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Transfer of cytoplasm from new *Beta* CMS sources to sugar beet by asymmetric fusion

1. Shoot regeneration from mesophyll protoplasts and characterization of regenerated plants

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Summary. For our program on the transfer of cytoplasmic male sterility (CMS) by cybridization in *Beta vulgaris* L. (sugar beet), we have developed a procedure for the isolation and culture of mesophyll protoplasts of sugar beet followed by shoot regeneration. A prerequisite proved to be the presence in the media of n-propylgallate (nPG), a lipoxygenase inhibitor. Sustained divisions were found in all accessions that were tested. Plating efficiencies and regeneration ability varied greatly from one experiment to the other and appeared to be accession-dependent. Shoots could be easily transferred to soil. A majority of the regenerants (72%) retained the diploid chromosome number. Somaclonal variation in phenotype was low (4.9%). Mitochondrial DNA probes, capable of discriminating different cytoplasms of *Beta* spp. showed no rearrangements due to the protoplast and in vitro culture phase, indicating that these probes can be used to identify cybrids after asymmetric fusions. The data presented here open up possibilities for genetic engineering using protoplasts in one of the world's most important arable crops.

Key words: *Beta vulgaris* – Protoplasts – Regeneration – CMS – mtDNA

Introduction

The maternally inherited inability to produce functional male gametes is called cytoplasmic male sterility (CMS). This trait has proven to be of great importance in the production of F_1 hybrid seed of a large range of crops on a commercial scale. In sugar beet (*Beta vulgaris* L.) the CMS source identified by Owen (1945) has also been applied to this purpose. However, the use of this one cytoplasm in most, if not all, breeding programs has led to a very narrow and vulnerable cytoplasmic base in sugar beet seed production. Cytoplasmic type-related sensitivity to specific pathogens, as was found in maize (Hooker et al. 1970; Miller and Koeppe 1971; Leaver and Gray 1982), could prove equally disastrous in sugar beet. Therefore, an increase in genomic variability at the cytoplasmic level would be of considerable benefit.

Recently, there have been several reports on the identification of CMS types different from the "Owen" type (Mikami et al. 1985; Boutin et al. 1987; Halldén et al. 1988). Most of the new cytoplasmic types (=cytotypes) originate from *B. maritima* sources. Since *B. maritima* can be sexually crossed with *B. vulgaris* and since these hybrids are fertile, the new CMS cytoplasms could be transferred to *B. vulgaris* nuclear backgrounds by conventional breeding programs. However, this is a tedious and time-consuming approach. Sugar beet is a biennial plant species, and it would require five to eight backcrosses to ensure elimination of the nuclear genome of the cytoplasm donor.

Transfer of the CMS trait through in vitro cell fusion (cybridization) would be much faster and has already been achieved in *Nicotiana* (Zelcer et al. 1978), *Brassica* (Barsby et al. 1987; Menczel et al. 1987), *Petunia* (Izhar et al. 1983), *Daucus* (Tanno-Suenaga et al. 1988), and possibly in *Oryza* (Akagi et al. 1989; Yang et al. 1989). Before sugar beet could be added to this list, protocols applicable to *B. vulgaris* had to be developed for the separate steps involved in CMS transfer by cybridization. New cytotypes suitable for transfer and the mtDNA probes to identify them have been found (Samitou-Laprade et al. 1990).

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Sugar beet is one of the world's most important crops, but despite intensive efforts, the application of genetic engineering techniques to sugar beet at the cellular level has been hampered so far by its notoriously recalcitrant behavior in vitro. Here we report for the first time the development of a reproducible method for the successful isolation and culture of sugar beet mesophyll protoplasts and the subsequent regeneration of shoots therefrom. The regenerants have been characterized with regard to somaclonal variation, ploidy level, and mtDNA organization.

Materials and methods

Plant material

Generally, the diploid, fertile SVP accession no. 31-188 (NF, *B. vulgaris*) was used. Conditions for the production of aseptic seedlings were as described earlier (Krens and Jamar 1989). Table 1 provides a list of accessions or cultivars that were tested in this study.

Protoplast isolation

Leaves from 6–8-week-old seedlings were cut into small pieces (approx. 5–10 mm^2) after removal of the main vein. Preplasmolysis was performed with 1.0–1.5 g of cut leaf material in the light in 10 ml of preplasmolysis mix for 6 h at 22 °C. The mix contained CPW salts (Frearson et al. 1973), 9% (w/v) mannitol (=CPW9M), 0.1 m*M* n-propylgallate (nPG, Sigma, St. Louis), and 3.8% (w/v) $CaCl_2 \cdot 2H_2O$. Osmolality is approximately 1180 mOsm/kg H_2O. After preplasmolysis, the mix was removed and replaced by 15 ml enzyme solution, which consisted of CPW9M + 0.1 m*M* nPG + 2% (w/v) cellulase Onozuka R-10 (Yakult Honsha Co., Tokyo) + 3% (w/v) macerozyme Onozuka R-10 (Yakult) (approx. 730 mOsm/kg H_2O). Incubation was performed overnight in the dark at 25 °C with continuous gentle shaking. Subsequently, the crude protoplast preparation was collected and passed through nylon sieves (297 and 88 µm mesh) for removal of undigested material. The protoplasts were washed two times with CPW9M + nPG (approx. 550 mOsm/kg H_2O) by centrifugation for 5 min at 700 rpm (MSE Centaur 2) in 12-ml polystyrene tubes, and were purified once on CPW supplemented with 15% (w/v) sucrose (CPW15S) + nPG (approx. 550 mOsm/kg H_2O). A 1-ml layer of culture medium was placed on top of the CPW15S. After centrifugation (5 min, 700 rpm) purified viable protoplasts were easily collected in this layer.

Table 1. Accessions tested for behavior in mesophyll protoplast culture under standard conditions

Accession/ cultivar	Divisions	Plating efficiency[a]	Regeneration[b] (%)
SVP no 31-188 (NF)	+	0.04–1.0	10–20
Dippe Ero	+	0.005	1–3
Zwaanesse I	+	0.01	ND
IRS I	+	0.005	0
N144	+	0.1	ND
B. maritima CMS (collected in France)	+	0.1 –1.0	0

[a] The plating efficiency (P.E.) is the ratio of the number of microcalli at 7 weeks to the number of originally plated protoplasts, × 100 (%)

[b] The percentage of calli transferred separately to PG1B (see text) showing shoot development

ND–Not determined

Protoplast culture and shoot regeneration

Isolated mesophyll protoplasts (pps) were plated at a density of 1.5×10^5 pps/ml culture medium in the dark at 25 °C. The culture medium was K8p (Kao and Michayluk 1975) without casein hydrolysate, and was supplemented with 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) + 1 mg/l naphthaleneacetic acid (NAA) + 0.5 mg/l benzylaminopurine (BAP), together with 0.1 m*M* nPG (approx. 500 mOsm/kg H_2O). Usually, aliquots of 4-ml protoplast suspension were transferred to petri dishes with a diameter of 6 cm. Every 2 weeks, 1 ml of fresh culture medium was added. When microcalli were formed and visible (after 4–5 weeks), 1 ml aliquots were plated on top of LGT agarose (0.8% w/v Seaplaque, FMC)-solidified PGo medium (De Greef and Jacobs 1979), supplemented with 3% (w/v) sucrose and 1 µ*M* BAP (=PG1B). After 2–3 weeks, separate calli were transferred to fresh plates with PG1B solidified with 0.8% (w/v) agar (Daichin, Brunschwig, Amsterdam). Here, shoot primordia were generated within 3 weeks. Only after the occurrence of regeneration were the dishes put into the light (16 h-photoperiod, 2,000 lx). Roots were induced on PGo medium supplemented with 3% (w/v) sucrose and 25 µ*M* indolebutyric acid (IBA). Rooted plants were put directly in soil and transferred to the greenhouse.

All media mentioned in the previous sections were filter sterilized except for the PG1B medium, which was autoclaved. Assays for viability (fluorescein diacetate = FDA staining) and for cell wall formation (calcofluor white staining) were performed as described by Krens et al. (1985).

Mitochondrial DNA analysis

Total DNA was extracted from leaves of regenerated plants that had been transferred to the greenhouse, essentially as described by Dellaporta (1983). DNA samples of 1 µg were digested by restriction endonucleases according to the recommendations of the manufacturers, except for the fact that generally the amount of enzyme added to the reaction mixture was doubled. In addition, the mixture was supplemented with 2.5 m*M* spermidine. Overnight electrophoresis at 1.5 V/cm in 0.8% (w/v) agarose gels in 40 m*M* TRIS-acetate, pH 7.8, 1 m*M* EDTA, and 0.5 µg/ml EtBr was used for separation of the fragments. Subsequently, the DNA in the gel was depurinated by soaking the gel for 5 min in 0.25 *M* HCl, and was denatured in 0.5 *M* NaOH, 1.5 *M* NaCl (15 min, twice). Finally, the gel was equilibrated twice for 10 min in blotting solution (1 *M* NH_4Ac) before capillary transfer of the DNA onto nitrocellulose (Kafatos et al. 1979). Filters were baked for 2 h at 80 °C in vacuo.

Both heterologous as well as homologous mitochondrial (mt) DNA sequences were used as probes in hybridizations. The maize cytochrome c oxidase subunit I (*CoxI*) gene was a kind gift from C. Leaver (University of Edinburgh). The alpha-subunit of the F_1 moiety of the ATP synthase complex was kindly provided by A. Brennicke (University of Tübingen). The homologous probe, pBv4, was isolated from a pUC19 library of EcoRI-digested *B. vulgaris* MS5 mtDNA (Samitou-Laprade et al. 1990; G. Rouwendal, personal communication). Probe DNA was labeled nonradioactively with digoxigenine-dUTP, hybridized to target DNA on the blots, and visualized as suggested by the manufacturer (Boehringer Mannheim) with minor modifications (Samitou-Laprade et al. 1990).

Ploidy level

Young leaves or young white root tips were taken as material for chromosome counting. They were pretreated in 2 m*M* 8-hydroxyquinoline for approx. 6 h, followed by overnight fixation in ethanol:acetic acid (3:1). The plant material was macerated in 1 *N* HCl at 60 °C (leaves 3 min; roots 7 min) and subsequently squashed in 45% (v/v) acetic acid. Finally, the preparations were stained by carefully lifting the coverslip and adding a drop of 1% (w/v) aqueous crystal violet. As an average, five cells at metaphase per preparation were counted with 3–5 leaf or root preparations per plant.

Results

Protoplast isolation

Many parameters were modified in the isolation and culture procedures leading to many different conditions and protocols being tested. In the isolation procedure, yield and viability of the protoplasts were greatly influenced by the preplasmolysis step and the enzyme preparation. The osmolality of the different isolation media and the presence of nPG did have a minor effect on yield and viability, but their main effect was observed on plating efficiencies and regeneration.

As demonstrated in Fig. 1, protoplast yields increased with increasing preplasmolysis periods, with an optimum at 6 h of incubation. Although actual yields varied between individual experiments, the overall trend remained with the 6-h preplasmolysis treatment giving, on average, 2.6 times more protoplasts than the controls without a preplasmolysis step.

The enzyme mix containing 2% (w/v) cellulase R-10 and 3% (w/v) macerozyme R-10 provided an average yield of 2.24×10^6 pps/gram leaves (SD = 1.33; n = 30). As a rule, decreasing the concentrations of the enzymes lowered protoplast production, while other enzyme combinations (Bhat et al. 1986; Lindsey and Jones 1987) did not give the same number of protoplasts. Generally, 80%–90% of the isolated protoplasts was viable as determined by FDA staining. Comparing different accessions of *Beta*, it was also evident that genetic background played an important role in determining protoplast yield (data not shown).

Protoplast culture and shoot regeneration

n-Propylgallate proved to be essential for retaining high viability in the first days after protoplast isolation (Fig. 2), with an optimal concentration of 0.1 m*M*. However, after a longer period in culture (12–14 days), the beneficial effect of nPG on viability was no longer discernible. By this time, only 5%–10% of the cells stained positive with FDA, irrespective of nPG presence. However, sustained divisions and regeneration were only observed when nPG (0.1 m*M*) had been present in all of the

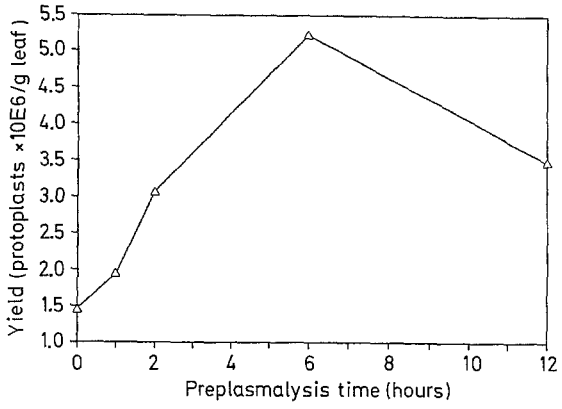

Fig. 1. The effect of different preplasmolysis periods on the yield of sugar beet mesophyll protoplasts

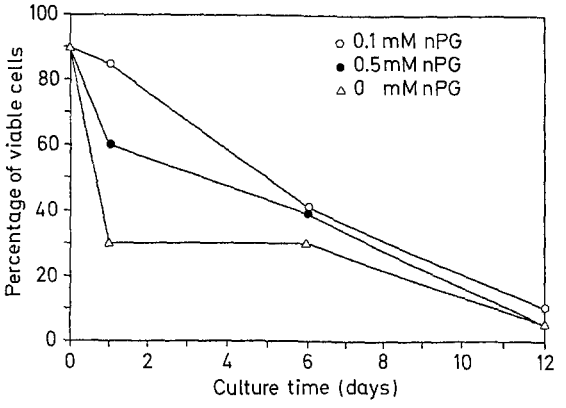

Fig. 2. The course in time of the viability of mesophyll protoplasts isolated and plated with different concentrations of nPG

isolation and culture media. Therefore, all further experiments were carried out in the presence of nPG. Figure 3 shows the effect of nPG on the morphology of the *B. vulgaris* NF protoplast-derived cells. Despite the advantageous effects of nPG, the majority of the cells in these cultures quickly turned brown and died. Synthetic and naturally occurring antioxidants such as 2,6-di-*tert*-butyl-4-methylphenol (BHT) and vitamin C, curcumin, and mepacrine could not mimic the beneficial action of nPG. In their presence, cells died after a few days and no divisions occurred.

Several plating densities were tested, i.e., 0.5×10^5, 1.0×10^5, and 1.5×10^5 pps/ml. Sustained divisions (Fig. 3 B) could only be observed at a density of 1.5×10^5 pps/ml. Transfer to the light at any stage before calli had developed completely inhibited divisions and microcallus formation. Using standard conditions as described in the "Materials and methods" section, the first divisions occurred after 7–8 days. Plating efficiencies were variable and ranged from 0.005%–1.0%, as shown in Table 1. Table 2 presents the data from a set of experiments carried out to determine the effect of the osmolal-

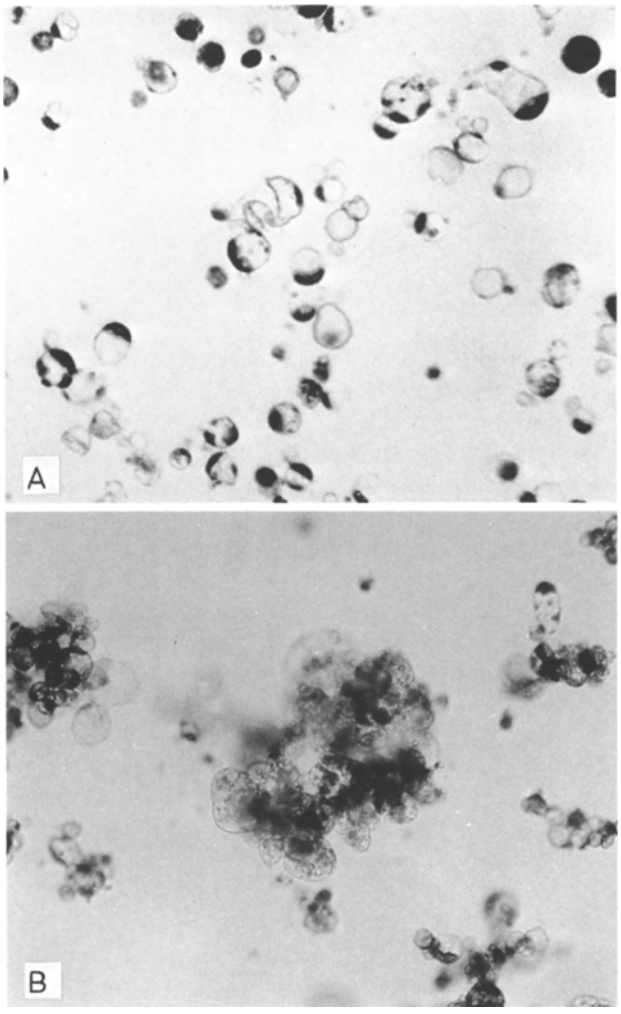

Fig. 3 A and B. The morphology of protoplast-derived cells, 3 weeks after isolation, **A** in the absence or **B** in the presence of nPG

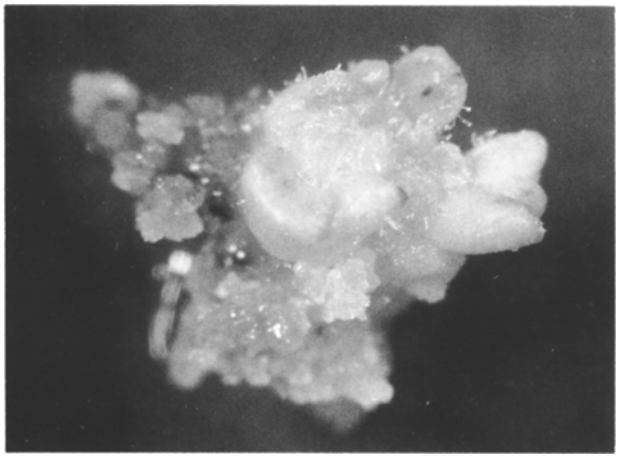

Fig. 4. Friable callus obtained from leaf mesophyll protoplasts showing the development of multiple shoot apices

Table 2. The effect of the osmolality on plating efficiency (see "Materials and methods" section for other, standard conditions)

Accession	Osmolality (mOsm/kg H_2O)	*N*	P.E.
NF	800	6	0.005
	550	4	0.040
B. maritima CMS	800	1	0.006
	550	1	0.073
N144[a]	800	4	0.950
	550	2	2.650

[a] Cell suspension protoplasts
N = number of experiments

Table 3. The occurrence of sustained divisions and regeneration related to the culture medium (other conditions were standard)

Medium	Divisions	Regeneration
K8p	+	+
mK8p[a]	±	−
PGo	±	−
RV	−	−

[a] mK8p = modified K8p (see text)

ity of the different media on the plating efficiency. It was found that an osmotic value of 550 mOsm/kg H_2O in the media gave significantly higher plating efficiencies compared to 800 mOsm. The increase appeared accession-dependent and varied from an increase of threefold to one order of magnitude.

The role of culture medium composition was assayed using K8p-, PGo-, and RV (Freytag et al. 1988)-based media. As Table 3 demonstrates, sustained cell division followed by shoot regeneration was only found when the rich K8p medium was supplied. Calli that produced shoots were always white and friable (Fig. 4). When the structure of the calli was different, i.e., more compact and solid due to the use of other culture conditions, phytohormones, or accessions, no regeneration occurred. The impact of phytohormones was studied further. The replacement of the hormones as formulated in the "Materials and methods" section by 0.1 mg/l IBA + 0.4 mg/l BAP or by 1 mg/l NAA + 5 mg/l BAP abolished all divisions. Substitution by 1 μM (= 0.22 mg/l) BAP, as present in later stages, gave similar plating efficiencies as the controls, but the regenerative capacity was lower. The same was found when microcalli were transferred from normal K8p to PGo medium supplemented with 5 or 10 μM BAP: less regeneration was observed, probably due to the formation of callus having an altered structure. Under standard conditions, regeneration frequencies of 10%–20% were obtained with the best accession (Table 1).

Fig. 5. Phenotypic variation in plants regenerated from mesophyll protoplasts after transfer to soil

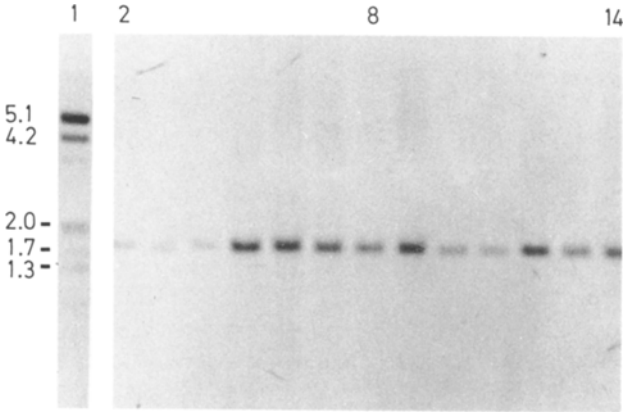

Fig. 6. Southern blot hybridization of total DNA isolated from sugar beet regenerants, digested with EcoRI and probed with the *CoxI* gene. *Lane 1:* molecular weight marker (lambda DNA cut with EcoRI and HindIII, and probed with labeled lambda DNA); *lane 2:* NF seedling (starting material); *lanes 3–14:* 12 different regenerants

Characterization of regenerants

To date, 82 regenerants have been rooted on IBA-containing medium and transferred to soil (Fig. 5), in order to allow further characterization of somaclonal variation in morphology, ploidy level, and mtDNA organization. More than 95% of the in vitro plantlets survived transfer to soil. Concerning an altered morphology (stunted growth or dwarfism), four individuals (4.9%) showed variation. No other forms of phenotypic abnormalities were observed in the regenerants. Chromosome counts were performed on root-tip or young leaf preparations of 25 plants. The diploid chromosome number of 18 was retained by 72%. The others were either tetraploid (16%, n=4) or chimeric, i.e., some roots were diploid, others from the same plant were tetraploid (12%, n=3). Total DNA of 12 regenerants was used in filter hybridizations with homologous and heterologous mtDNA probes, suitable to distinguish *Beta* cytotypes. As Fig. 6 shows no deviations in hybridization, patterns could be found taking seed-grown NF plants as a control, suggesting that no alterations in mtDNA were induced by the protoplast phase.

Discussion

New male-sterile cytoplasms in sugar beet have been isolated along with mtDNA probes that allow their rapid identification (Samitou-Laprade et al. 1990). Before specific nuclear genetic information can be successfully combined with these new cytoplasms, protocols for the separate steps that are involved, e.g., protoplast and cytoplast production, fusion, regeneration, etc., need to be developed. Preliminary data on cytoplast production and fusion conditions have been presented (Hall and Krens 1988). The present paper is the first in which a reproducible method is described for protoplast isolation, culture, and shoot regeneration in sugar beet.

A crucial role in the successful culture and regeneration was played by n-propylgallate, a lipoxygenase inhibitor. Although it did not have a direct effect on protoplast yield or viability immediately after isolation, it prolonged the period during which many cells remained viable to at least 7 days. As Fig. 2 demonstrates, viability after 12 days was almost down to levels without nPG. However, cells viable after that period continued with sustained divisions and subsequent shoot regeneration only when nPG had been present at the early stages. Other antioxidants were tested, but without any success. The leaf protoplast-stabilizing capacity of nPG has also been described for maize and has led to longer periods of viability (Saleem and Cutler 1987). It is thought that the formation of toxic peroxides by lipoxygenases is inhibited by nPG and that, thus, oxidative damage to membranes is reduced.

The yield of sugar beet mesophyll protoplasts was increased two- to threefold by preplasmolysis in a medium with a high osmotic value. Plasmolysis with $CaCl_2$ at 0.75 *M*, giving an osmotic value of 2230 mOsm/kg H_2O, has been reported in soybean (Lee-Stadelmann et al. 1985) to free protoplasts. Most of the accessions of *B. vulgaris* that were tested needed a relatively high macerozyme concentration of 3% (w/v) to allow release of mesophyll protoplasts. Qualitative and quantitative modifications to our standard enzyme mix proved unsatisfactory.

The osmotic values of both the isolation medium as well as the culture medium were found to play an important role in determining the plating efficiency. Nevertheless, plating efficiencies were still quite low at a maximum

of 1% (NF). Culture in the dark and at the right protoplast density proved essential for cell division. Other obvious factors involved were the culture medium composition and phytohormone levels. The rich K8p medium induced a lot of browning, but still allowed the remaining viable cells that had synthesized a cell wall to divide. Modification of the K8p medium, i.e., removal of most of the carbohydrates, organic acids, and vitamins (mK8p), led to less browning, but no sustained divisions were then observed. The latter was also found with other media. Changing hormonal regimes in the culture medium or increasing the BAP concentration in the PGo medium did not result in higher plating efficiencies or regeneration frequencies. In some cases the alterations gave a change in the structure of the resulting calli, making them more compact and solid. On these solid calli no shoots were generated. For shoot formation, the calli had to be white and friable. It would appear that, despite careful experimentation, there was some inherent cause of variation in these protoplast in terms of yield, plating efficiency, and regeneration frequency, which could neither be explained nor overcome. This hampered direct numerical comparison of the data of separate experiments. However, using the proper controls within each experiment it was obvious that yield, plating efficiency, and regeneration frequency were accession-dependent. Up until now regeneration has been found with two non-related *B. vulgaris* accessions. The general applicability of the technique is under further investigation.

It is well known that phenotypical alterations may occur in plants, induced by a tissue-culture phase such as callus or protoplasts. This phenomenon is called somaclonal variation. Too much somaclonal variation can be an obstacle to the application of cell or tissue culture techniques, as was observed, e.g., in certain potato varieties (Sree-Ramulu et al. 1983). It is most encouraging, therefore, that in our study somaclonal variation in morphological traits was low at 4.9%. Regenerants with a normal appearance could carry either a tetraploid or a diploid chromosome number, but diploid plants constituted the majority at 72%. All plants, both tetraploid as well as diploid were fully fertile after induction of flowering. Crossings are presently being made in order to provide further insight into the occurrence and heritability of variation. So far, the present method seems to give no aneuploidy, little polyploidy, and little phenotypic variation. The transfer of in vitro shoots to soil is successful in almost all cases (>95%) leading to fertile plants. Many of the phenotypic abnormalities disappear upon transfer to soil (data not shown). Similar observations were described by Detrez et al. (1989) using petiole regenerants of sugar beet.

For our goal, i.e., the transfer of CMS from wild beets to sugar beet, we developed molecular probes to monitor whether the desired combination of nucleus and cytoplasm had been achieved by the cybridization experiments. Also, recombination of mtDNA sequences can be identified in homologous areas with these probes. A prerequisite is that no alterations are induced by the protoplast and callus phases. In 12 regenerants that were examined, no such aberrations were detected in mtDNA using several probes that are well suited to discriminate between all of the different cytoplasm types in our research program. Since our probes did not cover the entire mitochondrial genome, we cannot exclude the possibility that some rearrangements did still take place. Brears et al. (1989), who used cosmid clones spanning the entire mitochondrial genome, found only 1 plant out of 30 where a single rearrangement had occurred. Their material was cytoplasmically male sterile, and the rearrangement reverted one of the differences in restriction fragments to the fertile configuration. However, the observed change did not convert the mitochondrial organization of their CMS plant material completely to the fertile cytotype, nor did it result in an altered phenotype. Their somaclones were not derived from protoplasts, but from callus cultures induced on flower buds. Our results suggest that molecular probes are appropriate for use in identifying the products of asymmetric fusions in sugar beet.

With this report on the techniques for successful protoplast isolation, culture, and shoot regeneration together with previous work on cytoplast production and identification of mtDNA sequences related to CMS, all steps necessary for cybrid production in *Beta vulgaris* have now been developed and can be integrated, in order to provide beet breeders with new plant material for inclusion in breeding programs.

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