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Biotechnological interventions for genetic improvement of safflower

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Abstract
This article reviews biotechnological options for genetic improvement of safflower. Recent years witnessed an interest from the industrial sector in safflower for production of plant made pharmaceuticals and industrial proteins through molecular farming. However, crop breeding programmes are not being complemented adequately with the biotechnological tools. The framework genetic map of safflower is not available till date. Safflower has enormous variability and several traits could be genotyped through the available molecular marker systems. Genotyping should invariably be carried out along with phenotypic trait evaluation for maximizing gain from selection. Rich genomic resources are available for other taxa of *Asteraceae viz., Lactuca sativa* and *Helianthus spp.* and safflower could be an ideal candidate crop to have advancements in genomics through comparative mapping. Safflower is amenable to manipulations *in vitro* and genetic transformation protocols through vector mediated gene transfer are in place providing scope for development of transgenics for desirable traits. In safflower, anthers and microspores are known to have great propensity for shoot regeneration *in vitro* and this offers possibilities in marker assisted breeding through development of doubled haploid population. Wild *Carthamus* species constitute a rich repertoire of genes for biotic and abiotic stresses besides oil quality traits and need to be utilized. Prebreeding through intraspecific and interspecific genetic enhancement coupled with marker aided selection will accelerate the breeding programmes. Nevertheless, safflower research is scattered and there is an immediate need for determining regional and international priorities and forming core work groups as in other crops for tapping the unexploited potential of safflower.

Key words: Transgenics - plant made pharmaceuticals - tissue culture - molecular markers - marker assisted breeding

Introduction
Safflower (*Carthamus tinctorius* L.) is one of the important oilseed crops and has been traditionally grown for its flowers as a source of dye for colouring food and fabrics. Subsequently, it is grown for edible oil, animal meal, bird feed, medicinal uses, as a potential candidate crop for production of plant made pharmaceuticals, biofuel and specialty type oils. India with an area of 0.42 million ha, production of 0.23 million tonnes of seed and average productivity of 547 kg/ha is the leading producer of safflower in the world (FAOSTAT, 2006). The average yield in different countries varied between 172 and 2500 kg/ha. Despite its vast potential and growth adaptability to a wide range of agro-ecological conditions, safflower remained as a neglected crop due to low seed oil content (28-36%), spininess, fibre rich seed meal and vulnerability to a number of diseases and pests. The major biotic stresses limiting production are powdery mildew (*Leveillula taurica*), aphids, wilts caused by *Fusarium oxysporum* f. sp. *carthami* and *Verticillium,* leaf spots caused by *Cercospora* and *Alternaria, Phytophthora* root rot and insects such as aphids and safflower fly. The abiotic stresses confronting safflower production include salinity, water logging, photoperiod insensitivity and frost. Genetic improvement mostly relied on conventional methods through germplasm augmentation, characterization and selection. Germplasm and wild species have been evaluated at different germplasm centers for their resistance to root rot caused by *Phytophthora dreschsleri,* rust (*Puccinia carthami*), fusarial and verticillium wilts, insects, drought, frost, oil content and oil quality, tocopherol levels and profiles. Safflower species are known to possess several desirable genes such as, drought hardiness, shattering tolerance, non-dormancy of seeds, resistance to safflower fly (*C. persicus* and *C. palaestinus*), rust, *Ramularia* leaf spot

and powdery mildew (*C. persicus, C. oxyacantha, C. lanatus* and *C. palaestinus*) and high □-tocopherol (*C. oxyacantha*). While traits from germplasm have been incorporated in the breeding material, not much headway has been made in transfer of genes from wild safflowers to cultivated safflower. In developed countries there is a continuing flow of new, genetically improved cultivars of major crops reaching the farmers. However, for small-acreage specialty crops like safflower grown under diverse production systems, support for crop improvement and biotechnological tools are lacking. Few isolated groups are working on the crop and access to biotechnological tools in terms of techniques, information, equipment, etc, are inadequate. The biotechnological developments made in the crop and futuristic perspective is presented.

I. Molecular markers

a. Genetic diversity analysis: Most of the earlier studies on safflower diversity were confined to morphological characterization, evaluation of agronomic traits, reaction to biotic and abiotic stresses and biochemical parameters pertaining to seed oil fatty acid profiles. In the 1980s and 1990s, limited studies were carried out on characterization of germplasm using isozymes. However, studies on isozyme polymorphism were restricted to few enzymes and cultivars owing to the technical difficulties in handling protein based gels. With the advent of molecular markers in 1990s, successful use and exploitation of DNA markers in genetic diversity analysis, genetic mapping and phylogenetic analysis has been reported for several crop plants. The advantages of DNA markers are mainly due to the technical ease, low cost, high reproducibility, and availability of a number of marker systems that produce either dominant or codominant markers. Despite the widespread use of molecular markers in other crops, their use in safflower remained dormant for until a decade. In the recent past, importance of molecular markers has been realized and have been used in germplasm assessment for understanding the geographic structure, molecular profiling of genotypes and in genetic mapping.

Molecular markers have been used primarily for evaluation of germplasm assessment of the local cultivars and land races or germplasm accessions and to partition genetic variation geographically (Sehgal and Raina, 2005; Johnson et al., 2007; Yang et al., 2007; Amini et al., 2008; Khan et al., 2008). The most commonly employed markers in safflower are random amplified polymorphic DNA (RAPD), intersimple sequence repeat (ISSR) markers and amplified fragment length polymorphism (AFLP). These are the markers of choice for crops with inadequate genomic resources, do not require prior sequence information and scan the genome including the repetitive sequences. Assessment of genetic diversity in safflower was mainly focused on estimating genetic variation by combining molecular polymorphism and phenotypic variation (Johnson et al., 2007; Yang et al., 2007; Amini et al., 2008; Khan et al., 2008). Johnson et al (2007) characterized 96 accessions representing 29 countries from seven world regions using AFLP markers. Yang et al (2007) determined relationships among 48 safflower accessions from 38 countries using 22 ISSR primers. Amini et al (2008) assessed genetic diversity in 16 Iranian landraces from Iran along with 4 exotic genotypes by combining agro-morphological traits and RAPD markers. Khan et al (2008) assessed the patterns of geographic diversity and the relationships between agro-morphological traits and fatty acid composition in 193 safflower accessions representing 40 countries in 9 eco-geographical zones. There was lack of congruence between agro-morphological and molecular matrices indicating the need for both measures for complete characterization of safflower diversity (Johnson et al., 2007; Yang et al., 2007; Amini et al., 2008; Khan et al., 2008). ISSR primers were more informative and disclosed more polymorphism than RAPD primers. Subsequently, studies of Sehgal and Raina (2005) and Johnson et al., (2007) indicated the robustness of AFLP marker system in terms of its high discriminating power, assay efficiency index, marker index, resolving power and genotype index and distinguished safflower diversity across broad geographic groups and in fingerprinting the genotypes. The average frequency of polymorphism was 2.4, 1.3 and 20.5 per primer and amount of polymorphism disclosed was 24.2, 17.8 and 61.1% with RAPD, ISSR and AFLP markers, respectively (Sehgal and Raina, 2005). Perusal of diversity analysis studies indicate close clusters in germplasm and higher genetic variation was obtained only when the study included exotic germplasm, land races or wild species. This clearly reflects on the narrow genetic base on which the breeding programmes are formulated. Diversity studies indicated higher genetic variation in accessions

from Asian continent and particularly from India (Yang et al., 2007; Khan et al., 2008). Yang et al (2007) suggests the availability of rich genetic diversity in China with its 2100 years of safflower cultivation history and long-term selection and breeding. However, studies of Johnson et al (2007) showed more diversity in accessions from American region. The relatively high diversity in American population was attributed to the wide range of genetic material used in US safflower breeding programs and the selection of progeny from a wide range of environments including hull characteristics, disease resistance and meal attributes. All the studies unequivocally establish the existence of wide genetic diversity in safflower germplasm including wild species and indicate considerable potential for genetic improvement of the crop for agronomic and quality traits. According to the IPGRI germplasm directory compiled by Zhang and Johnson, a total of 25,179 accessions of safflower including wild species are stored in 22 gene banks of 15 countries (safflower.wsu.edu/saff-dir.pdf)). Molecular markers will play a pivotal role in the management, characterization and utilization of germplasm.

b. Phylogenetic analysis: The genus *Carthamus* is comprised of 25 species and were classified into 5 sections based on chromosome numbers (2n=20, 22, 24, 44 and 64) (Dajue and Muendel, 1996). Species classification and phylogenetic relationships were predominantly based on morphological characters, crossability success, karyology, characterization of naturally occurring interspecific hybrids, plant habit, isozymes and biogeography. However, information with regard to the origin of cultivated safflower, the putative progenitor of cultivated safflower, origin of the polyploid species - *C. lanatus* (2n=44), *C. creticus* and *C. turkestanicus* (2n=64), evolution of dysploidy (variation in basic chromosome number), taxonomic status of the lone species, *C. divaricatus* (2n=22) and perennial species *C. arborescens, C. rhiphaeus, C. caeruleus* and *C. nitidus* is obscure. Use of molecular markers supported in resolving certain ambiguities.

Safflower exhibits dysploidy (x=12, 11 and 10) and dysploid evolution could be descending from x=12, ascending from x=10 or both ascending and descending from x=11. Analysis based on ITS sequences and karyology concluded that descending dysploidy is the main mechanism of karyological evolution in the genus *Carthamus* (Vilatersana et al., 2000a). Overlapping morphological characters are the main source of confusion in traditional classification of the Mediterranean complex, *Carduncellus-Carthamus.* Comparison of sequences of the internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA has resolved certain ambiguities with regard to the generic delimitations and phylogeny of the Mediterranean complex (Vilatersana et al., 2000b). RAPD markers have been useful in the study of sectional, species and subspecies classification in *Carthamus* (Vilatersana et al., 2005). While the genus *Carthamus* was classified into 5 sections based on chromosome numbers, correlation of molecular analysis data with morphological and karyological characters led to reduction of the number of sections from five to two viz., *Carthamus* and *Atractylis* (Vilatersana et al., 2005). The earlier classifications separated species with 2n=20 into two sections viz., Odontagnathius (*C. dentatus* spp. *dentatus*) and Lepidopappus (*C. glaucus, C. boissieri, C. tenuis* and *C. leucocaulos*) while molecular analysis supported grouping of all the species into a single section (Vilatersana et al., 2005). The proposed origin of the allopolyploids, *C. creticus* and *C. turkestanicus* through hybridization of *C. lanatus* (n=22) and species with n=10 has been confirmed through RAPD analysis which showed close relationships between the polyploids and species with n=10 viz., *C. leucocaulos* and *C. glaucus* (Vilatersana et al., 2005). Use of both nuclear and organelle specific markers could further help in confirmation of hybridity, identification of putative parents and the direction of the cross in polyploids and naturally occurring hybrids.

Chapman et al (2007) designed a set of universal markers using alignments of a conserved orthologous set (COS) of expressed sequence tags (ESTs) from lettuce and sunflower and genomic sequences of *Arabidopsis* for comparative mapping and phylogenetic analysis in the Asteraceae. All 10 loci were single locus and nine of the 10 loci were polymorphic with an average of 12.8 single nucleotide polymorphisms (SNPs) per Kb. Polymorphism data for these ten universal loci amplified in eight accessions of safflower in comparison with the internal transcribed spacers (ITS) region of the rRNA genes revealed five times greater nucleotide polymorphism with the universal primers than the ITS values and presumed to help in

phylogenetic restructuring (Chapman et al., 2007). Studies of Chapman and Burke (2007) on characterization of *Carthamus* species with 2n = 24 using universal markers specific to Asteraceae indicated *C. palaestinus* as the progenitor species of cultivated safflower. *C. oxyacanthus* and *C. persicus* appeared to be distantly related to cultivated safflower. Among the *Carthamus* species, cultivated safflower harboured the lowest levels of nucleotide diversity, *C. oxyacanthus* exhibited highest level of diversity while *C. palaestinus* was intermediate to these two species. On an average one single nucleotide polymorphism (SNP) was detected per 95 bp of sequence.

c. Marker assisted breeding: The importance of molecular markers in marker assisted selection (MAS) is realized. Recently, sequence characterized amplicon region (SCARs) markers based on RAPD polymorphism were developed for the closely linked recessive monogenic genes *Li* (controlling very high linoleic acid content) and *Ms* (controlling nuclear male sterility) (Hamdan et al., 2008). Bulked segregant analysis involving a population of 162 individuals from a cross between CLI (NMS) and CR 142 (high linoleic acid) led to construction of a linkage map with five RAPD-SCAR markers. The SCAR markers flanked both loci at 15.7 cM from the *Li* locus and 3.7 cM from the *Ms* locus. Recessive genetic male steriles are propagated through heterozygotes (*Msms*) which can be identified only by progeny testing. Mapping of nuclear male sterility gene allows early identification of lines carrying the male-sterile allele precluding the need for progeny testing. Markers linked to high linoleic acid content will facilitate marker assisted selection programme aimed at introgression of *Li* alleles into desirable agronomic background. This study serves as a prelude for development of markers linked to agronomically desirable traits in safflower for accelerating the breeding programmes.

d. Prebreeding and genetic enhancement: Knowledge, access and exploitation of available genetic diversity in domesticated and wild relatives are essential for broadening the genetic base of cultivars to increase crop stability and performance. In safflower, interspecific hybridization experiments were mostly confined to assessment of crossability relationships and characterization of F_1 hybrids. Wild *Carthamus* species possess a wealth of genetic diversity in terms of adaptation traits, resistance to biotic and abiotic stresses and oil quality traits. Introgression of desirable traits from wild safflowers to cultivated safflower is hampered by differences in basic chromosome number, day-length sensitivity, long rosette period and delayed flowering. However, reproductive isolation barriers are weak between cultivated safflower and species with chromosome numbers of 2n=24 and offer great potential for enhancement of genetic variation in safflower. Prebreeding through intra and interspecific hybridization coupled with molecular characterization will aid in gene tracking and successful gene introgression in cultivar germplasm.

e. Genomics: Safflower crop suffers from lack of well-developed genetic resources. As of now, 285 nucleotide sequences and 41,000 ESTs are reported in safflower through subtractive genomic library and compositae data base. The genome size of safflower is 2.70 pg (2C value). SemBioSys has a programme on safflower genomics for generating safflower bacterial artificial chromosome (BAC) library and seed expressed sequence tag (EST) library, isolation and characterization of seed specific promoters, oleosin and other seed storage protein genes and genes involved in lipid metabolism. There is a need to develop microsatellite markers for use in variety identification, DNA finger printing and genetic mapping studies. Microsatellites or simple sequence repeats (SSRs) are single locus markers and are characterized by their hypervariability, abundance, uniform distribution throughout the genome, codominant inheritance, reproducibility, ability to automate assays and transferability. Isolation of SSR markers is laborious, time-consuming and an expensive process. However, with the availability of ESTs for safflower it is possible to identify genic SSRs enabling the mapping of genes of known function. The vast genomic resources from other compositae members viz., sunflower and lettuce provide a potentially valuable source for mining SSR markers. The transfer success of *Helianthus* SSRs to safflower was 13% (http://cgpdb.ucdavis.edu/).

The safflower chloroplast genome is well characterized (Ma and Smith, 1985 and references therein). Safflower chloroplast DNA is about 151 kbp with a density of 1.700 g/cm^3, G+C content of 40.8%, Tm of 86 ^{0}C and genome size of 10^8 daltons. The physical map with relative

locations of *pst*I, *Sal*I, *Kpn*I, *Bam*HI, *EcoR*I and *Hind*III restriction sites and positions of *psbA*, *rbcL, atpA* and *rrnA* genes was constructed.

II. Tissue culture

Tissue culture protocols are established for both American and Indian cultivars using seedling tissues including roots and mature embryo explants. Safflower can be regenerated through organogenic and embryogenic pathways through direct and callus mediated methods (Reviewed in Sujatha, 2007). Shoot induction is rapid and shoots/embryos are observed within 10-15 days after culture initiation. The media requirements for evoking organogenic/embryogenic response from seedling tissues are simple and most studies employed Murashige and Skoog (MS) basal medium supplemented with benzyl adenine (BA) or thidiazuron (TDZ) in conjunction with naphthaleneacetic acid (NAA). Factors influencing shoot regeneration from seedling tissues include genotype, age of seedling, explant source and size, and polarity of explants. However, influence of these factors was found to be minimized on medium supplemented with TDZ+NAA. In the author's own experience, seedling age was found to be the most critical and best response can be obtained from 6-8-day-old seedlings. Androgenic response from cultured anthers is also favoured by BA+NAA combination and is influenced by genotype, growth condition of anther-donor plants, temperature pretreatment and hormonal concentration requiring a higher cytokinin to auxin ratio for evoking caulogenesis. Shoot regeneration frequencies reported from seedling tissues are fairly high (26-100%) with 8 to ~200 shoots per regenerating explants. Safflower tissues exhibit great propensity for multiple shoots but higher number of shoot formation is invariably associated with hyperhydricity and shoots without distinct nodes. Frequency of shoot regeneration from anthers is 31.8% with anthers from field-grown plants having better caulogenic response than greenhouse grown plants. Somatic embryogenesis from cotyledons and a maximum embryogenic response of 55% with a mean of 14.7 somatic embryos per responding explant and embryo to plantlet conversion of 70% is reported. Silver nitrate had pronounced effect on embryogenesis and incorporation of 50 µM silver nitrate enhanced both embryogenic response (83.3%) and number of somatic embryos induced per cotyledon (22.1). Rooting is reported on medium supplemented with either NAA, high sucrose concentration (6-9%), 2,4,5-trichlorophenoxy propionic acid (POP), IBA, IAA hormone-free MS salt medium, *Agrobacterium rhizogenes* or a combination of NAA with phloroglucinol. Shoot hyperhydricity could be reduced with inclusion of 1g/l of activated charcoal in rooting media. In most studies, rooting (>70%) has been successful on media supplemented with NAA and acclimatization success of more than 60% is reported. Rooting frequency is strongly influenced by genotype, shoot quality, medium to which the shoots were habituated and the period of culture.

The earlier studies were aimed at development and refinement of plant regeneration protocols for obtaining high frequency of shoot regeneration. Recently, the tissue culture technique has been extended for production of plants resistant to *Alternaria carthami* (Vijaya Kumar et al., 2008). In this protocol, embryogenic and organogenic calli were subjected to selection of shoots on medium supplemented with 40% fungal culture filtrate of *A. carthami* (5 x 10^5 conidia/ml). Resistance in selected plants was increased to 100, 97.6 and 84% over that of the control in R_0, R_1 and R_2 generations, respectively.

In safflower, callus and cell cultures have been established for production of tocopherols, Kinobeon A (an antioxidant), red and yellow pigments from flowers, isolation of callus lines resistant to *Fusarium oxysporum* f.sp. *carthami* and sodium chloride (reviewed in Sujatha, 2007).

III. Genetic transformation

Genetic engineering has the potential to accelerate crop improvement programmes and has yielded encouraging results in several crop plants (www.isaaa.org). In safflower, *Agrobacterium*-mediated transformation protocols have been reported for both Indian and American cultivars (Orlikowska et al., 1995; Rohini and Rao, 2000). In genetic transformation of American cultivars, tissue culture regeneration using cotyledons and primary leaves was

employed. For Indian cultivars, *in planta* transformation using embryo explants was developed. Transformation studies were limited to constructs harbouring commonly used reporter (*uidA*) and selectable marker genes (*npt*II). The studies indicated influence of co-cultivation conditions and the *Agrobacterium-tumefaciens* strain used. Transgene confirmation was done through transient assays by determination of GUS expression and molecular analysis through PCR and Southern hybridization assays of the primary transformants. Despite the high frequency of shoot regeneration from transformed tissues (15-34.3%), rooting of transformed shoots has been problematic. The protocols have not been exploited for development of transgenics with agronomically desirable traits.

IV. Plant made pharmaceuticals: In the last decade, plants have been exploited for production of large quantities of cost-effective recombinant proteins. Safflower being a less common crop and owing to its predominantly self-pollinated breeding behavior, lower production and capital costs, easy transformability, ability for accumulation of high levels of recombinant proteins in seeds has gained importance as a protein production factory for therapeutic proteins for metabolic and cardiovascular diseases and non-pharmaceutical products addressing human topical, nutritional oils and agricultural biotechnology markets (www.sembiosys.com, www.arcadia.com). Most of the proteins are targeted for accumulation in the seeds to enable easy extraction and for utilization of long term storage capabilities. The oilbody-oleosin technology is based on two proprietary capabilities of expression of recombinant proteins in seed oil bodies (Stratosome^{TM} Biologics System) and ability to extract oilbodies inexpensively from seeds (Affinity Capture System). Safflower has been successfully modified to express and produce apolipoprotein A1 and its variant apolipoprotein AI (Milano) collectively referred to Apo AI constituting the next generation cardiovascular drug that targets the removal of atherosclerotic plaque from arteries. SemBioSys is also engaged in production of insulin with accumulation levels of 1.2% of total seed protein. Safflower-produced insulin is physically, structurally and physiologically similar to pharmaceutical-grade human recombinant insulin and toxicological studies have been initiated in animals to demonstrate safety and compatibility to pharmaceutical grade human insulin. The other therapeutic product of interest in safflower is the □-linolenic acid (GLA). The high GLA safflower contains 35% GLA which is an omega-6 fatty acid with health benefits that are similar and complementary to the benefits of fish oil derived omega-3 fatty acids and has been shown to have significant anti-inflammatory effects with benefits for cardiac, joint, skin and neurological health. Arcadia Biosciences, Inc is expected to commercialize GLA-enriched safflower during the year 2008. Genetically engineered safflower expressing a carp growth hormone for stimulating the immune response of shrimps to enhance resistance to diseases is ready for field release in USA (www.aphis.usda.gov).

Conclusion and future perspective

Development of appropriate markers will support studies on origin and evolution of the genus *Carthamus,* map-based cloning, diversity analysis and marker assisted breeding programmes. The availability of extensive EST resources within the Asteraceae members will facilitate comparative mapping studies and in development of SSR markers. Focus on genetic diversity should be on germplasm of the Mediterranean region particularly the accessions existing wild rather than the accessions from genebanks to have accurate assessment of the genetic treasure in safflower. Several useful traits such as dwarfness, early maturity, resistance to biotic and abiotic stresses, high oleic (UC-1) types for enhancement of oil stability, high linoleic types and tocopherol variants are available in the cultivar germplasm and wild species that could be mapped to accelerate the breeding programmes. Hybrid vigour has been commercially exploited for production of hybrids in safflower through both genetic (GMS) and cytoplasmic male sterility (CMS) systems. Identification of suitable markers for identification of heterozygous maintainer in GMS and the fertility restorer genes in CMS system will add new dimension for hybrid breeding programmes. Unlike several other crops, development of mapping population through doubled haploid technique would be relatively easy in safflower owing to the propensity of safflower anthers for shoot regeneration. Micropropagation and *in vitro* conservation protocols could be developed for maintenance and multiplication of wild

Carthamus species. Shoot regeneration frequencies are very high and need to be converted to genetic transformation frequencies for development of transgenics aimed at input traits and high-value end products. Viable linkages need to be established between institutions involved in germplasm conservation, plant breeding activities and biotechnology for development and sharing of genetic and genomic resources and accelerating the breeding programmes.

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