ISOZYME AND CYTOGENETIC ANALYSIS IN *HELIANTHUS RESINOSUS*

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Abstract

Isozyme analysis and meiotic studies were done in order i) to examine the isozyme variation in the hexaploid *H. resinosus* in comparison to the diploid level, ii) to obtain useful markers for genotype identification of interspecific progeny, and iii) to associate isozyme segregation patterns to chromosome pairing behavior. ADH and PGI systems showed more complex zymograms in comparison with diploid species. Duplicated genes constitute molecular markers for hexaploid level and allow identification of hybrid plants in interspecific crosses. We observed asymmetric heterozygosity in GDH patterns and this points to the occurrence of tetrasomic inheritance. Meiosis in *H. resinosus* was found quite regularly and bivalents are the most frequent type of chromosome association. A low percentage of lagging chromosomes in dyads and tetrads together with more than 90% of pollen stainability revealed a high fertility for this polyploid species. Specific alleles, gene dosage effect and dominance of bivalent pairing support an autoallopolyploid origin with prevalence of autosyndesis.

Introduction

The genus *Helianthus* includes 49 species which have been classified in four sections and six series, using morphological and crossability data (Schilling and Heiser, 1981). The basic chromosome number is x=17 for diploid, tetraploid and hexaploid species. Due to its complex origin, polyploidy ancestry (particularly hexaploidy) among *Helianthus* species is not well delineated (Seiler and Rieseberg, 1997).

Helianthus resinosus Small is a perennial, hexaploid species (2n =102) placed in section Atrorubens, series Corona-Solis of the *Helianthus* genus (Schilling and Heiser, 1981; Seiler and Rieseberg, 1997). Hybrids and backcross generations between *H. resinosus* and cultivated lines of *H. annuus* L. have shown to be resistant to the parasitic plant *Orobanche cernua* Loefl., with relatively high pollen viability and seed set (Sukno et al., 1998). The cytoplasmic male sterility introduced from *H. resinosus* into sunflower inbred lines shows a stable expression and the existence of male-fertility restorer genes has been proved (Echeverría et al., 2003). Also *H. resinosus* is considered a source for resistance to *Sclerotinia sclerotiorum* (Lib.) de Bary (Serieys, 1987; Mondolot-Cosson and Andary, 1994).

Isozyme markers have largely contributed to elucidate the phylogenetic relationships within annual species of *Helianthus* (Rieseberg et al., 1990, 1991). Molecular markers are

suitable in verifying the hybrid status of F1, F2 or backcross generations at the seed stage. Morphological evaluation of progeny does not always allow the identification of hybrid plants. Individuals obtained from crosses between annuals and perennials show phenotypes resembling the parental species and a number of morphological intermediate plants (Gavrilova et al., 2000).

The aims of this study were i) to examine the isozyme variation in the hexaploid *H. resinosus* in comparison to the diploid level, ii) to obtain useful markers for genotype identification of interspecific progeny, and iii) to associate isozyme segregation patterns with chromosome pairing behaviour. Banding patterns were interpreted on the basis of zymograms of diploid species *H. petiolaris* (Carrera and Poverene, 1995), *H. argophyllus* (Carrera et al., 1996) and *H. annuus* (Pizarro et al., 2000, Carrera et al., 2002).

Materials and Methods

Plants. An *H. resinosus* population, accession PI 435864 (USDA), and F1 hybrids with *H. annuus* inbred lines, provided by the experimental station of INTA Balcarce, Argentina, were studied: HA 89 CMS PET-1 x *H. resinosus,* HA 89 (B) x *H. resinosus* and *H. resinosus* x B1.

Isozymes. Samples were prepared from seeds soaked for 24 hours using a 0.1M Tris-HCl-mercaptoethanol buffer, pH 7.5. The following enzymes were assayed: alcohol dehydrogenase (ADH), esterase (EST), glutamate dehydrogenase (GDH), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), phosphoglucoisomerase (PGI) and 6-phosphogluconate dehydrogenase (PGD). The isozymes were resolved on 12% horizontal starch gel; the buffer systems and staining methods are described in Carrera and Poverene (1995), after Soltis et al., (1983). The number of loci and alleles were interpreted according to Torres (1983), Rieseberg and Soltis (1989) and Carrera and Poverene (1995).

Cytological Studies. Immature heads of *H. resinosus* were fixed in Clarke's fluid (3:1). After 24 hours, the heads were transferred to 70% ethanol and were kept in a refrigerator at 10C until used. Meiotic stages were scored in pollen mother cells after staining them in carmine (Snow, 1963) and making the squashes in acetic acid 45% followed by aceto-hematoxylin 2%. Pollen fertility was estimated as percentage stainable according to Alexander (1980).

Results

Isozyme Markers. *Helianthus resinosus* zymograms proved to be more complex compared to the diploid level. However, we could make some inferences about the genetic control of bands, based on previous knowledge of these enzymes in diploid species.

ADH. This dimeric enzyme showed the common bands present in cultivated sunflower, designed *Adh-1* F/*Adh-2* S (Torres, 1983) and a third zone of higher mobility, close to *Adh-2* (Figure 1). The phenotypes in this zone consisted of individuals with two bands or no bands, and intensity variation. Similar patterns have been found in species where duplication events have taken place (Roose and Gottlieb, 1980). A duplicated locus with two alleles, one of them co-migrating with an allelic variant of another locus, explains the observed phenotypes. Individuals with two bands were considered homozygous aa or heterozygous ab, with

differential staining, and individuals lacking the bands were homozygous bb which overlaps *Adh-2*.

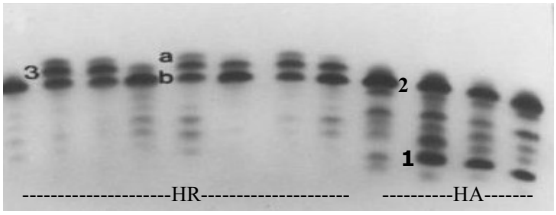

Figure 1. ADH zymograms of *H. resinosus* (HR) and *H. annuus* (HA). Numbers refer to loci at 2x-6x genotypes.

PGI. A notable increase in the number of bands in relation to diploid species was observed. Two genes code for PGI in diploid species, *Pgi-1* and *Pgi-2*, with polypeptides located in chloroplasts and cytoplasm. A minimal three-banded pattern in the *Pgi-2* zone indicates the existence of a third gene related to the cytoplasmic enzyme in *H. resinosus*. Two loci with allelic variants forming homo- and heterodimers can generate multiple-banded patterns (Gottlieb and Weeden, 1980).

GDH. Two activity zones have been found in 2x and 6x genotypes that have been assigned to *Gdh-1* and *Gdh-2* loci. GDH posses a tetrameric structure and heterozygous individuals display a five-banded pattern. Symmetric and asymmetric heterozygous could be differentiated. A particular variation in intensity was observed in *H. resinosus* that was attributed to gene dosage under a tetrasomic inheritance model.

The remaining enzymes displayed similar genic and allelic composition at both the 2x and 6x levels, except for one *Pgd-3* allele, common in cultivated sunflower but not present in *H. resinosus*.

F1 individuals in *H. resinosus* x *H annuus* crosses could not be clearly identified due to the alleles shared between parental species. *Helianthus annuus* alleles constitute a subseries of those found in *H. resinosus*. However, polymorphic loci should be useful if parents for crosses were selected for contrasting allelic variants. When *H. annuus* was used as the female parent, F1 individuals could be unequivocally recognized given that they showed the bands corresponding to duplicate genes from the hexaploid pollen donor. Many hybrid seedlings were obtained in crosses with either male sterile and fertile inbred lines. Hybrid seed production seems to be efficient even without emasculation of the B fertile line.

Meiotic Chromosome Analysis. Meiosis in *H. resinosus* was studied at the stages of diakinesis, telophase I and telophase II. Data presented on bivalents in Table 1 were collected from diakinesis, where most chromosome associations were clearly identified. The whole complement (51 II) was observed in only one well-spread cell; a variable number of chromosomes associated as bivalents could be scored in the remaining 19 cells. The number of bivalents per cell varied between 40 and 51, with an average of 44.2, which indicates that at least 87% of chromosomes were associated as bivalents. Two cells presented one quadrivalent, thus indicating that other configurations are possible in this species. In a low percentage of dyads (3.5%) and tetrads (3.7%), only one micronucleus was observed which probably represented a lagging chromosome. Ten anaphase I with normal disjunctions (51:51) were observed. The percentage of nonaborted pollen grains recorded in five plants was high, with an average of 91.25% over 2,300 pollen grains.

Table 1. Number of cells of *H. resinosus* in different meiotic stages.

	Scored bivalents									Dyads	Tetrads
	Diakinesis									Telophase I *	Telophase II*
	51	49	47	46	44	42	41	40	Total		
N of cells	1	1	3	3	5	2	2**	3**	20	328 (12)	215(8)

* Number of cells with one micronucleus in brackets.
** One cell with one quadrivalent.

Discussion

Isozyme Number at the Hexaploid Level. The number of bands in a zymogram depends on the number of genes, the genotype (homo-heterozygous), the quaternary structure of protein and subcellular location (Wendel and Weeden, 1989). There is a highly conserved number of isozymes in diploid plants (Gottlieb, 1982). Any increase in isozyme number is indicative of gene duplication at the diploid level or due to polyploidy. In this study, two enzymes, ADH and PGI, showed definite duplicated genes. These loci represent molecular markers for the hexaploid level which can be easily visualized. The isozyme systems expressing the same number of coding genes in both 2x and 6x species can indicate gene silencing. Current molecular techniques make it possible to identify the mutations responsible for nonfunctional proteins in these silenced genes (Ford and Gottlieb, 2002). Gene silencing can make a polyploid plant look isozymically like a diploid (Gastony, 1991).

Molecular Markers for F1 Identification. Crossing schemes make it necessary to identify true hybrid plants at an early stage. Morphology, pollen viability and chromosome numbers are not always enough for a whole characterization of the hybrids (Kraüter et al., 1991). RFLP (Faure et al., 2000), proteins (Anisimova et al., 1993) and RAPD markers (Cazaux et al., 1996) have demonstrated genomic rearrangements and loss of genetic material following hybridization between annual and perennial species. Different marker classes are useful tools for studying genomic incompatibility. The described polymorphic loci allow identification of hybrids using a relatively fast and simple technique.

Genome Relationships. High bivalent frequency, low number of micronucleis and high percentage of viable pollen grains suggest that meiosis in *H. resinosus* is quite normal and produces microspores with a balanced number of chromosomes. Meiotic studies in the close taxon *H. tuberosus* have revealed that this species, and probably all the hexaploid taxa, have the genomic constitution A1A1A2A2BtBt, where the A1 and the A2 genomes come from species in the perennial section Atrorubens and the Bt genome is closely related to the *H. annuus* genome (Kostov, 1939). If a similar genomic composition were proposed for *H. resinosus*, the GDH zymograms, distinguished by differences in banding intensity, could be related to A1 and A2 genomes. Thus, *Gdh-2* patterns would represent evidence of tetrasomic inheritance and would suggest a high homology between A genomes. Tetrasomic inheritance is an important criterion for recognizing autopolyploidy, but it has been difficult to demonstrate because of the few codominantly-inherited characters available for study (Crawford, 2000).

Based on RAPD fragments, Sossey-Alaoui et al. (1998) proposed the existence of four genomes in *Helianthus*, giving Helianthus and Atrorubens sections the formulae CH and CPA, respectively. Our results, comparing *H. annuus* and *H. resinosus*, agree with the expectancy of unique markers for perennials and annual species as well as common markers.

Pairing in the hybrids may be due to allosyndesis (i.e, pairing between chromosomes of the parental species) and/or autosyndesis (i.e., pairing within a parental complement). Considering an autoalloploidy origin for *H. resinosus*, multivalent meiotic figures should be expected. Our results show that bivalents are the most frequent type of chromosome association in *H. resinosus*. A high homology between A1 and A2 genomes has been demonstrated through meiotic studies in *H. annuus* x *H. resinosus* hybrids (Jan, 1997). The prevalence of bivalent pairing in male meiocytes indicates autosyndesis. The development of multivalent suppressors has been a key step in the success of polyploid angiosperms (Stace, 2000). Selection for higher seed-set in tetraploid stocks of corn and *Brassica campestris* resulted in a significant reduction of quadrivalent frequency over a period of ten years (Burnham, 1962).

Although tetrasomic inheritance is generally associated with multivalent formation at meiosis (Rieseberg and Doyle, 1989; Shore, 1991), this inheritance model can also be applied when bivalents are made up at random from the members of tetrasomes (Burnham, 1962). Tetrasomic inheritance has been documented in alfalfa, showing complete bivalent pairing (McCoy and Bingham, 1991). A limited chiasmata formation could explain the absence of multivalent figures.

Isozyme patterns in alloploids typically resemble heterozygous zymograms because the allozymes characteristic for diploid parental species do not recombine in the polyploid. Such "fixed" heterozygous phenotypes were not observed in *H. resinosus*. At least two allelic variants are segregating in the specific loci for hexaploid level. These results could be explained by two equally plausible hypotheses: 1) this polymorphism was already present in the founder perennial genomes of tetraploid species, or 2) multiple speciation events have occurred involving different ancestral genotypes (Werth et al., 1985).

Knowledge of genome relationships between plant species is very useful in planning effective breeding strategies to transfer desirable genes. Chloroplast DNA and nuclear ribosomal DNA produced phylogenetic trees largely concordant with the classification of Schilling and Heiser (1981) but insufficient DNA variation could not resolve the relationships among perennial species (Schilling, 2000). In this study most of the *H. annuus* loci and alleles were also present in the hexaploid species. It was suggested that *H. giganteus* and *H. mollis* contributed two of the three possible genomes for *H. resinosus* (Heiser et al., 1969). Unpublished preliminary enzyme studies in *H. mollis* are in agreement with the hypothesis that points this diploid perennial species as one of the parental species.

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