Phenotypic and molecular analyses of « sunflower x *Helianthus mollis* » interspecific crosses

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INTRODUCTION

Interspecific crosses between cultivated sunflower (*H. annuus L.*) and perennial species have been frequently reported with polyploid species (Seiler et al. 1992) and scarcely with a diploid species (Georgieva-Todorova, 1981, Skoric 1992, Christov 1991). However, the wild perennial species of sections Ciliares and *Atrorubentes* represent a huge genetic potential available to introgress disease resistance genes or other major agronomic traits in the cultivated sunflower. The control of the nature of hybridization products is essential for breeders, even if in some cases the use of male sterile plants facilitates the control of the cross. The direction of the cross remains an important aspect in connection with the use of nuclear or cytoplasmic components (Serieys 1994). In most of cases, phenotypical traits allow a clear characterization of the "hybrid" status of the material, nevertheless, this kind of identification becomes more delicate when occured selfing, chromosome set elimination (*Hordeum bulbosum* system), apomixy or limited chromosomic exchanges. The molecular markers offer a rapid way to characterize putative hybrids and to explain the involved mechanisms.

In Montpellier we have focused our work on *Helianthus mollis* Lamb, because this diploid perennial species displays tolerance traits to main sunflower diseases: *Plasmopara helianthi* (Seiler 1988), *Phomopsis* (Skoric & Rajcan 1989), *Sclerotinia sclerotiorum*, *Orobanche cumana* as well as restoration factors of CMS-PET1 (Christov, 1992). *H. mollis*, is naturally growing in the east of North America and has very distinct external characteristics and does not appear to be closely related with any other (Rogers et al. 1982). It hybridizes with some other species of the perennial group, but crosses are uncertain with the members of sect. *Helianthus* species (Georgieva-Todorova, 1967). Furthermore, through molecular diversity analyses (Sossey-Alaoui et al. 1993), *H. mollis* appears also as the more distant species in comparison with cultivated sunflower. The availability of "section specific" molecular markers provides a powerful tool for a rapid identification of the interspecific plants since the phenotypical aspect of the progenies do not allow a clear identification of the interspecific hybrids everytime. *H. mollis* x sunflower hybrids will serve as a model species to unravel the mechanisms which prevent usually crosses between these 2 distant species.

Among 94 F1 plants from 16 crosses we expected interspecific hybrid plants. We did not detect any such hybrid plants but we recognized 40 plants without any trace of the male parent, whereas in 54 plants we detected few fragments coming from the male parent. The occurrence of such fragments in the 54 plants is quite variable in a progeny. Two non exclusive explanations are suggested.

MATERIAL AND METHODS

Plant material

Wild helianthus species:

In order to hybridise the cultivated sunflower, we have used different individual plants from five accessions of the wild perennial *H. Mollis* (Table 1).These accessions: MPHE-286, MPHE-230, , MPHE 600, MPHE 673 and MPHE 742 were maintained at INRA-Montpellier Breeding Station. The last four have been introduced from USDA: MPHE-600, 673 and 742 are issued from PI 468761 and MPHE-230 from PI435749. The accession MPHE-286, introduced from the Vavilov Institute (St Petersbourg) is segregating for the male fertility.

Sunflower inbred lines

Six sunflower inbred lines were used in cross with *H. mollis* either as male parents (LA, WG, HA89, RHA274), or female parents (cms-HA89, cms-2603, cms-85A3). The LA, WG , 85A3, 2603 are maintainer or female INRA inbred lines, whereas HA89 and RHA274 are maintainer and restorer public lines for CMS-PET1, respectively (Table 1).

Interspecific hybridization

The interspecific crosses between *H. annuus* and *H. mollis* were performed in reciprocal way, in the field, during summer 1994. The wild and cultivated parental plants, individualy identified, were bagged before anthesis to prevent cross pollination. In all cases pollination was performed with freshly collected pollen. In the crosses where *H. mollis* was used as female parent, male sterile plants from MPHE-286, were used to control the pollination. In the other cases, we did not perform emasculation, since efficient self incompatibility systems prevent self-pollination. The reciprocal crosses were performed on cytoplasmic male sterile (CMS) inbred lines to avoid selfing.

Plant growth in the greenhouse.

The number of seeds produced in the interspecific crosses appeared low and strongly dependant of the considered genetic combination. The germination of the seeds was started in Petri dishes on a wet filter paper moistened with a solution of 40 ppm ethephon in distilled water, to break down the seed dormancy. The germinated seeds were then tranfered in 6 liters pots containing horticultural compost and daily irrigated with a commercial "Hakaphos" nutrient solution. The parental wild and cultivated genotypes were grown together with the interspecific material in the greenhouse. The mean growing conditions was day:25 ±1 °C night 18 ± 1 °C, 16 hours daylight supplemented with sodium lamps (Philips agro).

Phenotypic observations

In order to characterize the hybrid combinations, several peculiarities of branching, restoration, pollen fertility, seed set, anthocyans. The seed set was also measured by counting the number of akens produced on single heads, either in self pollinated, sib crossed or back-crossed plants. Chromosome counting was performed on rootips of all the plants of *H. annuus* x *H. mollis* progenies according to Bervillé et al (1992).

Molecular analyses

We used the method described in Sossey-Alaoui et al 1993, 1996 to amplify fragment unique to either the species of sect. *Helianthus* or the species of sect. *Atrorubentes*. These fragments have been checked on large samples of more than 40 *Helianthus* species (Sossey-Alaoui et al. 1996).

RESULTS

H. mollis x sunflower crosses.

The nine F1 progenies looked like the *H. mollis* parent as judged at the phenotypic level by wild branching, leaf and flower shape (Table 2). Moreover, because of the genetic diversity of the *H mollis* plants used in crosses a clear evidence that crosses occured was not founded. RAPD fragments were applied on the expected hybrid plants. Those RAPD fragments unique to sect. *Atrorubentes* were always found in all the *H. mollis* parent and the F1 progenies. In contrast those fragments unique to sect. *Helianthus* were not recovered in the F1 progenies except for few of them (Table 3). The individuals of one progeny were not identical for the presence of these fragments.

Sunflower x *H. mollis* crosses.

Progenies from seven crosses, i.e., 64 plant were studied. All the plants looked like cultivated sunflower but clear-cut evidences for the presence of foreign genes appeared in comparison with the female lines as controls (Table 2). Branching, restoration of the male fertility, anthocyanic colour of the corolla, ... were observed in most of the F1 plants whereas not present in the sunflower lines. We looked for RAPD fragments unique to species of sect. *Atrorubentes* in the F1 progenies (Table 4). All the controls clearly indicate that these fragments were present in the *H. mollis* plants of all the accessions but were absent in the sunflower lines. In progenies, the RAPD markers revealed the presence of *H. mollis* genomic fragments. In contrast, the phenotypic traits did not allow such a conclusion. All the crosses displayed at least one plant carrying either a sect *Atrorubentes* fragment or a trait from the *H. mollis* parent. This was the case for the HA89 x MPHE-230-10 cross lacking of phenotypic trait from *H. mollis* but which revealed RAPD fragments from *H. mollis* (tables 2 and 3). Our result of chromosome counting was homogenous for all the plants and progenies, we found 34 chromosomes.

DISCUSSION

In F1 progenies of the interpspecific hybrid progenies we expected an equilibrated contribution of the two diploid species on the basis of RAPD fragment patterns. Surprisingly there is a major contribution of the species taken as female and a poor contribution of the male. All the female fragments were recovered in each progeny as expected. In contrast, the majority of the F1 plants is completely deficient in fragments expected to be brought by the male gamete. However some F1 plants displayed a different set of male fragments even in one progeny. There is also a large variation for the male contribution in one progeny.

n the basis of RAPD fragment patterns any plant in *H mollis* x *H annuus* and *H. annuus* x *H. mollis* crosses displayed the sum of fragmentspresent in both parent. There is a strong avantage for the female genome and the male contribution was always a little. We recovered in the F1 progenies plants with 34 chromosomes suggesting that spontaneous chromosome doubling occured in the F1 plants. Two non exclusive mechanisms are therefore likely to explain our results:

1) The fertilization is normal (equal contribution of both female and male gametes) and then male chromosome elimination occurs as reported for *Hordeum* species favorising the female set (Jensen 1975). In this case traces of chromosomes which we observed should be male chromosome or fragment remains as we found them. Spontaneous doubling must occur to explain 34 chromosomes in all progenies. Dihaploid plants are therefore expected more or less with male contribution.

2) The fertilization is not normal: complete female set contribution plus none or only

few chromosomes from the male contribute to the egg. Then the female chromosome set must be doubled with random contribution of the male chromosome set. Dihaploid plants are therefore expected with more or less male contribution.

From a breeding point of view both mechanisms might be used to produce dihaploid plants. To introgress sunflower chromosome elimination in mechanism 1 may allow to screen for fragments carrying traits to improve sunflower. Chromosome doubling might be used to produce dihaploid sunflower in a very simple way. Recent genotypings of progeny revealed dihaploid structures but this remains to be confirm in a large scale.

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Table 1: Phenotypical traits of *H. mollis* populations and of *H. annuus* L.inbred lines.

Plant material	% branching	% male fertile plants	Pollen fertility %	Selfing Seed set [1].	Seed set Sib-cross
H. mollis MPHE-230	100 (wb) -	100	> 95	< 1	20.2[1]
H. mollis MPHE-286	100 (wb) -	60	> 95	< 1	9.2
H. mollis MPHE-600	100 (wb) -	100	> 95	< 1	43.7
H. mollis MPHE-673	100 (wb) -	100	> 95	< 1	69.8
H. mollis MPHE-742	100 (wb) -	100	> 95	< 1	63.4
Inbred line LA	0	100	> 95	> 200	
Inbred line WG	0	100	> 92	> 200	
Inbred line CMS-85A3	0	0	0	-	
Inbred line HA89	0	100	> 90	> 200	
Inbred line CMS-HA89	0	0	0	-	
Inbred line CMS-2603	0	0	0	-	
Inbred line RHA274	100 (ab)	100	> 95	> 200	

(*) male-sterile plant

Branching type : wb (wild), ab (recessive apical branching), [1] < 1 less than 1 seed /head

Table 2: Phenotypical traits of *H. mollis* and *H. annuus* interspecific progenies.

H mollis accession (= female)	*H annuus* line (= male)	Number of progenies	% branching	% male fertile plants	Pollen fertility %	Seed set Selfed	Seed set [1] backcross
MPHE-286.4	LA	1	100 (wb)	0	0	0	0.5
MPHE-286.9	LA	1	100 (wb)	100	77.5	0	nd
MPHE-742.7	LA	5	100 (wb)	100	78.7	0	0.4
MPHE-600.8	PAH3	2	100 (wb)	100	75.7	0	0
MPHE-286.5	WG	4	100 (wb)	0	0	0	nd
MPHE-286.1	HA89	2	100 (wb)	0	0	0	nd
MPHE-286.4	HA89	7	100 (wb)	0	0	0.1	0.6
MPHE-673.9	HA89	2	100 (wb)	100	97.7	0.0	nd
MPHE-286.1	RHA274	2	100 (wb)	0	0	0.	nd
Cms-85A3	MPHE-230-5	20	50.0	45.0	88.1	159.8	299
Cms-85A3	MPHE-742.1	14	13.3	35.7	81.6	105.6	7.6
Cms-85A3	MPHE-742.3	3	50.0	66.6	50.6	92.5	92.5
Cms-85A3	MPHE-742.5	5	0.0	20.0	99.3	240	0
Cms-2603	MPHE-230-7	12	33.3	9.1	97.5	282	153
Cms-2603	MPHE-600.10	4	0.0	50.0	98.8	248	151
Cms-HA89	MPHE-230.10	4	0.0	0.0	0	0	nd

([1]) mean number of seeds harvested on a single head; nd not determined

Table 3: Analyses of « *H. mollis* x sunflower line » interspecific progenies.

H mollis accession (= female parent)	*H annuus* line (=male)	number of plant in progenies	number of primers	plant missing *H mollis* fragments	plant with *H annuus* fragments	Obserevd H. annuus fragments	expected *H. annuus* fragments
MPHE-286.4	LA	1	8	0	1	2	31
MPHE-286.9	LA	1	8	0	1	2	31
MPHE-742.7	LA	5	8	0	4	1*-2**	31
MPHE-600.8	PAH3	2	7	0	2	2	31
MPHE-286.5	WG	4	8	0	4	6	26
MPHE-286.1	HA89	2	8	0	2	3	29
MPHE-286.4	HA89	7	8	0	6	3	29
MPHE-673.9	HA89	2	8	0	2	3	29
MPHE-286.1	RHA274	2	8	0	2	3	31

* is the lowest number of fragments from sunflower found in one plant
** is the highest number of fragments from sunflower found in one plant

Table 4: Analyses of progenies issued from (sunflower line x *H. mollis)* crosses.

H annuus line (= female parent)	*H mollis* accession (= male parent)	number of plants in progenies	number of primers applied	plant missing *H annuus* fragment	plant with *H mollis* fragments	observed *H mollis* fragments	Expected *H mollis* fragments
Cms-85A3	MPHE-230-5	17	18	0	12	1*- 5**	60
Cms-85A3	MPHE-742.1	13	11	0	3	1 - 2	45
Cms-85A3	MPHE-742.3	3	15	0	3	2 - 3	57
Cms-85A3	MPHE-742.5	5	11	0	1	4	40
Cms-2603	MPHE-230-7	12	13	0	4	1.- 4	48
Cms-2603	MPHE-600.10	4	12	0	4	1	39
Cms-HA89	MPHE-230.10	4	10	0	3	1	32

* is the lowest number of fragments from *H. mollis*r found in one plant
** is the highest number of fragments from *H. mollis* found in one plant