

BIOTECHNOLOGICAL METHODS APPLIED TO PRODUCE *Sclerotinia sclerotiorum* RESISTANT SUNFLOWER

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SUMMARY

Plant breeders are always interested in variability and new genetic sources. In the past, the sources have been limited to species able to be sexually crossed. Biotechnological methods applying cell biology, genetic engineering and tissue culture techniques now provide almost unlimited strategies to create additional breeding resources. In the past 10 years, we spent much effort using several biotechnological methods to achieve sunflower plants with a superior resistance to *Sclerotinia sclerotiorum*, which is the most important fungal pathogen of this crop worldwide. Symmetric and asymmetric somatic hybridizations were the most effective and viable alternatives to overcome the severe *Sclerotinia* infections in this crop. These results have been confirmed in preliminary field experiments of the hybrids.

Key words: Gene transfer, introgression, *Sclerotinia*, somatic hybridization, transgenic plants

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the fourth most important oil crop in the world. Successful sunflower production is endangered by fungal pathogens, e.g., *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Plasmopara*. Resistances against these diseases have been reported in wild species of the genus *Helianthus*. *Sclerotinia sclerotiorum* (Lib.) de Bary is a destructive fungus which causes severe economic damage to many crops (Fick and Miller, 1997). Currently, the commercial sunflower hybrid-cultivars are based on the same genetic background. Therefore, the existing variability within cultivated sunflowers prevents, in a short time, devel-

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opment of ideotypes for the different agroecological growth conditions. In contrast, pronounced variability within wild *Helianthus* species serves as a source of increasing genetic variability in the cultivated sunflower by using methods which provide gene flow across interspecific sexual barrier (Jan, 1997; Seiler and Riesberg, 1997). In recent years, serious efforts have been invested in developing biotechnological methods like *A. tumefaciens* transformation (Knittel *et al.*, 1994; Müller *et al.*, 2001), direct DNA transfer (Machlab, 1996), embryo rescue (Sukno *et al.*, 1999) and somatic hybridization (Krasnyanski and Menczel, 1995; Henn *et al.*, 1998; Binsfeld *et al.*, 2000) aiming at the transfer of elite resistance and quality traits to make the commercial production safer.

Sexual incompatibility between most wild and cultivated species as well as self-incompatibility and low fertility of the resulting F₁ hybrids limit the use of wild species in certain breeding methods (Jan, 1997; Sukno *et al.*, 1999). Interspecific hybridization between wild and cultivated sunflower species has been achieved by embryo rescue (Sukno *et al.*, 1999) and somatic hybridization (Krasnyanski and Menczel, 1995; Henn *et al.*, 1998; Binsfeld *et al.*, 2000). In these hybrids genetic exchange between homoeologous chromosomes upon meiotic pairing was possible.

This paper presents a successful method for getting interspecific gene flow across sexual barriers, providing a virtually unlimited potential to create additional variability for breeding programs.

Developed methods

In the past 10 years at our Institute, efforts have been made using several biotechnological methods to achieve transgenic sunflower plants with a superior resistance response to *Sclerotinia sclerotiorum*. These include:

1. Direct gene transfer into sunflower protoplast could be demonstrated employing electroporation and PEG (Kirches *et al.*, 1991). The recalcitrant genotype disabled a regeneration of a full transgenic plant.
2. Experimental studies on sunflower transformation using *Agrobacterium tumefaciens* were developed by Machlab (1996). Transgene calli and regenerated buds expressing GUS activity could be demonstrated. However, full transgenic plants were not obtained because shoots could not be rooted.
3. Successful fertile sunflower regeneration using protoplasts has been achieved using a regeneration competent genotype Florom-328 (Wingender *et al.*, 1996). This was an important milestone for hybridization experiments to follow.
4. For almost 30 years, much interest was shown in sunflower somatic hybridization between wild and cultivated species, but first authentic somatic hybrid plants between wild and cultivated sunflower species were not obtained until the success of Krasnyanski and Menzel (1995) and Henn *et al.* (1998a). The production of authentic and fertile interspecific somatic plants (SSH) has been accomplished using wild *Helianthus* species (Henn

et al., 1998b) which show resistance against *Sclerotinia sclerotiorum* stem infection (Henn *et al.*, 1997). Preliminary field experiments hint that 40% of the cybrid progenies score less than 10% infection.

- The most recent success was the production of asymmetric somatic hybrid plants (ASH) using microprotoplast technique (Binsfeld *et al.*, 2000). This technique consists of the transfer of single chromosomes carrying important genes between related, but sexually incompatible species. In this way, we are now able to produce hybrids for future breeding programs.

The production of ASH was done as schematically summarized in Figure 1. Protoplasts of the perennial sunflower species *Helianthus giganteus* and *H. maximiliani* ($2n = 34$) were isolated and cultivated as described (Binsfeld *et al.*, 1999). They were used as the donor source for micronuclei induction and isolation of microprotoplasts. As recipient of microprotoplasts, we used a regenerable annual sunflower genotype Florom-328 (*H. annuus*). The receptor protoplasts were isolated, as described by Schmitz and Schnabl (1989). Micronuclei induction, microprotoplast isolation, asymmetric fusion and plant regeneration were performed as reported by Binsfeld *et al.* (2000).

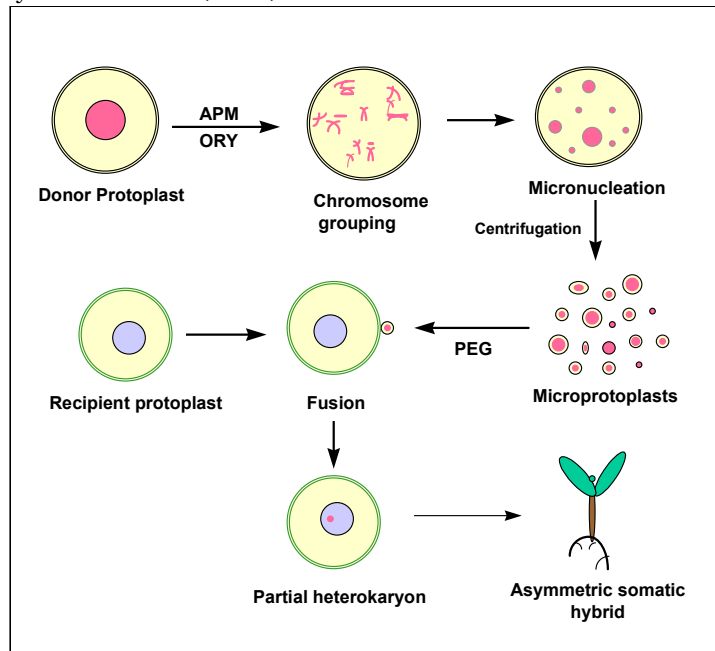


Figure 1: Schematic diagram showing the basic steps in asymmetric somatic hybridization using microprotoplast strategy for partial genome transfer. After inducing micronuclei by treatment of donor cells (from wild species) with anti-mitotic toxins (APM, ORY), microprotoplasts can be isolated from micronucleated protoplasts and fused with the recipient protoplast (*H. annuus*) in order to regenerate asymmetric somatic hybrids.

Asymmetric hybrids

The morphological variability of the regenerated ASH could be correlated to the presence of RAPD markers from wild genome. Out of 76 regenerated plants, more than 50% were positive for RAPD markers, indicating integration of genomic DNA from the wild sunflower (donor) species. The most expressive morphological differences were: leaf shape alteration, serrated leaves, early flowering, reduced growth, multiple flower buds per plant or variation of the ray flowers. Seeds resembled the crop seeds (*H. annuus*). Since the morphological variability and RAPD markers of the donor species persist in the progeny of ASH plants, it was the confirmation for sexual transmissibility of the transferred alien genome (Barone *et al.*, 2001).

The presence of additional alien genome in the regenerated ASH was also verified by chromosome counting and by flow cytometric analysis. On the basis of flow cytometric analysis, no variation at the ploidy level of the examined ASH could be detected. Additional genomic DNA was reflected in an increment of the total amount of DNA in the respective ASH. These results were similar to those reported for *Brassicaceae* somatic hybrids (Fahleson *et al.*, 1988), which showed a high correlation between chromosome number and DNA content of the somatic hybrids. Mitotic chromosome determination of ASH allowed the identification of addition lines, aneuploids and cytochimeras. Based on mitotic chromosome analysis of RAPD-confirmed ASH, we found in 75% of the plants the presence of 2 or more additional (alien) chromosomes. Even plants without additional chromosomes can be transgenic plants, as a result of chromosome recombination. This may be due to reciprocal, interstitial translocation or introgression, as observed on plants without additional chromosomes but showing RAPD markers for the wild genome.

Meiotic analysis of ASH showed that more than 85% of the meiocytes were normal, with bivalent chromosome pairing. Less than 15% showed irregular chromosomal behavior, with univalent or multivalent pairing, chromosome bridges or laggard chromosomes. This behavior is probably a result of different factors. The high percentage of bivalent chromosomes suggests the existence of a high homology between the chromosomes of the ASH (Jan, 1997). The fact that some of the bivalents were heteromorphic, open (rod bivalents) or chromosomes paired end-to-end, suggests that the bivalents did not possess complete chromosome homology, but showed only partial chromosome pairing (Sybenga, 1992; Jan, 1997). The formation of multivalents in ASH is also indicative of intergenomic homoeology, which would help in introgressing genes from perennial *Helianthus* species to *H. annuus*, as reported for somatic hybrids between *Brassica* (+) *Sinapis* (Gaikwad *et al.*, 1996).

Pollen viability and plant fertility are directly related to the presence of meiotic abnormalities caused by defective pairing, non-disjunction or unequal chromosome distribution (Jan, 1997). In ASH, decreased pollen viability was strongly negatively correlated ($r=-0.96$) with the chromosome number in root-tip cells. The lowest pollen viability was found in the ASH with the highest number of chromosomes in root-

tip cells. These results agree with those of many scientists who have attempted to correlate cytological behavior and meiotic irregularities with pollen viability, plant fertility or seed production (Sybenga, 1992; Gaikwad *et al.*, 1996; Jan, 1997).

Why this strategy?

For transgenic plants production, many different methods are available and the selection of the right strategy depends basically on the aims and the available tools to carry out the experiments.

In our case, the aim was to produce sunflower with superior resistance to *Sclerotinia sclerotiorum*. Since this resistance is a polygenically controlled trait, classical genetic engineering like cloning and transfer of individual genes would require the exact knowledge of genes involved as well as an efficient transformation system in order to transfer successive functional genes involved in this resistance. At the moment, transformation experiments in sunflower are characterized by a rather low efficiency that prevents the application of genetic engineering. Therefore, although powerful engineering strategies that are available, screening, isolation and molecular characterization of resistance genes should be anticipated on a relatively long-term basis. In this sense, for the transfer of genes which are not available (as a cloned gene), for transferring gene families (clusters) or production of addition lines, asymmetric somatic hybridization via microprotoplast fusion or microinjection of chromosomes or micronuclei represents a practical and viable alternative method aiming at partial genome transfer and introgression of resistance genes. This will help to overcome the severe *Sclerotinia* infections in cultivated sunflowers seen in preliminary field experiments.

In conclusion, the presented method makes it possible to obtain transgenic plants by a direct transfer of a single chromosome, *via* microprotoplasts technique, between sexually incompatible species. It is also a practical tool to transfer polygenetically determined traits or alien genes, for improvement and for gene pool extension of related species. The unique advantages of asymmetric somatic hybridization (microprotoplast fusion or microinjection) are that it allows the transfer of gene families for traits which are polygenically controlled or with unknown molecular background. It also allows the production of additional lines for genetic studies or even for recombination or introgression of economically important traits between sexually incompatible species.

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MÉTODOS BIOTECNOLÓGICOS EMPLEADOS PARA PRODUCIR GIRASOL RESISTENTE CONTRA *Sclerotinia sclerotiorum*

RESUMEN

Mejoristas de plantas siempre están interesados en la variabilidad y en nuevas fuentes de recursos genéticos. En el pasado, las fuentes se han limitado al las especies capaz de ser sexualmente cruzado. Com métodos biotecnológicos que aplican biología celular, ingeniería genética y técnicas de cultura de tejidos, proporcionan estrategias casi ilimitadas para crear recursos genéticos adicionales. Nos últimos 10 año, nosotros invertimos esfuerzos usando varios métodos biotecnológicos para lograr plantas de girasol con una resistencia superior a *Sclerotinia sclerotiorum*, que es la enfermedad más importante en esta cultura agrícola en todo el mundo. La hibridación somática asimétrica fue una de las alternativa más eficaces y viables para superar las severas infecciones de *Sclerotinia* en esta cultura, como podría observarse en experimentos del campo preliminares con los híbridos parciales.

MÉTHODES BIOTECHNOLOGIQUES ONT APPLIQUÉ POUR PRODUIRE LE *Sclerotinia sclerotiorum* RÉSISTANT TOURNESOL

RESUMÉ

Les breeders de la plante toujours s'intéressent à la variabilité et les nouvelles sources génétiques. Dans le passé, les sources ont été limitées à espèce capable pour être sexuel a traversé. Les méthodes biotechnologiques qui appliquent biologie cellulaire, génie génétique et techniques de la culture du tissu maintenant fournissent des stratégies presque illimitées pour créer des ressources de l'éducation supplémentaires. Dans les 10 années passées, nous avons dépensé grand nombre d'efforts qui utilisent plusieurs méthodes biotechnologiques d'accomplir des plantes du tournesol avec une résistance supérieure à *Sclerotinia sclerotiorum* qui est le fongique pathogène le plus important de cette récolte mondial. L'hybridation somatique asymétrique était une des alternatives plus efficaces et viables pour vaincre les infections *Sclerotinia* sévères dans cette récolte, comme a observé dans expériences du champ préliminaires des hybrides partiels.

