Theor Appl Genet (1986) 71:682–690

Somatic hybrids produced by protoplast fusion between *S. tuberosum* and *S. brevidens:* phenotypic variation under field conditions

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Received September 5, 1985; Accepted October 7, 1985
Communicated by G. Wenzel

Summary. Phenotypic and flowering characteristics of hybrid plants generated by protoplast fusion between a tetraploid *S. tuberosum* line and diploid *S. brevidens* were assessed under field conditions. Hybrids were compared to both clonal parental material and protoplast-derived plants of each parent. Almost all of the hybrids were hexaploid. A wide range of variation in morphological characters was observed for hybrids and protoclones. Flowering was markedly reduced in protoclones. The majority of hybrids flowered, had viable pollen and set tubers. Tuber and pollen characteristics of hybrids produced from individual fusion calli also varied. The potential usefulness of fusion hybrids in potato improvement is discussed.

Key words: *S. tuberosum* – Protoplast – Somatic fusion – Variation in hybrids

Introduction

Somatic fusions may provide a means by which traits from sexually incompatible wild species can be incorporated into crop plants. To this end, we have been examining somatic fusions between members of the genus *Solanum*. We have reported previously the production of somatic hybrids between *S. brevidens* (PI 245763), a non-tuber-bearing, diploid wild species of the Series Etuberosa and a selection from a *S. tuberosum* Gp. Phureja-Stenotomum population (Baer et al. 1984; Austin et al. 1985 b) Also, we have been able to fuse two dihaploid *Solanum tuberosum* Gp. Tuberosum lines (Austin et al. 1985 a).

A potential problem with fusion progeny is phenotypic variation. Such variation is a widely reported occurrence in protoplast and cell cultures of potato and is evident for several different genotypes (e.g. Wenzel et al. 1979; Shepard et al. 1980; Van Harten et al. 1981; Thomas et al. 1982; Austin and Cassells 1983; Cassells et al. 1983; Sree Ramulu et al. 1983). Plants produced via somatic fusion also possess a wide range of variation in morphological features (e.g. Binding et al. 1982; Maliga et al. 1978; Evans 1983). In most cases, however, analysis of the phenotypic variation among such materials has been hampered by the fact that relatively few fusion progeny were produced.

Recently, we have been able to fuse cells from another accession of *S. brevidens* (PI 218228) with a tetraploid *S. tuberosum* line. The hexaploid fusion plants from this fusion express the potato leaf roll virus (PLRV) resistance of the *S. brevidens* partner as well as the late blight (*Phytophthora infestans*) resistance of the *S. tuberosum* partner (Helgeson et al., in preparation). In this experiment, we were able to obtain a number of calli and, furthermore, our cell culture procedures enabled us to obtain high numbers of shoots from these individual calli. We are, therefore, able to assess phenotypic variation both between different somatic hybrids and within populations obtained from individual fusion calli. For comparisons, protoplast-derived materials as well as clonal copies of the individual fusion partners were included in the study.

Materials and methods

S. brevidens (PI 218228) and *S. tuberosum* Gp Tuberosum (PI 203900) were obtained from the Inter-Regional Potato Project, IR-1, at Sturgeon, Bay, WI, courtesy of R.E. Hanneman Jr. *S. brevidens* is a diploid, non-tuber-bearing wild species of the Series Etuberosa and has resistance to potato leaf roll virus (PLRV) as well as some frost tolerance. The accession used in this study generally yields protoplasts capable of limited cell division upon plating and poor regeneration on our standard differentiation (Dif) medium (Haberlach

et al. 1985). In preliminary experiments, this accession also failed to regenerate on J1 medium, an alternative regeneration medium (Austin et al. 1985 b). The *S. tuberosum* line used in the fusion is a tetraploid potato used as a differential for identification of races of *Phytophthora infestans.* This line has hypersensitive resistance to Race 0 but is susceptible to Race 4 of the fungus. Protoplasts isolated from this line generally divide and regenerate well on Dif medium (Haberlach et al. 1985).

Protoplast isolation and fusion

Leaf mesophyll protoplasts were isolated from shoot tip cultures following the method of Shepard (1980) as modified by Haberlach et al. (1985). For comparisons with fusion progeny, protoclones of *S. tuberosum* and *S. brevidens* were generated by separately culturing protoplasts from each line. Protoplasts isolated from the two species were fused by the previously reported procedure (Austin et al. 1985 b). Self-fusions between the two fusion partners themselves were also carried out by this method.

Culture of protoplasts and shoot regeneration

Protoplasts from the interspecific fusion experiment were plated at a concentration of 3×10^5 ml following the procedure used previously (Austin et al. 1985). After four weeks, wedges were taken from the plates and placed on the culture medium (Cul) given by Haberlach et al. (1985). Calli were moved to fresh Cul medium 1 week later. Calli remained on this medium until they had greened and were at least 2 mm in diameter (between 2–6 weeks depending on the individual callus). The calli were then transferred to J1 medium for shoot regeneration. Any calli with shoot initials were further proliferated by transfer to a high gibberellin medium (Austin and Cassells 1983 b). Calli from self-fusions were treated as above with the exception that regeneration was tested on Dif as well as J1 medium. Individual calli were numbered, as were any shoots taken from them. Care was taken to excise shoots at their point of origin on the callus to avoid taking nodal outgrowths of the same shoot if subsequent harvests of shoot were taken from the same callus. Rooting of shoots was achieved on the propagation (Prop) medium given by Haberlach et al. 1985).

Phenotypic assessment

Rooted shoots of potential somatic hybrids, protoplast derived parental lines (protoclones), and clonal copies of parental lines (produced by nodal cuttings, see Haberlach et al. 1985) were established in "Jiffy 7"[1] peat cylinders three weeks prior to planting at the Hancock Experimental Station, Hancock, Wisconsin. Field plants were spaced at 85 cm within rows with nine plants per row. Where possible, rows of fusion plants were alternated with rows of either clones of parental lines or protoclones. After an initial establishment period of four weeks, plants were assessed weekly for vigor and flowering (fully open blooms) for another eleven weeks. Vegetative characteristics were scored between weeks eight and eleven. At harvest, tubers from each plant were counted and weighed in the field. Assessment of tuber type was done one to two weeks later prior to storage. Morphological parameters and categories given by Huaman et al. (1977) in "Descriptors for the Cultivated Potato" were generally used for our analyses.

Ploidy estimation and pollen stainability estimation

Ploidy levels of plants from the fusion experiment were determined from anther squashes of floral buds collected from field-grown plants. Staining was done with a 1% acetocarmine solution (1% carmine in 45% aqueous acetic acid). Pollen stainability evaluations were done on pollen collected from field-grown plants in mid-August 1984. A minimum of 200 grains from each plant were counted.

Results

Callus culture and regeneration

After the interspecific fusion experiment, development of microcalli was slow and only a few calli were recovered. In total, 61 calli were transferred to Cul medium on wedges of CL/Res; 53 of these survived and were subsequently transferred individually to Cul. Of these calli, 39 greened during the following two to six weeks and shoots were obtained from 28 of these calli over a three to nine week period.

Shoots produced on the fusion-derived calli were distinctly different from those on calli of either fusion partner. The *S. tuberosum* partner had shoots with thin stems and poor leaf development. The shoots from unfused *S. brevidens* calli grew poorly and were generally pale green with short internodes. In marked contrast to either fusion partner, fusion-derived calli produced shoots with good leaf development and vigorous growth.

There were also obvious morphological differences between shoots on individual calli from the same source. Differences were more marked between calli of *S. brevidens* or between those from fusions than between calli derived solely from *S. tuberosum* protoplasts. Typically, abnormal shoots had distorted, crinkled, dark green or purple leaves, thick stems, and very short internodes. Root growth of these plants was generally poor.

No microcalli were produced after self-fusion of protoplasts from the *S. tuberosum* partner. *S. brevidens* protoplasts, however, appeared to be unaffected by the fusion step and gave microcalli and regenerative calli. Although, no regeneration occurred on J1, three of 41 calli on Dif gave shoots. Unfortunately, these were lost to contamination of the culture.

[1] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable

Table 1. Survival and establishment of fusion hybrids, protoplast derived parental material, and clonal parental material at different times during culture and evaluation

Plant group	Shoots from calli	Rooted shoots	Put into Jiffy 7's	Established in Jiffy 7's	Planted in field	Established in field
Fusion progeny	600	547 (91%)	367	352 (96%)	174	156 (90%)
Protoclonal *S. brevidens*	85	59 (69%)	57	51 (89%)	26	24 (92%)
Protoclonal *S. tuberosum*	165	128 (77%)	117	112 (96%)	89	72 (81%)
Clonal *S. brevidens*	N/A[a]	N/A	60	58 (97%)	45	43 (96%)
Clonal *S. tuberosum*	N/A	N/A	80	78 (97%)	63	60 (95%)

[a] N/A – not applicable since clonal material was maintained as nodal cutting

Table 2. Some morphological characteristics of fusion hybrids and clonal copies of the fusion partners

	S. tuberosum	*S. brevidens*	Hybrids
Stem cross section	solid	hollow	some with small hollow in stems
Stem color	green	green/purple	mainly green, slightly purple
Flower	cream/white	distinctive stripe on white upper surface lower surface purple	pigment distribution as *S. brevidens* but pigment pink
Calyx	green, pointed	purple with small points	intermediate
Tubers	round, medium fairly uniform	none	variable, tend to be small and elongate

Table 3. Flowering in field planted somatic hybrids, protoplast-derived parental material and clonal material

Plant group	No. assessed	Flowering	Non-flowering	Severely distorted flowers
Clonal *S. tuberosum*	57	57 (100%)	0 (0%)	0 (0%)
Protoclonal *S. tuberosum*	72	18 (25%)	54 (75%)	3 (16%)
Clonal *S. brevidens*	39	39 (100%)	0 (0%)	0 (0%)
Protoclonal *S. brevidens*	24	7 (29%)	17 (71%)	5 (71%)
Fusion progeny	156	123 (79%)	33 (21%)	18 (15%)

Plant establishment and phenotypic assessment

Shoots were taken from 18 calli obtained from the interspecific fusion experiment, from 24 *S. tuberosum* protoplast-derived calli and from four *S. brevidens* calli. Data on survival and establishment of these materials are given in Table 1. Roots were obtained on 91% of shoots from the fusion calli. Neither of the fusion partners yielded such high percentages.

Generally, shoots which had a grossly abnormal appearance failed to root. However, the converse was not always true, particularly for *S. tuberosum*. Some apparently normal looking shoots failed to root.

Of shoots transferred to "Jiffy 7's", only a few failed to become established and similarly high survival was noted upon transfer to the field. A notable exception was seen with the *S. tuberosum* protoclones; only 81% of the plants in "Jiffy 7's" survived after transplantation to the field plot.

Once plants were established in the field, it was obvious that fusion plants were a distinct morphological group from either parent. Some of the phenotypic characteristics of the fusion progeny and of parental lines are given in Table 2. All of the clonal parental lines flowered in the field (Table 3). However, flowering was markedly reduced in protoclones as compared to clonal plants. Only 25% of the *S. tuberosum* protoclones and 29% of the *S. brevidens* protoclones produced

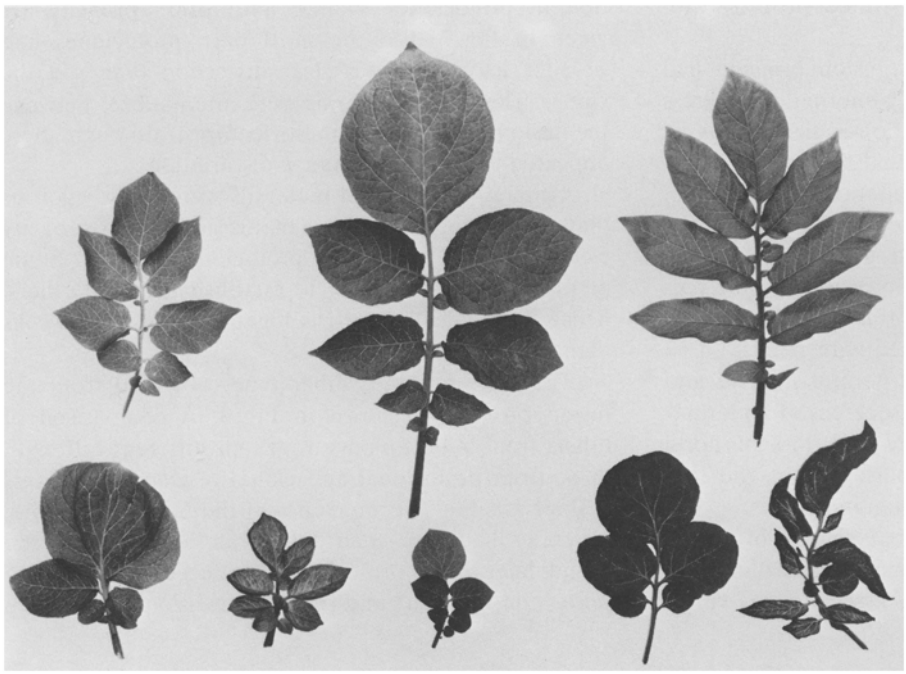

Fig. 1. Representative leaves of clonal *S. tuberosum* (*upper left*), clonal *S. brevidens* (*upper right*) and a typical fusion hybrid (*upper center*). Examples of other leaf types fusion hybrids are shown in the *lower row*

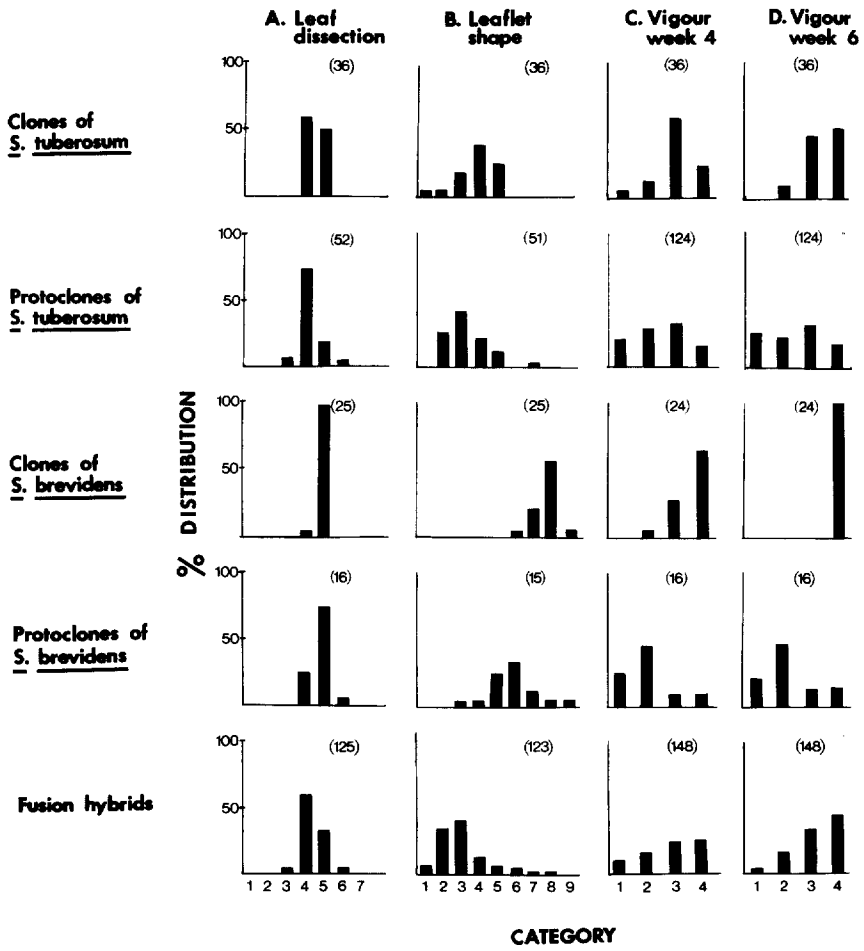

Fig. 2. Leaflet shape, leaf dissection and vigour of somatic hybrids, protoplast derived parental material and clonal material assessed under field conditions. Sample number for each histogram is given in parentheses. **A.** Leaf dissection: Category describing the degree of leaf dissection as given in the Descriptors for the Cultivated Potato (Huaman et al. 1977). *1* = Undissected; *2* = Pinnatilobed; *3* = Scarcely dissected; *4* = Weakly dissected; *5* = Medium dissected; *6* = Strongly dissected; *7* = Very strongly dissected. **B.** Leaflet shape: This is the ratio of length : width of the lateral leaflet immediately below the terminal leaflet of the 5th or 6th fully expanded leaf from the apex. *1* = 1.0–1.2; *2* = 1.2–1.4; *3* = 1.4–1.6; *4* = 1.6–1.8; *5* = 1.8–2.0; *6* = 2.0–2.2; *7* = 2.2–2.4; *8* = 2.4–2.6; *9* = 2.6 + +. **C.** Vigour: A subjective assessment of the growth rate and overall competance of the plants. *1* = slow growth rate, poor spread of foliage; *2* = slow-medium growth rate, reasonable foliage cover; *3* = medium-fast growth rate, good foliage; *4* = fast growth rate, excellent foliage cover

flowers. In contrast, 79% of the fusion progeny produced flowers.

Some of the protoclones and fusion progeny had severely distorted flowers. Typical abnormalities were a combination of greatly reduced flower size, fusion of petals and anthers and of individual flowers, split and distorted petals, loss of pigmentation and loss of pentate symmetry. Stamen abnormalities were also observed within both protoclone groups and fusion progeny. All but one of the flowering *S. brevidens* protoclones and all of the *S. tuberosum* protoclones had divergent stamens or stamens fused with petals. Of 67 fusion progeny examined, 13 had normal stamens and the rest had stamens with varying degrees of abnormalities. It would appear that there is a loss of floral competance as a result of protoplast culture and that this loss is greater in protoclones than in hybrids.

Illustrations of the various leaf shapes of fusion hybrids and representatives of clonal parental lines are shown in Fig. 1. The results from assessing this variation in protoclones, clones, and fusion progeny are given in Fig. 2. For the most part, protoclones had broader leaves and less leaf dissection than did the clones. The somatic hybrids were intermediate between the fusion partners for these features, although there appeared to be a greater range of variation.

Generally, the clonal materials were more vigorous than the protoplast-derived plants and fusion progeny were more vigorous than protoclones (Fig. 2). Some plants took a little longer to establish than did others, hence the tendency towards higher scores at six weeks than at four weeks.

The wide range of tuber types obtained from the fusion progeny is shown in Fig. 3. A comparison of tubers from hybrid plants from four different calli with those from protoclonal and clonal *S. tuberosum* plants is shown in Fig. 4. Protoclones of the *S. tuberosum* line generally had elongated tubers as compared to the clonal line. Some protoclones produced tubers which were non-uniform and slightly misshapen. Among

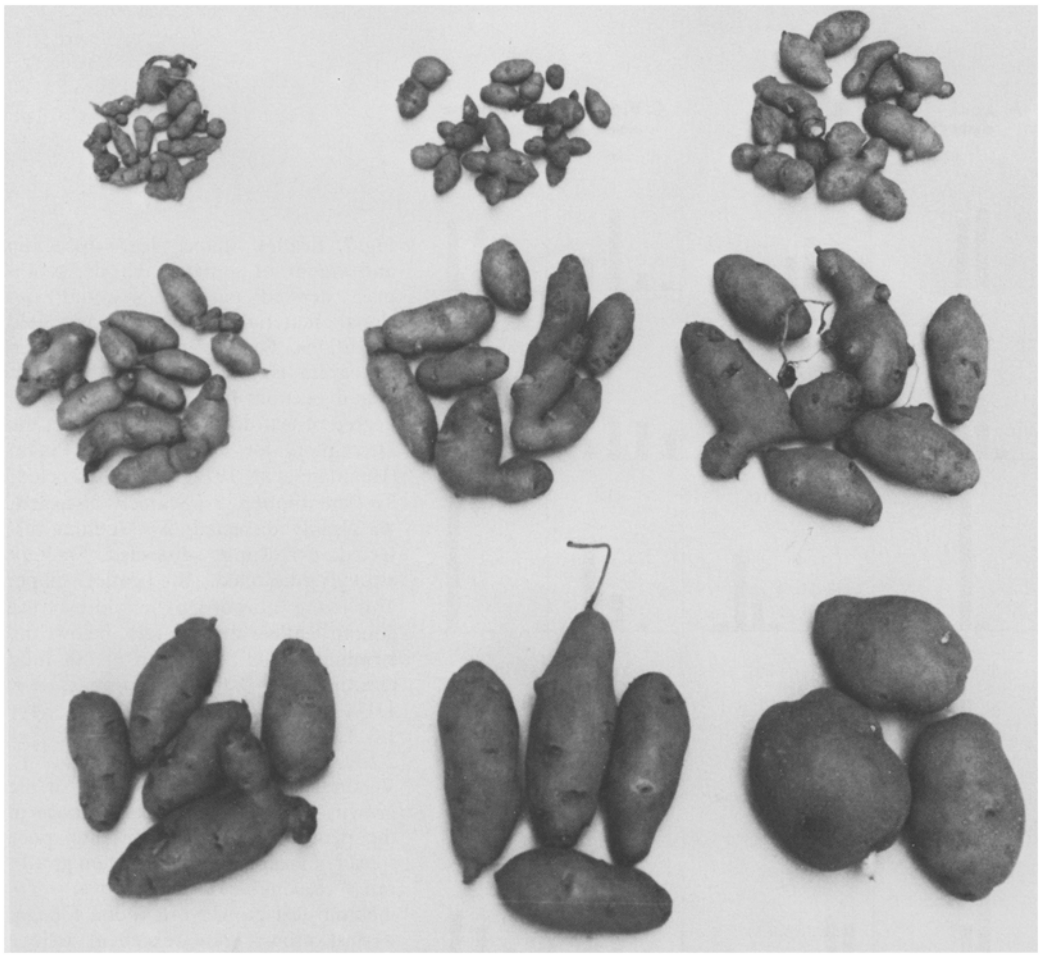

Fig. 3. Representative tuber types of hybrid plants and clonal *S. tuberosum;* fusion tuber types 2, 3, 4 (*upper row left to right*), types 5, 6, 7 (*middle row left to right*), types 8, 9 and clonal *S. tuberosum* (*lower row left to right*)

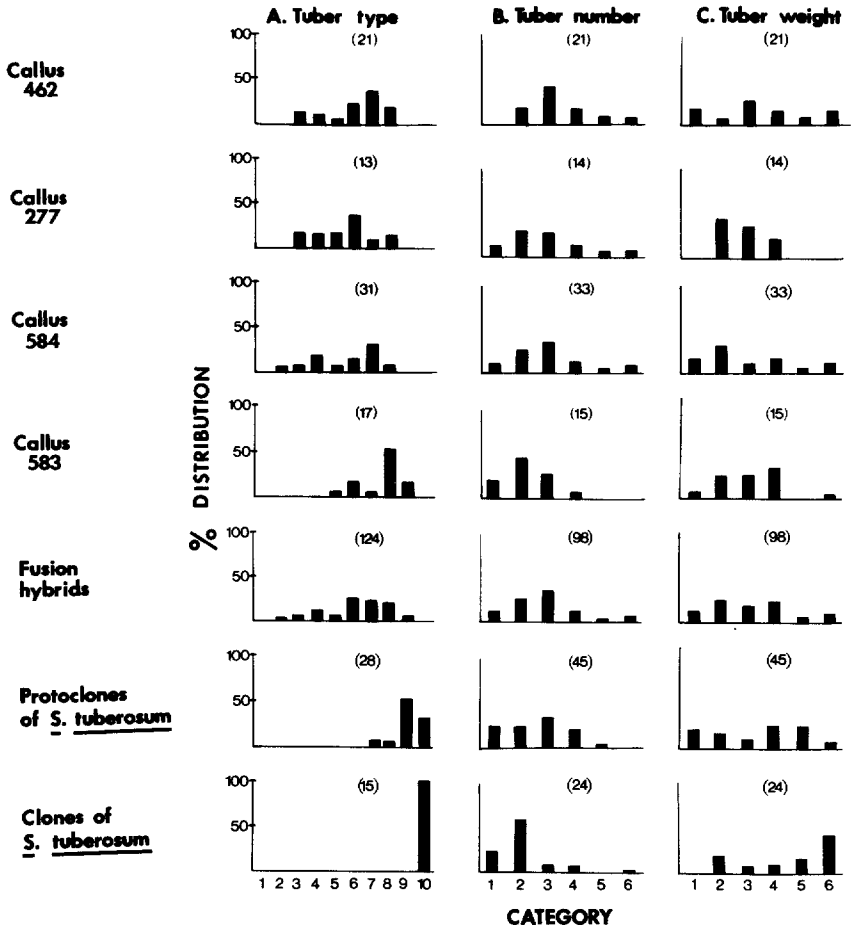

Fig. 4. Tuber characteristics of somatic hybrids derived from 4 individual fusion calli compared to those of protoplast derived and clonal parental material. Sample number for each histogram is given in parentheses. **A.** Tuber type: This was subjective approach to categorize the different types of tubers produced by the somatic hybrids. The higher the number the more closely the tubers approached the clonal parental material. *1*=No free tubers as such, enlarged stolons; *2*=A few [1–8] tiny misshapen tubers; *3*=Small misshapen tubes, low-medium numbers [1–20]; *4*=Mixture of different sizes and shapes, low-medium numbers [1–20]; *5*=Small and misshapen but high numbers [40+]; *6*=Small and medium sized tubers, some elongate with some misshapen; *7*=Most elongate and smooth, medium size with few exceptions; *8*=Elongate tubers, mainly smooth with good uniformity of size and shape; *9*=All smooth, slightly elongate, almost as clonal matrial; *10*=As clonal material. (Types 2 through 10 are illustrated in Fig. 3.) **B.** Tuber number: *1*=1–20; *2*=21–40; *3*=41–60; *4*=61–80; *5*=81–100; *6*=101+. **C.** Tuber weight: *1*=1–300 g; *2*=301–600 g; *3*=601–900 g; *4*=901–1,200 g; *5*=1,201–1,500 g; *6*=1,500+g

fusion calli, there were no striking differences in tuber types between individual calli, but one callus (No. 583) had tuber types more closely resembling the range from protoclonal material than did the other three calli. Clonal *S. tuberosum* plants tended to give fewer but heavier tubers than protoplast-derived plants, whereas protoclones tended to produce many small tubers. Hybrid tuber weight was also highly variable; fewer individuals had yields in the two highest weight categories than did individuals of the protoclone group. Plants from callus 583 produced fewer tubers than the other three calli.

A number of the plants from protoclones and from fusions were markedly abnormal (Table 4). Of the 156 hybrids assessed, a total of 18 individuals were considered grossly abnormal; 15 of 72 *S. tuberosum* protoclones and 13 of 24 *S. brevidens* protoclones were also judged to be abnormal. Abnormal plants typically were severely stunted, had small dark green, crinkled leaves with little or no dissection, and were generally heavily pigmented. Often these plants had grossly abnormal flowers or no flowers.

All of the protoclones of the *S. tuberosum* line produced tubers. However, 16 of the 148 somatic hybrid plants examined did not produce tubers. Of these 16, 10 did not flower and the remaining 6 had severely distorted flowers and 11 were grossly abnormal in their vegetative appearance.

The callus origin of plants with grossly abnormal features was traced (Table 4). Only one callus gave only (2 of 2) abnormal plants. The other calli produced both abnormal and normal looking hybrids. Some protoclones of each parents were also abnormal. Amongst the hybrids, however, one callus did give a much higher proportion of abnormal plants than any other callus. Of 13 plants tested from this callus, one died and 10 of the remaining 12 were grossly abnormal vegetatively. Eight of these abnormal plants failed to flower and 2 gave distorted flowers. Only 1 of the 2 vegetatively normal plants produced tubers; the remaining eleven plants in this group failed to tuberize.

Ploidy counts

Ploidy was estimated through chromosome counts at anaphase I to telophase I of microsporogenesis in 94 fusion hybrids. Within this group of hybrids, 91 were at

Table 4. Callus relationship of hybrid plants with abnormal characteristics

Abnormal feature	Plants with feature	No. of calli giving plants which were:		
		1) all abnormal types	2) both normal and abnormal types	3) all normal types
1. No flowers	33/156	0	9	9
2. Distorted flowers	18/123	0	8	10
3. Grossly abnormal vegetatively	18/156	1[a]	6	11
4. No tubers	16/148	0	7	11

[a] 2/2 plants

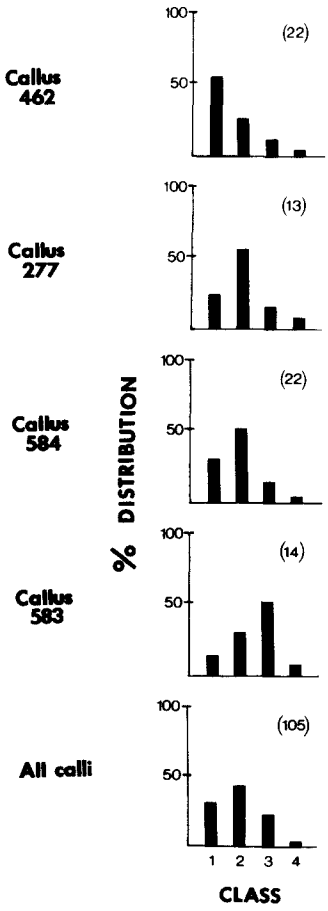

Fig. 5. Pollen stainabilities of plants from fusion calli. Histograms of percentages of plants in each of four different classes. Sample number for each histogram is given in parentheses. Class 1 = < 1% stainable, class 2 = 1–10% stainable, class 3 = 11–20% stainable, class 4 = 21–30% stainable

or near the expected hexaploid level, two others were found to be tetraploid and one was found to be octaploid. These three anomalous plants had abnormal vegetative morphologies compared to most of the other hybrids. However, they still retained the distinctive morphology of fusion hybrids. Thus, they did not appear to be the result of either self-fusion or regeneration of unfused cells.

Pollen stainability

Pollen stainabilities were determined for 105 fusion clones representing 20 different calli. These values for pollen stainability ranged from 0% to 25%. It is notable that overall 30% of the fusion progeny had pollen stainabilities of less than 1%, and 43% had values only between 1 an 10%. Nonetheless, a substantial number (27%) had stainabilities greater than 10%, and for some lines (i.e. 583), the majority of plants produced fell in this range. Clonal lines of the parents had stainabilities of 82% and 11% for *S. brevidens* and *S. tuberosum,* respectively. All hybrids examined had micro-pollen, an indication of pairing problems in meiosis. In some cases a substantial amount of micro-pollen was seen. Micro-pollen was not seen in the parents. Protoclonal material was not assessed.

The variation seen in the summed data is also observable in data on groups of plants from individual calli. As in the summed data, if a callus produced a significant number of shoots which ultimately flowered, it generally produced a plant or two with stainability values in excess of 20%. The stainability of pollen from the rest of the plants was distributed over the range from 0 to 20% (Fig. 5).

Differences between groups of plants from different calli were also observable. Although ranges of pollen stainability were similar in several different calli, the distribution of stainabilities across this range varied. Callus 462 had a modal class value of < 1%; callus 583 had a modal class value of 11–20% (Fig. 5). Some calli, from which fewer shoots were obtained, had a reduced range of pollen stainabilities among their hybrids, often having none with more than 10% stainable pollen.

Discussion

Plants derived from this fusion experiment all had the phenotypic appearance of somatic hybrids and the majority of those assessed for ploidy were at or near the hexaploid level. It would appear that a selection for hybrid callus tissue occurred based on the ability of protoplasts of the fusion partners to differentially survive the fusion procedure and to regenerate on selective

media. Sensitivity of the *S. tuberosum* clone may be due to the PEG used in the fusion protocol. Such sensitivity in potato lines has been reported by Binding and his co-workers (1982). A selection of hybrids after protoplast fusion that was based on cultural procedures and increased vigour of hybrid material has been reported by other workers (see reviews by Evans 1983; Schieder and Vasil 1980).

We were fortunate to have a large number of hybrid plants from several different calli to examine and to compare to both clonal and protoplast-derived clones from parental material. The protoclones gave an indication of phenotypic changes in parental material which may arise as a consequence of tissue culture. As a group, hybrids had reduced flowering as compared to clonal material. However, flowering incidence of the fusion progeny was much higher than with protoplast-derived protoclones from unfused materials. Therefore, lack of flowering was not necessarily a feature of the hybrids per se. This point would appear to be important as production of flowers and viable pollen are essential features if the hybrids are to be useful to plant breeders. It appears that if one screens progeny from fusions for individuals with high pollen viability, work with these hybrids will not be hampered by lack of either flowers or viable pollen. However, it may be useful to test fusion partners for their ability to retain flowering and viable pollen through tissue culture procedures prior to attempting fusion experiments. Some lines may be better than others in this respect.

The majority of hybrids from our fusion experiment gave chromosome counts at or very close to the hexaploid level. Since non-flowering plants were not assessed, we do not know if these plants were also hexaploids. Some abnormal potato protoclones have been shown to have increased ploidy levels or even to be aneuploids (Wenzel 1980; Sree Ramulu 1983). This was also the case in our study and it is, perhaps, the basis of the other aberrant plants within our hybrids and protoclones. The results for other characteristics (Figs. 2 and 4) indicate that the wide range of phenotypic features seen in the hybrids was also evident in the protoclones.

Tuber characteristics of hybrids were highly variable but, in general, better tubers were produced by the hexaploids than by the previously reported tetraploid fusion progeny of *S. brevidens* and a diploid *S. tuberosum* line (Austin et al. 1985 b). We hypothesize that the improved tuberization resulted from a gene dosage effect of a 2 : 1 ratio of *S. tuberosum* to *S. brevidens* as compared with the equal dosages that result from the diploid-diploid fusion. Preliminary results with tetraploid plants of a *S. brevidens, S. tuberosum* cv. Superior haploid fusion would support this view. The tubers produced were merely slightly enlarged stolons.

In view of results obtained by others with protoplast-derived plants, the range of variation seen in our fusion-derived material was not surprising. Individual protoplasts and single cell-derived calli from several potato protoclones have given shoots which differ phenotypically and/or in chromosome number from other plants derived from the same callus (Karp et al. 1982; Sree Ramulu et al. 1983; Austin and Cassells 1983 a; Austin et al. 1985 a). In our observations, one callus did give predominantly aberrant plants, but it also yielded two phenotypically normal plants. This suggests that the gross variants arose during the cultural steps, perhaps from a sector of abnormal callus, and are not the result of an unusual fusion event. One other callus gave only vegetatively abnormal plants, but only two plants were field tested. Still another callus gave more uniform plants than other calli, and these plants had tuber and pollen characteristics more closely resembling those of the protoclones of the *S. tuberosum* partner. These results indicate that the progeny from fusion experiments can differ substantially from each other. Thus, it is useful to have several calli available from a given experiment and to examine a number of the progeny from each callus. This may ultimately aid in selection of clones for further breeding work.

Preliminary results (Helgeson et al. 1985) show that most, but not all, of the hybrids from this fusion were resistant to potato leaf roll virus (PLRV), as were the hybrids reported earlier (Austin et al. 1985 b). Also, the hybrids appeared to be more resistant to Race 0 of *Phytophthora infestans* than is *S. brevidens* and, perhaps, as resistant to the fungus as the *S. tuberosum* partner. These preliminary findings indicate that the resistances of both fusion partners may be expressed in the progeny. Tests on disease resistance of the fusion progeny are being repeated in greater detail.

The potential usefulness of these fusion hybrid plants is also dependent on their ability to cross sexually and to transmit their desirable characteristics. Preliminary crossing data are very encouraging and progeny plants are currently being examined for the transmission of the traits of the progeny through meiosis. In at least one case, resistance to PLRV appears to be transmissible. The fact that we have been able to obtain hybrids with viable pollen will give us the opportunity to fully examine and test these essential parameters.

Acknowledgements. The authors would like thank Beth Howman-Combs and Pamela Kazmierczak for excellent technical assistance, R.E. Hanneman Jr. for tubers and helpful discussions, Steve Vicen for photographs and Danica Gakovich for drawings.

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