Mapping QTL specifying pollen viability in an interspecific cross of sunflower with *Helianthus* argophyllus.

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Introduction

It is important to better understand the reproductive barriers between cultivated sunflower and wild Helianthus species to utilise genetic resources in breeding programs. Several *Helianthus* species are of great interest for the genetic improvement of the cultivated sunflower (Seiler 1988). Interspecific progenies could serve as an important variability resource, unfortunately little is known about fertility and recombination efficiency with cultivated sunflower or their consequences on the maintenance of variability.

Helianthus argophyllus T. & G. is closely related to wild *H. annuus* L. (Heiser *et al.* 1969). The 2 species display common morphological characteristics such as general plant architecture and large leaves in contrast to other annual species with small leaves. Several genetic pools have been derived from interspecific hybrids between *H. annuus* and *H. argophyllus* in order to introgress wild traits into the cultivated background (Morizet *et al.* 1984; Miller *et al.* 1992; Seiler 1992). However, the role of reproductive barriers has not been studied and furthermore, correlations between the quantitative contribution of the *H. argophyllus* genome to these pools and recombination frequency have not been examined. With molecular markers, QTLs controlling wild traits of agronomic interest can be determined. In addition, loci which correlate with such traits as interspecific sterility can be identified provided that a map is available in the appropriate crosses.

To address these problems, we have constructed a genetic linkage map in a backcross population derived from *H. annuus* and *H. argophyllus*, and identified genetic factors limiting pollen viability of interspecific BC1 plants. Pollen viability has been analysed in the light of cytogenetic data from this BC1 progeny.

Material and methods

Plant material

The genetic map was constructed on the basis of 133 backcross (BC1) progeny obtained from the cross [*H. annuus* cv PEF1-RHA274 x (*H. argophyllus* n°92 x *H. annuus* cv RHA274)]. The *H. argophyllus* parent was a unique plant from the accession FRAINRAMPG n°92 (IBPGR code). The BC1 population was obtained by crossing a unique F1 interspecific hybrid plant onto the cytoplasmic male sterile (CMS) line PEF1-RHA274. RHA274 maintains the PEF1-CMS whereas *H. argophyllus* accession n°92 restores it. BC1 progeny and 10 plants of each parental genotype (RHA274 and *H.*

argophyllus x RHA274) were sown in a greenhouse. They were transplanted in the field at the first leaf stage.

The relation between pollen viability and chromosome end arrangements at meiosis was evaluated on 15 BC1 male fertile plants which were full sibs of the ones used in the linkage map. These BC1 plants were sampled to represent variability for pollen viability in the mapping population. Cytogenetic analyses were performed on parental genotypes RHA274, *H. argophyllus* and *H. argophyllus* x RHA274.

Pollen viability (i.e. non aborted pollen) was estimated according to the staining procedure of Alexander (1969). Pollen viability was noted as the percentage of stained nuclei, on a minimum of 300 pollen grains per sample. Floral buds at the R2 stage, according to the Schneiter and Miller (1981) scale, were harvested in late morning, fixed in Carnoy's solution and maintained at 4°C prior to observation. An average of 20 PMCs per plant were scored for chromosome end arrangements.

Phenotypic and enzymatic markers

Stigmata colour (locus *Stigp*) and CMS *fallax* restoration (locus *RF1-PEF1*) were used as genetic markers because of their monogenic inheritance in the BC1 population. The segregation of six isozyme loci was scored: *Mdh1*, *Mdh2* (malate dehydrogenase), *Pgm1* (phosphoglucomutase), *Sdh1* (shikimate dehydrogenase), *Acp1* (acid phosphatase) and *Me1* (malic enzyme). Isozyme preparation was performed on cotyledons of 8 day- old plants. Electrophoreses were carried out on starch gels in histidine-citrate pH 5.7 buffer (Quillet *et al.* 1992).

RAPD markers

Total DNA was isolated according to Gentzbittel (1990). Random 10-bp primers were obtained from Bioprobe (France). Amplification reactions were carried out according to Quillet et al 1995. Of 102 primers screened, 15 amplified 2 to 4 fragments unique to the *H. argophyllus* parent. The 133 BC1 progeny were divided into 4 sets of about 33 individuals. Amplification reactions were performed independently on each set of plants. Each segregating fragment in the BC1 population was assigned to one locus. The presence of a RAPD fragment among BC1 plants was considered as the heterozygous state and the absence of fragment was assigned to the homozygous state. Data concerning primer sequences and fragment nomenclature are available from the authors.

Linkage map construction and detection of factors affecting pollen viability

Segregation at each locus was checked against the expected 1:1 ratio using a Chi-square test. A map was constructed using MAPMAKER version 3.0b (Lander *et al.* 1987) establishing linkage groups with recombination fraction values less than 0.35 and the LOD score threshold of 3.0. Map distances in centiMorgans were calculated using Kosambi's mapping function (Kosambi 1944). For details of map construction see Quillet et al. (1995).

Association between genetic markers and pollen viability were detected by analysis of variance with the SAS GLM procedure (SAS Institute Inc. 1987). Marker loci effects were declared significant when the probability levels associated with *F*- values were less than 0.01.

Results

Genetic marker segregation and linkage map

Segregation data for genetic markers were analysed for 133 BC1 progeny. Eleven loci (10 RAPD marker loci and the *Me1* isozyme locus) displayed segregation distortion at the 5% level. Eight out of these 11 loci displayed a deficit and 3 an excess of the enzymatic allele or RAPD fragment from the *H. argophyllus* parent. A total of 40 RAPD fragment segregations were scored for the mapping of the BC1 population.

The basic map with 37 non distorted markers had 8 linkage groups and 9 isolated markers with a threshold LOD score of 3 and a maximum recombination fraction of 0.35. Relaxing the mapping conditions to include LOD score variation between 3 and 2 and recombination fraction variation between 0.35 and 0.45 did not place any isolated marker on an existing linkage group. After corrections according to models 1 and 2, the addition of the 11 marker loci showing segregation distortion led to 8 linkage groups and 9 isolated markers (Fig. 1). Ten of these 11 markers were placed on 3 linkage groups. Model 1 was applied to correct marker orders for linkage group 2 because some pairs of linked markers were distorted in opposite orientation. The hypothesis of selection against the parental heterozygous genotypic class (model 2) led to the construction of linkage group 1 with 11 markers covering 196 cM. The final linkage map represented 390 cM with a mean distance between linked markers of 14.4 cM and 13 of the 31 intervals of less than 5 cM. On linkage group 3, 7 markers were clustered on approximately 15 cM (Fig. 1).

Genetic markers associated with interspecific pollen viability

The BC1 progeny was produced on the PEF1 male sterile cytoplasm. The *H. argophyllus* n°92 accession restores fertility to this cytoplasm and we observed segregation for this trait in the BC1 population. About half of the plants (74 plants out of 133 BC1 progeny) used for linkage map construction, were male fertile as expected for the segregation of one dominant male fertility restorer gene. These plants displayed a mean pollen viability of 58.2% ranging between 23.6% and 96.5 % (Fig. 2). Parental genotypes of the BC1 population (RHA274 and *H. argophyllus* x RHA274) displayed extreme values for this trait with low intra-genotype variability. The trait distribution in the population was significantly different from normality with at least 2 modes, therefore, the data were transformed by a logarithm function for further analysis.

One-way analysis of variance revealed significant effects for 3 markers on linkage group 1. The most important effect was displayed by the *A11_8* locus which explained 39.2% of pollen viability variation. Two additional genomic regions were detected on linkage groups 2 and 3. The most informative markers, *D17_12* on linkage group 2 and *Me1* on linkage group 3 explained 18 % and 18.3 % of total phenotypic variation, respectively. A multiple regression model analysis, including all the markers with significant effects, explained 82.9 % of the total phenotypic variation for pollen viability. For each of these genomic regions, the heterozygous state of markers conferred a negative effect on pollen viability. Moreover, markers strongly linked to pollen viability systematically displayed segregation distortion with a deficit in heterozygous genotypes. In fact, out of the 17 plants homozygous for the loci *A11_8, D17_12* and *Me1*, 16 exhibited more than 75% viable pollen (Fig. 3). Only 3 plants were found heterozygous for these 3 loci and their pollen viability was less than 30%. The disequilibrum between the number of heterozygous and homozygous plants for these 3 loci explained in part the non normal distribution observed for this trait. Plants with 1 or 2 of these 3 loci in the heterozygous state had pollen viability ranging from 29 to 62 percent.

Relation between pollen viability and meiotic behaviour of F1 and BC1 hybrids

In the F1 hybrids, 5% of PMCs had one tetravalent and less than 1 % had 2 tetravalent (Fig. 4) or univalent configurations. The number of rod bivalents varied from 3 to 14 chromosome pairs in interspecific hybrids instead of 0 to 5 in parental species. The meiotic behaviour of the 15 BC1 plants was quite variable but the range for the number of rod bivalents, univalents, and the presence of one or two tetravalent configurations was intermediate between that exhibited by the parental species and F1 interspecific hybrids.

Discussion

Genetic map

The unsaturated genetic map has 39 markers assigned onto 8 linkage groups. In addition there are 9 unlinked markers. The map covers less than 400 cM and represents a minimum of 20-25% of the genome according to the intraspecific nearly saturated maps for sunflower (Gentzbittel *et al.* 1995; Berry *et al.* 1995) or *Helianthus anomalus* (Rieseberg *et al.* 1993).

About one fourth of our markers displayed segregation distortion. The distortion was generally low (only 3 out of the 11 loci showed a significant deviation at $P< 0.001$) but to place some of them on the linkage map required the use of corrected models to estimate unbiased genetic distances and linkage probabilities. Marker positions in the frame of model 1 did not interact with pollen viability. Model 2 was applied when segregation distortion was considered as a consequence of selection against the heterozygous parental genotypic class in locus pair segregation. This model led to a robust significant linkage between the *A11_4* and *D17_20* loci (LOD score =5.5, r = 0.25) and to the construction of linkage group 1 which displayed a disproportional length in comparison to other linkage groups. Linkage group 1 covered 196 cM and represented half the total map length. This situation could be attributed to clustering of polymorphic markers in this genomic region because of reduced recombination, non random distribution or structural heterozygosity. In this last case, it is possible that the 2 genomic segments on opposite sides of a translocation break-point exhibit linkage even though they are on different chromosomes in one of the parental species. Such explanations have already been hypothesised for the banana (*Musa acuminata*) linkage map (Fauré *et al.* 1993).

Genetic factors controlling interspecific pollen viability

According to Chandler *et al.* (1986), *H. argophyllus* and *H. annuus* karyotypes differ only by 2 reciprocal translocations. Interspecific hybrids are highly vigorous but female and male fertilities are reduced. From a breeding point of view, *H. argophyllus* contains several disease resistance factors (Rogers *et al.* 1982; Skoric 1985; Griveau *et al.* 1992, Quresh *et al.* 1993), and is a source for cytoplasmic male sterility and male fertility restoration genes (Christov 1990; Serieys 1994). The meiotic behaviour of *H. annuus* x *H. argophyllus* hybrids has already been analysed by several authors (Heiser 1951). These different studies have reported the presence of a single tetravalent in a few percent of PMCs. We observed a high number of rod bivalents in hybrid meiosis whereas few were observed in parental species. Partial pairing in F1 interspecific hybrids might be due to early partition of 2 tetravalents. The abnormalities in the meiotic behaviour of BC1 plants were correlated with a reduction in pollen viability. These results support the hypothesis that chromosome rearrangements are the preponderant factors for the observed reduction in pollen viability in this interspecific progeny.

Using the genetic map, 3 genomic regions were detected which, in combination, explain about 80% of the pollen viability variation in the BC1 population. A genetic factor having a major effect on pollen viability is located close to the *A11_8* locus on linkage group 1. This location is consistent with the hypothesis of structural heterozygosity explaining the abnormal length of this group and the decrease in pollen viability due to reciprocal exchanges. Markers associated with pollen viability on linkage groups 2 and 3 displayed less effects. Although these linkage groups did not have a particular structure, we suggest that these genomic regions are also located on opposite sides of a second

translocation break-point because of the strong relation between pollen viability and chromosome end rearrangements.

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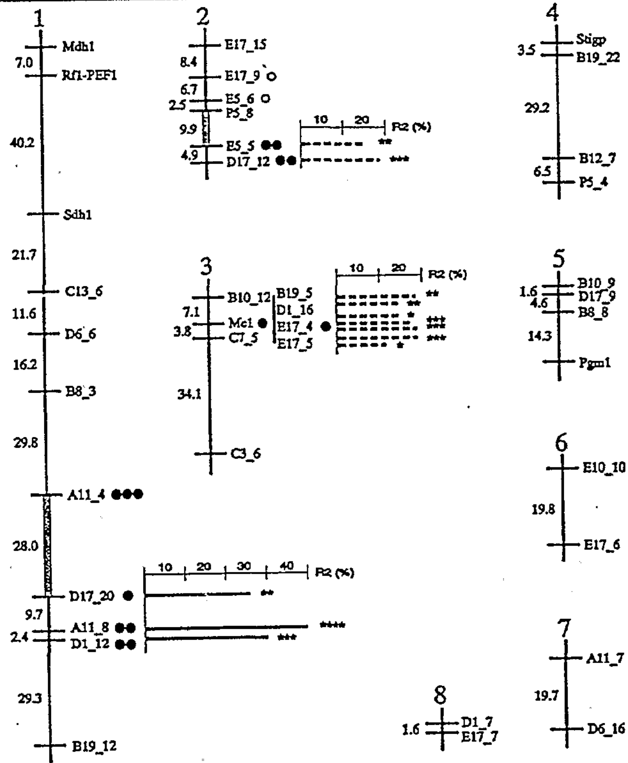
Figure 1: Linkage map based on 48 genetic markers derived from the cross [*H. annuus* cv PEF1-RHA274 x (*H. argophyllus* n°92 x *H. annuus* cv RHA274)] and location of putative factors controlling pollen viability. Marker loci names are on the right and the Kosambi map distances on the left of the linkage groups numbered from 1 to 8. Grey vertical bars indicate corrected interval distances in the frame of model 1 for linkage group 2 and significant linkage between loci *A11_4* and *D17_20* in the frame of model 2 for linkage group 1. Segregation distortion with a deficit of the heterozygous genotypes (● P<0.05, ●● P<0.01, ●●● P<0.001) or excess of the heterozygous genotypes (O P<0.05, OOO P<0.001) are indicated on the right of locus names. Loci listed on the right of linkage group 3 could not be ordered with likelihood support ≥ 100:1. Horizontal lines indicate the percentage of phenotypic variation (R^2) for pollen viability explained by the individual locus and asterisks represent probability levels (* P< 0.01, ** P<0.001, *** P<0.0001). Continuous lines correspond to results of one-way analysis of variance and dashed lines to two-way analysis of variance with locus *A11_8* as a fixed factor.



Unlinked markers: Mdh2, Acp1, B19_10, C3_4, C7_4, D17_6, E10_2, E17_11, C13_5 OOO

Figure 2: Pollen viability distribution for the 74 restored male fertile BC1 plants according to the homozygous or heterozygous state of the 3 loci *A11_8*, *D17_12* and *Mel* which display the strongest significant R^2 for pollen viability on linkage groups 1, 2 and 3.

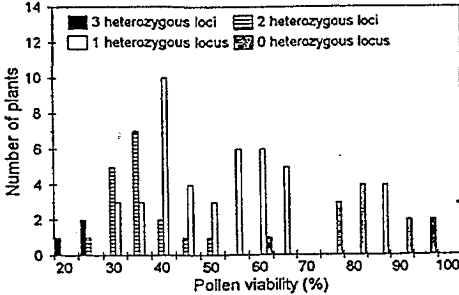


Figure 3: Correlation between mean number of ring bivalent per PMC at metaphase I and pollen viability of interspecifc BC1 plants, *H. argophyllus* x *H. annuus* cv RHA274 (F1), *H. argophyllus* (arg) and *H. annuus* cv RHA274 (ann). Plants which displayed at least 1 tetravalent (▼) or 2 tetravalents (●) among the PMCs scored are indicated.