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Advances in Safflower Biotechnology

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ABSTRACT

Safflower is a versatile crop with several desirable attributes and multiple uses. Genetic improvement mostly relies on the variability available in the cultivar germplasm. Introgression of desirable traits from wild species is hampered by differences in basic chromosome number, asympatry and strong barriers to sexual crossability thus, necessitating the use of biotechnological tools. Reliable and reproducible protocols for direct as well as callus-mediated shoot regeneration from both somatic and gametic tissues through organogenic and embryogenic pathways are established. Compared to other Asteraceae members, the development of molecular and genomic resources for safflower has been limited. AFLP, ISSR and RAPD markers are used in assessment of genetic diversity in land races and germplasm and also the extent of outcrossing of cultivated safflower with weedy relatives. Noteworthy progress is being made in the development of a wide array of genetic engineering technologies for recombinant protein production using an oleosin-fusion protein system and metabolic engineering of oilseeds for production of high value lipids like γ-linolenic acid (GLA), eicosopentaenoic acid (EPA) and docosahexaenoic acids (DHA) for increased health and economic benefits. Owing to the self-pollinated nature of the crop, lower production and capital costs, greater production flexibility, safflower is used as a target for biopharming for production of plant made pharmaceuticals, novel proteins and industrial enzymes. The immediate challenge is to genetically engineer safflower for input (agronomic) traits and also for modification of safflower oil to high-value oil for specific market needs.

Keywords: genetic engineering, molecular farming, molecular markers, tissue culture

Abbreviations: AFLP, amplified fragment length polymorphism; **BA**, N^6-benzyladenine; **DHA**, docosahexaenoic acid; **EPA**, eicosopentaenoic acid; **GLA**, γ-linolenic acid; **GUS**, β-glucuronidase; **IBA**, indole-3-butyric acid; **ISSR**, inter simple sequence repeat; **MS**, Murashige and Skoog basal salt medium; **NAA**, α-naphthaleneacetic acid; **RAPD**, random amplified polymorphic DNA; **TDZ**, 1-phenyl-3-(1;2;3-thiadiazol-5-yl) urea (thidiazuron)

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INTRODUCTION

Safflower (*Carthamus tinctorius* L.; tribe Cynareae; subfamily Tubuliflorae; family Asteraceae) is one of humanity's oldest crops and is grown commercially in India, the USA, Mexico, Ethiopia, Kazakhstan, Australia, Argentina, Uzbekistan, China, while the Russian Federation, Pakistan, Spain, Turkey, Canada, Iran and Israel grow safflower to a limited extent. Mexico and India with a production of 422,765 (MT) are the leading producers and contribute 54% of the total safflower production in the world (FAOSTAT 2005). Safflower is a multipurpose crop and is grown for the use as edible oil (linoleic rich types), industrial oil (oleic rich types), cake, animal meal, birdseed and safflower dye while it is predominantly cultivated in China for the flowers for use in traditional medicines (Dajue and Mundel 1996; Zhang 1997). Safflower oil rich in polyunsaturated fatty acids (75% linoleic) is known to reduce blood cholesterol level and demands a premium price among edible oils and is competitive from a health viewpoint with canola and olive oil. Health concerns regarding use of synthetic food colorants has increased interest in safflower-derived food colouring, which is natural and non-toxic. The safflower florets contain the water-soluble yellow coloured carthamidin constituting 28-36% and an alcohol soluble carthamin or safflower red constituting 0.3-0.6%. Carthamin is the only chalcone-type pigment and is used in colouring silk and cotton fabrics. Both the pigments are found safe and are used for colouring processed foods, beverages, cosmetics and drug additives (Dajue and Mundel 1996; Zhang

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1997). These pigments have been experimentally shown to enrich blood, to decrease fatigue and to promote menstruation (Akihisa *et al.* 1994). The growing awareness of the importance of all the plant parts for various clinical uses and more particularly the use of dried florets in herbal medicine has rekindled the interest in the crop. Safflower is also being exploited in horticulture over the past twenty years for its colourful inflorescences (Uher 2005) and in production of plant-made pharmaceuticals, such as, human insulin, apolipoprotein A1, γ-linolenic acid, docosahexaenoic acid and eicosopentaenoic acid.

Conventional breeding efforts were aimed at genetic improvement of agronomically desirable traits such as high seed yield, high oil content and resistance to biotic and abiotic stresses. Plant breeding efforts launched in India since 1970s resulted in the identification and development of several high yielding varieties of specific regional and multi-regional importance in safflower. The reports of the existence of significant heterosis to an extent of up to 177% for seed yield and 50 to 80% for total oil in safflower led to the development of hybrids based on genetic (both dominant and recessive) and cytoplasmic male sterility systems (Sujatha 2006; Singh and Nimbkar 2007). In the USA, plant breeding programs have centered on developing disease resistant cultivars, altering lipid and amino acid composition, removal of deleterious glucosides for use of the meal as feed for monogastric animals, cold tolerance, male sterility, polyploidy, interspecific hybridization and cytogenetic studies (Knowles 1980). Genetic improvement of safflower mostly relied on the existing variability in the cultivar germplasm and to a limited extent through crosses with closely related species from Section I of Ashri and Knowles (1960). Successful cultivation of the crop in the safflower growing regions worldwide is beset with problems of low seed yields besides the vulnerability of the released cultivars to biotic (aphids, safflower fly, vascular wilts, foliar diseases) and abiotic (salinity, water logging, photoperiod insensitivity, frost) stresses. Another major problem is the low oil content in the released varieties (28-36%). Approaches for elevating the oil content by combining mutant hull characteristics such as thin hull, striped hull and reduced hull possessing 42 to 46% oil content proved largely unsuccessful due to the pleiotropic effect of the *th* gene, low penetrance of *rh* gene and the multiple allelic nature of the *stp* gene (Ebert and Knowles 1966; Ramachandram 1985). There is also a need for breeding work of ornamental varieties with spinelessness, early maturity, delayed physiological drying of lower leaves and tolerance to *Colletotrichum* and *Fusarium* (Uher 2005). This review highlights the progress in biotechnological research in safflower and the future strategies that could be followed for genetic upgradation of the crop.

ORIGIN OF SAFFLOWER AND GENOMIC RELATIONSHIPS

Wild safflowers are distributed across the Mediterranean, the Nile valley to Ethiopia, South-West Asia and parts of Pakistan and India. The genus *Carthamus* is comprised of 25 species according to the classification of Ashri and Knowles (1960) and Hanelt (1963) while the reclassification of the genus by Lopez-Gonzalez (1990) and Vilatersana *et al*. (2005) has separated the perennial subshrubs from the annual herbs and restricted the species to 18. Earlier species classifications were based on the karyology, morphological characters, crossability success, degree of hybridization, biogeography and isozyme studies (Ashri and Knowles 1960; Hanelt 1963; Estilai and Knowles 1976) and the species were categorized into four/five sections (**Table 1**). Alot of inconsistencies existed with regard to the taxonomic classifications and the use of molecular markers in clarifying a few of the ambiguities is presented.

- **Origin of cultivated safflower:** Vavilov (1951) proposed three areas of origin for cultivated safflower. These include i) India (his center II) based on variability and ancient culture, ii) Afghanistan (his center III) based on variability and proximity of wild species, and iii) Ethiopia (his center IV) for the presence of wild safflower species in that area. However, Ashri and Knowles (1960) and Hanelt (1963) placed the center of origin in the near East based on the similarity of cultivated safflower to two closely related wild species, *C. flavescens* and *C. palaestinus*.
- **Progenitor of cultivated safflower:** One of the species

Table 1 Classification of *Carthamus* species.

***Carthamus* species**	**Chromosome number (2n)**	**Ashri and Knowles (1960)**	**Hanelt (1963)**	**Lopez-Gonzalez (1990)**	**Vilatersana *et al.* (2005)**
C. tinctorius L.	24	Section I	*Carthamus*	*Carthamus*	*Carthamus*
C. palaestinus Eig	24	Section I	*Carthamus*	*Carthamus*	*Carthamus*
C. oxyacantha M. Bieb	24	Section I	*Carthamus*	*Carthamus*	*Carthamus*
C. flavescens Spreng (Syn *C. persicus* Willd)	24	Section I	*Carthamus*	*Carthamus*	*Carthamus*
C. gypsicola Iljin	24	--	*Carthamus*	*Carthamus*	*Carthamus*
C. arborecsens L.	24	Not grouped	*Thamnacanthus*	*Phonus*	--
C. rhiphaeus Font Quer and Pau	24	Section I	*Thamnacanthus*	*Phonus*	--
C. curdicus Han.	24	--	*Carthamus*	*Carthamus*	--
C. nitidus Boiss	24	Section I	*Lepidopappus*	Not grouped	*Atractylis*
C. caeruleus	24	*Carduncellus*	--	--	--
C. divaricatus Begg. and Vaccari	22	Section V	*Atractylis*	*Odontognathius*	--
C. dentatus Vahl ssp. *dentatus*	20	Section II	*Odontognathius*	*Odontognathius*	--
C. alexandrinus Boiss. and Heldr	20	Section II	*Lepidopappus*	--	*Atractylis*
C. tenuis Boiss and Blanche (Syn *C. glaucus* ssp. *tenuis*)	20	Section II	*Lepidopappus*	*Odontognathius*	*Atractylis*
C. glaucus M. Bieb ssp. *glaucus*	20	Section II	*Lepidopappus*	*Odontognathius*	*Atractylis*
C. boissieri Halacsy	20	Section II	*Lepidopappus*	*Odontognathius*	*Atractylis*
C. leucocaulos Sibth and Sm	20	Section II	*Lepidopappus*	*Odontognathius*	*Atractylis*
C. glaucus ssp. *anatolicus* Boiss	20	Section II	*Lepidopappus*	--	*Atractylis*
C. glaucus ssp. *glandulosus* Hanelt (Syn *C. syriacus*)	20	Section II	*Lepidopappus*	--	--
C. ambiguus Heldr	20	Section II	--	--	--
C. rechingeri Davis	20	Section II	--	--	--
C. dentatus ssp. *ruber* Link	20	Section II	*Odontognathius*	--	*Atractylis*
C. sartori Held	20	Section II	--	--	--
C. lanatus L. ssp. *lanatus*	44	Section III	*Atractylis*	*Atractylis*	*Atractylis*
C. lanatus L. ssp. *montanus*		--	*Atractylis*	--	--
C. baeticus Boiss and Reuter (Syn *C. creticus*)	64	Section IV	*Atractylis*	*Atractylis*	*Atractylis*
C. turkestanicus Popov	64	Section IV	*Atractylis*	*Atractylis*	*Atractylis*

-- Species not included in the study

of Section I *viz., C. palaestinus* (Ashri and Efron 1964), or *C. flavescens* (Ashri and Knowles 1960; Imrie and Knowles 1970) is proposed to be the progenitor of cultivated safflower.

- **Origin of polyploid species:** The species *C. lanatus* (x=22) could be an allopolyploid (x=10 + x=12) (Ashri and Knowles 1960) or an autopolyploid originating from *C. divaricatus* with x=11 (Estilai and Knowles 1976). Likewise the allopolyploid species, *C. creticus* and *C. turkestanicus* (x=32) could have resulted from the hybridization of *C. lanatus* (x=22) and species with x=10 and probably *C. leucocaulos* and *C. glaucus* ssp. *glaucus*, respectively (Khidir and Knowles 1970).
- **Taxonomic status of the lone species *C. divaricatus* with x=11:** This species could probably be due to crosses with species with x=10 and 12 chromosomes or hybridization of *C. alexandrinus* (x=10) with *C. lanatus* and backcrossing with species with x=10 (Estilai and Knowles 1976).
- **Uncertain placement:** Taxonomic status of *C. arborescens*, *C. rhiphaeus, C. caeruleus* and *C. nitidus* all with x=12 is obscure (Ashri and Knowles 1960; Knowles and Schank 1964; Estilai and Knowles 1976).
- **Dysploidy:** The evolution of dysploidy (x=10, x=11, x=12) in the genus *Carthamus* (Estilai and Knowles 1976).
- Generic limits were not described and the sectional classification of the genus *Carthamus* remains elusive.

Some of the issues related to taxonomical classification were resolved through use of molecular sequences and the studies used the classification of the genus *Carthamus* proposed by Lopez-Gonzalez (1990) based on anatomical characteristics, biogeographic distribution and biosystematic information. In this system of classification, the genus *Carthamus* was grouped with three other genera *viz*., *Carduncellus*, *Phonus* and *Lamottea*. The genus *Carthamus* included the annual species with 2n = 20, 22, 24, 44 and 64 chromosomes including the polyploid species while the other three genera included perennial species with 24 chromosomes. The genus *Carthamus* was further subdivided into three sections: i) Section *Carthamus* composed of species with 24 chromosomes; ii) Section *Odontognathius* consisted of species with 20 or 22 chromosomes, and iii) Section *Atractylis* is indicated to have n=11 chromosomes producing the polyploid species.

Taxonomic classification of the Mediterranean complex, *Carduncellus-Carthamus* has proven difficult and none of the numerous classifications based on structural or morphological characters have been generally accepted. The problem of generic limits of this complex has been clarified with the use of sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA (Vilatersana *et al*. 2000a). The ITS classification corresponded with biogeography than the morphological characters and the complex was classified into four genera viz., *Carduncellus*, *Femeniasia* and *Phonus* (the western group) and *Carthamus* (the eastern group).

Subsequently, Vilatersana *et al*. (2000b) correlated taxonomic classification and karyology with the generic classification that was based on molecular analysis and suggested interesting differences between the genera *Carduncellus*, *Phonus* and *Carthamus*. The genus *Carduncellus* showed a constant base chromosome number, x=12 with no dysploidy and it included diploids, triploids and polyploids. The genus *Phonus* was found to be closer to *Carduncellus* than to *Carthamus* and included only diploids (x=12) and with no dysploidy. The genus *Carthamus* had three well-defined groups. All the representatives of the section *Carthamus* were diploid with x=12. Members of the section *Odontognathius* exhibited dysploidy with x=12 (*C. nitidus*), x=11 (*C. divaricatus*) and x=10 (all other species). The third section *Atractylis* was formed by species of purported hybrid origin.

Taxonomic problems in the sectional classification were resolved through RAPD markers (Vilatersana *et al*. 2005). The RAPD analysis was found to be complementary to DNA sequence analysis and the data was correlated with morphological and karyological characters and the number of sections was reduced from five to two. The section *Thamnocanthus* (Hanelt 1963) that was delineated from *Carthamus* as a separate genus, *Phonus* by Vilatersana *et al*. (2000a) based on ITS sequences continued to be classified separately with RAPD markers as well. The section *Carthamus* was retained in its original delimitation while the section *Atrcatylis* was redefined and included three sections viz., *Atractylis*, *Lepidopappus* and *Odontognathius*. *C. nitidus* with 12 pairs of chromosomes but having morphological relatedness to species with 10 pairs of chromosomes has been regarded as a "link species" between sections *Atractylis* and *Carthamus*. Molecular analysis strongly favoured a separate species status for *C. glaucus* ssp. *alexandrinus* (Hanelt 1963), *C. glaucus* ssp. *tenuis* (Schank and Knowles 1964), C. *lanatus* ssp. *creticus* and *C. lanatus* ssp. *turkestanicus* (Hanelt 1963) as the four purported species failed to group with the species to which they were subordinated.

Safflower exhibits dysploidy (variation in basic chromosome number) and the diploid chromosome numbers of the species are 2n = 20, 22, 24, 44 and 64. Estilai and Knowles (1976) proposed three different hypotheses to explain the dysploid evolution (x=12, 11 and 10) in *Carthamus*: i) descending dysploidy from the base chromosome number, x=12, ii) ascending dysploidy from x=10, and iii) ascending and descending dysploidy from x=11, resulting in species with x=12 and x=10. Analysis based on ITS sequence analysis and karyology support the first hypothesis and it is concluded that descending dysploidy is the main mechanism of karyological evolution in the genus *Carthamus* (Vilatersana *et al*. 2000a, 2000b).

Despite the progress being made in the taxonomic classification of the genus *Carthamus* through correlation of karyological, morphological and molecular studies, several issues still remain to be resolved with regard to the utilization of wild safflowers. Sunflower is another economically important oilseed crop of the family Asteraceae. Genetic improvement of cultivated sunflower has greatly benefited through introgression of desirable traits, such as, male sterility, restorer lines, oil quality and resistance to biotic stresses from wild sunflowers to cultivar background. Wild *Carthamus* species constitute a rich repertoire of genes for agronomically desirable attributes such as drought hardiness, resistance to biotic stresses, shattering tolerance, self-incompatibility and non-dormancy of seeds (Ashri 1971; Kumar 1991). Accessions of *C. persicus* and *C. palaestinus* were reported to be free of the safflower fly (Ashri 1971). Sources of resistance to rust, *Ramularia* leaf spot and powdery mildew were identified in *C. persicus*, *C. oxyacantha*, *C. lanatus* and *C. palaestinus* and in other more distantly related wild safflower species (Ashri 1971; Kumar 1991). Introgression of desirable traits from wild species into cultivated safflower through conventional methods has not been successful owing to differences in basic chromosome numbers and strong barriers to sexual crossability (Dajue and Mundel 1996). The other major problems in the use of *Carthamus* species for interspecific hybridization are the daylength sensitivity, long rosette period and delayed flowering leading to asynchrony in flowering of the wild and cultivated species. There is an immediate need to widen the genetic base of cultivated safflower through interspecific hybridization by employing the techniques of embryo rescue for recovery of intersectional F_1 hybrids and chromosome doubling techniques (*in vivo* and *in vitro*) for facilitating crosses between species with 22 and 44 chromosomes.

The origin and the putative progenitor of cultivated safflower remain ambiguous. Likewise the origin of polyploid species and the putative parental species involved in polyploidization are not clearly understood. The chloroplast genome of safflower is well characterized (Ma and Smith 1985a, 1985b, 1985c) and Southern blot techniques could be

employed for characterization of the chloroplast genome of wild safflowers. Use of molecular markers and specifically the mitochondrial and chloroplast specific microsatellite primers and DNA barcodes of the organellar genomes will unequivocally explain the origin of polyploid species and natural hybrids, confirm the hybridity in artificial hybrids, unravel the progenitor of cultivated safflower and maternal origin of allopolyploids besides elucidating the taxonomical and phylogenetic relationship of the genus.

ASSESSMENT OF GENETIC VARIATION IN GERMPLASM THROUGH BIOCHEMICAL AND MOLECULAR MARKERS

A total of 25,179 samples of safflower germplasm accessions are stored in 22 gene banks of 15 countries (Zhang and Johnson 1999). The World collection as well as the germplasm at respective national gene banks has been evaluated for morphological characters, their reaction to diseases and for variations in fatty acid profiles. The importance of neutral markers for characterization of the germplasm has been realized and studies have been carried out with isozymes and PCR-based molecular markers.

Prior to the advent of molecular tools, isozyme polymorphism was used for assessment of genetic variation in safflower due to the codominant nature of allelic expression, absence of environmental or epistatic effects and expression in a range of plant tissues. Bassiri (1977) reported the use of peroxidase and acid phosphatase isozymes for identification of 14 safflower cultivars and seven ecotypes of *C. oxyacantha*. The study indicated close genetic relationships between cultivated safflower and *C. oxyacantha*. Studies of Carapetian *et al.* (1994) and Carapetian and Estilai (1997) revealed isozyme polymorphism with regard to menadione reductase, 6-phosphogluconate dehydrogenase, phosphoglucoisomerase and triosephosphate isomerase. The studies on variation and inheritance of isozymes demonstrated the potential of isozyme markers as tools for identification of hybrid individuals in this predominantly inbreeding species. Zhang (2001) demonstrated the usefulness of isozymes in characterization of safflower genetic resources by assessing 89 accessions originating from 17 countries. The study revealed 87.5% polymorphism among 8 loci identified and 60% polymorphism among 25 alleles identified with four isozymes. The germplasm from East Asia had the highest value for both the mean allele frequency and the mean gene diversity. Accessions from India possessed high diversity while the accessions from Turkey were closely related to those from the other Middle-east countries.

DNA markers have the advantages of scanning the entire genome depending on the marker system and provide precise and authentic markers of diversity. Sujatha (2002) reviewed the status of *in vitro* techniques and biotechnology in safflower. At that juncture, there was virtually no information with regard to use of molecular markers in safflower. Subsequently, the importance of molecular markers in germplasm characterization particularly for identification of duplicate accessions and development of genetic core collections and also in genetic diversity and genetic mapping studies has been realized. RAPD polymorphism was used to detect genetic diversity of 28 safflower genotypes, which included Iranian landraces, wild, and several exotic genotypes (Yazdi-Samadi *et al*. 2001). Use of RAPD, ISSR and AFLP markers on 14 Indian cultivated varieties revealed the high discriminating power with least confusion probability of AFLP system as compared to the other two molecular marker systems (Sehgal and Raina 2005). In this study, two AFLP primer combinations could fingerprint all the 14 cultivars while 36 RAPD and 21 ISSR primers could differentiate a maximum of eight and four cultivars, respectively. AFLP polymorphism generated using 12 selected primer combinations could distinguish 28 Chinese safflower populations (Zhang *et al*. 2006). Ninety six accessions from seven regions (America, China, east Africa, eastern Europe, The Mediterranean, South Central Asia and South West Asia) representing 29 countries worldwide were analyzed with AFLP markers to compare within and among regional molecular variation (Johnson *et al*. 2006, 2007). AFLP markers distinguished safflower diversity across broad geographic groups. Accessions from Americas overlapped with those from Europe and Asia and to a lesser extent with the Mediterranean and China, while South West Asia was excluded from all other regions. Correlation of AFLP distance matrix with a phenotypic distance matrix with 16 attributes revealed weak correlation between the two matrices indicating the necessity of using both morphological characters and molecular polymorphism for complete characterization of safflower diversity. Yang *et al*. (2007) distinguished 48 safflower accessions with 22 ISSR primers. The polymorphism was 82.7% and maximum genetic diversity was recorded for accessions from Asia. The genetic variation was relatively higher among accessions from Indian and Middle-eastern safflower diversity centers.

In an interesting study of Ash *et al*. (2003), ISSR polymorphism was employed for detection of genetic variation in populations of *C. lanatus* (a troublesome weed) from New South Wales for successful deployment of fungal biological control agents, which are often specific in terms of their host range, and the genetic variation within the host.

The molecular studies demonstrated that detection of genetic variation at DNA polymorphism level holds promise for the classification of safflower germplasm and identification of safflower landraces. Signature molecular markers have to be established for released cultivars and parental lines of hybrids. Molecular markers should be also used to characterize the germplasm and wild species available at various centers to eliminate duplicates and also to utilize the information in marker assisted breeding programmes.

MOLECULAR CYTOGENETICS

Molecular cytogenetics plays an important role in the study of phylogenetic pathways, evolution of constituent genomes and also in introgressive breeding. Raina *et al*. (2005) isolated two novel repetitive DNA sequences, pCtkpn1-1 and pCtkpn1-2 of 343-345 bp and 367 bp, respectively with 37% sequence similarity between the two. Fluorescence *in situ* hybridization on metaphase chromosomes of safflower using the two sequences revealed exclusive localization of pCtkpn1-1 at subtelomeric regions on 22 of the 24 chromosomes while pCtkpn1-2 clone was distributed on two nucleolar and one non-nucleolar chromosome pairs. Variability in the number, size and location of the two repeated sequences provided identity to most of the chromosomes. The study of Raina *et al*. (2005) is just a beginning and demonstrates the usefulness of FISH in identification of specific chromosomal regions. There is an immediate need to gain knowledge of the cytogenetic and taxonomic relationships among species of *Carthamus* through molecular cytogenetics which would help in the assessment of the extent of introgression of the wild genome in interspecific derivatives and provides a basis for effective introgression of characteristics from wild and weedy relatives to cultivated safflower.

Genomics

The family Compositae (Asteraceae) is divided into three major subfamilies that are represented by lettuce, sunflower and safflower. Under the Compositae Genome Program (http://cgpdb.ucdavis.edu/), EST data bases are being developed for lettuce and sunflower and 40,000 reads are in progress for safflower. The genomic tools and resources should accelerate the discovery of genes underlying economically important traits, biochemical pathways and developmental and physiological processes in the Compositae. Cross taxa utility of sunflower markers on safflower is only

13%. SemBioSys has initiated a programme on safflower genomics with the objectives of generating safflower BAC genomic library and seed EST library, isolation and characterization of oleosin and other seed storage protein genes and also genes involved in lipid metabolism and identification of high expressing seed specific promoters.

Plant genome size is an important biological characteristic and has relationships to systematics, ecology and distribution. Using flow cytometry, Garnatje *et al*. (2006) assessed the nuclear DNA content of 16 *Carthamus* species with the objective of improving the knowledge base on interspecific variation and its implications for the infrageneric classification of the genus *Carthamus* besides assessing the variation in plant genome size in the process of formation of allopolyploids. The 2C values ranged from 2.26 pg for *C. leucocaulos* to 7.46 pg for *C. turkestanicus* while the monoploid genome values ranged from 1.13 pg in *C. leucocaulos* to 1.53 pg in *C. alexandrinus*. The 2C value for cultivated safflower was 2.70 pg. Between the two sections as circumscribed by Vilatersana *et al*. (2005), viz., *Carthamus* and *Atractylis*, the mean 2C values were significantly different and were 2.70 pg and 4.33 pg, respectively while the 1C values were 1.35 pg and 1.32 pg and were not statistically significant. With regard to the allopolyploid species, the nuclear DNA content was in accordance with the exact sum of the putative parental species for *C. creticus* while there was slight reduction than the sum of the parental species for *C. turkestanicus*.

CHARACTERIZATION OF THE ORGANELLAR GENOMES

Restriction fragment analysis has been carried out for characterization of organellar genomic DNA of safflower. A study on chloroplast biogenesis involving the interaction and expression of nuclear and chloroplast genomes requires both the physical and genetic maps and the genomic clones containing the relevant genes. Methods have been standardized for isolation of pure organellar DNA free of contaminating DNA (Pay and Smith 1988). A chloroplast genomic library has been constructed in the *BamH*I site of λ EMBL3 (Tippetts *et al*. 1991). The safflower chloroplast genome has been completely cloned and the 16S and 23S rRNA genes were characterized (Ma and Smith 1985a; Tippetts *et al*. 1991). Safflower chloroplast DNA has a density of 1.700g/cm^3 with a G+C content of 40.8%, T_m of 86ºC and a kinetic complexity and genome size of about 10^8 daltons (Ma *et al*. 1984). The plastid genome is circular (151 kbp) and contained a repeated sequence of about 25 kbp containing structural genes of 16S and 23S rRNAs and small and large single copy regions of approximately 20 and 81 kbp, respectively. The physical map of safflower chloroplast DNA has been constructed, the relative location of *pst*I, *Sal*I, *Kpn*I, *BamH*I, *Eco*RI and *Hind*III restriction sites was determined and the positions of *psbA*, *rbcL*, *atpA* and *rrnA* genes were located (Ma and Smith 1985b, 1985c; Smith and Ma 1985; Tippetts *et al*. 1991).

The mitochondrial genome of CMS and restorer lines of safflower was characterized (Pay and Smith 1991). The size is 252 kbp and restriction analysis of DNA from both the lines revealed differences in copy number and substoichiometric amounts. About 75% of the mitochondrial genome was common to both the fertile and CMS plants while the remaining 25% could be accounted for the unique restriction fragments observed in only one or the other plant types. However, detailed investigations are required with regard to the mitochondrial genome rearrangements and the involvement of nuclear genes in determination of such reorganizations.

TISSUE CULTURE AND *IN VITRO* TECHNIQUES

Safflower tissues, particularly the juvenile tissues including roots are amenable to manipulations *in vitro*. Direct as well as callus mediated shoot regeneration systems through organogenic and embryogenic pathways are reported for both spiny and non-spiny genotypes (**Table 2**). Of the various basal media tried, Murashige and Skoog salt medium (Murashige and Skoog 1962) proved superior for induction of morphogenic response in both somatic and gametic tissues (Prasad *et al*. 1991; Chatterji and Singh 1993). Most of the media that promoted shoot regeneration either through organogenesis or embryogenesis included BA singly or in combination with NAA (George and Rao 1982; Tejovathi and Anwar 1984; Chatterji and Singh 1993; Mandal *et al.* 1995). Anthers of safflower also elicited morphogenic potential on medium supplemented with 2.0 mg/l BA and 0.5 mg/l NAA and haploids were recovered with a frequency of 64% (Prasad *et al*. 1991). Other cytokinins such as kinetin, 2-isopentenyl adenine and zeatin singly and in combination have been tried but with little success (George and Rao 1982; Radhika *et al*. 2006). The best regeneration in terms of frequency of responding explants, response from all tissue types and tested genotypes, number of shoots per responding explant has been obtained with TDZ+NAA combination for both the American and Indian genotypes (Orlikowska and Dyer 1993; Radhika *et al*. 2006). Shoot regeneration up to 98.5% with countless number of shoots per responding explant were recorded on media supplemented with TDZ+NAA (Radhika *et al*. 2006). Strong influence of genotype and explant type was observed for organogenic competence on media supplemented with BA+NAA, while on media supplemented with TDZ+NAA, differences due to genotype and explant type were less significant.

The most challenging aspect of safflower tissue culture is the rooting of regenerated shoots and post-acclimatization survival. Improvements have been made to enhance the frequency of rooting of the regenerated shoots. These include high sucrose levels up to 9% in the rooting medium (Tejovathi and Anwar 1984; Nikam and Shitole 1999), increased sucrose (9%) along with 1 mg/l each of NAA and riboflavin (Orlikowska and Dyer 1993), incorporation of 2,4,5, trichlorophenoxypropionic acid (Tejovathi and Anwar 1993; Baker and Dyer 1996) and root induction with *Agrobacterium rhizogenes* (Baker and Dyer 1996). Bayer and Dyer (1996) reported that a 7-day exposure to 10 mg/l IBA in root induction media, followed by incubation in media with 15 g/l sucrose and 1 g/l activated charcoal for 21-days enhances rooting frequency and reduces shoot hyperhydricity. Roots could be induced in different treatments at frequencies ranging from 10 to 95% in various experiments but post-acclimatization survival was successful only with shoots exhibiting less hyperhydricity and those that developed good tap roots with branches *in vitro*. Despite these improvements, the problem of rooting still persisted and frequency of rhizogenesis varied with genotype, shoot quality, the medium to which the shoots were habituated and the period of culture. The problem was further aggravated in genetic transformation experiments where the regenerated shoots were subjected to exposure to the bacteriostats and selective agents (antibiotics/herbicides) for selection of putative transformants. Extensive studies on improvements in rooting resulted in the identification of a hormonal combination involving IBA and the compound phloroglucinol, which gave >70% rooting with high rate of survival following acclimatization (M Sujatha, unpublished results).

One interesting feature of safflower tissue culture is the *in vitro* induction of capitula on media with appropriate combination of growth regulators (Tejovathi and Anwar 1984; Radhika *et al*. 2006). Growth regulators type and concentration and genotype were found to have strong influence on flower formation *in vitro*. Among the cytokinins tested, capitula were induced frequently on media supplemented with BA+NAA and at a low frequency on media fortified with kinetin (Tejovathi and Anwar 1984). Concentration of BA+NAA should be optimum for production of flowers (Seeta *et al*. 1999). *In vitro* produced flowers were normal with normal pollen production and seed set. Studies of Seeta *et al*. (1999) revealed that *in vitro* produced pollen serve as a useful source for creation of genetic variation in

Table 2 Tissue culture studies in safflower.

Explant	Type of morphogenetic response	Medium used (mg/l)	Reference
Hypocotyl, cotyledons	Multiple shoots	MS+0.2-2.0 BA+0.5 NAA	George and Rao 1982
Cotyledons	Induction of capitula	MS+0.5 BA+0.1 NAA MS+0.5 Kn+0.1 NAA	Tejovathi and Anwar 1984
Leaf	Oil accumulation	MS+1.0 BA+0.25 NAA +5% sucrose+1g/l CH+10% coconut water	Singh and Chatterji 1991
Primary seedling explants of *C. tinctorius*	Shoot regeneration from leaf	MS+8.0 BA+0.5 NAA+5.0 adenine sulphate	Singh 1991
Shoot apices of *C. oxyacantha*	Shoot proliferation	MS+0.5 NAA+20 GA_3+5.0 ascorbic acid	
Anther	Shoot regeneration	MS+2.0 BA+0.5 NAA	Prasad *et al.* 1991
Cotyledons	*In vitro* rooting	MS+1.0-2.0 2,4,5-Cl_3POP	Tejovathi and Anwar 1993
Leaf	Shoot buds	MS+5.0 BA+0.25 NAA	Chatterji and Singh 1993
Primary seedling explants	Adventitious shoot regeneration	MS+0.5 BA+0.1 NAA MS +0.1 TDZ+0.1 NAA	Orlikowska and Dyer 1993
Immature embryos	Shoot regeneration	MS+0.01 TDZ+0.1-10.0 NAA	
Cotyledons	Somatic embryos	MS+0.5 BA +1.0-2.0 NAA	Mandal *et al.* 1995
Primary seedling explants	Adventitious shoots Histological analysis	MS+0.5-5.0 BA+0.1-0.5 NAA	Zhanming and Biwen 1993
Cotyledons, hypocotyl	*In vitro* rooting	MS+10.0 IBA for 7days followed by MS+1.5% sucrose+1g/l AC for 21 days	Baker and Dyer 1996
Hypocotyl, cotyledons	Shoot regeneration	MS+0.25 BA+0.1 NAA	Jhansi Rani *et al*. 1996
Primary seedling explants	Selection of calli and shoots resistant to *Fusarium oxysporum*	MS+1.0 BA+1.0 NAA for callus MS+1.0 BA+0.1 NAA for shoots	Suganya *et al*. 1997
Cotyledons	Selection of calli resistant to sodium chloride	MS+0.5 BA+1.5 NAA	Nikam and Shitole 1997
Primary seedling explants	Direct shoot regeneration	MS+1.0 BA+10.0 CH	Nikam and Shitole 1999
Cotyledons	Adventitious shoots	MS+0.5-2.0 BA	Mandal and Gupta 2001
Cotyledons	Somatic embryos	MS+0.5 BA+1.0 NAA	Mandal and Gupta 2003
Cotyledonary node, stem node	Shoot buds	MS+B5 vitamins+4.5 BA+1.5 Kn	Vijaya Kumar and Kumari 2005
Primary seedling explants including roots	Shoot regeneration	MS+0.5-5.0 TDZ+0.1-0.5 NAA	Radhika *et al*. 2006

AC, activated charcoal; **B5**, Gamborg medium; **BA**, N^6-benzyladenine; **CH**, casein hydrolysate; **2,4,5-Cl_3POP**, 2,4,5-trichloro phenoxy propionic acid; **GA_3**, gibberellic acid; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **MS**, Murashige and Skoog basal salt medium; **NAA**, α-naphthaleneacetic acid; **TDZ**, 1-phenyl-3-(1;2;3-thiadiazol-5-yl) urea (thidiazuron)

safflower.

Protocols for *in vitro* multiplication using shoot apices are available for cultivated safflower, which could be extended for multiplication and conservation of wild safflowers and in maintenance of genetic purity of the self-incompatible species. The phenomenon of *in vitro* flowering could be exploited for recovering interspecific hybrids through *in vitro* fertilization and in overcoming the problems of asynchronous flowering. Protoplast isolation and culture techniques need to be established for obtaining interspecific hybrids from crosses limited by sexual crossability barriers. Protoplast fusion in conjunction with molecular biology constitutes an entirely new tool for characterization of the organelle genomes in safflower. Protocol for induction of somatic embryos has been reported (Mandal *et al*. 1995). There is an immediate need for optimization of parameters for enhancing embryo to plantlet conversion frequency, somatic embryo maturation and encapsulation not only for use in large-scale plant propagation but also in genetic transformation experiments due to a very low probability of recovery of chimaeric tissues.

Cell cultures

Plant cell cultures are being widely used in studies on the physiology, biochemistry and molecular biology of primary and secondary metabolism, developmental regulation and cellular responses to pathogens and stress. In safflower, callus and cell cultures have been established for production of tocopherols, which are essential dietary components that possess the physiological action of vitamin E and an antioxidant action. Tocopherol production was effectively stimulated by administration of biosynthetic precursors (homogentisic acid, phytol) and casamino acids (Furuya *et al*. 1987; Wang *et al.* 1999). Phytol increased the total tocopherol content by 18-fold and the α-tocopherol content by 11-fold. Incorporation of 0.1% casein in to MS medium supplemented with 0.3 mg/l 2,4-D and 1.8 mg/l BA resulted in accumulation of tocopherol 57 times higher than that in control (Wang *et al.* 1999). Cell cultures of safflower have been used for production of an antioxidant, kinobeon A (Wakayama *et al.* 1994). Kinobeon A with its potent tyrosinase activity suppressed the free-radical induced damage of cell and microsomal membranes, which indicated its role in protecting biological systems from oxidative stress (Kanehira *et al.* 2003). Kambayashi *et al.* (2005) demonstrated quenching of singlet-oxygen by kinobeon A and presumed that this red pigment could have a preventive effect on single oxygen related diseases of the skin and eyes.

Flower pigments of safflower are valued for their use as food and drug additives. Plant cell culture as an alternative method for production of red and yellow pigments has been investigated and several factors were found to influence pigment formation. Commercially available polysaccharides such as xanthum gum, fucoidan, cellulose, chitin and extracellular polysaccharide from a culture of unknown fungus strain SE-801, cell walls of blue-green algae, increased sucrose concentration (up to 12%) elicited pigment formation approximately 6 to 13 times that of the control (Hanagata *et al.* 1994b; Gao *et al.* 2000). Pigment formation was improved with use of cell aggregates <1.0 mm in the production medium (Hanagata *et al.* 1993), periodical removal of pigment from the reaction and reuse of the production medium in the second culture stage (Hanagata and Karube 1994), and growth of cell cultures under light conditions (Gao *et al*. 2000). Increase in concentration of $CaCl_2 2H_2O$, $MgSO_4.7H_20$ and KH_2PO_4 to 2-, 3- and 8- fold, respectively that of the MS medium led to an increase in red pigment formation by 5-fold that of MS medium (Hanagata *et al.* 1994a). A 10 times higher concentration of the cytokinin (kinetin) than the auxin (NAA) increased pigment production (Hanagata and Karube 1994; Gao *et al.* 2000). All the results were produced with cell cultures grown in 100- to 300 ml Erlenmeyer flasks while Hanagata and Ka-

rube (1994) and Gao *et al.* (2000) carried out pigment production experiments in bioreactors. Most of the studies have focused on red pigment production with the exception of the studies of Gao *et al.* (2000) where both pigment types were produced. The yellow and red pigments originate from the same precursors and detailed investigations are required to investigate the biosynthetic routes of yellow and red pigments of safflower for production of the desired pigment type.

Callus and cell cultures have been used for selection of resistant cell lines. Suganya *et al*. (1997) demonstrated the use of cell cultures for identification of safflower genotypes resistant to *Fusarium oxysporum* f. sp. *carthami* and also in selection of cells resistant to the incitant. Studies of Nikam and Shitole (1997) revealed tolerance of callus cultures to sodium chloride, which was retained even after eight passages (30 d each).

GENETIC TRANSFORMATION

In safflower, genetic transformation using *Agrobacterium tumefaciens*-mediated method has been developed for the American and Indian cultivars. The methods of transformation include callus-mediated regeneration from transformed seedling tissues (Ying *et al*. 1992; Orlikowska *et al*. 1995; Rao and Rohini 1999) or whole plant development of transformed embryos through a method termed as *in planta* approach (Rohini and Rao 2000). For tissue culture based transformation experiments, Ying *et al.* (1992) used tissues from 3-4 week seedlings while Orlikowska *et al.* (1995) and Rao and Rohini (1999) used explants from 3-6-day old and 10-12-day old seedlings, respectively. In studies of Ying *et al.* (1992) and Rao and Rohini (1999), cocultivation was for 2 days and shoot induction was on medium supplemented with BA and NAA while in studies of Orlikowska *et al.* (1995), explants cocultivated for 3 days were transferred to medium supplemented with TDZ and NAA for shoot induction. Growth of *Agrobacterium* was checked with use of 500 mg/l carbenicillin (Ying *et al.* 1992; Orlikowska *et al.* 1995) or 250 mg/l cefotaxime (Rao and Rohini (1999). All these genetic transformation experiments were carried out with gene constructs harboring the selectable marker (*nptII*) and reporter (*uidA*) genes. Different selection levels of kanamycin were used and were 25 mg/l (Orlikowska *et al.* 1995), 50 mg/l (Ying *et al.* 1992) and 100 mg/l (Rao and Rohini 1999). Orlikowska *et al* (1995) replaced kanamycin with 25 mg/l of geneticin in shoot elongation and rooting media. The *vir* gene induction treatments for improvement of transformation efficiency included the use of acetosyringone (Orlikowska *et al.* 1995) and tobacco leaf extract (Rao and Rohini 1999).

Presence of the introduced gene in these experiments was initially confirmed through GUS histochemical assay. Molecular confirmation was done through Southern analysis (Ying *et al.* 1992*),* PCR analysis with *uidA* and *nptII* gene specific primers and Southern analysis (Orlikowska *et al.* 1995), PCR analysis with *uidA* and *virC* region specific primers, neomycin phosphotransferase activity in the total proteins by non-denaturing PAGE, western blotting and dot blot hybridization (Rao and Rohini 1999). Molecular analysis was confined to kanamycin resistant calli due to problems of low frequency of shoot regeneration and poor rooting (Ying *et al.* 1992; Rao and Rohini 1999). In studies of Ying *et al.* (1992), regeneration from transformed leaf calli was 15% as compared to 26% from untransformed calli. All the six calli subjected to Southern analysis showed the presence of the introduced gene while only 50% were found positive for GUS activity. Orlikowska *et al.* (1995) subjected six geneticin-resistant shoots to PCR analysis of which 3 and 4 shoots were positive for the presence of *uidA* and *nptII* genes, respectively. In Southern analysis, five of seven geneticin-resistant shoots showed hybridization with *gus* gene fragment. Rao and Rohini (1999) reported a recovery of 34.3 and 23.3% transformed shoots from shoot apices and cotyledons, respectively. Thus, tissue culture based transformation experiments were limited to the characterization of the primary transformants due to the problems associated with rooting of the putative transformants, genotype dependence of the shoot regeneration and transformation systems, inhibition of shoot growth due to antibiotic selection pressure and the susceptibility of regenerated shoots to media water content leading to hyperhydricity.

To avoid difficulties in a tissue culture-based system, the entire embryo axes of 2-day old germinating seedlings were used as the target tissues for transformation and were directly grown into transformed shoots in the *in planta* method (Rohini and Rao 2000). The transformation efficiencies in terms of presence of both the *uidA* and *nptII* genes in two of the tested genotypes viz., A-1 and A-300 were 5.3 and 1.3%, respectively. Southern analysis of T_0 plants showed the integration of both the marker genes and T_1 plant analysis confirmed transmission of the introduced genes. The method of *in planta* transformation overcomes the difficulties associated with tissue culture and to some extent is genotype independent but has the possibility of recovery of chimaeras.

Leaf spot of safflower incited by *Alternaria carthami* causes significant yield losses of the crop on a world-wide basis. *A. carthami* produces three phytotoxins in culture viz., brefeldin A (BFA), 7-oxobrefeldin and zinniol – all of which produce symptoms when applied to safflower tissue

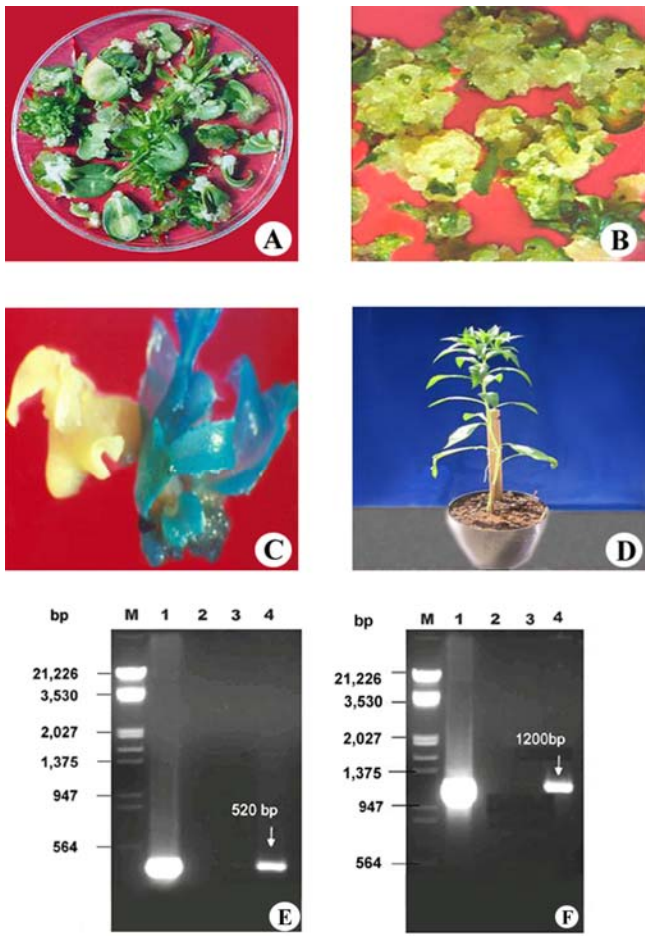

Fig. 1 Tissue culture and transformation in safflower. (**A**) Shoot regeneration from cotyledonary leaves on MS medium supplemented with 0.5 mg/l TDZ and 0.2 mg/l NAA. (**B**) Selection of transformed shoots from hypocotyl explants on medium with 15 mg/l hygromycin. (**C**) Transformed shoot showing blue colouration after staining for GUS. (**D**) An acclimatized primary transformant. (**E**) PCR analysis for the presence of the selectable marker gene *hpt*. Lanes: M, marker; 1, plasmid DNA; 2, no DNA control; 3, untransformed plant; 4, transformed plant. (**F**) PCR analysis for the presence of the reporter gene *uidA*. Lanes: M, marker; 1, plasmid DNA; 2, no DNA control; 3, untransformed plant; 4, transformed plant.

(Tietjen *et al*. 1983). Studies employing safflower suspension cultures revealed the pivotal role of BFA for the infection of safflower by *A*. *carthami* through suppression of phytoalexin accumulation (Tietjin and Matern 1984). Owing to the difficulties in development of BFA resistant cell lines, recombinant DNA technologies were used to confer resistance to *A*. *carthami* of safflower. The strategy adopted was to clone the esterase gene from *Bacillus subtilis* that detoxifies BFA rapidly by a one-step hydrolysis of the macrolide ester bond (Kneusel *et al*. 1990). The esterase gene was cloned, sequenced and characterized for use in *Agrobacterium*-mediated transformation of safflower (Matern and Kneusel 1993). However, the experiments were discontinued as the efforts at transformation of safflower with the isolated brefeldin A esterase gene were not successful.

In the author's laboratory, highly efficient protocols for genetic transformation of safflower based on callus mediated regeneration of seedling explants on media supplemented with TDZ+NAA have been developed (**Fig. 1**). Genetic engineering of male sterility by deploying the mitochondrial gene(s) from sunflower (*orf H522*-male sterility causing factor) and also the unedited versions of the mitochondrial genes (*atp 9* and *nad 3*) from safflower that are under the control of tapetum specific (*TA 29*) promoter is under progress. The proof of concept of induction of male sterility has been confirmed in tobacco transformants and several independent transformants harbouring the three candidate genes are being evaluated in safflower.

Safflower is amenable to manipulations *in vitro* in terms of obtaining high frequency of regeneration as well as transformation. The advancements in genetic transformation techniques coupled with functional genomics provide ample opportunities for development of cultivars resistant to both biotic and abiotic stresses under an agronomically acceptable background. In safflower, incorporation of resistance to diseases assumes utmost priority and most of the diseases are single gene controlled providing scope for vertical resistance. There is also an immediate need for using the protocols of genetic transformation for development of transgenics for resistance to aphids and herbicides and in development of lines with altered tocopherol content.

MOLECULAR FARMING

Plants as pharmaceutical and chemical industries (bioreactors) use field crops as platforms for production of therapeutic proteins, drugs and vaccines. Biopharming or molecular farming stimulated by advances in genetic engineering and biotechnology offers tremendous economic and health benefits. Safflower has attracted interest due to advantages it offers with respect to the technology and also the containment aspects. With regard to technology, safflower is easily transformed with *Agrobacterium*, recombinant protein levels in seeds are high and is amenable to large-scale production and purification. As concerns with regulatory issues, it is non-allergenic, has low tendency to weediness, highly self-pollinated (>90%), low acreage grown in N. America and can be geographically segregated from other safflower production and from the main food and feed crops and has the status of generally regarded as safe (GRAS).

SemBioSys Genetics Incorporation (www.sembiosys.ca), a Canadian plant biotechnology company, has formed strategic partnerships with several firms to utilize safflower as a production vehicle for various pharmaceutical, cosmaceutical and neutraceutical applications. The products of interest are human insulin, apolipoprotein A1, growth hormones, healthy fatty acids, antibody production and capture and safflower oilbodies for use as cosmetic ingredient. SemBioSys has developed proprietary genetic engineering technologies for recombinant protein production, oilbody-based products and metabolic engineering for production of high value fatty acids such as n-6 γ-linolenic acid (GLA), *n*-3 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).

Oilbodies are protein-coated lipospheres that naturally form in plant seeds to function in oil (triglycerols) storage. The utility of an oleosin fusion protein to facilitate the production of a protein of interest in an oilseed has been described by Parmenter *et al.* (1995) and the structure and isolation of the oleosin protein in safflower have been demonstrated by Lacey *et al*. (1998). SemBioSys has comercialized the oilbody-oleosin technology and has built it on two basic proprietary capabilities: i) capability to express recombinant proteins in seed oilbodies (Stratosome^{TM} Biologics System), and ii) the ability to extract oilbodies inexpensively from seeds (Affinity Capture System). Transgenic technology of oilbodies and oilbody-associated proteins (Oleosins) is positioned to enable the development of recombinant proteins and face cost and capacity challenges to commercialization. These technologies are being extended for production of human biotherapeutics, animal health products, purification reagents and industrial applications and also for a broad range of therapeutic actives.

GLA is an ω-6-fatty acid with health benefits that are similar and complementary to the benefits of fish oil derived ω-3 fatty acids, DHA and EPA. GLA is a natural anti-inflammatory with benefits for cardiac, joint, skin and neurological health (Flider 2005). Use of GLA supplements is currently constrained by high production costs, low productivity and the need to consume 10 or more capsules per day of existing GLA supplements from evening primrose oil and borage oil to receive optimal health benefits. Arcadia Biosciences has successfully demonstrated 65% GLA in the oil from genetically transformed safflower seeds. These high levels of GLA hold promise of providing consumers with a more cost effective and convenient source of the health promoting ω-6-fatty acid. Canola is also considered as a candidate crop for production of high value oils but it produces significant quantities of linolenic acid, which competes with linoleic acid for D-6-desaturase and produces stearidonic acid at the expense of γ-linolenic acid thus, leaving the platform for safflower.

SemBioSys in collaboration with Martek Biosciences Inc has genetically engineered safflower for production of oil to contain the essential long chain polyunsaturated fatty ω-3 fatty acid, DHA for use as a nutritional supplement, food ingredient and as an animal feed ingredient. DHA in combination with arachidonic acid has been demonstrated to be essential for normal brain development and function in infants and adults and also in maintaining cardiovascular health. Safflower has also been transformed with the *Apo AI* (apolipoprotein) gene for use in cardiovascular therapies to reduce plaque associated with acute coronary syndrome and stroke. Likewise, human insulin is produced in transgenic safflower with accumulation levels of 1.2% of total seed protein. Safflower produced insulin is supposed to reduce capital costs by 70% and product costs by 40% as compared to synthetic insulin traditionally manufactured by pharmaceutical companies using genetically engineered bacteria and yeast. Genetically engineered safflower expressing a carp growth hormone fused to an *Arabidopsis* oleosin is ready for field release in the USA (www.aphis.usda.gov). The product has been developed for use as a supplement in aquaculture meal for enhancing resistance of shrimps against diseases by stimulating their immune system.

With the gaining importance of safflower for the production of plant made pharmaceuticals, the need for assessment of gene flow from cultivated safflower to wild relatives was realized. McPherson *et al.* (2004) evaluated the potential for transgenic gene flow from *C*. *tinctorius* to wild and weedy relatives in western Canada; their studies indicated the possibility of gene flow from cultivated sunflower to *C*. *oxyacanthus* and *C*. *creticus*. Keeping in view the distribution of these two species in the New World, it has been concluded that locations where wild species of *Carthamus* have not been naturalized should provide biologically isolated locations for cultivation of transgenic safflower.

BIOENGINEERING LIPID PROFILE AND OIL COMPONENTS

Stobart *et al*. (1986) demonstrated deposition of oil *in vitro* in safflower microsomes. Safflower provides an excellent model system for studies on triacyl glycerol biosynthesis from *Sn*-glycerol 3-phosphate and linoleoyl-CoA and is particularly important in experiments designed to elucidate the control and enzymology of seed-oil formation for plant breeding and genetic engineering programmes concerned with modification of oil quality.

Tocopherols (α, β, γ and δ) are oil-soluble compounds present in oilseeds that exert an antioxidant action both *in vivo* and *in vitro*. α-Tocopherols have high vitamin E activity but show weak antioxidant potency *in vitro* while γ-tocopherol is a powerful antioxidant *in vitro*. The β and δ derivatives of tocopherol exhibit intermediate properties. Safflower is rich in α-tocopherol (95%) and there is a need for genetic modification of tocopherol composition for improvement of *in vitro* oxidative stability of the seed oil. Towards this goal, wild safflowers were screened for the total tocopherol and the constituent tocopherols and a mutant with high γ-tocopherol (85%) has been identified in *C. oxyacantha* (Velasco *et al*. 2005). Significant progress has been made in sunflower for development of lines with modified tocopherol through conventional and molecular marker approaches using mutants exhibiting variation in tocopherol content. The genes determining high β-tocopherol content (*Tph1*) and high γ-tocopherol content (*Tph2*) have been mapped in sunflower to linkage groups 1 and 8, respectively. Utilization of the mutant lines being identified in *C. oxyacantha* or characterization of tocopherol biosynthesis pathways will provide information for such modifications in safflower.

CONCLUSIONS

Zhang (1997) quotes safflower as the most versatile and "hot point" plants in the world underlining the importance of the plant as medical herb. Progress in genetic engineering over the past decade has firmly established ground for safflower as an ideal crop for production of plant made pharmaceuticals and high-value end products. Molecular markers were successfully employed for characterization of germplasm and proved useful for resolving generic delimits and sectional classification of the genus *Carthamus*. However, all these account for promotion of safflower as a medicinal and industrial crop. Greater emphasis should be laid on extending the advances in biotechnological research for genetic enhancement of safflower for increasing the crops' productivity in various production systems and improving oil quality characteristics for both edible and industrial uses. Safflower exhibits great propensity for manipulations *in vitro* and provides great opportunities to use haploid technique (double-haploids), somatic hybridization and genetic transformation for improvement of safflower cultivars. Molecular markers utilization in safflower has been initiated and more efforts are needed for identification of useful DNA markers for important traits and development of safflower specific markers such as, simple sequence repeats (SSRs) and target region amplified polymorphic (TRAP) markers to facilitate gene introgression from wild safflowers and in marker assisted breeding programmes. Molecular cytogenetic tools have to be developed to establish genomic relationships among the *Carthamus* species and aid in introgressive breeding. Research on safflower is scattered and confined to few laboratories across the world and it would be difficult to accomplish all these objectives by an individual organization. International networks exist for all the major oilseed crops and such Consortium need to be established for safflower in order to facilitate the conservation of genetic resources, development of molecular markers and biotechnological tools, sharing of genetic and gene resources for accelerating the breeding programmes and making safflower a commercially viable crop.

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