A Simple and Efficient Method for Identification of Hybrids Using Nonradioactive rDNA as Probe

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A simple and efficient method for identification of hybrids after cell fusion was established by combining a method for the efficient extraction of DNA from very small amounts of plant tissue and Southern blotting with non-radioactively labeled rDNA from rice as a probe. One hundred milligrams of leaf or callus tissue, are sufficient for identification of the hybrid, and the amount of material can be reduced to as little as 10 mg. Since this method permits selection of parasexual hybrids not only at the plantlet stage but also at the callus stage, hybrids can be identified at the early stages of the development of the products of cell fusion.

KEY WORDS: Brassica, Lycopersicon, Nicotiana, cell fusion, hybrid identification, rRNA gene.

Introduction

Protoplast fusion facilitates the transfer of nuclear genes between plant species and is becoming an important tool in plant breeding. The technique allows the hybridization of sexually incompatible species (Melchers et al. 1978; Gleba and Hoffmann 1980). This technique has been applied not only to dicot plants but also to monocot plants such as the Gramineae (Hayashi et al. 1988; Toriyama et al. 1988).

One of the most important and difficult steps in protoplast fusion experiments is the selection or identification of hybrids. Various methods have been reported for the identification of parasaexual hybrids. Such methods include the analysis of multiple molecular forms of enzymes (Dudits et al. 1980; Evans et al. 1981; Gleba et al. 1983; Vallesios 1983); complementation of existing mutants (Melchers and Labib 1974); the identification of specific secondary substances (Rodnick and Melchers 1985) and volatile substances (Ninnemann and Juttner 1981); and the detection of genetic tumors (Carlson et al. 1972), resistance markers (Pental et al. 1986) and species-specific DNA sequences (Uchimiya et al. 1983; Saul and Potrykus 1984; Sala et al. 1985; Schweizer et al. 1988). However, these methods are not always suitable because they need relatively large amounts of sample, they are restricted to specific species, or they require facilities for the handling of radioactive materials.

The method introduced in this report can overcome the difficulties mentioned above. It is based on the method for the detection of specific DNA sequences reported by Uchimiya et al. (1983). Genes for ribosomal RNA (rDNA) which consist of highly conserved coding sequence and of species-specific intergenic spacers (Gerbi 1985; Dover and Flavell 1984; Flavell et al. 1986) are arranged in long, tandemly repeating units and are maintained in the nucleus of all cells (Appels and Honeycutt 1986). Plants generally carry from 500 to 40,000 copies of rDNA per diploid cell (Rogers and Bendich 1987). Therefore, a small
amount of plant tissue is sufficient for the detection of such sequences. Furthermore a modified method for the isolation of DNA and the use of a system for the nonradioactive labeling and detection of DNA permit the identification of parasexual hybrids at the DNA level by a simple and convenient procedure.

Material and Methods

Plant material

*Lycopersicon esculentum* Kagome 70 and *L. pervianum* LA1274, as well as their parasexual hybrid, were a gift from Dr. K. Oono, Kagome Co., Ltd. *Brassica oleracea* cv. Young (cabbage) and *B. campestris* cv. Goseki bansei (a variety of rape, also known as Komatsuna), and their parasexual hybrid, *B. campestris* Ooyabukabu (turnip) and the parasexual hybrid of this plant with *B. oleracea* cv. Young (NISHIO et al. 1987) were provided by M. HIRAI, National Research Institute of Vegetables, Ornamental Plants and Tea. *Nicotiana glauca*, *N. gossei*, the offspring (designated as G') produced by a backcross [♀ (*N. gossei* × ♂ *N. glauca*) × ♂ *N. glauca* (AKADA and HIRAI 1986), *N. langsdorffii*, and the parasexual hybrid between G' and *N. langsdorffii* were grown in a greenhouse.

Extraction of DNA

A modified version of the method of DELLA PORTA et al. (1983) was employed. One hundred mg of fresh leaf tissue or callus was homogenized with 600 μl of extraction buffer (100 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 10 mM 2-mercaptoethanol) in a glass homogenizer. The homogenate was transferred to a 1.5-ml polypropylene microtube, 40 μl of 20% SDS were added, and the solution was incubated at 65°C for 10 min. Two hundred μl of 5 M potassium acetate were added and the mixture incubated on ice for 20 min. After centrifugation at 15,000 xg for 20 min, the supernatant was transferred through one layer of Miracloth (Calbiochem) into a new microtube, mixed with 400 μl of isopropanol, and incubated at −70°C for 15 min or −20°C for 2 hrs.

The mixture was centrifuged at 15,000 xg for 15 min, and the pellet was dried in a vacuum desiccator. The DNA was redissolved in 140 μl of 50 mM Tris-HCl, 10 mM EDTA, pH 8, and centrifuged at 15,000 xg for 10 min. The supernatant was transferred to a new microtube and mixed with 15 μl of 3M sodium acetate and 100 μl of isopropanol, then centrifuged at 15,000 xg for 1 min. The pellet was rinsed with 80% ethanol and dissolved in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). When 10 or 20 mg of tissue were used, a scaled-down extraction procedure was used with half of the volume of each reagent. DNA in solution was stored at −20°C.

Hybridization and Detection

Aliquots of 200 ng of DNA were digested separately with several restriction enzymes under the conditions suggested by the supplier (Takara Shuzou Co., Ltd.). Restriction fragments were separated by electrophoresis in 0.7% agarose gels that contained ethidium bromide (0.5 μg/ml). After electrophoresis, DNA was visualized under a UV transilluminator. Pre-transfer steps, denaturation for 10 min, and neutralization for 10 min, and southern blotting to nylon membrane (Hybond-N, Amersham) for 70 min were carried out using the VacuGene vacuum blotting system (Pharmacia LKB). Similar results were also obtained by the standard
method of Southern (1975). After drying of the membrane, DNA was efficiently bound to the membrane by cross-linking with a UV transilluminator for 5 min. Non-radioactive labeling of DNA, hybridization and detection were performed almost exactly as suggested by the manufacture, using the DNA Labeling and Detection Kit Nonradioactive from Boehringer Mannheim.

Plasmid pRR217, containing ribosomal DNA from rice in the EcoRI cloning site of pBR325 and generously provided by Dr. F. Takaiwa, National Institute of Agrobiological Resources, was used as probe. Approximately 1μg of the template DNA was labeled using the Kit described above. However hybridization was carried out with 2.5ml per 100-cm² filter of hybridization solution which contained one twentieth of the total labeled DNA, i.e., an amount of DNA equivalent to 50ng of the template. The color reaction was allowed to proceed for 90 minutes. The bands of interest were detectable within a few minutes after the start of the color reaction, but the color was allowed to develop for 90 minutes for better sensitivity.

Fig. 1. a, Restriction patterns after digestion with EcoRI or BamHI of total DNA equivalent to 2mg of tissue from cabbage (1), the parasaexual hybrid (2) and rape (3). The DNA fragments were separated by electrophoresis in a 0.7% agarose slab gel. M, DNA digested with HindIII; b and c, hybridization of labeled pRR217 that contained genes for rRNA from rice to EcoRI and BamHI digests, respectively. Prominent bands specific to each species are marked by arrowheads.
Results

Total DNA was extracted from 100mg of leaf tissue of cabbage, rape, and their parasaexual hybrid, and digested with EcoRI or BamHI. Complete digestion was checked by staining with ethidium bromide, as shown in Fig. 1a. Hybridization of digoxigenin-labeled pRR217, which contained the genes for rRNA, to Southern blots of digested total DNA from the three plants generated both common and species-specific bands (Fig. 1b) The parasaexual hybrid had both parental bands. This result demonstrates that this procedure is suitable for the detection of the parasaexual hybrids.

In the case of *Lycopersicon* plants, both BamHI and XbaI produced appropriate profiles for identification of the parasaexual hybrid, generating species-specific bands, whereas EcoRI did.
not (Fig. 2). This result demonstrates that rRNA genes from rice can be used in the case of *Lycopersicon* plants.

Analysis with BamHI of cabbage, turnip, and their parasaexual hybrids generated patterns similar to that in Fig. 1, and analysis with HindIII was also performed (Fig. 3). Hybrid A had a phenotype that was more or less intermediate between the parents, while hybrid B had predominantly a cabbage-type phenotype. The patterns from Southern hybridization

![Image](image_url)

**Fig. 3** Hybridization of labeled pRR217 that contained genes for rRNA from rice to BamHI or HindIII digests of total DNA from cabbage (1), the parasaexual hybrid A (2), the parasaexual hybrid B (3) and turnip (4). Other details are the same as in the legend to Fig. 1. Asterisks placed on the right side of lanes indicate weaker bands from hybrids than those from turnip.
reflected the morphology of the hybrids. In the case of hybrid A, almost all the species-specific bands were found at nearly the same intensity as those observed in the respective fusion partners, but in the case of hybrid B, turnip-specific bands were weaker than those from hybrid A or turnip. Analysis with HindIII clearly showed that the single turnip-specific band was weak in hybrid B.

When leaf tissues of *N. glauca*, *N. gossei*, *N. langsdorffii* and G' (obtained by first crossing female *N. gossei* and male *N. glauca*, then backcrossing with *N. glauca*) and callus tissues of the parasexual hybrid between G' and *N. langsdorffii* were analyzed with *Xba*I, sexual or parasexual hybrids were identified from specific parental bands as shown in Fig. 4.

In order to elucidate the minimum amount of plant tissue that can be used for detection in this system, total DNA was extracted from 10 and 20 mg of leaves of *N. gossei* and *N. langsdorffii* by halving volumes of reagents, but using the same procedure as used for 100 mg of tissue. DNA equivalent to that extracted from a sample of only 2 mg tissue was digested with *Xba*I and subjected to the detection procedure described above. As described in Fig. 5, the same pattern and intensity of bands as those observed from 100 mg of tissue were obtained. In short, the efficiency of the extraction of DNA was almost identical between 10 mg-scale and 100 mg-scale.

**Discussion**

Identification of genuine parasexual hybrids from among non-fused or escaped lines in the selection of hybrids is an essential step in cell fusion experiments with plants. The method described here is characterized by the following important points: it is easy and simple, neither special equipment nor facilities for the handling of radioactive materials are

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**Fig. 4** Hybridization of labeled pRR217 that contained genes for rRNA from rice to *Xba*I digests of total DNA from *N. glauca* (1), *N. gossei* (2), G' (3), the parasexual hybrid between G' and *N. langsdorffii* (4) and *N. langsdorffii* (5). Other details are the same as in the legend to Fig. 1.
necessary; and the probe is labeled not with radioactive isotopes with a short half life but by digoxigenin which is stable. We tested the stability of the digoxigenin-labeled DNA, and found that it was stable for at least one month at room temperature (data not shown).

In addition to the advantages just mentioned, this new technique requires a very small amount of tissue for the analysis. We isolated DNA from 10mg of tissue and obtained good

Fig. 5 Hybridization of labeled pRR217 that contained genes for rRNA from rice to Xbal digests of total DNA from *N. gossei* (1-3), or *N. langsdorffii* (4-6). DNA was extracted from 100mg (1, 4), 20mg (2, 5) or 10mg (3, 6) of tissue, and DNA equivalent in each case to 2mg of tissue was applied to each lane for analysis.
restriction patterns. Since identification is based on the DNA itself and not on the expressed
gene products, it is possible to compare leaf tissue and callus tissue. Therefore, this method
can be used for the detection of parasexual hybrids at a relatively early stage after protoplast
fusion.

Furthermore, the technique for the extraction of DNA is simple but very efficient. Spectro-
photometric quantification of DNA following removal of RNA showed that yields ranged
from 100–200 μg per gram of tissue. The DNA isolated by this method was completely
digested at least by the treatment with EcoRI, BamHI, XbaI or HindIII.

The heterogeneity in rDNA repeats is in subrepetitive elements, which are generally be-
tween 100 and 200 base pairs (bp) in length, and it is species-specific with respect to se-
quence (see review by ROGERS et al. 1987). Recently, such elements have been cloned and
used as probes for identification of parasexual hybrids between Lycopersicon esculentum and
Solanum acaule (SCHWEIZER et al. 1988). In this latter case, species-specific elements of the
respective fusion partners must be cloned for identification of parasexual hybrids. By con-
trast, a cloned gene for rRNA from rice which contains the coding regions for 17S, 5.8S
and 25S rRNA (TAKAIWA et al. 1984, 1985a, 1985b) is used as a probe in our method.
Since these coding regions are highly conserved among all organisms (GERBI 1985), most
parasexual hybrids, if not all, should be identifiable if these regions are used as probes.
Another convenient feature of our methods is that the inserted fragments need not to be
cut out of vectors for labeling. The patterns of hybridization allow us to detect not only
parasexual but also sexual hybrids and, moreover, to characterize their chromosomal state
to some extent as well (see Fig. 3 and Fig. 4). We assume that the intensities of the
species-specific bands found after hybridization of DNA from parasexual hybrids have some
relation to the relative contribution made by the chromosomes from the two fusion partners.

Acknowledgments

We are indebted to Drs. K. OONO and M. HIRAI for generous supplies of parasexual
hybrid tissue. We also thank to Dr. F. TAKAIWA for the gift of pRR217. This work was
supported by Grants-in-Aid from the Ministry of Education, Science, and Culture of Japan.

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非放射性のrDNAプローブを用いた簡便かつ能率的な体細胞雑種同定法

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細胞融合は，安配不可能な異種植物間における遺伝子の導入を可能とし，育種上重要な手段を提供している。この際，体細胞雑種の同定，選抜は不可欠のステップであり，アイソサイムパターンの解析をはじめ，様々な方法が用いられている。しかし，それらの多くは，比較的多量のサンプルを必要とすること，特定の種の組み合わせに限られること，あるいは，取扱い上の困難さなどから必ずしも有効ではなかった。そこで，種特異的な塩基配列を持つrDNA遺伝子（rDNA）に着目し，UCHIMIYAらの方法を基により簡便で能率的な方法を開発を試みた。

Brassica，LycopersiconおよびNicotianaに属する植物を材料として用いた。まず，DELLPORTAの方法を改良し，より微量の葉（100 mg）から全DNAを抽出した。この抽出法は，細胞崩壊液からタンパク質や多糖類を酢酸カリウムにより除去し，さらにイソプロパノールによりDNAを特異的に沈殿させるものである。操作は単純で，塩化セシウムによる超速心のような複雑な操作は不要であり，短時間で済む，收量も良く，得られたDNAは制限酵素で切断することが出来た。次に，抽出したDNAの1/50量（葉2 mgからのDNAに相当）を適当な制限酵素で3時間処理し，0.7％アガロースゲルで電気泳動を行った。キャベツ，コマツナおよびその体細胞雑種の泳動パターンに示したように（Fig. 1 a），完全に切断された場合，EtBrで染色したDNAはほぼ均一なsmear bandsとなって現れた。DNAをナイロンメンプレントにトランスファーした後，クロマシ化されたイネのrDNAを非放射性のdigoxigeninでラベルしたものをプローブとして，ハイブリダイゼーションを行った（Fig. 1 b），その結果雑種植物は融合親特有のバンドを併せて有することにより，体細胞雑種としての同定が可能だった。トマト栽培種と野生種の組み合わせにおいても同様に同定できた（Fig. 2）。

キャベツ，カブおよびその体細胞雑種の結果から，雑種における種特異的なバンドの強度が，その種の表現型と一致することもわかった（Fig. 3）。雑種Aが両親の中の表現型を示したのに対し，雑種Bはキャベツに近い表現型を示したが，実際，キャベツ特有のバンドに対しカブ特有のバンドが薄くなっていた。rDNAの解析により，雑種内の2種の染色体の状態について，ある程度の知見が得られると考えられる。

タバコ属を用いた解析結果から，交配による雑種の同定も可能であり，また，体細胞雑種はカルスの段階で同定できることもわかった（Fig. 4）。さらに，より微量の葉（10 mg）から全DNAを単離し，100 mgの葉と同様，2 mg相当のDNAで解析したところ，感度の低下はみられなかった（Fig. 5）。以上のことから，比較的早い時期に同定が可能であることことが示唆された。

この同定法の長所として，先に述べたことの他，操作にラジオアイソトープ専用の施設を要せず，被曝の危険を伴わないこと，ラベルしたDNAが室温でも安定であること，制限酵素の種類を変えることにより多くの情報が得られること，さらに，かなり近縁でないあらゆる種間で解析が可能であることなどがあげられ，有効な利用が期待できる。