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THE ROLE OF BIOTECHNOLOGIES IN THE DEVELOPMENT OF SUNFLOWER CULTURES IN THE WORLD

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SUMMARY

Sunflower improvement by conventional breeding is severely restricted by the availability of a rather limited gene pool owing to natural incompatibilities, even between related species, and by the time scale of most breeding programs. Therefore, much attention has been directed recently to the newly emerging and novel technologies of plant cell and molecular biology that provide a powerful means to supplement and complement the traditional methods of plant improvement. The concept of DNA-based markers has revolutionized our ability to directly access any part of the plant genome, and has led to new opportunities such as map-based cloning and directed plant breeding. Efficient regeneration of fertile plants from cultured cells and protoplats, combined with novel methods of DNA delivery and selection of transformed cells, has resulted in the production of transgenic sunflower. Agronomically useful genes, which confer resistance to insect pests and pathogens, have been introduced. However, further molecular improvement of sunflower could be limited most by the lack of our knowledge about, and access to, important and useful genes (e.g., those controlling multigenic traits like yield, and resistance to biotic and abiotic stresses). Therefore, high priority should be given to the development of ultra high density (UHD) linkage maps and the development of new tools for high-throughput genome and expression analyses.

Key words: ***Helianthus annuus*, genetic transformation, genomics, molecular markers, somatic hybridization**

INTRODUCTION

The past decades has seen tremendous advances in plant biology, including the biochemical dissection of developmental process, molecular assays of gene expres-

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sion and the development of stable transformed plants. The integration of rapidly developing molecular techniques with conventional methods of plant breeding can speed up the process of developing new cultivars with desirable characteristics. The common sunflower (*Helianthus annuus* L.) is cultivated on every continent and is one of the four major annual crops grown for edible oil: it is likely to be one of the first beneficiaries of these technological advances (Knapp *et al.*, 2000; Pugliesi *et al.*, 2000; Alibert *et al.*, 2001; Gentzbittel *et al.*, 2001).

New tools for breeding and genome manipulation in sunflower

Molecular biology over the past 30 years has greatly expanded knowledge about the genetic architecture of living organism and the communication system by which a gene or genotype exercises control over phenotype. Historically, plant breeders have had to rely on phenotypic traits alone to breed for valuable traits. The development of the molecular marker concept offers a significant opportunity for applying linkage or "Mendelian" genetic approach to agriculturally important crops complementing classical breeding techniques. Simple-sequence repeats (SSRs), amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs), insertions-deletions (INDELs) and single nucleotide polymorphisms (SNPs) are just a few of the different molecular markers that are available today and potentially meaningful for construction of ultra high density (UHD) linkage map for facile positional cloning, expressed sequence mapping, trait mapping, marker-assisted selection, studies on biodiversity and phylogenetic relationships.

The public DNA sequence databases for sunflower are limited. However in the last years several leader groups have begun mass sequencing cDNA clones from developing cDNA libraries (Knapp *et al.*, 2000; Gentzbittel *et al.*, 2001) and are working toward producing database of specifically expressed sequence tags (ESTs) from cDNA (Alibert *et al.*, 2001), large inserted DNA and bacterial artificial chromosome (BAC) libraries of cultivated and wild sunflowers (Knapp *et al.*, 2000; Gentzbittel *et al.*, 2001; Horn *et al.*, 2001). These resources should accelerate gene discovery through data mining and cDNA micro-array analysis and create an access for DNA marker development in sunflower (Alibert *et al.*, 2001; Gentzbittel *et al.*, 2001; Tamborindeguy *et al.*, 2001).

Advances in plant cell culture research have played an increasingly critical role in the development of modern plant biotechnology. Widely used protocols for the regeneration of plants from cultured cells of dicotyledonous species were already available in the early 1980's, when *Agrobacterium*-based methods for DNA delivery and integration were developed, leading to the production of the first transgenic plants in the 1983 (Fraley *et al.*, 1983; Zambryski *et al.*, 1983). Although in the genus *Helianthus* several procedures to regenerate plants from tissue and cell cultures have been established (Alibert *et al.*, 1994), an effort to improve both somatic hybridization and genetic transformation techniques is need (Pugliesi *et al.*, 2000).

Linkage maps and marker assisted selection (MAS)

Construction of a detailed genetic map will make available a precise but vast amount of information that plant breeders can use to identify, manipulate, and complement traits to their maximum advantage. The concept of using genetic markers to identify specific regions of the genome was established long ago using morphological characters, but developments in molecular biology have increased the repertoire of polymorphic assay procedures available. In sunflower, a major effort has been devoted towards creating linkage maps, based on RFLP and AFLP markers (Berry *et al.*, 1995, 1996; Gentzbittel *et al.*, 1995, 1999; Jan *et al.*, 1998). In particular, AFLPs allowing the generation of a large number of data points in a short time are especially suited for recurrent or donor parent analysis. The next step in this field should be the generation in sunflower of linkage maps based on multiallelic SSR assays that could facilitate the transfer of map data to different populations and genotypes.

One of the immediate benefits to plant breeding from genome mapping is using DNA markers that are linked to single genes in order to select for important qualitative traits. Moreover, DNA markers can be used to obtain information about: 1) the number, effect, and chromosomal location of each gene affecting a trait; 2) effect of multiple copies of individual genes (gene dosage); 3) interaction between/among genes controlling a trait (epistasis); 4) whether individual genes affect more than one trait (pleiotropy); and 5) stability of gene function in different environments (Dudley, 1993; Paterson and Tanksley, 1997).

In the course of plant improvement, plant breeders deal with several qualitative traits; however, the most difficult problem facing breeders is the manipulation of metric traits with complex inheritance. Many strategies are available which rely upon the statistical analysis of field data to evaluate what has occurred on the genotypic level, but these inferences are often very imprecise as to the number of gene involved and their mode of action. Tracking polygenes with genetic markers can be traced back to the early 1920's when Sax (1923) reported the association of quantitatively inherited seed size with monogenes controlling seed coat pigmentation and pattern in bean (Dudley, 1993; Paterson and Tanksley, 1997). Effort to construct high-density linkage maps of molecular genetic polymorphism (marker loci) is currently underway for sunflower (Jan *et al.*, 1998; Gentzbittel *et al.*, 1995, 1999). Soon it should be possible to routinely score large numbers of such polymorphism on many individuals in a population. Statistical associations between alleles at molecular marker loci and alleles at quantitative trait loci (QTLs) can be used to select indirectly, but with potentially very high accuracy, for DNA segments containing favorable QTL allele, effectively increasing the hereditability of economically important agronomic characters such as yield, plant status and its components, quality traits, resistance and environmental stresses (Dudley, 1993; Paterson and Tanksley, 1997).

Molecular markers provide a mechanism for applying linkage genetic techniques to complex inheritance problems that almost reduces them to the level of studying single gene traits, although both the experimental design and phenotypic measurements are much more critical. QTLs can be followed in a segregating population with the help of molecular markers. The selection for QTLs using genetic markers can be effective if a significant association is found between the quantitative trait and the genetic markers and using these associations to develop improved lines or populations (MAS). QTL regions obtained from one population can later be introgressed into other varieties, which may be more suited for specific environments (Dudley, 1993). These studies helped to bring forth the potential of exploiting non-adapted and wild germplasm using backcrossing QTL analysis for the enhancement of elite crop varieties (MAB: marker-assisted backcrossing). The quick discovery and transfer of these QTLs from non-adapted to adapted germplasm ultimately opens the door for the expansion of the genetic base of sunflower (Vischi *et al.*, 2001). Within the broad field of genomics, the QTL approach can be further validated or supported by other areas such as transcriptional profiling, physical mapping, and other functional genomics technologies (Alibert *et al.*, 2001).

Genomics

Genomics can be described as "high-throughput genetics" or a synthesis of three disciplines: molecular biology, automation and bioinformatics. Genomics mainly involves the isolation of genes. Traditionally, this has operated *via* a "one gene at a time" approach. In contrast, genomics adopts a global approach to gene discovery, isolation (i.e., on a very large scale) and parallel assays of gene expression. Functional genomics include EST programs, expression analysis *via* high-density arrays, genome sequencing, promoter trapping, large-insert DNA libraries, BAC libraries, physical mapping and positional cloning, automated DNA sequence processing. The development of new tools for high-throughput genome and expression analyses is one of contemporary main goals of molecular biology in sunflower (Gentzbittel *et al.*, 2001; Horn *et al.*, 2001). A sunflower BAC library corresponding to 1.7 haploid genome equivalents has been constructed using the restorer line RHA325 and pBeloBAC11 as vector for isolation and characterization of restorer (*Rf*) genes (Horn *et al.*, 2001). Moreover, the utility of a BAC library consisting of an estimated 4-5-fold coverage of the sunflower genome has been recently evaluated by screening for the presence of putative transmembrane receptor genes sharing epidermis growth factor (EGF) and integrin-like domains (Fabre *et al.*, 2001).

Somatic hybridization

Sunflower has been primarily selected for high yields, oil content and, where necessary, adaptation to certain environmental conditions. This "selection pressure" over the centuries has severely disrupted the co-evolutionary relationships between plants and pathogens with the consequence that the cultivated sunflower

has not retained the degree of resistance (pathogens and abiotic stress) exhibited by their wild relatives (Škorić, 1992). Moreover, the wild ancestors of the cultivated sunflower are potentially useful for modifications of oil quality (for example, reduction of saturated palmitic and stearic fatty acid) or modification of plant architecture (e.g., floral architecture) that could allow the exploitation of new markets.

However, the use of interspecific hybridization has been limited by difficulties to overcome the incompatibility barriers and to remove undesirable genes of the donor species (Seiler, 1992). Advances in the application of cell fusion techniques (Krasnyansky and Menczel, 1995; Wingender *et al.*, 1996, Binsfeld *et al.*; 2000) can offer an opportunity for the recombination of genomes and in transferring of chromosome fragments from an alien species and/or genera to cultivated sunflower (Binsfeld *et al.*, 2000, 2001b; Varotto *et al.*, 2001).

In *Helianthus* ssp., protoplasts can be easily isolated from hypocotyls (Lénée and Chupeau, 1986; Moyne *et al.*, 1988; Krasniansky *et al.*, 1992; Krasniansky and Menczel, 1993; Petitprez *et al.*, 1995; Trabace *et al.*, 1995; Wingender *et al.*, 1996; Laparra *et al.*, 1997; Bolandi *et al.*, 1999; Vallee *et al.*, 1999; Varotto *et al.*, 2001), cotyledons (Fischer *et al.*, 1992; Laparra *et al.*, 1995) or mesophyll cells (Guilley and Hanhe, 1989; Kirckes *et al.*, 1991; Keller *et al.*, 1994) of young seedlings. In addition, regenerated plants from protoplast-derived calli have been obtained in several laboratories (Krasniansky and Menczel, 1993; Trabace *et al.*, 1995; Wingender *et al.*, 1996). Protoplast fusion has resulted in cybrid/hybrid callus (Krasniansky and Menczel, 1995; Binsfeld *et al.*, 2000) and intergeneric hybrid plants have been obtained from fusion between chicory and *H. annuus* protoplasts (Varotto *et al.*, 2001).

However, in the genus *Helianthus* the general lack of an efficient and reproducible plant regeneration system is still hampering exploitation of protoplast fusion and gene transfer to protoplast. A number of studies have revealed the influence of genotype in protoplast culture. In experiments conducted to identify the genetic factors controlling protoplast division, organogenesis and somatic embryogenesis, a high hereditability has been shown in sunflower for the two protoplast division parameters: total division per 100 protoplasts (TOTD) and asymmetric division per 100 protoplasts (ASYD) (Berrios *et al.*, 2000). Twelve putative loci associated with total division per 100 protoplasts were identified. Eleven QTLs were also detected for asymmetric division per 100 protoplasts. The QTLs identified in three linkage groups should be involved in cell division and in early events associated with cell differentiation (Berrios *et al.*, 2000).

As pointed out by Binsfeld *et al.* (2001a), symmetric and asymmetric somatic hybridization *via* microprotoplast fusion or microinjection of chromosomes or micronuclei represent an amenable method for the transfer of genes which are not available as cloned DNA sequences or for transferring blocks of genes of agronomic interest. Asymmetric somatic hybrid (ASH) plants have been obtained by PEG-mediated mass fusion of microprotoplasts from perennial *Helianthus* species and

hypocotyl protoplasts of sunflower (Binsfeld *et al.*, 2000). The formation of micronuclei in perennial sunflower cell cultures was induced, at early log phase, by addition of the herbicides amiprophosmethyl or oryzalin (Binsfeld *et al.*, 2000). Subdiploid microprotoplasts were isolated by high-speed centrifugation and the smallest enriched by sequential filtration through nylon sieves of decreasing pore size. Fusion products were cultured and DNA analysis using RAPD markers revealed that about the 50% of regenerated plants were asymmetric hybrids phenotypically similar to *H. annuus*. Moreover, flow-cytometric analysis of nuclear DNA showed that these plants had a higher DNA content than the receptor *H. annuus*, suggesting that they represent addition lines (Binsfeld *et al.*, 2000). Cytological investigation of the metaphase cells of several cybrids revealed an addition of 2-8 extra chromosomes in these plants. Pollen viability of the ASH plants ranged from 79.2 to 95% with a strong negative correlation to chromosome number, which varied between 34 and 42. Over 85% of the ASH meiocytes showed regular bivalent chromosome pairing; however, several anomalies like anaphase bridges, laggard chromosomes, univalent and multivalent pairing were reported (Binsfeld *et al.*, 2001a). Molecular investigation of ASH progeny using RAPD markers revealed the presence of donor genotype markers in 68% of the offspring (Binsfeld *et al.*, 2001a).

Male-sterile ASHs between *Cichorium intybus* and a sunflower male-sterile cytoplasmic line have been obtained fusing mesophyll chicory protoplasts inactivated with iodoacetic acid with hypocotyl sunflower protoplasts irradiated with gamma-rays (Varotto *et al.*, 2001). A cytological analysis of root-tip cells from regenerated plants indicated that most of them had 18 chromosomes, the same number as chicory (Varotto *et al.*, 2001). Through molecular analyses three plants were identified as cytoplasmic asymmetric hybrids. The morphology of the cybrids resembled the parental chicory phenotype, and at anthesis their anthers produced fewer pollen grains, which could not germinate either *in vitro* or *in situ*. Cybrid plants grown in the field produced seeds when free-pollination occurred (Varotto *et al.*, 2001).

Genetic transformation

The general requirements for a transformation system are: i) the target tissue should be easily available; ii) it should regenerate with high frequency; iii) it should be uniform in response. Progress in the sunflower transformation has been restricted for many years by the limitation of available regeneration systems. Although the recent development of efficient procedures for the successful culture of somatic cells and protoplasts (Alibert *at al.*, 1994; Trabace *et al.*, 1995; Wingender *et al.*, 1996; Binsfeld *et al.*, 2000), the most efficient regeneration systems are direct, without an intervening callus phase (Power, 1987; Pugliesi *et al.*, 1991; Alibert *et al.*, 1994). Prolonged culture in the non-differentiated state appear to be incompatible with the selection of transformed calli able to plant regeneration

(Laparra *et al.*, 1995) or limited to particular genotypes (Everett *et al.*, 1987; Robinson and Everett, 1990).

***Agrobacterium tumefaciens*-mediated gene transfer**

Sunflower is naturally susceptible to infection with *Agrobacterium tumefaciens* (Braun, 1941) so that foreign genes have been introduced easily into sunflower tumor cells through *Agrobacterium* (Murai *et al.*, 1983; Matzke *et al.*, 1984; Helmer *et al.*, 1984; Goldsbrough *et al.*, 1986). Using a genotype that retained high embryogenetic potential also after prolonged culture (Paterson and Everett, 1985), Everett *et al.* (1987) first selected kanamycin-resistant calli capable of plant regeneration, after infection with an *A. tumefaciens* strain carrying the coding sequence for neomycin phosphotransferase II (NPT II). This protocol of genetically stable transformation of sunflower showed however, limited applications to other sunflower genotypes (Peerbolte and Dek, 1991). More recently, using hypocotyl explants of the inbred line HA300B, stable transformation (0.1%) were obtained after co-cultivation with *A. tumefaciens* carrying a *gfp* (green fluorescent protein) and *nptII* genes (Muller *et al.*, 2001).

From the procedures reported for other species, shoot apical meristems of sunflower were dissected from seeds and co-cultivated with an *A. tumefaciens* strain harboring a binary vector carrying genes encoding β-glucuronidase (GUS) and NPT II activity (Schrammeijer *et al.*, 1990). Transformation of shoot meristem cells occurred at low frequencies and chimeric expression of the two genes was observed in a few transformed shoots. Although the low transformation frequency of the meristematic cells limits the applicability of this procedure (Schrammeijer *et al.*, 1990), technical modifications, that involved secondary culture of nodal meristems excised from transformed sector of *Agrobacterium*-infected intact meristems (Bidney *et al.*, 1998: WO 98/51806), allowed the production of transgenic *Sclerotinia*-resistant plants of sunflower (Scelonge *et al.*, 2000). In addition, it has been demonstrated that the overexpression of the cytokinin-synthesizing gene *ipt* of *A. tumefaciens*, improved the induction of adventitious shoots from embryonic axes, increasing the regeneration efficiency and the rate of recovery of transgenic shoots after *Agrobacterium*-mediated transformation (Molinier *et al.*, 2001).

Despite of the high regenerative potential of sunflower immature embryos (Bohorova *et al.*, 1985; Alissa *et al.*, 1986; Finer, 1987; Witrzens *et al.*, 1988; Freyssinet and Freyssinet, 1988; Wilcox-McCann *et al.*, 1988; Espinasse and Lay, 1989; Espinasse *et al.*, 1989; Jeannin and Hahne, 1991), co-cultivation with *A. tumefaciens* gives high frequencies of transformed calli but no plant regeneration (Voronina *et al.*, 1991) or regeneration of chimeric transformed shoots (Tassie *et al.*, 1991; Dek and Peerbolte, 1991; Peerbolte *et al.*, 1992).

Another explant that gives relatively high frequency to regenerate fertile plants is the cotyledon from ungerminated seeds and/or young plantlets (Pugliesi *et al.*, 1991; Chraibi *et al.*, 1991, 1992; Ceriani *et al.*, 1992; Sarrafi *et al.*, 1996; Deglene

et al., 1997; Fiore *et al.*, 1997), but, unlike other sunflower tissues (Escandón and Hahne, 1991), this organ is not easy to be transformed (Laparra *et al.*, 1995). A detailed analysis of the interaction of *Agrobacterium* with cotyledon explants showed that transformation efficiency is critically dependent on the culture conditions: in particular, the percentage of transformed explants is influenced by the mineral and hormonal composition of the medium (Laparra *et al.*, 1995). In addition, histological analyses of cotyledonary explants co-cultivated with *Agrobacterium* showed that the transformed cotyledon cells are not necessarily competent for regeneration (Damm *et al.*, 1991; Laparra *et al.*, 1995). Moreover, their regeneration potentiality is swiftly lost whether during either *in vivo* development (Pugliesi *et al.*, 1991) or *in vitro* culture (Knittel *et al.*, 1991). On the other hand, regeneration from cotyledonary explants is direct and eventually, the induced callus shows low organogenetic potentiality, so that the selection of eventually transformed competent cells results very arduous. Thus, transformations of cotyledons were only suitable for the production of transgenic callus and, occasionally, of shoots with chimeric expression of transgenes (Biasini *et al.*, 1992; Pugliesi *et al.*, 2000).

Although in the genus *Helianthus* leaves explants are not able for prominent adventitious regeneration (Pugliesi *et al.*, 1993a; Bianchi *et al.*, 1999) a remarkable embryogenic potential is displayed by leaf explants of regenerated plants of the interspecific hybrid *H. annuus* x *H. tuberosus* subjected to a second culture cycle *in vitro* (Pugliesi *et al.*, 1993a; Fambrini *et al.*, 1996, 1997). This morphogenetic competence allowed the stable genetic transformation by co-cultivation of leaf disk explants with *A. tumefaciens* carrying the *nptII* and the *uidA* genes (Pugliesi *et al.*, 1993b) but the polyploid nature of the hybrid confine the applicability of this transformation method.

Recently, a simple *A. tumefaciens*-mediated transformation system has been developed to eliminate the *in vitro* regeneration component from the transformation protocol (Rao and Rohini, 1999). Two-day-old seedlings with one cotyledon detached were infected with an *A. tumefaciens* strain harboring *uidA* and *nptII* genes. Following co-cultivation, seedlings, grown aseptically for 5 day on a growth regulator-free basal medium, were screened for transient GUS expression and the shoot portions of the putative transformants were utilized for propagation as transgenic plants. The excised shoots that initiated roots following selection were subsequently transferred to a glasshouse. This transformation technique allowed rapid generation of up to 2% phenotypically normal fertile plants containing functional transgenes (Raho and Rohini, 1999).

Direct gene transfer to protoplast

Protoplast-transformation has been obtained by DNA uptake mediated by chemical treatments (Moyne *et al.*, 1988; Kirches *et al.*, 1991; Laparra *et al.*, 1995) or electroporation (Burrus *et al.*, 1990; Kirches *et al.*, 1991; Laparra *et al.*, 1995;

Burrus *et al.*, 1996a); nevertheless, achievement of fertile transformed plants was never reported.

The crucial point in the chemical transformation is the treatment of the protoplasts suspended in the medium containing DNA and PEG that promotes DNA uptake. In sunflower protoplasts, the best results were obtained with PEG 6000 (Moyne *et al.*, 1989; Laparra *et al.*, 1995). The PEG method allowed the recovery of transformed sunflower calli at the frequency of 4 calli for 106 treated protoplasts (Moyne *et al.*, 1989). However, after 6 months of culture, calli degenerated and failed to develop into mature embryos and/or shoots (Moyne *et al.*, 1989).

In electroporation protocols, freshly isolated protoplasts are suspended in a salt solution with a particular electric resistance that produces transient pores in the plasma membrane, facilitating penetration of macromolecules into the protoplast. In sunflower, application of this technique was performed by Burrus *et al.* (1990) using a DNA construct carrying *uidA* and *nptII* genes. However, no transgenic plants were regenerated, probably due to the poor regenerative potential of the genotype used (Burrus, 1991: cited in Alibert *et al.*, 1994; Laparra *et al.*, 1995). In any case, regeneration from protoplast of *H. annuus* is tightly genotype dependent and fertile plants have been obtained only at low frequency (Schmitz and Schnabl, 1989; Burrus *et al.*, 1991; Chanabé *et al.*, 1991; Trabace *et al.*, 1995; Wingender *et al.*, 1996).

Particle bombardment

In the first report on the introduction of a foreign gene by particle bombardment of sunflower meristem explants, the regenerated plants showed GUS expressing sectors, indicating that chimeric plants had been produced (Bidney, 1990). Transient expression of the *uidA* gene has been induced in sunflower cotyledonary explants and immature zygotic embryos at different developmental stages after microprojectile bombardment (Hunold *et al.*, 1995). Small embryos of approximately 1.5-2.0 mm in diameter were the most suitable for efficient transient GUS expression (Laparra *et al.*, 1995; Hunold *et al.*, 1995) and multiple shoot formation (Hunold *et al.*, 1995); but, the conversion rate of transient to stable transformation was shown to be very low (Hunold *et al.*, 1995). The limited success of DNA transfer into sunflower cotyledons by microprojectile bombardment is likely due to the strong cuticle (Hunold *et al.*, 1995).

Combination of particle bombardment with *A. tumefaciens* co-cultivation

Although the biolistic technique allows only transient expression of foreign gene (Hunold *et al.*, 1995) and/or selection of transformed chimeric shoots (Bidney, 1990; Hunold *et al.*, 1995; Burrus *et al.*, 1996b), its application has been decisive in sunflower transformation. With the combination of bombardment of shoot apical meristems or embryonic axes of immature embryos and the successive co-cultivation of the treated explants with *A. tumefaciens* strains, fertile transgenic plants

have been obtained in several laboratories (Malone-Schoneberg *et al.*, 1991, 1994; Bidney *et al.*, 1992a; Knittel *et al.*, 1994; Lucas *et al.*, 2000; Hewezi *et al.*, 2001). Microprojectiles (Bidney *et al.*, 1992b; Malone-Schoneberg *et al.*, 1994; Knittel *et al.*, 1994; Lucas *et al.*, 2000; Hewezi *et al.*, 2001) or glass beads (Grayburn and Vick, 1995) were used as a wounding mechanism to enhance *Agrobacterium* transformation frequencies. Chimeric transgenic plants were recovered after the screening of putatively transformed cells with a short (4 days) (Grayburn and Vick, 1995) or long (2-5 weeks) (Malone-Schoneberg *et al.*, 1994) incubation on selective medium. Solid transformants were recovered after self pollination (Malone-Schoneberg *et al.*, 1994; Grayburn and Vick, 1995; Lucas *et al.*, 2000).

The chimeric nature of primary transformants is the major complication of these protocols. The applications of selective cycles on medium containing kanamycin allow the enrichment in transformed cells: a considerable number of shoots isolated at the end of three cycles were solid transformants (Knittel *et al.*, 1994). Other problems are the occurrence of premature flowering, and the long time of selection on medium containing cytokinins that inhibit rooting of regenerated and/or microprogated shoots (Lupi *et al.*, 1987; Cavallini and Lupi, 1992). Thus, the survival of transgenic plants and the number of filled achenes can be greatly improved by *in vitro* grafting (Malone-Schoneberg *et al.*, 1994; Grayburn and Vick, 1995).

Field performances of transgenic plants

Field testings are necessary to determine that yield and other agronomic characteristics have been maintained in transgenic plants and to test the expression of the introduced gene under field conditions. The heritable changes in both quantitative and qualitative traits, described in regenerated plants of *H. annuus* (Pugliesi *et al.*, 1991; Natali *et al.*, 1995), could have detrimental effects on transformation experiments. Genetic transformation in sunflower is documented (Everett *et al.*, 1987; Malone-Schoneberg *et al.*, 1994; Knittel *et al.*, 1994; Graiburn and Vick, 1995) and the transgenic phenotypes were inherited in expected Mendelian segregation ratios confirming that they were stably transformed (Everett *et al.*, 1987; Knittel *et al.*, 1994; Malone-Schoneberg *et al.*, 1994, Lucas *et al.*, 2000; Muller *et al.*, 2001). However, many problems on *Agrobacterium*/sunflower cell interaction are largely unsolved, including plant regeneration, *Agrobacterium* virulence induction, T-DNA activation, transfer and integration: these aspects make extremely arduous to establish the optimal conditions for a reliable transformation method. Thus, field performance tests of transformants are limited indicating that further improvements of the sunflower transformation protocols are necessary to obtain enough materials for field evaluation.

Recently, Bazzalo *et al.* (2000) have reported the field screening for resistance to *Sclerotinia* of transgenic inbred lines and hybrids containing the wheat oxalate oxidase gene, ascertaining that the transgenic genotypes were more resistant to the pathogen than non-transgenic isolines and corresponding isogenic hybrids. In addi-

tion, transgenic plants for the *Cry1F* gene of *Bacillus turingiensis*, obtained using a modified meristem culture protocol (Bidney *et al.*, 1992b) have been evaluated for resistance to two insect pests: *Rachiplusia na* and *Spilosoma virginica* that have a significant economic impact on the Argentina sunflower production. *Bt* material showed to be resistant to *Rachiplusia na*, even at high levels of attack (Pozzi *et al.*, 2000).

SOME APPLICATIONS OF BIOTECHNOLOGIES IN SUNFLOWER

Disease resistance

Developing methods for diagnosis of plant pathogens, as well as preventing or limiting infection by plant pathogens and pests have clear relevance to sustainable and reduced input systems and the quality of products for several market sectors. Limiting infection is especially important with the global desire to use benign practices that are more environmentally friendly. The primary objectives should be direct to: 1) elucidate the genetic, physiological and biochemical events underlying host-pathogen interactions in compatible and incompatible responses (Roeckel-Drevet *et al.*, 1997; Virányi and Walcz, 2000); 2) determine the pathways involved in early recognition by plants of pathogens or pests and to develop strategies to produce germplasm with enhanced disease resistance (Vear and Touvieille de Labrouhe, 1988; Rashid, 1993; Mouzeyar *et al.*, 1994; Langar *et al.*, 2000a, 2000b; Maširević, 2000); 3) develop genetic, physical and transcriptional maps of the genomes of important prokaryotic and eukaryotic sunflower pathogens and pests to better understand the basis of pathogenicity and host range; 4) develop a conceptual understanding of the aetiology, epidemiology and population biology of the target pathogens, and to develop the necessary diagnostics (Viguie *et al.*, 1999; Chaillou *et al.*, 2000; Delos *et al.*, 2000; Tourvieille de Labrouhe, 2000).

During the last decade, many important results have been obtained concerning the molecular aspects of disease resistance to several fungal disease, both on the localization of genomic regions involved in this resistance and on some genes and proteins accumulating following infection (Mouzeyar *et al.*, 1995; Roeckel-Drevet *et al.*, 1996; Besnard *et al.*, 1997; Vear *et al.*, 1997; Gentzbittel *et al.*, 1998; Mazeyrat *et al.*, 1998, 1999; Mestries *et al.*, 1998). The discovery of novel genes and the understanding of the complete molecular mechanisms leading resistance of sunflower require the merging or the exchange of the tools available worldwide. To speed up the localization and the cloning of resistance genes, molecular probes (Gentzbittel *et al.*, 1998; Brahm *et al.*, 1999) and libraries (Gentzbittel *et al.*, 1995; Berry *et al.*, 1995; Jan *et al.*, 1998) are probably the most important that need to be exchanged between scientific teams. It is noteworthy that almost all the plant resistance genes have been cloned using either transposon tagging or map-based cloning strategies (chromosome walking or chromosomes landing). Transposon

tagging often involves the maize *Dissociation* (*Ds*) element and unfortunately this technique is not applicable or available in sunflowers. However, major genes for resistance to insect and pathogens or also related to morpho-agronomical and physiological characters (e.g., drought tolerance) can be tagged with tightly linked molecular markers will allow to establish the presence of these genes by assaying plants for the markers (Gentzbittel *et al.*, 1998; Brahm *et al.*, 1999; Panković *et al.*, 2000, 2001; Vasile *et al.*, 2001) and to clone genes, about which only the effect on phenotype is known (Weeden, 1991). In *Helianthus* ssp. the construction of RFLP and AFLP linkage maps (Berry *et al.*, 1995; Jan *et al.*, 1998; Gentzbittel *et al.*, 1999) has allowed the identification and isolation of resistance genes (Mouzeyar *et al.*, 1995; Roeckel-Drevet *et al.*, 1996; Besnard *et al.*, 1997; Vear *et al.*, 1997; Gentzbittel *et al.*, 1998; Mazeyrat *et al.*, 1998; Mestries *et al.*, 1998) giving the opportunity of the incorporation of these traits in cultivated sunflower (Scelonge *et al.*, 2000).

Two RAPD markers linked to the *RAdv* gene conferring resistance to most of the pathotype of *Puccinia helianthi* have been identified (Lawson *et al.*, 1998). Moreover, two sequence-specific markers (SCAR), developed from the sequences of the RAPD marker (Lawson *et al.*, 1998), could be used in MAS programs and to cloning the *RAdv* gene.

Seven QTLs for resistance to *Phoma macdonaldii*, a casual agent of black stem disease, has been defined using a collection of Recombination Inbred Lines (RILs) (Alibert *et al.*, 2001). These QTLs justified 92% of the phenotypic variability of the trait.

To clone genes giving resistance to *Plasmopara halstedii*, many molecular markers have now been identified and mapped to the genomic region containing the major locus *Pl6* for resistance to all the known races of *P. halstedii* (Mouzeyar *et al.*, 1995; Roeckel-Drevet *et al.*, 1996; Vear *et al.*, 1997). In addition PCR based markers for *Pl2*, *Pl6* and *Plarg* useful for MAS were recently reported (Brahm *et al.*, 1998, 1999, 2000). *Pl6* locus seems more complex than expected and may contain more than one *Pl* gene (Mouzeyar, 2000). The screening of a sunflower large insert library could allow the identification of different alleles conferring resistance to five race of *P. halstedii*. Using a PCR-based methods Mouzeyar *et al.* (2000) have cloned some cDNA and genomic fragment, from an inbred line of sunflower, containing the *Pl1* locus. Sequence comparison of these clones has showed that *Pl* genes belong probably to the large TIR-NBS-LRR class of plant resistance gene. In the incompatible interaction, sunflower seedlings develop a hypersensitive-like reaction within the hypocotyl (Mazeyrat *et al.*, 1999). It has been suggested that the induction of chitinase transcripts following infection of seedlings by *P. halstedii* occurs at the transcriptional level (Mazeyrat *et al.*, 1999). Different methods such as differential screening or subtractive libraries can be used to cloning these defense-related genes (Mazeyrat *et al.*, 1998; Klein *et al.*, 2001).

In the temperate regions of the world, white rot caused by the polifagous fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is considered the most serious disease, since it is widespread, persists for many years in the soil, and has a very wide host range (Maširević and Gulya, 1992). It can attack many parts of the plant: roots, base of the stem and capitulum (Bazzalo *et al.*, 1991; Castaño *et al.*, 1992, 1993).

To date, complete resistance to *Sclerotinia* is not available in cultivated sunflower, but only in its wild relatives (Škorić and Rajčan, 1992; Cerboncini *et al.*, 2001). Moreover, in each part of the plant the level of this poligenic trait may be quite different (Degener *et al.*, 1999a, 1999b). Analyses of QTLs associated with resistance to exstension of *S. sclerotiorum* mycelium on sunflower capitula and leaves using molecular marker have been reported (Mestries *et al.*, 1998; Hahn *et al.*, 2001). Seventy-three RFLP probes were used to construct a genetic map where four QTL loci were demonstrated for leaf resistance and two for capitulum resistance. One of these zones appears to be involved in resistance to both type of *S. sclerotiorum* attack while the others appear specific for resistance of one part of the plant.

Actually the classical breeding utilize costly and time-consuming artificial infection as resistance tests, thus it would be very useful to find early techniques for screening genotypes and to find markers of different resistance mechanisms which would make it possible either to reduce the number of test or to increase the efficiency of these carried out (Mestries *et al.*, 1998). Recently, it has been proved that the exposure of sunflower to toxic metabolites of *Sclerotinia* can result in plant wilting, tissue injury, increase in the plant tissue of the levels of oxalic acid and shikimate dehydrogenase (SKDH) enzymatic activity and synthesis of new proteins (PR) (Tamhasebi Enferadi *et al.*, 1998a, 1998b, 2000). The increase in SKDH enzymatic activity, related to the biosynthesis of shikimic acid, seems involved in the synthesis of lignin for cell wall, a typical mechanical reaction defense against fungal attack (Carrera and Poverene, 1995). *Sclerotinia* secretes toxins, including oxalic acid (Callahan and Rowe, 1991) which, acting as a toxin, causes pH variations, stem lesions and complete and irreversible plant wilting (Marciano *et al.*, 1983). The reaction of sunflower genotypes to oxalic acid treatment is similar to their reaction to *Sclerotinia* attack in the field. A wheat oxalate oxidase gene (mentioned earlier) was incorporated into a transformation cassette, driven by the SCP1 promoter (Lu *et al.*, 2000), used to transform an inbred restorer line of sunflower with low *Sclerotinia* head rot resistance (Bazzalo *et al.*, 2000; Scelonge *et al.*, 2000). In addition, oxalic acid has been used as a screening agent for *Sclerotinia* resistance in laboratory conditions (Vasić *et al.*, 1999, 2001).

Drought resistance

During the last 20 years, crops of sunflowers have extended in EU Mediterranean area due to its capacity to adapt to dry environments. Although sunflower was moderately tolerant of drought, production is strongly influenced by the presence of

water stress, which is found fairly regularly. In fact, drought is a permanent feature in many developing areas and may also periodically influence the economy of the Europe and USA. The late stress is typical of Mediterranean environments and the crop during the flowering-grain filling period is affected by drought during the development of the dry season. The threats from the trend in the climate render this property ever more important. Unfortunately, useful knowledge concerning the improvement of the species to environments with trophic, especially water; limitations are still scarce and fragmentary. In particular, genetic improvement for drought resistance has been limited, by the lack of simple screening technologies to assess the drought tolerance of genotypes, obliging the breeders to utilize methods involving selection for yield and its stability, with repetitions over many locations and years, which are expensive, laborious and time consuming (Blum, 1987). During the last few years many morpho-physiological characteristics related to yield under limited water availability have been identified (Baldini *et al.*, 1992, 1993; Acevedo and Fereres, 1993; Panković *et al.*, 1998, 1999), but in most cases no correlation has been found between these indexes and an increase in yield (Schonfeld *et al.*, 1988; Sloane *et al.*, 1990). This may be due to the fact that all these indexes have been generated from measurements made on single tissues or organs during a particular phenological stage, whereas achene yield under drought conditions springs from very complex mechanisms, deriving from genotype-environment interactions that develop throughout the whole plant cycle.

In order to analyze germplasm in relation also with agronomic and physiological observations on population offspring in field or controlled conditions, several molecular markers could be used either for SNP or for general mapping. SNP will help to detect polymorphism hidden until now but potentially important in *Helianthus* bio-diversity and candidate genes functional properties. This will result in a candidate gene approach combined with QTLs mapping for the exploitation of desirable traits issued from yet under-exploited germplasms either from cultivated (Panković *et al.*, 2000; Hervé *et al.*, 2001) than wild *Helianthus* species (e.g., *Helianthus argophyllus*) crossed with sunflower. Introgression of traits conferring higher drought tolerance would therefore lead to an improvement of this crop into a more economically optimized and ecologically well-adapted system for the production of renewable resources.

Oil quality

Traditional sunflower oil has been the major polyunsaturated oil used for many years in human nutrition to replace saturated fat, in an attempt to reduce cardiovascular diseases (Carmena *et al.*, 1996). The quality of sunflower oil is generally associated to the relative content in linoleic fatty acid. However, it has been demonstrated that diets including high consumption of monounsaturated oils are as effective as those rich in polyunsaturated oil in lowering cholesterol (LDL-C), but in contrast to the effect of polyunsaturated diets, the monounsaturated diets do not

lower HDL-C (Delplanque, 2000). Thus the development of new selected sunflower seeds producing enriched in oleic acid (reviewed in Lacombe and Bervillé, 2000) at the expense of linoleic acid has made sunflower oils highly competitive compared with other traditional predominant monounsaturated oils (Delplanque, 2000). Moreover, the present trend in human diet is to decrease the consumption of the saturated palmitic and stearic fatty acid. To identify sunflower germplam with reduced saturated fatty acid composition, a total of 884 cultivated sunflower accessions has been screened (Vick *et al.*, 2001). The genetic analysis of selected plants with low saturated fatty acid content indicates that the trait is dominant (Vick *et al.*, 2001). In addition, preliminary data suggested that the content of these fatty acids can be reduced introducing genes from wild perennial progenitors (e.g., *H. giganteus*) into cultivated sunflower (Seiler, 2001).

The increase of alternative fatty acid contents in the oil has stimulated new markets, thus providing new interest in growing sunflower (Gielen, 1992). Mutants affecting seed oil fatty acid composition are of great value leading to novel oil composition (Lacombe and Bervillé, 2000). In sunflower several different mutants have been isolated using chemical and physical mutagens (Soldatov, 1976; Ivanov and Ivanov, 1985; Garcés *et al.*, 1992; Osorio *et al.*, 1995; Miller and Vick, 1999; Peréz-Vich *et al.*, 1999a, 1999b, 2000a), however, little is known about the molecular nature of these mutations. Nevertheless, candidate genes from the fatty acids biosynthetic pathway have been recently mapped using molecular markers (Hongtrakul *et al.*, 1998; Lacombe *et al.*, 2000; Peréz-Vich *et al.*, 2000b; Lacombe and Bervillé, 2001) that can serve as starting point for chromosome walking or chromosome landing to clone the corresponding gene. The modification by genetic transformation of fatty acids composition of sunflower seed oil can be also prospected in a near future.

Seed storage protein

Sunflower seeds can also provide a source of proteins in the diets of livestock. Unfortunately, the values of sunflower proteins are lowered by their unbalanced amino acid compositions. The protein of sunflower is hardly lacking in lysine, and has a low intestinal digestibility due to the presence of phenolic compounds (chlorogenic and caffeic acids). The *in vitro* mutagenesis of seed storage protein genes to increase the content of high valuable amino acids and/or the introduction of heterologous genes encoding proteins containing high lysine can allow to improve the nutritive value of the sunflower seeds.

Phylogenetic studies

The genus *Helianthus* belongs to the *Compositae (Asteraceae)* family and includes about 100 species (Watson, 1929), the majority of which are native to North America. The genus provides two food plants, *H. annuus*, the sunflower, and *H. tuberosus*, the topinambour or Jerusalem artichoke. Several varieties of *H.*

annuus, as well as other species of the genus, are sometimes cultivated as ornamentals (Rogers *et al.*, 1982).

Molecular phylogenetic studies have contributed significantly to our understanding of the phylogenetic relationships of *Helianthus*, although several problems remain unresolved. The availability of molecular data has made it possible to refine with confidence the circumscription of *Helianthus*, the relationships of *Helianthus* to other member of subtribe *Helianthinae* and the divergence within the genus (Schilling and Panero, 1996; Schilling, 1997, 2000). However, the results obtained from molecular data set based on restriction site analysis of chloroplast DNA (cpDNA) (Schilling and Jansen, 1989; Schilling, 1997) and data set from sequencing of the nuclear ribosomal internal transcribed spacer region (ITS) (Schilling and Panero, 1996) are mostly but not entirely congruent. Thus, it is needed to identify molecular markers that show the appropriate, high level of variation that will be required to resolve fully relationships within *Helianthus*, overall for the polyploid species (Schilling, 2000).

Genetic variability

Although sunflower is an important crop species, present knowledge of its basic genetics lags behind other genetically well-known species. Methods have been developed for the generation of novel mutants in crop species. In sunflower, mutants have been induced by a variety of chemical mutagens and ionizing/non-ionizing radiation (Wallace and Habermann, 1959; Soldatov, 1976; Ivanov and Ivanov, 1985; Garcés *et al.*, 1992; Osorio *et al.*, 1995; Miller and Vick, 1999; Peréz-Vich *et al.*, 1999a, 1999b; Triboush *et al.*, 1999). The recent progress on *in vitro* tissue culture and genetic transformation could make sunflower a suitable candidate for the use of alternative mechanisms for genome modifications and plant breeding applications.

The first relates the exploitation of the genetic variability induced by *in vitro* tissue culture (Pugliesi *et al.*, 1991; Roseland *et al.*, 1991; Encheva *et al.*, 1993; 2001; Fambrini *et al.*, 1993, 2001; Barotti *et al.*, 1995; Fambrini and Pugliesi, 1996). Moreover, since plant tissue culture causes alterations in DNA methylation, this mutagenic system may be unique and perhaps will generate some novel types of variants. Interestingly, the activation of transposable elements has been correlated with hypomethylation of certain DNA sequences (Fedoroff *et al.*, 1989; Dennis and Brettel, 1990), and active transposable elements have been induced *via* the tissue culture process (Peschke *et al.*, 1987).

Other methods include the insertion of transposable elements to induce stable and unstable mutants forms and the use of antisense RNA to down-regulate the gene expression (Schuch, 1990). Therefore, these methods could also allow the identification of previously unknown biochemical functions of plant genes.

Transcription factors

The developmental processes of higher plants are thus complex that might appear intractable, even to the powerful investigative tools of modern molecular biology, but remarkably good progress toward understanding them has been made

in recent years in model species (Goodrich *et al.*, 1997). Genes that control the ontogeny are often homeotic or heterochronic genes, and member of a limited number of multigene families (homeobox genes, MADS-box genes) which encode transcription factors (Ma, 1998; Percy *et al.*, 1998; Reiser *et al.*, 2000). The isolation and characterization of these genes, also in sunflower, might add news insight in important developmental process such as pattern formation, cellular differentiation and organogenesis (Meyerowitz, 1997; McSteen and Hake, 1998).

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PAPEL DE LOS METODOS BIOTECNOLOGICOS EN LA CREACION DEL GIRASOL EN EL MUNDO

RESUMEN

La mejora del girasol con los metodos de la seleccion convencional es considerablemente limitada por la base genetica estrecha, incompatibilidad natural aun entre la especies semejantes, y los limites temporales del programa de selleccion. Por eso, se presta grande atencion a nuevas tecnologias de la biologia celular y molecular, que representan las armas poderosas que completan los metodos tradicionales de la seleccion de plantas. El concepto del marcador DNK ha aumentado considerablemente nuestra capacidad para el acceso directo a qualquier parte del genoma vegetal, lo que presenta nuevas posibilidades como clonar a base de las mapas de genes y la seleccion de plantas dirigida. La regeneracion eficaz de plantas fertiles a partir del cultivo de celula y protoplasto en combinacion con nuevos metodos de introduccion de DNK y de seleccion de las celulas transformadas, llevo a la creacion de las plantas de girasol transgenicas. En el girasol cultivado fueron introducidos los genes agronomicamente utiles que aseguran la resistencia a los insectos y patogenos. Entretanto, la mejora ulterior del girasol al nivel molecular puede ser limitada por la ignorancia o la imposibilidad del acceso a los genes utiles (por ejemplo, a los genes que controlan los poligenes de las propiedades siendo el rendimiento y la resistencia a los efectos bioticos y abioticos). Por eso, la prioridad es dada al desarrollo de mapas detalladas de los genes enlazados y a la creacion de nuevos instrumentos para analisis muy eficaces del genoma y su expresion.

RÔLE DES BIOTECHNOLOGIES DANS LE DÉVELOPPEMENT DE LA CULTURE DU TOURNESOL DANS LE MONDE

RÉSUMÉ

L'amélioration du tournesol par les méthodes de culture conventionnelles est significativement réduite par la pauvreté de la base de gènes qui est due à des incompatibilités naturelles, même entre des espèces semblables et par le manque de temps de la plupart des programmes de sélection. C'est pourquoi, ces derniers temps, une grande attention a été accordée aux nouvelles technologies de biologie cellulaire et moléculaire qui représentent un outil puissant pour compléter et parfaire les méthodes traditionnelles d'amélioration des plantes. Le concept des marqueurs basés sur l'ADN a révolutionné notre aptitude à accéder directement à quelque partie que ce soit du génome de la plante et a conduit à de nouvelles opportunités comme celle du clonage basé sur carte génétique. Ce concept a aussi révolutionné notre aptitude à diriger la culture. La régénération efficace de plantes fertiles à partir de cellules cultivées et de protoplastes en combinaison avec de nouvelles méthodes d'introduction de l'ADN et de sélection des cellules transformées a eu pour résultat la production de tournesols transgéniques. Des gènes utiles assurant la résistance envers les insectes et les pathogènes ont été introduits. Cependant, l'amélioration moléculaire ultérieure du tournesol pourrait être limitée par un accès limité à ces gènes utiles importants et par notre manque de connaissances (par exemple, sur le contrôle de traits multigéniques comme le rendement et la résistance aux stress biotique et abiotique). C'est la raison pour laquelle la priorité devrait être donnée au développement de cartes détaillées de chaînes de gènes et à l'élaboration d'outils permettant une analyse plus efficace des génomes et de leurs expressions.