Progress Towards the Development of a General Somatic Hybridization Protocol for *Beta*

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ABSTRACT

Beta vulgaris **is notoriously recalcitrant concerning biotechnological techniques. However, recently we have been able, for the first time, to report on the obtention of normal, diploid regenerants from mesophyll protoplasts of sugarbeet. This encouraging breakthrough lead to consideration of the application of somatic hybridization techniques to beet. The ability to exchange cytoplasm between genotypes could prove of great benefit in this species, where CMS is routinely exploited for plant breeding and seed production purposes. Detailed experiments have thus been carried out to determine the most appropriate techniques to use to this aim. Different protocols for protoplast pretreatments, fusion, culture, regeneration and DNA analysis have been tested and optimum conditions for each determined. The applicability of the chosen protocols for the production of asymmetric beet hybrids has been investigated. The results of this research will be presented and discussed in the context of other work being carried out in this field on *Beta* and on other species.**

Additional Key Words: cell fusion, protoplast culture, plant regeneration

In recent years many *in vitro* techniques have been the subject of detailed investigations to assess their applied value. One technique which has received considerable attention is that of somatic hybridization where the aim is to produce novel hybrids via the *in vitro* fusion of plant protoplasts. Interest in this technique stems from a gained ability either to overcome the limitations of conventional breeding (e.g. natural crossing barriers), or to provide a more rapid alternative to traditional breeding methods. Somatic hybridization can be exploited either to produce symmetric hybrids, where two complete genomes are combined, or alternatively, asymmetric hybrids, where only a fraction of one plant genome is combined with the complete genome of another (Gleba and Shlumukov, 1990). Such a fraction can involve either nuclear or cytoplasmic genetic material. This potential ability to treat the three individual portions of the total plant genome as individual entities allows for the realisation of all possible mitochondrial/plastid/nuclear combinations and thus represents a unique feature of *in vitro* somatic hybridization techniques. Recently, a number of reports have confirmed the value of these techniques by detailing the production of symmetric and asymmetric hybrid plants for a range of crop species such as rice, *Citrus* spp., potato, and *Brassica* spp., and the incorporation of these plants into new or existing breeding programmes (for detailed reviews see, Gleba and Shlumukov, 1990, Glimelius et al., 1991).

For sugar beet and fodder beet our interest in somatic hybridization is centred on its use as a potentially rapid method to transfer cytoplasm (and in particular, mitochondria) between genotypes. However, once developed, a somatic hybridization protocol can of course be used for a number of aims. The ability to change a plant cell cytoplasm, *e.g.* from a cytoplasmic male sterile (CMS) to a male fertile (MF) type, or *vice versa,* would greatly assist our breeding programmes in, for example, allowing the creation of the perfect maintainer line or permitting the production of F_1 hybrid seed. While such a goal in beet could be achieved, at least with related lines, via conventional breeding, the time required to carry out the necessary backcrosses to regain the original nuclear genome is prohibitively long in this biennial species. Furthermore, in beet, a potentially high-risk situation exists regarding the very limited cytoplasmic genetic variation which is present, which has resulted from the almost exclusive use of a single (Owen) CMS cytoplasm for breeding and seed production purposes (Kaul, 1988). This problem could be significantly eased through the rapid introduction and assessment of some of the potentially new CMS cytoplasms identified recently (see Saumitou-Laprade et al., 1993 and refs. therein).

In this paper, the results of our detailed investigation into the development of a complete (a)symmetric somatic hybridization protocol for *Beta* will be summarised and discussed. For the first time, the results of this study have been combined in order to detail the entire protocol which has been chosen. Realistic conclusions will be made with regard to the potential application of this protocol for the production of *Beta* hybrids and cybrids in the light of the results from other groups working on equivalent systems.

REQUIREMENTS FOR A SUCCESSFUL SOMATIC HYBRIDIZATION PROTOCOL

A protocol for plant cell somatic hybridization involves a number of individual steps for which the methodology has to be determined and optimised if the overall protocol is to be successful (Figure 1). The necessary requirements for (a)symmetric hybridization in beet are thus

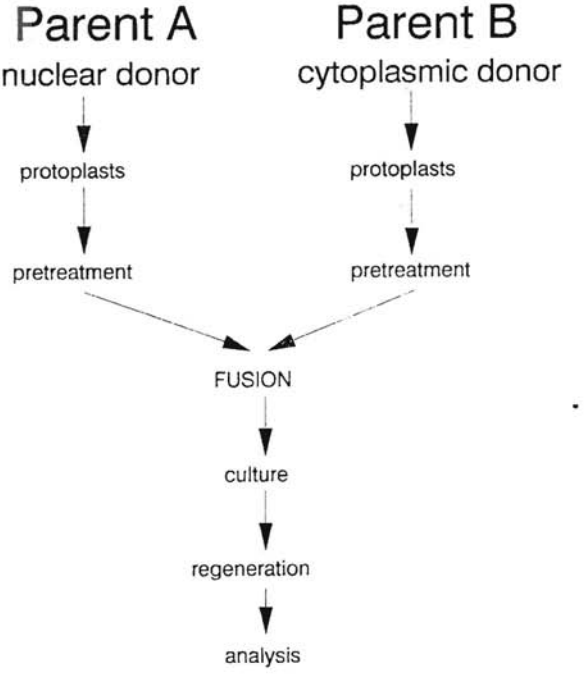

Figure 1. General scheme for asymmetric somatic cell hybridization in plants.

listed as follows:

(a) Suitable culture conditions for the donor plant material.
(b) A method for the isolation of large numbers of viable protoplasts from appropriate plant material.
(c) The development of a suitable selection technique for hybrid cells.
(d) A cell fusion protocol.
(e) Appropriate culture techniques (also for low density culture) ultimately leading to plant regeneration.
(f) Techniques for the molecular analysis of the putative hybrids and cybrids to confirm their genetic origin.

Each of these points will now be taken in turn and the results of our experiments will be summarised and presented along with detailing the best techniques which have arisen from this work.

(a) The source material

Our early attempts using greenhouse-grown plants revealed not only the problem of obtaining sterile isolations but also a subsequent poor protoplast survival rate. Divisions were never observed (unpublished observations). Aseptically-germinated seedlings (see Krens and Jamar, 1989) proved a very reliable source of mesophyll protoplasts (Krens et al., 1990). However, shoot cultures are the most favoured choice as these provide a uniform and re-usable supply of leaf material for which sterility can be guaranteed. Growth on hormone-free medium is preferred as this leads to essentially normal plant morphology. However, for some lines *(e.g.* subsp. *maritima* lines) sustained growth in the absence of a cytokinin was not possible. The chosen conditions for the culture of *in vitro* plant material, which were suitable for a range of genotypes, are summarised in Table 1.

(b) Protoplast isolation techniques

Attempts to obtain large numbers of viable protoplasts from leaf blades and petioles (from seedlings or shoot cultures) and from cell suspension cultures have been very successful. CPW-based salt solutions (Frearson et al., 1973) proved appropriate, although for petiole protoplasts the additional use of W5 medium (Menczel et al., 1981) during washing was essential due to the unusual specific densities of these cells (Pedersen et al., 1993). In all cases a cell viability after purification of ≥ 90 %, as measured using fluorescein diacetate (FDA), is usual. Medium osmolality was greatly influential in successful protoplast isolation and the inclusion of the anti-oxidant

n-propyl gallate (nPG) at a concentration of 0.1 mM proved critical not only for the isolation but also for the subsequent culture of beet protoplasts (Krens et al., 1990). Preincubation, after finely chopping up the leaf tissue in a medium with a high calcium concentration (CPW salts, 3.8% $CaCl_2 \cdot 2H_2O$, 9% mannitol, 0.1 mM nPG), for 4 - 6 h prior to enzyme incubation was also beneficial. The most suitable enzyme cocktails and the yield of protoplasts which can be realistically expected from beet plant material have been summarized in Table 2.

Table 1. Suitable conditions for the culture of *Beta vulgaris* plant material for use as a source of leaves for protoplast isolation.

Subspecies	Type†	Material	Medium‡	Gelling agent	Culture conditions	Subculture period
vulgaris	sb/fb	seedlings	½ MS 30	Gelrite	22°C, 3000 lux, 16 h day	21 d
vulgaris	sb/fb	shoot cultures	½ MS 30	Gelrite	22°C, 3000 lux 16 h day	21 d
maritima	sea beet	shoot cultures	½ MS 30 1 μM BAP	Agar	22°C, 3000 lux, 16 h day	21 d

† sb = sugarbeet; fb = fodderbeet.
‡ half strength Murashige and Skoog (1962) medium supplemented with 30 g/l sucrose. BAP = 6-benzylaminopurine

Table 2. Appropriate enzyme combinations for protoplast isolation from in vitro *Beta vulgaris* plant material. Enzymes were dissolved in CPW medium supplemented with 9% mannitol, 0.1 mM nPG, pH 5.8. Expected protoplast yield represents the yield after an overnight incubation (25°C, darkness), followed by appropriate washing / purification steps (see Hall et al., 1993 and Pedersen et al., 1993).

Subspecies	Type†	Tissue	Source‡	Enzymes	Expected yield (g FW)$^{-1}$
vulgaris	sb / fb	leaves	seedlings / sh. cultures	2 % Cellulase R-10 3 % Macerozyme R-10	± 4×10^6
maritima	sea beet	leaves	sh. cultures	0.5 % Cellulase R-10 0.75 % Macerozyme R-10	± 2×10^6
vulgaris / *maritima*	sb / fb / sea beet	petioles	seedlings / sh. cultures	1 % Cellulase R-10 1 % Cellulase TC 0.5 % Macerozyme R-10 0.05 % Driselase	± 1×10^6
vulgaris	sb / fb	-	suspensions	1 % Cellulase R-10 0.5 % Macerozyme R-10 0.05 % Driselase	± 15×10^6

† sb = sugarbeet, fb = fodderbeet
‡ sh = shoot

(c) Selection techniques

Before even considering the initiation of protoplast fusion experiments, a means must be available to permit the subsequent preferential isolation of the heterofusion products from the mass of unfused and autofused parental cells remaining in the protoplast mixture. Generally, one of three methods has been used: (i) physical selection, at the protoplast stage, for combined morphological features of both parents *(e.g.* fluorescence patterns, Mattheij and Puite, 1992); (ii) selection at the callus stage, again through the exploitation of morphological characters *(e.g.* form/colour, Derks et al., 1992) or alternatively, of introduced selectable markers *(e.g.* herbicide/antibiotic resistance, Ichikawa and Imamura, 1990) or (iii) parental cell pretreatment so that only the heterofusion products can survive. For beet, only the last method is feasible at present. The generally poor plating efficiencies and regeneration frequencies for beet protoplasts (see (e) below) restrict the use of method (i). Regarding method (ii), we are unaware of any generally-applicable characters which could conveniently be exploited. However, the recent publication of the first successful transformation method for sugarbeet (D'Halluin et al., 1992) opens up possibilities for the introduction of suitable marker genes for selection.

Protoplast pretreatments (method iii) are necessary to inhibit either nuclear or cytoplasmic function so that only heterofusion products can survive through complementation by the functional organelles of the other partner. Unfused or autofused parental cells eventually die. In beet, nuclear inactivation is possible either through irradiation (Hall et al., 1992a, b, c) or through the physical removal of the nucleus by centrifugation to produce cytoplasts (van Ark et al., 1992; Hall and Krens, 1988). A dose of 30 kRad gamma radiation is sufficient to prevent all colony formation in sugarbeet suspension and mesophyll protoplasts without having any significant effect on initial protoplast viability, as determined by FDA staining. Alternatively, using a sucrose/mannitol density gradient, cytoplasts can be obtained in sufficient numbers and at suitable degrees of purity to permit them to be included in asymmetric hybridization experiments.

For cytoplasm inactivation, an iodoacetamide treatment was found to be the most reliable (Hall et al., 1993b). However, great caution is necessary concerning the choice of concentration to use and the period of exposure. Optimum concentrations were dependent not only on the genotype and cell type but also on the subsequent culture method to be used. Inclusion of conditioned medium or a feeder system during culture require that a higher iodoacetamide concen-

tration be used. For mesophyll protoplasts, 10 mM iodoacetamide for 10 min at 4°C in darkness was usually sufficient to prevent any subsequent colony formation in unfused protoplasts. However, the optimum concentration must always be determined for each individual protoplast isolation.

(d) Protoplast fusion

Cell/cell fusion is possible using either electrical or chemical (polyethylene glycol, PEG) fusion techniques. Protoplasts are first brought into contact, after which their plasmamembranes can be induced to fuse together to form ultimately a single cell. In beet, high fusion frequencies can be obtained using electrical methods although, for the obtention of binary fusion products (involving just two cells) there were clear optima (Figure 2). Under optimal conditions (100 V/cm AC alignment, 1 MHz; 1500 V/cm DC pulse, 2 x 50 μsec) up to 15% binary fusion products per 100 aligned cells is obtainable. However, for certain lines (namely, subsp. *maritima* genotypes) electrofusion techniques had a definite detrimental effect on subsequent cell development (Table 3). In contrast, PEG fusion techniques had a stimulatory effect on plating efficiencies of these genotypes. The choice of fusion method must therefore be made only after taking into account the genotypes to be used. The selected protocol for PEG fusion is

Table 3. The effect of the fusion method upon the subsequent development of *B. vulgaris* ssp. *maritima* mesophyll protoplasts. PE = plating efficiency, defined as the % of originally-plated protoplasts giving rise to viable colonies as determined after 28 d.

Genotype	Fusion protocol	PE (%)
Bm-F	unfused	0.68
	electrofusion†	0.02
	PEG fusion	1.12
Bm-64	unfused	0.55
	electrofusion†	0.09
	PEG fusion	0.86

† Mildest possible conditions used: alignment - 100 V/cm AC, 1 MHz; fusion - 1 pulse, 50 μsec, 1050 V/cm DC

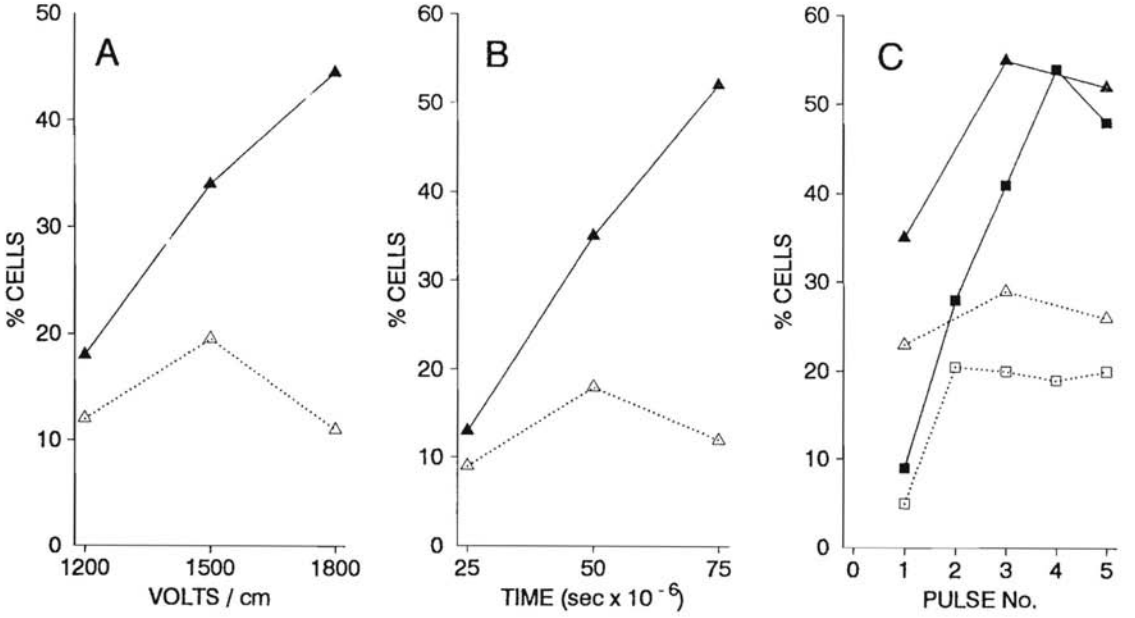

Figure 2. [A] Fusion frequency of *B.maritima* (Bm-F) mesophyll protoplasts suspended in 9 % mannitol containing 1 mM calcium chloride. Closed triangles = % aligned cells involved in all fusion events; open triangles = % aligned cells involved in binary fusions. (Alignment voltage, 100 V/ cm AC, 1 MHz, 1 pulse DC, 50 μsec. [B] As [A] but at a fixed pulse size of 1500 V / cm with varying pulse durations. [C] The influence of pulse number on the frequency of cell fusion in aligned mesophyll protoplasts of *B.vulgaris* (line Bv-NF). Closed symbols = % cells involved in all fusion events: open symbols = % cells involved in binary fusions; triangles = 100 V / cm AC alignment field, 50 μsec DC pulse, 1500 V / cm; squares = as triangles but with a pulse size of 1300 V/cm.

detailed in Figure 3. Using this method, approximately 5% of the cells available for plating out were found to be heterofusion products. Furthermore, this procedure proved suitable for mesophyll - mesophyll, mesophyll - suspension and mesophyll - cytoplast fusion combinations (unpublished results).

(e) Protoplast culture and plant regeneration

In beet, successful plant regeneration from protoplasts has only been achieved when using leaf material. Both seedlings and shoot cultures provide appropriate starting material and the majority of the genotypes tested so far have yielded plants (Hall et al., 1993b). The chosen isolation method and the osmolality of all of the media used are strongly influencial in the success of protoplast culture (Krens et al., 1990). For culture, media based on those of de Greef and Jacobs (1979), Murashige and Skoog (1962) and Schenk and Hildebrandt (1972) could not support cell division with our genotypes (unpublished observations). Only a K8p-based medium (Kao and Michayluk, 1975) led to the successful establishment of regenerable callus (Krens et al., 1990). For colony formation this medium was supplemented with 6.84% glucose, 0.1 mM n-propyl gallate, 0.2 mg/l 2,4-D, 1 mg/l NAA and 0.5 mg/l BAP. All totipotent calli had a distinct morphology, being very friable with a watery appearance. Calli deviating from this morphology were never observed to regenerate shoots and only occasionally formed roots (Pedersen et al., 1993).

While regeneration frequencies of up to 20% have been observed (Krens et al., 1990), plating efficiencies of *Beta vulgaris* L. mesophyll protoplasts are always very low and usually are $\leq$ 1%. Furthermore, plating densities often need to be prohibitively high (125000 protoplasts/ml) before cell division takes place (Hall et al., 1993a). However, the use of 25% conditioned medium or the inclusion of a feeder system (protoplasts or suspension cells) can significantly reduce the critical plating density to just 3000-4000 protoplasts/ml culture medium (Hall et al., 1993a). Improving plating efficiencies has unfortunately proven more difficult. Embedding cells in calcium alginate prior to culture or switching from mesophyll to petiole protoplasts, results in greatly enhanced plating efficiencies, with values $>$10% (Pedersen et al., 1993). Unfortunately however, both of these methods result in the preferential enhancement of a compact, non-regenerable callus type and act, therefore, to the detriment to the desired goal.

Shoot regeneration from callus of the appropriate type occurs relatively rapidly, usually within 6-8 weeks of the transfer of the

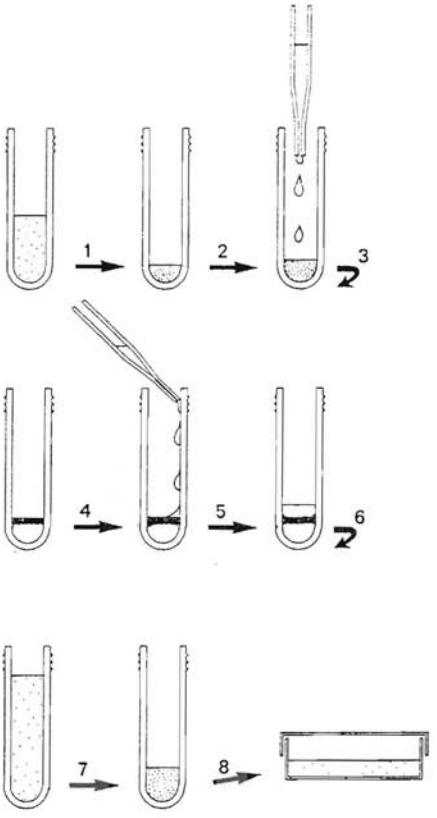

Figure 3. Polyethylene glycol (PEG) fusion method (after Gilmour et al., 1989).

1. In each centrifuge tube (Tissue Culture quality) add 1.5 x 10^6 protoplasts, suspended in CPW9M, from each parent. Centrifuge @ 35 x g for 5 min and remove sufficient medium to leave a total final volume of 300 μl. Resuspend the pellet. 2. Add, dropwise, 300 μl (10 drops) PEG solution. 3. Leave untouched for 10 min. 4. Add, very carefully, 800 μl of the high pH / calcium solution along the side of the tube. 5. Leave untouched for 20 min. 6. Gently mix the contents of the tube and add 8 ml washing medium. Centrifuge @ 35 x g for 5 min and wash a further two times with 10 ml washing medium, taking care to gently but thoroughly resuspend the pellet each time. 7. Resuspend the pellet after the third wash in culture medium and determine the protoplast density. 8. Plate out at the desired density for subsequent culture.

Solutions needed:

1. PEG solution (pH 5.7)
   30% PEG 6000, 4% sucrose, 0.147 % $CaCl_2 \cdot 2H_2O$

2. High pH/Calcium solution (pH 10.5)
   0.375 % glycine, 5.4% glucose, 0.735% $CaCl_2 \cdot 2H_2O$

3. Washing medium (CPW9M, pH 5.8)
   CPW salts, 9% mannitol, 0.735% $CaCl_2 \cdot 2H_2O$

microcolonies to solid regeneration medium. This medium is much simpler than that used for protoplast culture and consists of PGo medium (de Greef and Jacobs, 1979) supplemented with 3% sucrose, 1 μM BAP and 0.8% agarose. Shoots could be rooted with almost 100% success on the same medium but with the BAP being substituted by 25 μM IBA.

(f) Genetic analysis

Examination of the plants regenerated from leaf protoplast culture after transfer to soil revealed that $\geq 95\%$ appeared morphologically normal. Most ($>70\%$) were diploid, as determined by chromosome counting (Krens et al., 1990). For analysis at the molecular level, a suitable DNA isolation/digestion/electrophoretic/blotting and detection protocol has been determined. To this end, standard techniques with slight modifications proved very effective (Hall et al., 1992b, Saumitou-Laprade et al., 1993). In addition, mtDNA RFLP's have been identified which can be used to confirm the genetic origin of mitochondria in hybrid tissue obtained from cell fusion. In our experiments, it was possible, using two mtDNA probes *atp* 6 and *pBv* 4 (see Saumitou-Laprade et al., 1993), to distinguish between the mtDNA's from 8 different cytoplasm types (Figure 4).

SOMATIC HYBRIDIZATION EXPERIMENTS

In order to determine the optimum conditions for each procedure, it was necessary to take the individual steps separately for experimentation. Testing the entire protocol could thus only be performed after combining all of these chosen procedures in one experiment. In our study those conditions found to be optimal, as detailed above, have since been tested in a complete protocol using a range of plant materials. Fusion experiments have been carried out using the following combinations:

Nuclear donor		Cytoplasm donor
subsp. *vulgaris* (sugarbeet) mesophyll[1]	(+)	subsp. *maritima* (sea beet) mesophyll[2]
subsp. *vulgaris* (sugarbeet) suspension[1]	(+)	subsp. *maritima* (sea beet) mesophyll[2]
subsp. *vulgaris* (fodderbeet) mesophyll[1]	(+)	subsp. *vulgaris* (sugarbeet) mesophyll[2]
subsp. *vulgaris* (sugarbeet) mesophyll[1]	(+)	subsp. *vulgaris* (fodderbeet) mesophyll[2]
subsp. *vulgaris* (fodderbeet) mesophyll[1]	(+)	subsp. *vulgaris* (sugarbeet) suspension[3]
subsp. *vulgaris* (fodderbeet) mesophyll[1]	(+)	subsp. *vulgaris (sugarbeet) suspension*[2]

[1] iodoacetamide-treated; [2] gamma-irradiated; [3] cytoplasts

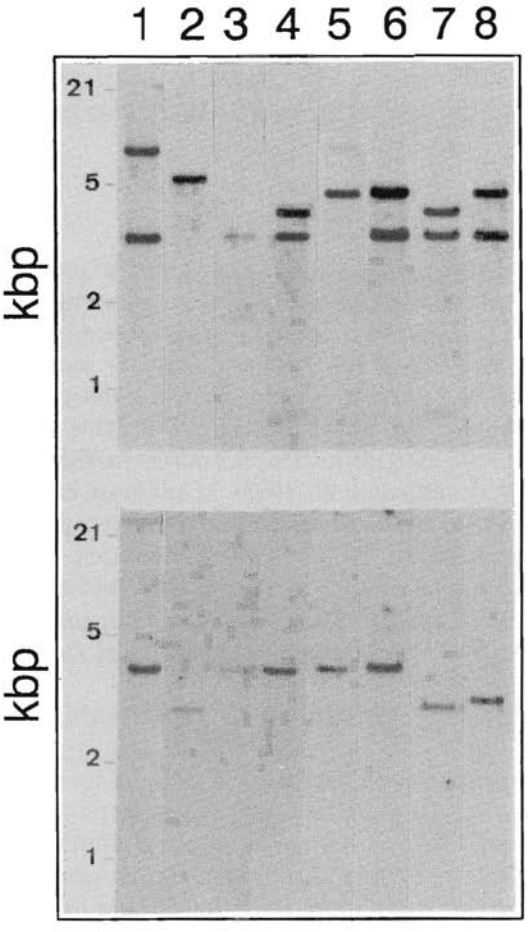

Figure 4. Southern blot analysis of *Eco*RI digests of total DNA from various *Beta* accessions. Upper series probed with the mtDNA probe *pBv*4; lower series probed with the mtDNA probe *atp*6. Accessions: Lane 1 = *B. vulgaris* subsp. *vulgaris* (fertile sugarbeet line SVP31-188, maintainer for [2]); Lane 2 = *B. vulgaris* subsp. *vulgaris* (CMS, Owen-type); Lane 3 = *B. vulgaris* subsp. *maritima* (CMS line, Bm-F); Lane 4 = maintainer for [3]; Lane 5 = *B. vulgaris* subsp. *maritima* (male sterile phenotype, CGN 870906/64); Lane 6 = *B. vulgaris* subsp. *maritima* (male sterile phenotype, CGN WB510/67); Lane 7 = *B. vulgaris* var. *atriplicifolia* (male sterile phenotype, CGN WB196/72-1); Lane 8 = *Beta vulgaris* subsp. *orientalis* (male sterile phenotype, CGN 218185 / 77-1).

However, to date, it has not proven possible to confirm the production of asymmetric hybrid callus, or plants. From most combinations, small numbers of calli were obtained (usually <10/dish) which, on reaching a suitable size, were subjected to DNA analysis to test for the presence of mtDNA from the cytoplasm donor. In every case, the mtDNA, as checked using two DNA probes, proved to be that of the nuclear donor and thus were presumably parental escapes.

DISCUSSION AND CONCLUSIONS

Beta is notoriously recalcitrant regarding most *in vitro* techniques and to some extent, remains so. However, in the course of this work we have made very substantial progress in determining suitable protocols for use with beet genotypes not only in somatic hybridizations, but also for a wide range of other biotechnological applications. It is now possible to regenerate plants from mesophyll protoplasts from a range of sugar beet and fodder beet lines. Furthermore, essentially standard techniques have been determined for plant culture, protoplast isolation, fusion, DNA analysis etc. Consequently, these results place us in a very good position to exploit *Beta* crop species for further biotechnological aims.

Concerning our ultimate goal, *i.e.* the production of asymmetric somatic hybrids in beet, and based on the results described here, two limiting factors remain which, in combination, have restricted the success of the overall protocol. The iodoacetamide treatment proved to be the most difficult to apply in a way which routinely gave reproducible effects. Despite taking great care to ensure that freshly-made solutions were used, at a precise temperature and for exact periods, considerable interexperiment variation was experienced. For this reason, per experiment, three different concentrations of iodoacetamide were tested in an attempt to ensure that one would be exactly right. Nevertheless, it appeared that in all cases, it was this treatment which proved 'leaky' and resulted either in parental escapes or in hybrid cells in which the mitochondria from the nuclear donor exclusively were able to colonise the cytoplasm. This problem was compounded by the fact that beet protoplasts still have, even in the untreated state, a low potential for cell division in regenerable systems. Had this not been the case, it would perhaps have been possible to apply more stringent iodoacetamide treatments in order to prevent escapes. There is however, at least one report in the literature where somatic hybrid cells have indeed been isolated when using protoplast cultures with a low plating efficiency of ca. 1% (Creemers-Molenaar et al., 1992). In any case, the low plating efficiencies of regenerable beet protoplast (mesophyll) systems clearly

remains the most important factor determining the success or failure of this technique. Before this fundamental limitation is removed it would appear unlikely that somatic hybridization for this species will be successful.

To date, our attempts to improve plating efficiencies with regenerable *Beta* mesophyll protoplasts have either proved generally ineffective or have resulted in a preferential enhancement in the frequency of nontotipotent calli. With the exception of the system described here, no other regenerable beet protoplast culture system has ever been described. While we are aware that several research groups have also been working on this problem for a considerable period, no success has yet been published. The known recalcitrance of this genus remains a significant barrier to its further exploitation for biotechnological applications (Hall et al., 1993b). The reasons, however, remain unknown. Further research is necessary before regeneration protocols and hence, somatic hybridization protocols, can be improved.

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