RFLP APPLIED TO INTERSPECIFIC PROGENIES REVEALED CROSS FAILURE AND TRUE HYBRIDISATION BETWEEN SUNFLOWER AND *HELIANTHUS* PERENNIAL SPECIES.

Nathalie Faure, Hervé Serieys★, Yves Griveau★, François Kaan, André Bervillé.✉

INRA-UR-GAP, Laboratoire du Tournesol, 2 place Viala, 34060 Montpellier Cedex 1
(★) Domaine de Melgueil, 34130 Mauguio. France.
✉ berville@ensam.inra.fr

RÉSUMÉ

Les espèces sauvages pérennes du genre *Helianthus* possèdent de nombreux caractères agronomiques d'intérêt (résistance aux maladies, vigueur, sources de stérilité mâle). Les croisements interspécifiques avec le tournesol cultivé ont donc été beaucoup étudiés.

Plusieurs croisements interspécifiques ont été réalisés en utilisant un hybride F_1 mâle-stérile et deux espèces pérennes: *H. salicifolius=H. orgyalis* et *H. mollis*. Les résultats des croisements sont conditionnés par les distances génétiques entre les espèces et les incompatibilités génomiques dues aux différences de constitution chromosomique. C'est pourquoi peu de graines sont produites ce qui nécessite de recourir au sauvetage d'embryon. Les observations phénotypiques ne sont pas toujours suffisantes pour valider les croisements. Nous avons donc utilisé les marqueurs RFLP pour caractériser les descendances.

Dans le croisement (*H. annuus* x *H. orgyalis*), plusieurs fragments RFLP du parent sauvage sont détectés, mais ne sont pas retrouvés dans les descendants. Ce résultat suggère des réarrangements génomiques entraînant une hybridation partielle entre les deux génomes. Nous avons observé qu'un des fragments introgressés est corrélé à la restauration de la fertilité mâle des plantes.

L'analyse montre que les individus issus du croisement (*H. annuus* x *H. mollis*) possèdent un allèle de chaque parent, suggérant que ce soient de vrais hybrides. Une de ces plante recroisée avec le tournesol a engendré une descendance dont l'analyse par les marqueurs RAPD révèle qu'à chaque locus étudié, les marqueurs pérennes ségrègent selon le ratio attendu (1:1).

SUMMARY

Due to numerous promising agronomic traits (diseases resistances, vigour and male sterility), interspecific crosses between sunflower and wild relatives were studied to broaden the genetic basis of cultivated sunflower.

Controlled crosses in the greenhouse were made using a cultivated sunflower male-sterile F1 hybrid and one of the two perennial *Helianthus* species: *salicifolius=orgyalis* and *mollis*. Few seeds were obtained using embryo rescue, since the success of the crosses depends on the genetic distances between the two parents. Phenotypic observations were not sufficient to characterise plants obtained. We used RFLP marker analysis to determine whether the different progenies were hybrids or not.

In the cross (*H. annuus* x *H. orgyalis*), several RFLP markers of the wild species were detected, but most of the markers were not recovered in the offspring. These results suggested that these hybrids originated from genomic rearrangements following the hybridisation between the two genomes, leading to partial hybridisation. We observed that one of the introgressed markers was correlated with the male fertility restoration of the plants.

The RFLP profiles of the progenies (*H. annuus* x *H. mollis*) displayed both *H. annuus* and *H. mollis* alleles, indicating that the plants were true hybrids. One plant was backcrossed with the cultivated sunflower. Analysis with RAPD markers revealed wild perennial fragments in the progenies according to the expected (1:1) allelic segregation at each locus.

INTRODUCTION

Interspecific crosses between crop species and their wild relatives are of great potential for broadening the genetic base because they may lead to gene transfer or the creation of a new species.

Wild species of the genus *Helianthus* carry several desirable agronomic traits. Distant hybridisation has been widely studied (Seiler, 1992). Results depend upon the genomic incompatibilities due to genetic constitution of the species (Georgieva-Todorova, 1984). We report here the analyses of two interspecific crosses resulting from pollination of a cultivated sunflower (*H. annuus*) hybrid with two wild perennial species. *H. mollis* that belongs to the *Corona-solis* series, and *H. salicifolius=orgyalis* that belongs to the *Microcephali* series. The two series are part of the Atrorubentes section, one of those constituting the *Helianthus* genus (Schilling and Heiser, 1981). Genetic distances are about the same between *H. annuus* and *H. mollis* and between *H. annuus* and *H. orgyalis* (Sossey-Alaoui, 1998). Morphological characterisation was not always useful to characterise the plants since their phenotypes can vary from a female parent to an intermediate hybrid appearance. We used molecular co-dominant markers to determine the genetic constitution of the plants.

MATERIAL AND METHODS

Plant material. The inbred male sterile line cmsHA89 (Cms-PET1, named A line) was crossed with the fertile male line AA724 (Cms-PET1, Leclercq, 1969, named B line) to produce a male sterile F_1 hybrid. This hybrid was used as a female parent and pollinated with two different wild perennial species to obtain interspecific progenies. We used an accession of *Helianthus mollis* MPHE-230 (PI435749), and one accession of *Helianthus salicifolius=orgyalis* MPHE-108. Regarding the interspecific cross with *H. mollis*, one plant of the F_2 generation (plant 0) was backcrossed twice with the inbred line 90HR15 to produce 34 offspring in which wild markers were searched for.

Embryo rescue. Only a few seeds were obtained so embryo rescue was used to increase efficiency of the cross. Five days after pollination, the immature embryos were excised and cultivated according to the technique used by Asad *et al.* (1986).

Restriction fragment length polymorphism (RFLP) analysis. DNA preparation, DNA restriction with *Eco*RI and *Hind*III, and Southern blotting were made according to the methods described in Lacombe *et al.*, 1999.

Probes used are sunflower cDNA obtained from Kabbaj *et al.* (1996), Ouvrard *et al.* (1996), and Sarda *et al*. (1997). One cDNA hybridising beside the Rf_1 locus on the map of cultivated sunflower developed by Gentzbittel *et al.* (1995) was used as a probe allowing the determination of the sterile/fertile status of the plant analysed (Lacombe *et al.*, 1999).

Random Amplified Polymorphic DNA. We used twelve random 10-base primers obtained from Bioprobe (Montreuil, France) or GibcoBRL. Amplification reactions were carried out according to Williams *et al.* (1990), with the following modifications: 30 ng DNA, 0.5 μM primer, 1.2 U Taq DNA polymerase (Appligène). Amplifications were performed using a Biometra (Eurogentec, France) thermocycler according to the following conditions: 94°C for 3 min, followed by 35 cycles at 93°C for 1 min, 38°C for 1 min and 72°C for 1 min, and a final extension cycle at 72°C for 6 min.

Electrophoresis of the RAPD products, DNA staining, and photographs were made according to Quillet *et al.* (1995).

RAPD fragment hybridisation. In the BC_2 progeny, fragments specific to the wild parent and present in some of the offspring were isolated from the agarose gels. DNA was purified

using a Wizard PCR Preps Purification System kit, and labelled as described above to be used as probes. Amplification products in the agarose gels were Southern blotted onto a Nylon membrane for further hybridisation with the specific probe to check the wild origin of the fragments.

RESULTS

• **Interspecific cross between the F_1 male sterile hybrid and *Helianthus mollis*.**

Pollination of one cultivated sunflower F_1 hybrid with *H. mollis* pollen produced nine plants after embryo rescue. Two plants (number 71 and 72) exhibited a cultivated sunflower phenotype and seven (number 73 to 79) were of an intermediate phenotype between the two parents with reduced pollen fertility, less than 5%. The ten probe/enzyme combinations detected polymorphism between the two sunflower lines and the wild parent. Except numbers 71 and 72, the DNA analysis revealed bands derived from the cultivated sunflower parents and fragments from the wild species.

As shown in figure n°1, plants number 73 to 79 possessed bands derived from one of the two cultivated lines and fragments coming from the wild species. Depending on the enzyme and the locus, we observed that several fragments from the wild species were detected, and that offspring did not possess the same wild fragments. All these individuals exhibited a hybrid phenotype and were almost male sterile. With some combinations, several bands present in the progeny and absent in the three parents were observed. When the DNA were digested with *Hind*III and probed with *Sdi*-6, a 6 kb band appeared in the individuals 73, 76, 77, 78 and 79. Additional bands were also detected: 8 kb with *Sdi*-10/*Eco*RI; 7.5 kb; 5.7 kb; 4.8 kb with *Sdi*-10/*Hind*III and 4.3 kb with Δ12. In some cases, one parental band was replaced by an additional band. For example, when DNA of the plant number 75 was digested with *Eco*RI and probed with *Sdi*-10, the RFLP pattern obtained was made up of one wild fragment, only one of the two sunflower AA724 line fragments and the 8 kb band neither present in the cultivated nor the wild forms.

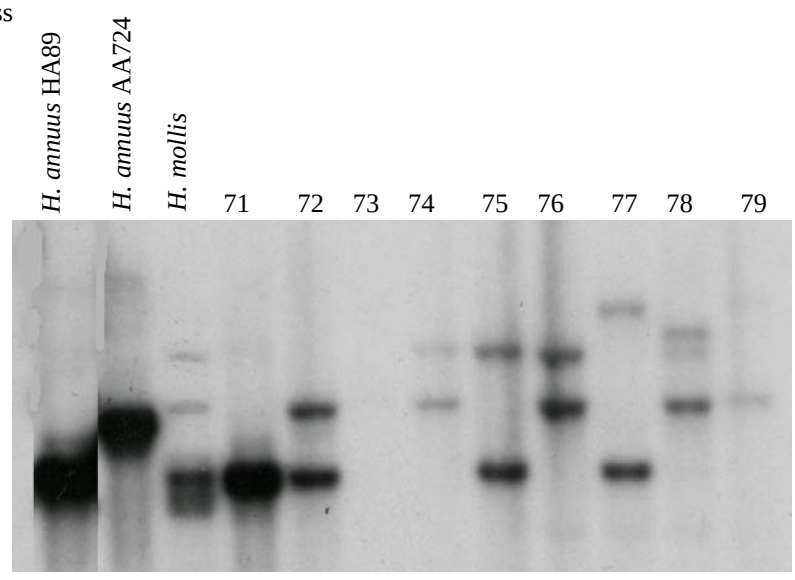

Figure n°1: RFLP analysis of the cross between *H. annuus* and *H. mollis*. DNAs were digested with *Eco*RI

Case of the plant number 71: With the different probes used, this plant was found to be homozygous (bands of one of the two lines were detected with *Sdi*-6, *Sdi*-10 and Δ12) or displayed hybrid patterns (with *Sdi*-8, *Sdi*-9 and Tip). No wild allele was detected in this plant. Since self-fertilisation of the F_1 hybrid was impossible due to the male sterile status of

the plant, we assumed that this plant resulted from an illicit fertilisation by foreign pollen.

Case of the plant number 72: The DNA analysis showed homozygous sunflower profiles (with *Sdi*-8, *Sdi*-9, Δ12 and Tip) or hybrid patterns (with *Sdi*-6). Moreover, the couple *Sdi*-10/*Hind*III revealed abnormal patterns for this individual: a 2.8 kb band was present in plant 72, but absent in the three parents. But, the pattern also showed a band similar to the 3.6 kb wild one. We observed that this plant exhibited a sunflower phenotype and was male fertile. Finally, we noticed that the global pattern was similar to another cross found that resulted from an illicit pollination (data not presented). Thus, in this case, we suspected fertilisation by foreign pollen carrying such a fragment.

Back-crosses on plant 78: After two back-crosses (plant 78 x 90HR15) x 90HR15 twelve RAPD primers revealed that, in the BC_2, three fragments were specific to the *H. mollis* and also present in some of the offspring. The amplification products obtained with the primer C04 showed a 1000 bp fragment present in thirteen of the thirty-four descendants analysed, present in the plant 78, but absent in the sunflower line 90HR15. With the primer C07, a 950 bp fragment was revealed only in plant 78 and in thirteen plants of the offspring. The third 450 bp fragment was generated with the primer B18. It was present in plant 78 and fourteen of the offspring. Molecular hybridisation therefore confirmed their presence and that the three fragments originated from the wild species. In all cases, about half of the progeny carried the wild fragments (thirteen of thirty-four in the case of C04-1000 and C07-950, and fourteen of thirty-four for B18-450). These data correspond to a classical 1:1 allele segregation (χ^2=1,23 for fragments C04 and C07, and χ^2=0,64 for fragment B18. For df=1, χ^2 (α=0,95)=3,84, and χ^2 (α=0,99)=6,63).

- **Interspecific cross between the F_1 male sterile hybrid and *Helianthus salicifolius=orgyalis.***

The cross was made using one head of cultivated sunflower that generated thirteen offspring after embryo rescue. Twelve enzyme / probe couples revealed different fragments specific to the wild species and present in eight of the offspring (numbers 82 to 86, 88, 90 and 92). Depending on the locus analysed, the profiles obtained were homozygous (line A or line B) or similar to the hybrid patterns or combined sunflower fragments with bands of *H. Orgyalis*. As in the previous cross with *H. mollis*, we detected some fragments present only in the progeny but absent in the 3 parents (3.4 kb and 3.8 kb bands with *Sdi*-10/*EcoR*I and a 3 kb band with *Sdi*-10/*Hind*III).

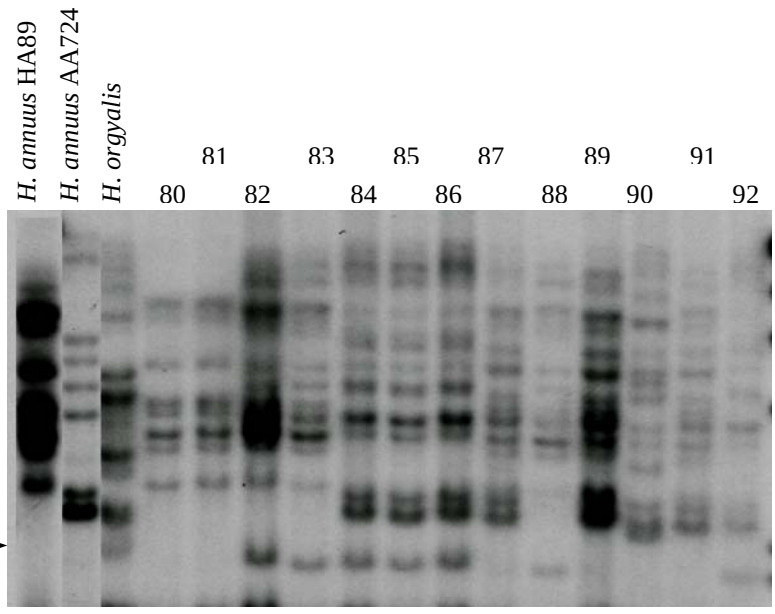

Figure n°2: RFLP analysis of plants from the cross between *H. annuus* and *H. orgyalis.* DNA were digested with *Hind*III and probed with *Sdi*-9.

All offspring displayed a cultivated phenotype except number 90, which exhibited a hybrid "sunflower- *H. orgyalis"* phenotype. We compared the male fertile status of the plant and the profiles generated with the couple *Sdi*-9/*Hind*III. We observed that the offspring (82 to 86, 88 and 92, cf. fig. n°2) which carried the *Sdi*-9/*Hind*III-2300 bp fragment were fully male fertile. We checked that plants without the fragment were male sterile (numbers 80, 81, 87, 89 and 91). Number 90 did not possess this fragment, but was partially fertile.

Five descendants did not have any fragment originating in the wild species. RFLP analysis only showed fragments, which were also present in the inbred sunflower lines. These plants had homozygous parental profiles or heterozygous (between line A and line B) profiles.

DISCUSSION

The eight cDNA used as probes with the two enzymes revealed polymorphism between the parental sunflower lines, and between the cultivated and the wild species. RFLP profiles obtained allowed us to check the origin of the distant crossing products, and to explain the possible mechanisms involved. In the case of *H. mollis*, allele segregation led to two types of heterozygous profiles: line A-*H. mollis* and line B-*H. mollis*. These descendants did not only carry some wild species introgressed fragments, but resulted in a complete distant hybridisation. We concluded that the cross generated true hybrids between the two species. These hybrids represent a new and promising material for sunflower improvement. In a previous work, Cazaux *et al.* (1996) used molecular RAPD markers specific to annual or perennial *Helianthus* species to analyse interspecific crosses. A similar cross was analysed, and the authors showed that the progenies were partially hybrids. The *H. mollis* plant employed for crossing was not genetically fixed, for this reason, the eight descendants obtained were different. Due to numerous gamete combinations and meiotic recombination, progenies displayed different genotypes. Then, hybridisation between the two *Helianthus* species led to several genetic combinations, and in each of the offspring, the wild fragments detected in the RFLP analysis were different.

We noticed that at some loci, the profiles were homozygous (for example, number 77 with *Sdi*-8). Since the hybridisation occurred between two different genetic entities, (genome HC for the wild species and CPA for the sunflower, Sossey-Alaoui, 1998), we supposed that chromosome rearrangements led to genetic instability in the hybrids.

After two back-crosses, the wild fragments were detected in the offspring according to the expected segregation 1:1 (significant Chi-square test). These data confirmed that plants were true hybrids between *H. mollis* and *H. annuus*. We concluded that this cross generated seeds formed by germ cells hybridisation. They were not derived from an apomictic process, which might have been disturbed by the wild genome (translocation or retention of a portion of the *H. mollis* genome). Cytological analysis may be carried out to check the number and origin of the chromosomes.

In the second cross between *H. annuus* and *H. salicifolius=orgyalis*, wild fragments were detected in eight of the thirteen plant progeny. In this case, RFLP profiles generated were not heterozygous *H. annuus-H. salicifolius=orgyalis* regardless of the probe. Each cDNA revealed several wild fragments; each one present in some of the offspring. We were unable to determine the allelic relations between the different fragments detected. We assumed that this distant cross led to partial hybridisation, that is the introgression of diverse wild parental fragments in a sunflower genetic back-ground. Each of the descendants came from a particular fertilisation process, including chromosomal breakage, translocation, or retention, and some unusual means allowing introgression to occur. All these rare events might explain why some offspring had sunflower heterozygous or only homozygous patterns. We noted that

plant number 90 seemed to be more introgressed by the wild genome than other progenies. All the probes used revealed introgression, whereas only some wild fragments were detected with some probes in the remaining plants.

In the two different crosses, the few new bands in the RFLP analysis that belongs to any of the parents could be further evidence for chromosome rearrangement. In the cross with *H. mollis*, analyses showed that the additional bands may replace in some cases one of the parental fragments detected. Recombined fragments may be due to "genomic shock" (Mc Clintock, 1984) that occur during interspecific crosses. Such quantitative and qualitative DNA variations have already been studied by Natali *et al.* (1998) in a cross between *Helianthus annuus* and *H. tuberosus*. In the cross with *H. orgyalis*, cytological observations could determine that either the alien DNA was integrated into the sunflower genome or if it was constituted in some fragments trapped in the nucleus. So, these progenies might have been generated by a haploidisation process during which the wild genome would not have been completely eliminated, and caused chromosome rearrangements, as in the case of the cross *Oryza alta* x *O. sativa*, reported by Mao *et al.* (1995). But, we can not conclude this based on the present molecular analysis.

An *a priori* interesting introgressed wild fragment was correlated to the restoration of the PET1 male fertility. Several male fertility restoration sources had already been discovered in wild species, and reports suggested *Helianthus salicifolius=orgyalis* as Rf source (Christov *et al.*, 1996).

In some RFLP profiles, (plant n°71 of the cross *H. annuus* x *H. mollis*, and plants n°80, 81, 87, 89, 91 of the cross *H. annuus* x *H. salicifolius=orgyalis*), did not have any wild fragment detected. All the probes revealed homozygous or heterozygous sunflower profiles. No foreign fragment was displayed. We concluded that they originated from an illicit fertilisation with foreign pollen carrying similar alleles to the sunflower parents.

This study showed that distant hybridisation can induce different genetic structure in materials (even in a same progeny): with partial to complete hybridisation. In some cases, data suggested the occurrence of a possible haploidisation mechanisms. The utilisation in sunflower breeding is of interest for different and complementary purposes (introgression, partial introgression, haploidisation and the use of molecular markers was particularly efficient to control the nature of hybridisation products.

REFERENCES

Asad A. *et al.* 1986. C. R. Acad. Sc. Paris, t. 302, série III, n°5: 161-164.
Cazaux E. *et al.* 1996. Proc. of 14th Sunflower Conference, Beijing, China, 1093-1098.
Christov M. *et al.* 1996. Helia,19 (24): 65-72.
Genzbittel L. *et al.* 1994. Theor Appl Genet, 89: 419-425.
Georgieva-Todorova J. 1984. Zpflanzenzücht, 93: 265-279.
Kabbaj A. *et al.* 1996. Helia, 19 (25): 1-18.
Lacombe S. *et al.* 1999. Helia, 22 (30): 19-28.
Leclercq P. 1969. Ann. Amélior. Plant., 19, 99-106.
Mc Clintock B. 1984. Science, 226: 792-801.
Mao L. *et al.* 1995. Genome, 38: 913-918.
Natali L. *et al.* 1998. Theor Appl Genet, 97: 1240-1247.
Ouvrard O. *et al.* 1996. Plant Mol Biol, 31: 819-829.
Quillet MC. *et al.* 1995. Theor Appl Genet, 91: 1195-1202.
Sarda X. *et al.* 1997. Plant J, 12: 1103-1111.
Seiler G. J. 1992. Field Crops Research, 30: 195-230.
Schilling E. and Heiser C.B. 1981. Taxon, 30: 393-403.
Sossey-Alaoui K. *et al.* 1998. Theor Appl Genet, 97: 00-00.
Williams J. G. K. *et al.* 1990. Nucleic Acids Res, 18: 6531-6535.