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Rapid hybridization-based assays for identification by DNA probes of male-sterile and male-fertile cytoplasms of the sugar beet *Beta vulgaris* L.

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Summary. Methods are described whereby hybridization of mitochondrial (mt) DNA with different DNA probes can definitely distinguish male-fertile and and male-sterile (cms) cytoplasms of sugar beet *Beta vulgaris* L. We have developed two types of miniassays. (1) Comparative methods requiring the isolation and restriction of total cellular DNA, hybridization with cloned mtDNA fragments from either fertile or male-sterile cytoplasms, and comparison of the hybridization patterns to the fertile- and sterile-specific patterns of mtDNA of sugar beet for the given mtDNA probe. For these analyses, we routinely used 1 g of plant material to determine the type of cytoplasm. (2) Noncomparative ("plus-minus") methods requiring neither the isolation of pure DNA nor restriction, electrophoresis, or Southern blotting. Instead, alkaline-SDS plant extracts from as little as 50 mg of plant material were dot-blotted and hybridized with fertile-specific (mitochondrial minicircular DNA) and/or cms-specific probes (consisting of a 2.3-kb mtDNA sequence exclusively occurring in the cms cytoplasm). The assays are simple to perform, give definitive results, are nondestructive to the plants, and may be used in mass screening of sugar beet populations for hybrid production or in in vitro culture processes.

Key words: *Beta vulgaris* – Cytoplasmic male sterility – mtDNA probes – Miniassays

Introduction

Hybrid seed production of sugar beet is facilitated by the use of cytoplasmic male sterility (cms). cms in the sugar beet is based on one type of cytoplasm in combination with recessive alleles of at least two nuclear genes (Owen 1942, 1945). This type of cytoplasm is the only one commercially used worldwide by plant breeders. Several lines of evidence suggest that cms is encoded by the mitochondrial genome (reviewed by Weihe and Börner 1984; Lonsdale et al. 1988; Levings and Brown 1989). The mtDNAs of the fertile (N) and the male-sterile (cms or S) cytoplasm of the sugar beet differ substantially in their restriction fragment patterns (Powling 1982; Mikami et al. 1985; Weihe et al. 1985; Duchenne et al. 1989). The different restriction fragment patterns are the result of extensive inter- and intramolecular rearrangements within the mitochondrial genome, which is a common phenomenon of higher plant mitochondria (Lonsdale et al. 1988; Levings and Brown 1989). Furthermore, mtDNA sequences may occur specifically within the one or the other type of cytoplasms which are not simply a result of rearrangements (Fauron and Havlik 1989). Such sequences may reflect the origin of the cms from a particular source of cytoplasm, e.g., as a result of interspecific crosses. Sugar beet mitochondria also contain minicircular DNAs in addition to the high-molecular-weight (HMW) mtDNA: mc a (1.62 kb), mc b (1.5 kb), mc c (1.45 kb), and mc d (1.31 kb) (Powling 1981; Hansen and Marcker 1984; Thomas 1986). These plasmid-like DNAs do not seem to be involved with the occurrence of cms, although one of these minicircular DNAs, mc c, appears specifically associated with the N type of cytoplasm (in most, but not all, cases reported) (Hansen and Marcker 1984; Mikami et al. 1986; Duchenne et al. 1989). The sugar beet minicircular mtDNAs could be used as molecular "markers" of cytoplasms independently of their still unknown role in cms.

Our studies were directed to the establishment of methods for the identification of the N and S cytoplasms of sugar beet. Plant breeders are interested in fast and simple techniques to identify the specific type of cyto-

plasm in the plant material they use in breeding programs or in the search for new sources of cms. As male-fertile or -sterile plants can only be detected at the flowering stage, the development of simple screening methods relying on molecular probes provides a powerful tool to plant breeders. The miniassays we describe here can be established as routine screening procedures. They are nondestructive to the plants and definitively identify the type of cytoplasm.

Materials and methods

Plant material

Plants of any age grown under greenhouse, field, or in vitro conditions can be utilized in the assays. We used 6-day-old seedlings, green leaves, dry seeds, and a callus culture. The specific lines used were: N-KW (fertile cytoplasm); cms-KW (male-sterile); a commercial Swedish cultivar, "Regina," carrying the S cytoplasm; a cms hybrid of Japanese origin, JAP; and a number of dihaploid lines with N and S cytoplasms maintained in in vitro culture, all from the collection of the Institute for Beet Research, Klein Wanzleben.

Isolation of DNA

Total cellular DNA was isolated from 100–200 µg (seedlings) or 1–2 g (leaf, dry seeds, callus) of plant tissue with cetyltrimethylammonium bromide (CTAB), according to Rogers and Bendich (1985). The DNA pellet was dissolved in 300 µl distilled water and mixed with 150 µl 7.5 *M* ammonium acetate to remove RNA and other contaminants. After incubation on ice for 30 min and centrifugation for 10 min in a microfuge, the DNA (200–450 µg per gram of tissue) was precipitated out of the supernatant by adding 1 ml ethanol.

The mtDNA was isolated from mitochondria purified by differential centrifugation steps and DNase I treatment as described (Dörfel et al. 1989). The mtDNA was purified by CTAB (Rogers and Bendich 1985) and RNA was removed by ammonium acetate precipitation. For molecular cloning, mtDNA was additionally purified by cesium chloride/ethidium bromide centrifugation (Maniatis et al. 1982).

Restriction of DNA, electrophoresis, transfer, and hybridization

Total cellular DNA and mtDNA was digested with restriction endonucleases (Boehringer, Mannheim) according to the manufacturer's instructions. Digests (4–5 µg) were electrophoresed on horizontal agarose gels in TAE buffer and run at 50 mA for 5 h (Maniatis et al. 1982). The gels were photographed on a UV transilluminator (312 nm) and the DNA was subsequently transferred from the gels onto nylon membranes (Compas, Genofit S.A.), using vacuum blotting and an alkaline transfer protocol as recommended by the supplier of the membrane. Radioactively labelled probes were prepared by hexamer priming to a specific activity of 5×10^8 cpm/µg, according to Feinberg and Vogelstein (1984). For Southern blots of total DNA digests, hybridization was performed in 0.8 *M* NaCl; 50 m*M* TRIS-HCl (pH 7.4); 1% SDS; 0.5% nonfat dry milk, at 65 °C overnight. The filters were washed at 65 °C once in 2 × SSC-1% SDS, once in 1 × SSC-1% SDS, and once in 0.5 × SSC-1% SDS, followed by an additional wash in 0.1 × SSC-0.1% SDS. For dot blots, an alternative hybridization protocol was used: filters were hybridized in 7% SDS, 1% bovine serum albumine, 0.5 *M* sodium phosphate (pH 7.2), 1 m*M* EDTA at 65 °C overnight (Church and Gilbert 1984). Washes were as for Southern blots. All filters were exposed to X-ray film ORWO HS11 at –80 °C for 1–4 days using intensifying screens.

Preparation of plant extracts and dot blots

Approximately 50 mg of plant tissue was homogenized in an Eppendorf tube with a plastic micropistill (Eppendorf) in 500 µl 0.25 *N* NaOH, 1% SDS and incubated for 15 min at 65 °C; 250 µl 3 *M* ammonium acetate was added, the tubes were incubated for 20 min on ice, and finally centrifuged in a microfuge for 10 min at 12,000 × *g* and 4 °C. The supernatant containing 1–2 ng DNA/µl, as determined by ethidium bromide fluorescent quantitation on agarose plates (Maniatis et al. 1982), was used for dot blotting: 10–80 µl was blotted by low vacuum via the wells of a blotting device (Biodot-Apparatus, BioRad) onto a positively charged nylon membrane (Compas, Genofit S.A.) presoaked in 0.25 *N* NaOH. The wells were rinsed two times with 500 µl 0.25 *N* NaOH. The filter was neutralized in 2 × SSC for 2 × 10 min and air dried.

Molecular cloning of mtDNA probes

Two DNA libraries were established from BamHI digests of sugar beet mtDNA from N and S cytoplasms in the plasmid vector pBR322 (Meixner et al. 1989). Ligated DNA was used to transform *E. coli* strain HB101. Ampicillin-resistant colonies were transferred to tetracycline plates and positive clones were identified on the ampicillin master plates. Plasmid DNA was isolated according to standard techniques (Maniatis et al. 1982). Random clones were selected from the two libraries and used as hybridization probes (clones pN245 and pN340 are from the fertile library, clones pS035, pS567, and pS598 are from the sterile library). Clone pSX2.3 is a 2.3-kb XhoI subclone from the sterile library. pZd2.5 is a 2.5-kb EcoRI clone of the *coxII* gene from *Zea diploperennis* and was kindly provided by C. S. Levings, III, Raleigh/NC. Minicircular mtDNA pO (= mc c cloned into pBR322) was a generous gift from Dr. A. Holm Rasmussen, Aarhus, Denmark.

Results

The mtDNAs of N- and S-type cytoplasms of sugar beet differ remarkably in their restriction fragment patterns (Fig. 1). Only two types of patterns are known for sugar beet mtDNA: type 1 (N, fertile cytoplasm) and type 2 (S, male-sterile cytoplasm) (Powling 1982). On this basis, in a comparative manner, an identification of cytoplasms is always possible. However, the procedure of mtDNA isolation is too time-consuming and labor-intensive and requires a relatively large amount of plant tissue, in order to be useful for the identification of cytoplasms in a large number of individual plants. It proves not applicable, for instance, for dry seeds. The two types of cytoplasms can be distinguished in a simpler way if one uses mitochondrial gene probes. Southern hybridization with mitochondrial genes as probes is sensitive enough to allow the use of total cellular DNA rather than mitochondrial DNA as the target, even if heterologous probes are used. Figure 2 shows the hybridization patterns for *coxII* with EcoRI-digested total DNA using a clone from *Zea diploperennis* as probe. The hybridization patterns (frag-

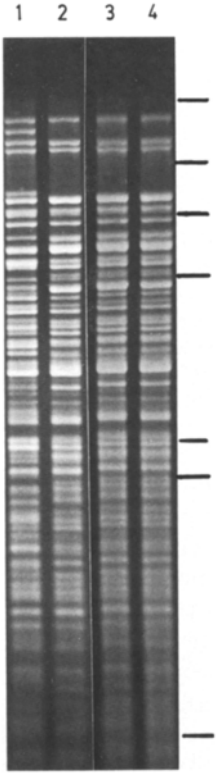

Fig. 1. EcoRI restriction pattern of mtDNA from fertile (*1* KW-N/89) and male-sterile lines (*2* KW-S/89; *3* "Regina", a commercial Swedish cultivar; *4* JAP, a hybrid plant of Japanese origin carrying the S cytoplasm) of *Beta vulgaris* L. *Horizontal bars* indicate positions of lambda-HindIII molecular weight markers (23.1; 9.4; 6.7; 4.4; 2.3; 2.0; 0.5 kb)

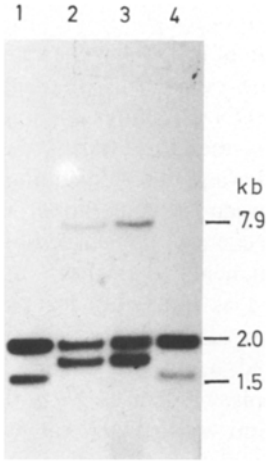

Fig. 2. Hybridization of a heterologous *coxII* probe (pZd2.5) with total cellular DNA from two fertile (*1*; *4*) and two male-sterile (*2*; *3*) lines of sugar beet. DNA (6 µg) was digested with EcoRI and blotted to nylon filters as described in "Materials and methods." Exposure to ORWO X-ray film 24 h

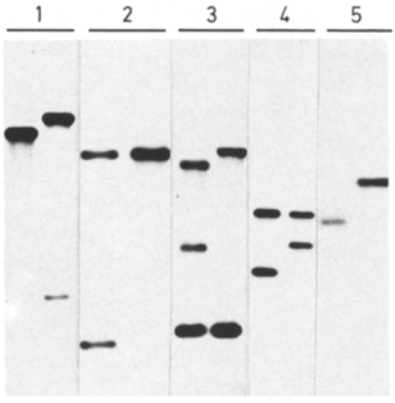

Fig. 3. Hybridization of random clones (*1* pN245; *2* pS035; *3* pN340; *4* pS567; *5* pS598) selected from mtDNA libraries of *Beta vulgaris* (see "Materials and methods") with total cellular DNA from plants with fertile (*left lanes*) and sterile (*right lanes*) cytoplasms digested with EcoRI. Exposure to ORWO X-ray film 16 h

ments of 1.5 and 2.0 kb for N cytoplasm, and 1.7, 2.0, and 7.9 kb for S cytoplasm) coincide with those obtained with purified mtDNA (Weihe et al. 1991). By using total cellular DNA, the requirement for plant material can be scaled down to 1 g or less. We routinely use leaves or seedlings, but taproot tissue, callus, or seeds may be utilized as well. The DNA preparations obtained by the CTAB method (Rogers and Bendich 1985), followed by an additional ammonium acetate precipitation step, are of sufficient quality to be fragmented by restriction endonucleases and yield clear hybridization patterns. Different restriction enzymes can be employed, but we recommend EcoRI because of its low price and stable performance.

We established DNA libraries of BamHI fragments of mtDNA isolated from N and S plants to obtain homologous probes for the hybridization-based identification of cytoplasms. Cloned fragments were tested for their capacity to discriminate between total DNA of N and S plants digested with EcoRI in Southern hybridizations. Out of 30 randomly selected clones tested, 23 revealed distinct differences. A representative example of hybridization patterns is shown in Fig. 3.

Although the procedure of hybridization of mtDNA fragments to total DNA is a reliable method to determine the type of cytoplasm, we were interested in developing an assay on a noncomparative ("plus-minus") basis. For this purpose, mtDNA sequences are required that are specific to only one type of cytoplasm. We used the cloned mitochondrial minicircular DNA p0 (mc c) (Hansen and Marcker 1984; Thomas 1986) as a probe specific for the fertile cytoplasm. Clone pSX2.3 served as a probe specific for the sterile cytoplasm. It contains a 2.3-kb XhoI subfragment from the sterile mtDNA library hybridizing exclusively to the mtDNA from S cyto-

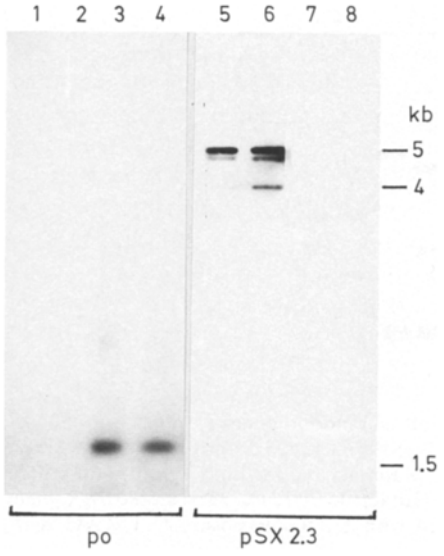

Fig. 4. Hybridization of minicircular mtDNA p0 and sterile-specific probe pSX2.3 with total cellular DNA from plants with male-sterile (*1, 2, 5, 6*) and fertile (*3, 4, 7, 8*) cytoplasms. For hybridization with pSX2.3 the DNA was digested with EcoRI. Exposure to ORWO X-ray film 16 h

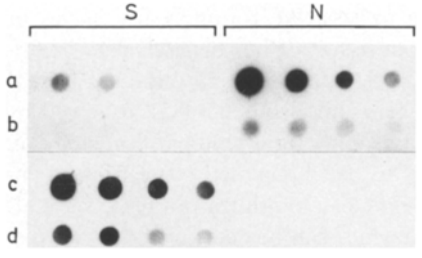

Fig. 5. Dot blot hybridization of minicircular mtDNA probe p0 (*a, b*) and sterile-specific probe pSX2.3 (*c, d*) with plant extracts from green leaves (*a, c*) and taproot tissue (*b, d*) from male-sterile (S) and fertile (N) plants. Decreasing amounts of extract were applied to the filter in each row: 80 µl, 40 µl, 20 µl, 10 µl. Exposure to ORWO X-ray film 48 h

plasm. Figure 4 shows the hybridization pattern of fertile and male-sterile cytoplasms with mc c and pSX2.3, respectively, as probes. pSX2.3, the sterile-specific probe, showed no signals or unspecific background in the fertile lines, whereas in the case of the fertile-specific probe, p0, faint, "unspecific" hybridization sometimes occurred also in the sterile lines (not shown), probably due to very low homology with HMW mtDNA sequences.

In a further step towards simplifying the method, we used plant extracts enriched in DNA rather than pure DNA, and dot blots instead of Southern blots, thereby ommitting gel electrophoresis. We had to use another hybridization protocol employing 7% SDS (Church and Gilbert 1984) to supress unspecific hybridization, which probably occurred because of contaminating proteins or

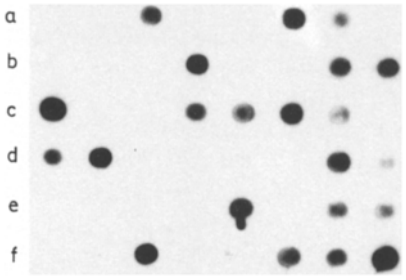

Fig. 6. Dot blot hybridization of sterile-specific probe pSX2.3 with plant extracts form single plant green leaves (*a, b*), callus (*c*), single seedlings (*d, e*), and dry seeds (*f*) from fertile and male-sterile plants. Exposure to ORWO X-ray film 48 h

other debris on the filters. Figure 5 shows the hybridization of dot blots of plant extracts with p0 and pSX2.3 as probes. The unspecific hybridization with extracts from plants with sterile cytoplasms could not be completely suppressed in the case of p0. Nevertheless, if approximately the same amounts of DNA are immobilized on the membrane, a discrimination between N and S cytoplasm is possible (see Fig. 5). Clone pSX2.3 proved to be the most powerful probe for identifying sugar beet cytoplasms in this simple, noncomparative, "plus-minus" miniassay. No background or unspecific hybridization was observed with the fertile cytoplasms, and even an eightfold difference in the amounts of fertile and sterile extracts still allowed a clear discrimination (see Fig. 5). The miniassay with pSX2.3 definitively identified cytoplasms, not only when leaves or taproot tissue were used as the source of the extracts, but also using callus, seedlings, and even dry seeds (Fig. 6).

Discussion

Restriction enzyme fragmentation of mtDNA is the simplest way to distinguish N and S cytoplasms of sugar beet. However, isolation of mtDNA requires a large amount of plant tissue (and may therefore destroy the donor plants) and is labor-intensive and time-consuming. The remarkable differences between the mitochondrial genomes of N and S cytoplasms can also be revealed by hybridization with mtDNA fragments as probes, and total cellular DNA can be used as the target for the hybridizing sequence(s). We have developed a simple, comparative, hybridization-based assay for definitive identification of sugar beet cytoplasms. It is usable with 1 g or less of plant material, from which DNA can be isolated that is pure enough for restriction and subsequent electrophoresis and Southern transfer. DNA probes can be obtained from mtDNA libraries from fertile and/or sterile plants. Most randomly selected clones from such libraries will yield specific hybridization pat-

terns for N and S cytoplasms and thus serve as molecular tools in screening programs. The large number of clones that are suitable for the identification of the cytoplasms does not seem to depend upon coding sequences on the cloned fragments (unpublished results), but rather reflects the high degree of differences between the mitochondrial genomes of the N and S type, as a result of intensive rearrangements, as well as the presence of genome-specific sequences (Brears and Lonsdale 1988).

One disadvantage of this method is that it requires DNA pure enough to be digestable by restriction enzymes and that it functions only in a comparative manner. We have therefore optimized procedures for isolating plant extracts enriched in DNA, using dot blotting for subsequent hybridization with N- and S-specific probes hybridizing exclusively with N and S cytoplasm, respectively. As little as 50 mg of plant tissue is required for this miniassay. As a fertile-specific probe, minicircular mtDNA "c" (p0) can be used, although there are reports in the literature that it may occur also in some sterile lines of sugar beet (Mikami et al. 1986; Duchenne et al. 1989). In the plant material used for commercial sugar beet breeding in Klein Wanzleben, Germany, mc c was not detected during screening of more than 800 sterile plants (Budahn et al. 1989; H. Budahn, personal communication). A low "unspecific" hybridization of p0 with sterile cytoplasms, which may occur even if one uses cesium chloride purified DNA as the target (results not shown), has no effect on the reliability of the assay, as long as the amounts of the DNAs immobilized on the filters do not differ by a factor of more than approximately two (see Fig. 5a, b). Specific probes can be found and constructed by screening mtDNA libraries and subcloning sequences that occur exclusively in one type of cytoplasm. Clone pSX2.3, which was employed in the present study, had been isolated from a mtDNA library of the S cytoplasm. Sequences like that of pSX2.3 definitively distinguish between the fertile and sterile cytoplasms, and the stringent hybridization conditions allow considerable differences of the DNA concentrations in the plant extracts, without influencing the reliability of the assay (see Fig. 5c, d). This is an important benefit for mass screening processes, where it would not be practicable to determine routinely the exact DNA concentrations. Fertile- and sterile-specific probes of this type should also be easily isolated from mtDNA libraries from other higher plants where cms plays a role in the breeding process, since considerable parts of the sterile mitochondrial genomes may consist of specific sequences peculiar to that type of cytoplasm (Fauron and Havlik 1989).

The assays described here can unambiguously identify the type of cytoplasm of sugar beet, they are nondestructive and simple, they allow screening of large numbers of plants independently of the tissue to be used, at an early stage of development, even in seed grains, so that, e.g., the degree of seed contamination in batches of commercial seed can be determined. The simplest procedure we suggest for mass screening of plants or seeds is the noncomparative, "plus-minus" miniassay with a cytoplasm-specific probe. In cases where genetic variation is expected, as in in vitro programs or if the success of organelle transformation is to be analyzed, the comparative method using Southern hybridization with total cellular DNA and mitochondrial gene probes would be preferable. The principles of the methods outlined in this report should be applicable to most plant species with cms.

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