions. The early, unambiguous identification of somatic hybrid colonies is of great importance to avoid the unnecessary culture, regeneration and characterization of non-hybrid clones. Selection of somatic hybrids at early culture phases is often based on biochemical and/or tissue culture complementation, or double inactivation of parental cells (as review see Gleba and Shlumukov 1990).

The possible use of hybrid vigor in the early selection of somatic hybrids has been proposed by Schieder (1978). However, there are only a limited number of reports on the identification of interspecific somatic hybrids on the basis of vigorous growth of their calluses (Handley et al. 1986, O'Connel and Hanson 1987, Preiszner et al. 1991). This strategy has been successfully used in the selection of fusion products of dihaploid potato clones as well (Debnath and Wenzel 1987, Waara et al. 1989).

Expression of hybrid vigor at plant level after *S. tuberosum* x *S. brevidens* somatic hybridization has been described (Austin et al. 1985, Fish et al. 1988, Preiszner et al. 1991). Moreover, we have reported previously, that the vigor can also be expressed at the callus level and can be used for the early selection of hybrids (Preiszner et al. 1991). However, there is no report on the efficiency of this selection method. Here we describe our attempts to verify and to improve the reliability of this approach via the use of "squash blot" hybridization with a species- specific *S. brevidens* probe.

Materials and methods

Plant material: *In vitro* shoot cultures of tetraploid *Solanum tuberosum* breeding lines designated as Ke79 and Ke59 and *Solanum brevidens* were maintained on MS medium (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg et al. 1968), 1% sucrose and 0.6 % agar (pH 5.8) in Erlenmeyer flasks with cotton wool stoppers. The temperature was 22/18 °C (day/night), the illumination was 9000 lux (12 h daily).

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Protoplast isolation: Leaves from 4-5-weeks old plants were cut to 1-2 mm wide strips and were digested (in the dark) overnight with 1% cellulose R 10 (Onozuka)+ 0.1% macerozyme R 10 (Onozuka) dissolved in MWSS protoplast culture medium (see below), pH 5.6. Protoplasts were washed in W58 solution ($CaCl_2x2H_20$ 1.8 g/l, NaCl 16 g/l, KCl 0.4 g/l, glucose 1 g/l, pH 5.6).

Fusion and protoplast culture: The fusions were carried out as described by Menczel et al. (1981). Protoplasts were cultured in liquid WSS medium (Sidorov 1985) with a reduced concentration of NH_4NO_3 (300 mg/l) and an elevated concentration of $CaCl_2$ and $MgSO_4$ (1100 and 950 mg/l) and with 0.03 M sucrose + 0.46 M mannitol instead of glucose (MWSS). Every second week, the cultures were diluted with an equal volume of the same culture medium.

Isolation of hybrid colonies: Selection was carried out either in liquid culture at one and a half month after fusion -"early selection"- or one month later after transferring the colonies onto the "greening" medium (Shepard and Totten 1977),-"late selection". For molecular analyses, three types of calluses (largest ones Type I, middle sized Type II, smallest ones Type III) were selected based on their relative size.

Nuclear DNA analysis: DNA was isolated according to Gill et al. (1991). A species-specific probe was isolated from *S. brevidens* genomic library via differential hybridization with potato and *S. brevidens* total genomic DNA (Preiszner et al. unpublished). Dot blot analysis was carried out by squashing approximately 10 mg callus or leaf tissue on Hybond N nylon membrane under continuous vacuum using a dot blot apparatus. Filters were prewetted with denaturation solution for 10 min prior squashing. After drying of the filters, the DNA was fixed on the filter by 30 s UV illumination.

RFLP was detected using the probe TG 46 kindly provided by S.D.Tanksley (Tanksley et al. 1987). Ten microgram of genomic DNA was digested with EcoRI and the Southern analysis was carried out as described previously by Fehér et al. (1992).

Chromosome counting: Approximately 5-10 mg pieces of calluses was pretreated with 21 mM (w/v) 8-hydroxyquinoline for 5 hours and fixed in ethanol: glacial acetic acid (3:1, v/v) for 24 hours, and the tissues were stained in carbol fuchsin (Kao 1975).

Results and Discussion

Culture and selection of fusion products

Three independent fusion experiments were carried out with leaf protoplasts from *S. brevidens* and *S. tuberosum* Ke79 or Ke59 as fusion partners. Protoplasts were cultured in an improved WSS protoplast culture medium that provided 60% of division frequency with the above mentioned potato genotypes (Polgár 1992). In the same medium, *S. brevidens* protoplast derived cells failed to divide and no colonies were formed. Therefore, a discrimination between colonies from parental potato and fused protoplasts was required that was based on the vigorous growth of the fusion products.

As far as the size of colonies is concerned, a highly heterogeneous population of microcalluses developed from the protoplasts after one month culture of fusion products in the liquid culture medium. The most vigorously growing calluses reached the size of 1-2 mm at that time. In comparison, the control potato protoplasts reached a similar colony size only after an additional culture for at least two more weeks (data not shown). After six weeks of culture, the colonies were transferred onto the surface of agar solidified Shepard's medium "C" (Shepard and Totten 1977). This medium supported the growth and the greening of the colonies. The manual selection according to colony size was carried out at two time points. In the so called early selected cultures (experiment B and C, Table 1), the size fractionation was performed one and half month after fusion before transferring the colonies onto agar surface. At this time, two size groups (Type I > 2mm and Type II 1-2mm) of calluses were identified. Microcalluses representing the smallest size class (Type III < 0.5 mm) were cultured in the liquid MWSS medium for an additional two weeks prior transferring to medium "C". Two and half month after fusion ("late selection", experiments A,B,C), calluses of the Type I (large > 4 mm), Type II (middle 2-4 mm) and Type III (small < 2 mm) size classes were selected manually from Shepard's medium "C" and cultured separatedly. In total, 178 callus tissues of Type I calluses and 244 belonging to the Type II class could be selected in three experiments. From the smallest (Type III) calluses only a representative sample of 278 microcalluses was selected and cultured further.

"Squash blot" hybridization to analyze the efficiency of the selection

Since leaf protoplasts served as fusion partners, the determination of frequency of heterokaryons could not be carried out by microscopic study. Small portions of the callus colonies, representing the different size classes, were used for "squash blot" analysis using a species-specific repetitive DNA sequence (pSb 4/1, Preiszner et al. unpublished) as a probe (Fig.1/a). This sequence element is clustered on at least 20 out of the 24 *S. brevidens* chromosomes (Preiszner et al. unpublished) and since it is only detectable in the *S. brevidens* genome, it could serve as a molecular marker for the identification of hybrids. Table 1 summarizes the results of the squash blot tests with representative (exp. A, and early selections of exps. B and C) or total (late selection in exps. B and C) numbers of selected calluses from three independent experiments. All together 511 calluses have been tested so far, of which 424 were hybridization positive.

Table 1. Efficiency of vigor based hybrid selection after the fusion of *S. brevidens* (S.b.) and *S. tuberosum* (Ke79 or Ke59) protoplasts as confirmed by squash blott hybridization with a *S. brevidens* specific repetitive DNA probe

Exp.	Combination	Selection[a]	Calluses[e] type	Calluses No.	Hybrid calluses[b] No.	Hybrid calluses[b] %
A	Ke79 x S.b.	late	I	20	20	100
			II	20	19	95
			III	20	14	70
B	Ke79 x S.b.	early	I	15	15	100
			II	15	12	80
			III	15	5	33
		late	I	34	29	85
			II	49	45	92
			III	44	35	79
C	Ke59 x S.b.	early	I	15	14	93
			II	15	12	80
			III	15	9	60
		late	I	26	21	81
			II	85	75	88
			III	123	109	89

a: Selection was based on the inability of *S. brevidens* protoplasts to form colonies and the vigorous growth of fusion products.

b: Number of calluses showing positive signal in squash-blot hybridization with a *S. brevidens* species-specific repetitive DNA probe.

c: Two and half months after fusion, from agar surface.

d: One and half months after fusion, from liquid medium.

e: Type I largest, Type II middle sized, Type III smallest calluses.

In general, it can be concluded from the data that an unexpectedly high percentage of the calluses showed a hybridization signal, irrespective of the selection time and fusion combination, when probed with the pSb 4/1 *S. brevidens*-specific DNA sequence. Detection of this high frequency of putative hybrids suggests a possible role for other factors influencing the composition of the cell population in addition to the vigorous growth of hybrid colonies.

The following possibilities can be considered as likely explanations for the observed high percentage of outgrowing calluses exhibiting the *S. brevidens* specific repetitive sequence:

1 - colony formation of *S. brevidens* protoplasts through cross feeding in mixed cultures.
2 - extremely high fusion efficiency that was not detected in other systems.
3 - much higher colony formation capability of hybrid than parental potato protoplasts.
4 - suppression of growth of potato protoplasts by the hybrid cells.

The first possibility can lead to the suggestion that 80 % of the colonies are either *S. brevidens* or a mixture of fusion derived and *S. brevidens* clones. On the basis of our experience, it is rather unlikely that most of the colonies would be of *S. brevidens* origins, as we have never seen any outgrowing *S. brevidens* colonies in MWSS medium. Moreover, none of the selected colonies has regenerated *S. brevidens* shoots so far, although we could regenerate *S. brevidens* protoclones to plants with 40% frequency in unrelated experiments, but using the same regeneration media (Shepard's C,D and E). It means that if there were outgrowing *S. brevidens* clones in the mixed cultures, they were eliminated somehow at the regeneration step. Considering the second possibility, it has to be taken into account that there is no report on a heterofusion efficiency above 10 % with the used method in other systems (Deák et al. 1988, Dudits et al. 1987, Fehér et al. 1992). Therefore we propose, that the growth of potato colonies is restricted in mixed cultures by fusion products in an unknown manner. Only a small percentage of them can reach that size which is suitable for manual selection in liquid culture and allows them to survive on "greening" medium. This is supported by the fact that the ratio of non-hybridizing, putative potato colonies within the smallest (Type III) size class was significantly increased (to 40-67%, Table 1) via the culture of this type of calluses for two more weeks in liquid culture after picking up the larger Type I and Type II calluses and before transferring them manually onto medium "C". One possible explanation for this suppression could be a limited nutrient supply for potato protoplasts caused by the vigorously growing hybrid colonies, since the used protoplast culture medium is relatively poor in N- and C- sources (see Materials and Methods).

Characterization of fusion products by RFLP analysis and chromosome counting:

Six independent regenerants were randomly chosen for RFLP analysis. The results of Southern hybridization with radioactively labelled TG 46 probe are shown on Fig. 1/b. Each of the selected clones carried the species-specific bands of both parents.

To see whether the regeneration step eliminates any outgrowing *S. brevidens* clones, 22 representative calluses were chosen from the three calluses types for chromosome counting. Five days after the induction of cell division on a medium containing 26.85 μM NAA and 2.22 μM BA, mitotic chromosomes were counted. From 22 calluses tested, 13 (4:7:2, from Type I:Type II: Type III, respectively) showed a hexaploid chromosome set while 5 (2:2:1 from Type I: Type II: Type III, respectively) had 96 chromosomes. All of these carried the *S. brevidens* species-specific DNA sequence. Four callus lines not carrying the *S. brevidens* specific sequence (1 from Type II and 3 from Type III) also had a higher number of chromosomes likely to have originated from potato homofusions. Thus, RFLP and chromosome counting also verified the hybridity of the selected colonies.

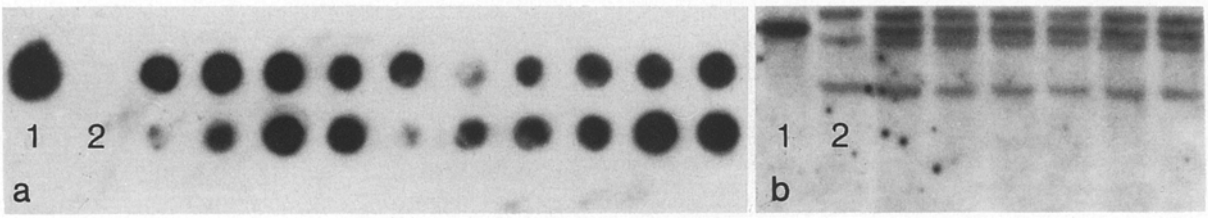

Figure 1. Characterization of *S. tuberosum* Ke79 and *S. brevidens* somatic hybrids

a: Squash blot hybridization of twenty vigorously growing (Type I) calluses derived from a protoplast fusion experiment between *S. brevidens* (1) and *S. tuberosum* Ke79 (2) using a *S. brevidens* specific repetitive DNA probe. Hybridization signal differences are due to the different amounts of the callus samples used.

b: RFLP patterns (TG46/EcoRI) of parents (1,2) and six hybrid clones of *S. tuberosum* Ke79 and *S. brevidens*.

The described fusion experiments demonstrate the possibility for the early selection of somatic hybrids between *S. brevidens* and *S. tuberosum* that is simply based on the vigorous growth of hybrid colonies. Till this time, 120 independent hybrid colonies have been regenerated to plants. All of them showed the main morphological characters previously shown to be typical for *S. tuberosum* x *S. brevidens* hybrids (see e.g. Preiszner et al. 1991).

Considering the accuracy in identification of hybrids, this selection strategy can be useful to decrease the time and labour costs of somatic hybrid selection. There is no need to use specific genotypes with selectable genetic markers or any refined techniques. However, the present studies provide only an example and further studies will be needed to analyze the potential use of this approach in other fusion combinations with cultivated and wild species as partners.

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