Chapter 7
Neglected Oil Crop Biotechnology

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Abstract Global food security has become increasingly dependent on only a handful of crops cultivated intensively leading to crop replacement and a massive reduction in the number of species and diversity of crops. This poses a threat to local and global food security because the replaced indigenous crops are often essential for low input agriculture, have unique nutritional value, and contain diversity of locally adapted genotypes with resistance to a wide array of biotic and abiotic stresses. Most of these plant species are important locally or regionally only, and are known as 'minor', 'neglected', 'underexploited' or 'underutilized' crops. Like many other crops, production of oilseeds has not improved significantly due to their susceptibility to pests, sensitivity to abiotic stresses and low nutrient use efficiency. An approach for meeting the increasing demand for vegetable oils will be to introduce new or underutilized oilseed crops that are more suited for cultivation on less fertile land that do not support production of major oilseed crops. A need also exists for dedicated non-food oilseed crops that can be used for metabolic engineering of novel oil compositions for industrial applications. A number of oilseeds have recently received attention for their potential to fill one or more of these niches. These include Ironweed (*Vernonia galamensis*), crambe (*Crambe abyssinica*), desert mustard (*Lesquerella fendleri*), niger (*Guizotia abyssinica*), camelina (*Camelina sativa*), the Ethiopian mustard (*Brassica carinata*) and Sesame (*Sesamum indicum*). In this chapter emphasis has been given to current biotechnology research and progress for the improvement of these neglected oil crops. Agricultural biotechnology is creating new tools to tackle the problems of crop improvement, rural poverty, employment and income generation by helping to enhance farm productivity and production, improve quality, and explore marketing opportunities in newer ways. Technology like tissue culture provides the means for the culture of protoplasts, ovules and embryos used to create new genetic variation by overcoming reproductive barriers between distantly related crop species and haploid production by the culture of anthers and microspores to shorten the selection cycle in a breeding programme. Characterization of genetic

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diversity by molecular markers is important for devising effective sampling and conservation strategies. Molecular markers can also be used to certify varieties, to determine the presence or absence of diseases and development of linkage maps for identifying quantitative trait loci and marker assisted selection. Transferred genes through genetic engineering may contribute to a range of properties, including resistance/tolerance to biotic and abiotic factors, improved nutritional status and better management options.

Abbreviations

NAA	α- napthaleneacetic acid
BA	Benzyl adenine
MS	Murashige and Skoog
TDZ	Thidiazuron
2-ip	N^6-[2-isopentenyl] adenine
KN	6-furfurylaminopurine
IBA	Indole-3-butyric acid
2, 4-D	2, 4-dichlorophenoxyacetic acid
AFLP	Amplified fragment length polymorphism
MAS	Marker assisted selection
EST	Expressed sequence tags
DGAT	Diacylglycerol acyltransferase
GISH	Genomic in situ hybridization

7.1 Introduction

An unintended consequence of the intensive agriculture has been a massive reduction in the number of species and diversity of crops. This process of crop replacement is a threat to local and global food security because the replaced indigenous crops are often essential for low input agriculture, have unique nutritional and cultural value, and contain diversity of locally adapted genotypes with resistance to a wide array of biotic and abiotic stresses. Global climate change and degradation of once productive lands have further heightened the demand for crops that perform well in harsh and/or changing environments (http://www.botany.ubc.ca/noug/).

Global food security has become increasingly dependent on only a handful of crops. Over 50 % of the global requirement for proteins and calories are met by just three—maize, wheat and rice. Only 150 crops are traded on a significant global scale. Yet, surveys indicate there are over 7,000 plant species across the world that are cultivated or harvested from the wild for food (www.underutilized-species.org). Most of these plant species are important locally or regionally only,

and are known as ‘minor’, ‘neglected’, ‘underexploited’ ‘orphan’ species or ‘underutilized’ crops. These terms are often used interchangeably to characterize the range of plant species that are the focus of this chapter. Neglected crops are those grown primarily in their centers of origin or centers of diversity by traditional farmers, where they are still important for the subsistence of local communities. Some species may be globally distributed, but tend to occupy special niches in the local ecology and in production and consumption systems. While these crops continue to be maintained by cultural preferences and traditional practices, they remain inadequately characterized and neglected by research and conservation. In contrast, underutilized crops were once more widely grown but are falling into disuse for a number of reasons. Farmers and consumers are using these crops less because they are in some way not competitive with other crop species in the same agricultural system. The decline of these crops may erode the genetic base and prevent the use of distinctive useful traits in crop adaptation and improvement. Biotechnological approaches may be employed in these neglected crops to overcome slow pace of their improvement (Tiwari et al. 2011).

Agricultural biotechnology is creating new tools to tackle the problem of rural poverty, employment and income generation by helping to enhance farm productivity and production, improve quality, and explore marketing opportunities in newer ways. This offers a considerable promise as a means of improving food security and reducing pressures on the environment. Biotechnology is a rapidly developing field that, in an attempt to meet the current and emerging challenges facing agriculture—such as poor nutrition, unstable and limited food production, and restricted fuel availability—has received considerable attention for the improvement of major crops. In fact, agricultural biotechnology can be considered to cover at least four areas of work: (i) tissue culture and micropropagation, (ii) molecular marker characterization of genetic diversity, (iii) marker assisted selection (MAS), genomics and the related disciplines of proteomics and metabolomics; and (iv) the production of transgenic crops (FAO 2004).

7.1.1 Tissue Culture and Micropropagation

Tissue culture provides the means through in vitro techniques for the culture of protoplasts, anthers, microspores, ovules and embryos used to create new genetic variation in breeding lines, often via haploid production to overcome reproductive barriers between distantly related crop relatives. Micropropagation is in vitro vegetative multiplication through somatic embryogenesis or organogenesis being used to clone large numbers of plants from genotypes of particularly desirable characteristics, allowing these types to be distributed and used more widely. The culture of single cells and meristems is being effectively used to avoid diseases from germplasm, for in vitro transfer of breeding material and conservation of germplasm through cryopreservation. Cell and tissue culture has also produced somaclonal and gametoclonal variants with crop improvement potential.

7.1.2 Molecular Marker Characterization of Genetic Diversity

Characterization of genetic diversity by molecular markers is important for devising effective sampling strategies: e.g., in order to determine diverse material for pre-breeding programmes. It may also be important for developing conservation strategies: e.g., in rationalizing ex situ germplasm collections. Molecular markers can also be used to certify varieties, determine the presence or absence of diseases and assess the reproductive biology of species, among other applications. There is a wide range of markers with different characteristics available. Common markers include: isozymes, random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats, or microsatellites (SSRs); and, more recently, SSRs from expressed sequence tagged sites, derived from transcribed DNA (EST-SSRs).

7.1.3 Marker-Assisted Selection and Genomics

Since 1990s, significant attention has been given to the development of linkage maps for identifying quantitative trait loci (QTL), which represent statistical associations between markers and genes that control a proportion of the variation of a trait. By establishing an association, markers can be used to understand complex traits and assist in selection; when combined with more traditional breeding methods, Marker-Assisted Selection (MAS) has great potential to accelerate genetic improvement. Genomics, a 'second-generation' biotechnology, the ultimate goal of which is to identify all genes and their functions in an organism, has burgeoned over the last decade. By revealing gene sequence similarities and common arrangements of genes (synteny), genomics raises the prospect of information gathered on one species benefiting work on other less researched taxa. Genomics involves a wide range of activities, including: the production of expressed sequence tags (ESTs), genome sequencing, gene function determination, comparative analysis (exploring synteny, cross-identification of candidate genes, etc.), physical mapping and the discipline of bioinformatics. The information gathered is then incorporated into selection and breeding programmes. Genomics, proteomics and metabolomics are disciplines that, among others, can be combined together into a biotechnology meta-analysis, the basis of 'systems biology'.

7.1.4 Genetic Engineering and the Production of Transgenic Crops

Genetic Engineering (GE) is the use of recombinant DNA and asexual gene transfer methods to alter the structure or expression of specific genes and traits in an organism. Active research in this area has been ongoing since 1980s. The product of GE, a transgenic, is one that has been transformed by the insertion of one or

more transgenes from another, often unrelated organism. Transferred genes may theoretically contribute to a range of properties, including: resistance/tolerance to biotic and abiotic factors, improved nutritional status and better management options. In future, GE holds the potential for the production of nutraceuticals, oral vaccines and new biofuels in plants. To date, however, commercial deployment of GE has been limited to a narrow range of traits only, herbicide tolerance and insect resistance being the most important (James 2009).

Despite considerable discussion regarding the potential uses of biotechnology for meeting global agricultural challenges, practical deployment for underutilized plants is currently limited. There are relatively simple applications such as tissue culture that do already realize some significant benefits in few crops. However, more advanced techniques may result in significant value in the future (Dowson et al. 2009). It is clear that biotechnology is not a panacea for promoting underutilized species, and the cost and effort involved in realizing successful interventions will often be much greater than many researchers have first considered, with time scales of decades rather than years being the norm (CGIAR 2006).

Vegetable oils have historically been a valued commodity for food use and to a lesser extent for non-edible applications such as detergents and lubricants. Many plants are cultivated for their oil content which is used for cooking and industrial purposes. The production of oilseeds has not improved significantly due to their susceptibility to pests, pathogens and sensitivity to abiotic stresses and low nutrient use efficiency. For qualitative and quantitative improvement, the breeder has to rely upon the extent of genetic variability present in the base population. The lack of genetic variability has been considered a major limiting factor to the progress made in the improvement of these crops. The desired goals can be achieved by incorporating additional genetic variability in the existing germplasm. However, in addition to the conventional methods of plant breeding, like introduction, selection and hybridization, recent advances in biotechnology have opened several new possibilities for the creation of genetic variability and selection of desired traits.

Vegetable oils consist principally of energy-dense triacylglycerols that are composed of three fatty acids bound to a glycerol back bone. These molecules are an important source of calories in human and animal diets and are also used in the preparation of margarines, salad oils and fried foods. The energy density of triacylglycerols has also made these molecules an attractive source of biodiesel that is produced by trans-esterification of their component fatty acids. Furthermore, the immense diversity of fatty acid structures that can be found in triacylglycerols in the plant kingdom opens up opportunities for the use of vegetable oils in a variety of bio-based industrial formulations, including lubricants and drying oils. Biotechnological improvement of fatty acid composition will certainly lead to additional demand for vegetable oils in food, feed, and bio-based industrial materials. One of the most notable recent breakthroughs in oilseed biotechnology is the complex metabolic engineering of oilseeds to produce fish oil-type omega-3 poly unsaturated fatty acids. Progress has also been made in the identification of genes for the synthesis and metabolism of novel fatty acid structures, including fatty acids with hydroxy and epoxy residues that are well-suited for industrial

applications such as lubricants, plasticizers, and nylon precursors. These genes have typically been isolated from species with limited agronomic potential and transferred to established oilseed crops to generate vegetable oils with new functionality (Lu et al. 2011).

An approach for meeting the increasing demand for vegetable oils is to introduce new or underutilized oilseed crops that are more suited for cultivation on less fertile land or in arid and semi-arid climates that do not support production of major oilseed crops, such as soybean and canola. A need also exists for dedicated nonfood oilseed crops that can be used for metabolic engineering of novel oil compositions for industrial applications. The use of such crops would preclude the real or perceived risks of mixing industrial and food traits that could arise, for example if soybean was used for production of industrial oils with novel fatty acid compositions (e.g. hydroxy fatty acids). A number of oilseeds have recently received attention for their potential to fill one or more of these niches. These include Ironweed (*Vernonia galamensis*), crambe (*Crambe abyssinica*), desert mustard (*Lesquerella fendleri*), niger (*Guizotia abyssinica*), camelina (*Camelina sativa*), the Ethiopian mustard (*Brassica carinata*) and Sesame (*Sesamum indicum*).

In this chapter, a detailed account of biotechnological approaches is given in context with major neglected oil crop species listed as neglected or underutilized (NUS) species by three organizations that are active in NUS crop promotion in the tropics and subtropics: the Global Facilitation Unit for Underutilized Species (http://www.underutilized-species.org/), the International Centre for Underutilized Crops (http://www.icuc-iwmi.org/) and Bioversity International (http://www.bioversityinternational.org/). These institutions help to set priorities based on the potential impacts of promotion in addressing agricultural challenges, while also considering issues such as cultural importance, the cost of intervention, the feasibility and sustainability of activities, and ethical concerns.

7.2 *Vernonia galamensis* (Cass.) Less.

Vernonia galamensis is an annual plant in the Asteraceae family, known for its use as an oilseed. This species, often called 'Ironweed', is a new potential industrial oilseed crop, which originates from Eastern Africa. It is the largest source of vernonia oil, which is rich in a useful epoxy fatty acid called vernolic acid and is used to make plastics, rubbery coatings, and drying agents. Use of this oil as a replacement for traditional plasticizers and binders in the production of paints and PVC shows promise as a method of reducing smog pollution (www.underutilized-species.org). It produces high quantities of epoxy fatty acids (at least 60 %) in a trivernolin form, useful in the reformulation of oil-based paints to reduce emission of volatile organic compounds (Perdue et al. 1986). About 38 % of the Vernonia seed is oil of which about 72 % is vernolic acid. Vernonia has "reactive diluents" oil properties to serve as solvents that become part of the dry paint surface and do not evaporate

to pollute air. Other potential markets for the fatty acids include plasticizers, additives in polyvinyl chloride (PVC), coatings, cosmetic, and pharmaceutical applications (Carlson et al. 1981). Preliminary investigations also showed that the meal after seed oil extraction is a valuable source of crude protein (43.75 %); it also consists of crude fiber (10.90 %), ash (9.50 %) and the carbohydrate fraction (6.57 %) with sucrose (2.36 %), fructose (1.90 %) and glucose (0.77 %). The major mineral elements, calcium (11.08 mg/g), potassium (14.18 mg/g), magnesium (6.90 %) and high phosphorus (644 mg/g) not only meet the nutritional requirements but also are higher than in most other oilseeds (Ologunde et al. 1990). The lipase activity found in the ungerminated seed and the characteristics that the lipase shows make *Vernonia galamensis* an attractive oilseed crop not only as an industrial oil source but also as a source of low-cost lipase (Ncube and Read 1995).

In 1950, the Agricultural Research Service (ARS) of US Department of Agriculture (USDA) made an extensive search to identify plants not competing with existing crops as new sources of industrial raw materials (Perdue 1988). Among many species examined *V. galamensis*, native to East Africa, emerged as one of the most important due to its high triacylglycerols (TAGs) contents with vernolic acid (cis-12-epoxyoctadeca-cis-9-enoic acid) (Baye et al. 2005). Ethiopian Vernonia has the highest oil content, up to 41.9 % with up to 80 % vernolic acid. Apart from Ethiopia it is also grown in many other African regions as an industrial oilseed. Its seed production is poor when it is grown outside of the equatorial region hence, cultivation in other places is not always economically worthwhile. Further studies in the breeding of this species to generate more productive varieties may show promise. Biotechnological studies conducted to this crop is scanty, despite its importance, it has remained a neglected oil crop so far.

7.2.1 Plant Tissue Culture

Belay et al. (1989) in their preliminary work reported Vernonia immature seed callus production to ascertain its oil producing potential. They initiated callus from immature seeds of Vernonia on semisolid MS medium supplemented with 0.05 mg/l 2,4-D. For oil extraction and subsequent chemical analysis, they selected approximately 22 weeks old callus tissues from the fourth subculture on the initiation medium. However, they could not find vernolic acid formation at this stage in the callus.

7.2.2 Molecular Marker Analysis

RAPD analysis among ten selected Ethiopian *V. galamensis* lines showed moderate genetic diversity as shown by the resulting four different groupings using 13 RAPD primers. OPA10 was most informative one. This suggested that for a given number of Vernonia lines tested for polymorphism about 40 % would show polymorphism or genetic variations (Ramalema et al. 2010).

7.2.3 Genomics

Limited commercial supply of epoxy fatty acids, such as vernolic acid has commenced considerable interest for genetic engineering of oil crops to produce high levels of this acid (Kinney 2002). However, there is only limited information as to how plants such as *V. galamensis* accumulate high levels of vernolic acid. Thus, a better understanding of the mechanism for the effective channeling or selective accumulation of vernolic acid into triacylglycerols felt necessary.

Molecular techniques have been used to clone the genes encoding epoxygenase enzyme responsible for vernolic acid synthesis in *V. galamensis* using RT-PCR, heterologous probes and Northern blotting. Seither et al. (1997) isolated two cDNA clones from the cDNA library constructed at a stage in seed development postulated to have abundant transcripts for the epoxygenase. These clones shared highest homology to plant cytochrome P-450s and they reported that epoxygenase in the *V. galamensis* was a cytochrome P-450. In their experiment, total RNA was isolated from seeds, reverse transcribed and a cDNA library was constructed. A PCR was performed with cDNAs by using a highly degenerate 5′ primer designed for the heme binding motif shared among cytochrome P-450s and 3′ oligo dT primer for the poly-A tail. The products were TA cloned and sequenced and a putative candidate was used for screening from cDNA library. A full length clone of 1.6 kb was obtained with highest homology to several plant cytochrome P-450s. The cDNA was excised and used to probe a Northern blot of total RNA from leaf and three stages of seed development. The expression was most abundant in the mid-mature seed. The same cDNA library was also screened using a probe for allene oxide synthase (AOS) from flax. This heterologous probe facilitated the isolation of a cDNA which showed highest homology to several plant cytochrome P-450s. Subsequently, the *V. galamensis* epoxygenase gene was cloned by Hitz (1998). However, transgenic *Arabidopsis* and soybean with epoxygenases gene expressed only low levels of vernolic acid in mature seeds (Hatanaka et al. 2004; Kinney 2002).

Seed microsome assays of *V. galamensis* demonstrated that diacylglycerol acyltransferase (DGAT), an enzyme for the final step of triacylglycerol synthesis, has a strong substrate preference for vernolic acid bearing substrates including acyl-CoA and diacylglycerol (Hatanaka et al 2003). There are two classes of DGATs known as DGAT1 and DGAT2. Yu et al. (2008) reported the isolation, characterization, and functional analysis of two DGAT1 cDNAs from *V. galamensis* (VgDGAT1a and VgDGAT1b). VgDGAT1a and VgDGAT1b were expressed in all plant tissues examined with highest expression in developing seeds. Enzymatic assay using isolated microsomes from transformed yeast showed that VgDGAT1a and VgDGAT1b have the same DGAT activity levels and substrate specificities. They concluded that the two VgDGAT1s are functional, but not likely to be responsible for the selective accumulation of vernolic acid in *V. galamensis* seed oil.

Triacylglycerol (TAG) is the main storage lipid in plants. DGAT1, DGAT2 (Acyl-CoA: diacylglycerol acyltransferase) and PDAT (phospholipid:

diacylglycerol acyltransferase), encoded by three separate gene families, are all capable of catalyzing the final acylation step during TAG synthesis. Li et al. (2010a) investigated the expression patterns of DGAT1, DGAT2 and PDAT in relation to the accumulation of oil and epoxy and hydroxy fatty acid in developing seeds of *V. galamensis*, Euphorbia lagascae, Stokesia and castor that accumulate high levels of these fatty acids in comparison with soybean and *Arabidopsis*. The expression patterns of DGAT1, DGAT2 and the PDAT were consistent with all three enzymes playing a role in the high epoxy or hydroxy fatty acid accumulation in developing seeds of these plants. PDAT and DGAT2 transcript levels were present at much higher levels in developing seeds of Vernonia than in soybeans or *Arabidopsis*. DGAT1 appeared to be a major enzyme for seed oil accumulation at least in Arabidopsis and soybeans. For the epoxy and hydroxy fatty acid accumulating plants, DGAT2 and PDAT also showed expression patterns consistent with a role in the selective accumulation of these unusual fatty acids in seed oil.

7.3 *Crambe abyssinica* Hochst. ex R.E. Fries

Crambe abyssinica, commonly known as "Abyssinian mustard or Abyssinian Kale" is an oilseed crop and belongs to the Brassicaceae family. It is a native to the Mediterranean region and has a short life cycle; 40–50 days for flowering and 75–90 days for seed maturity. One of the most important advantages of this crop is that it does not outcross with any food oil seed crops (Wang and Peng 1998), thus eliminating the problem of gene flow. Besides, the seed oil of Crambe contains 55–60 % erucic acid that makes the oil non-edible, but can be used as starting material for producing oils used in chemical industry. Crambe has thus been considered as a very promising industrial oilseed crop in recent years and it has already been commercially cultivated on small scale with an acceptable yield potential. *C. abyssinica* is a fast growing high biomass crop with significant potential for biofuel production and for phytoremediation of heavy metal contaminated soils and sediments.

There is growing interest in Europe and the United States for the use of crambe as a renewable industrial feedstock, biofuels, lubricants and bioplastics. Crambe seed meal contains 45–58 % protein with a well-balanced amino acid content, with especially high in lysine and methionine levels and thus could be used as high value feedstock protein. However, crambe seed meal has high levels of sulfur-containing glucosinolates known to cause toxicity from ingestion by swine and poultry. Therefore, reducing the levels of expression of the key genes in the glucosinolate biosynthesis pathway could decrease the glucosinolate contents and make crambe seed meal palatable to ruminant animals. Developing and producing quality feed will not only be advantageous to the health of livestock, which will improve the meat quality and thus will also have indirect positive effects on human nutrition.

7.3.1 Tissue Culture

The lack of regeneration protocols for Crambe has hampered the development of transgenic Crambe plants. Gao et al. (1998) reported a regeneration frequency of 45 % from single cell culture, but without further transformation attempts. Li et al. (2010b) achieved a similar regeneration frequency of 43.7 % using hypocotyls of *C. abyssinica* cv. Galactica as explants cultured on a Murashige and Skoog medium supplemented with various plant growth regulators (PGRs). Among the different PGR combinations tested, 10 μM thidiazuron (TDZ) and 2.7 μM α-naphthaleneacetic acid (NAA) promoted the highest frequency of regeneration. However, this regeneration frequency is still very low for developing an efficient transformation system since the regeneration frequency is often dramatically reduced after *Agrobacterium* infection. Li et al. (2011) reported an efficient regeneration protocol for Crambe in which the regeneration frequency reached over 95 % using hypocotyls as explants. They investigated the effects of N-source, C-source, $AgNO_3$, cultural conditions as well as the concentration and combination of plant growth regulators (PGR) on the regeneration frequency of *C. abyssinica*. The results showed that all these factors, especially the N-source and PGR concentrations and combinations, played an important role in shoot regeneration. Among all the factors tested, the combination of using hypocotyls from *C. abyssinica* cv. galactica, the Lepiovre basal medium supplemented with 16 g l^{-1} glucose, 0.5 g l^{-1} $AgNO_3$, 2.2 mg l^{-1}TDZ, 0.5 mg l^{-1} NAA, 2.5 g l^{-1} Gelrite, seeds germinated in dark for 3 days and explants cultured in light, gave the best regeneration frequency (over 95 %). The results also suggest that reducing the content of NH_4^+ or keeping a suitable NO_3^-/NH_4^+ ratio in the regeneration medium would be crucial to Crambe shoot regeneration. Chhikara et al. (2011) described the development of an efficient method of plant regeneration through indirect shoot organogenesis from hypocotyl explants and transformation.

7.3.1.1 Protoplast Fusion

PEG-induced asymmetric somatic hybridization between *B. napus* and *C. abyssinica* was accomplished with the fusion of UV-irradiated mesophyll protoplasts of *C. abyssinica* cv 'Carmen' and cv 'Galactica' fused with hypocotyl protoplasts of different genotypes of *B. napus* cv 'Maplus' and breeding line '11502'. Shoot regeneration frequency varied between 6.1 and 20.8 % among the different doses of UV-irradiation, ranging from 0.05 to 0.30 J/cm^2. In total, 124 shoots were regenerated, of which 20 asymmetric somatic hybrids were obtained and verified by nuclear DNA content and AFLP analysis. AFLP data showed that some of the characteristic bands from *C. abyssinica* were present in the hybrids (Wang et al. 2003). The chromosomes of *B. napus* and *C. abyssinica* origin could be clearly discriminated by genomic in situ hybridization (GISH) in mitotic and meiotic cells. Analysis of cleaved amplified polymorphic sequence (CAPS) markers

derived from the fae1 gene showed novel patterns different from the *B. napus* recipient in some hybrid offspring (Wang et al. 2004). The investigation into the fertility of asymmetric somatic hybrids indicated that the fertility increased with increasing UV-doses. All of the hybrids were cultured to full maturity, and could be fertilized and set seeds after self-pollination or backcrosses with *B. napus*. The analysis of the fatty acid composition in the seeds showed significantly greater amounts of erucic acid than *B. napus*. These studies indicated the use of UV-irradiation for the induction of asymmetric somatic hybrids to promote the fertility of the hybrids and their sexual progeny via chromosomal elimination. Protoplast fusion technique also facilitates the introgression of exotic genetic material into crop species.

7.3.1.2 Development of Intergeneric Hybrids Through Tissue Culture

An intergeneric hybrid between *B. juncea* × *C. abyssinica* was obtained for the first time through the conventional crossing method combined with ovary culture, when *C. abyssinica* was used as a paternal parent. The hybridity was confirmed by morphology, cytology and isozyme analysis (Wang and Peng 1998). The intergeneric hybrid from a cross between *B. chinensis* and *C. abyssinica* was observed with 2n = 55 chromosomes in the original progenies. After several generations of in vitro propagation by tissue culture, the chromosomes of the intergeneric hybrid were remarkably reduced, varying from 25 to 28, averaged at 26. The reduction of chromosomes in the hybrid and the high numbers of bivalents were possibly due to the chromosome of *C. abyssinica* eliminating and the genome of *B. chinensis* doubling in the hybrid cells (Tang et al. 2006).

7.3.1.3 Microspore Culture

Wang et al. (2006) regenerated twenty-seven microspore-derived plants from the *B. napus–C. abyssinica* monosomic addition lines obtained from the F2 progeny of the asymmetric somatic hybrid. Fourteen seedlings were determined to be diploid plants (2n = 38) arising from spontaneous chromosome doubling, while 13 seedlings were confirmed as haploid plants. Doubled haploid plants produced after a treatment with colchicines. The lines are potentially useful for molecular genetic analysis of novel *C. abyssinica* genes or alleles contributing to traits relevant to oilseed rape breeding.

7.3.1.4 Somatic Embryogenesis

Palmer and Keller (2011) investigated somatic embryogenesis in *C. abyssinica* cv. Prophet using cotyledon, hypocotyl and root explants from 8-day-old seedlings cultured with levels of NAA and 2,4-D ranging from 2.2 to 39.0 μM, combined

with 6-benzyladenine (BA) to achieve an auxin:cytokinin ratio of 20:1. Callus formation frequency for cotyledon and hypocotyl explants was 100 % for levels of 2,4-D from 4.5 to 33.9 μM. The response was similar with NAA levels of 13.0 to 39.0 μM. Root explants were less responsive. When calluses were transferred to a medium containing 0.56 μM each of thidiazuron and BA with 1.0 μM indole-3-butyric acid (IBA), somatic embryos were induced. Embryos were induced from calluses grown on media containing either 11.3 μM 2,4-D or 13.0 μM NAA, or higher. On a medium without plant growth regulators, embryos were induced but at a much lower frequency. For all three explants, a combination of 22.6 μM 2,4-D and 26.0 μM NAA was optimal for embryogenic callus induction. Hypocotyl-derived calluses were superior to cotyledon- and root-derived calluses for embryo induction. The best embryo formation response was with medium containing 5.0–6.0 % sucrose. The highest average number of embryos per callus (36) was obtained from hypocotyl calluses from medium with 22.6 μM 2,4-D. Somatic embryos germinated best on half-strength B5 or MS medium with 3 % sucrose, and plantlets were successfully established under greenhouse conditions. The results indicate that high levels of auxins are required for the induction of embryogenic calluses from explants of *C. abyssinicia*, while cytokinins are critical for somatic embryo formation.

7.3.2 Genetic Transformation

Since, a high efficiency genetic transformation system for crambe has recently been developed, it can effectively be manipulated for lower erucic acid content for palatability, high erucic acid for pharmaceuticals, as well as for the production of functionalized fatty acids, increased biomass and seed oil yield for bioenergy, bioplastics or other industrial purposes.

Li et al. (2010a, b) evaluated six *Agrobacterium* stains, each harbouring the cloning vector containing the neomycin phosphotransferase (nptII) and β-glucuronidase (gus) genes. EHA101 and AGL-1 yielded the highest transformation frequencies of 1.3 and 2.1 %, respectively. They successfully recovered putative transgenic lines and confirmed as transgenic by Southern blot analysis. Subsequently, *Agrobacterium*-mediated transformation of hypocotyls of cv. Galactica with constructs harbouring the wax synthase and fatty acid reductase genes have also successfully recovered confirmed transgenic plants carrying these transgenes.

Recently, Chhikara et al. (2011) developed an efficient *Agrobacterium*-mediated transformation system for *C. abyssinica* cv. BelAnn using β-glucuronidase (gus), bacterial arsenate reductase (ArsC), and γ-glutamylcysteine synthetase (γ-ECS) genes with the highest transformation efficiency. Pre-cultured hypocotyl explants were infected with *A. tumefaciens* strain LBA4404 harboring binary vector pCAMBIA1300 containing gus, ArsC, and γ-ECS genes under the control of CaMV35S, leaf-specific SRS1p and constitutive Act2p promoters, respectively. Following co-cultivation and selection, regenerated shoot buds were sub-cultured on MS medium containing GA3 for shoot elongation. Elongated

shoots were transferred to root induction medium for 1 week. Semi-quantitative RT-PCR analysis confirmed the expression of mRNA transcripts for gus, ArsC and γ-ECS genes in T0 generation transgenic plants. Histochemical assays showed the gus expression in both vegetative and reproductive tissues of stably transformed T1 generation plants. Germinating seeds from T1 transgenic plants grown on MS medium containing hygromycin revealed a 3:1 Mendelian inheritance pattern for each transgene. This method achieved an overall frequency of 50–70 % regeneration and 6.7–8.3 % transformation with three different gene constructs.

7.3.3 Molecular Marker Analysis of Genetic Diversity and Relatedness

Crambe is an 'Old World' genus with a disjunct distribution among the four major centers of species diversity. A phylogenetic analysis of nucleotide sequences of the internal transcribed spacers (ITS) of the nuclear ribosomal repeat conducted with 27 species of Crambe and 18 related genera using weighted and unweighted parsimony supported Crambe as a monophyletic genus with three major lineages (Francisco-Ortega et al. 1999). *C. abyssinica* (n = 45) is most closely related to *C. hispanica* L. (n = 30) and *C. glabrata* DC. (n = 15). The species complex extends throughout the Mediterranean region, Ethiopia and East Africa. *C. abyssinica* is endemic to Ethiopia, *C. glabrata* to Spain, Portugal and Morocco, and *C. hispanica* is distributed in the Mediterranean region and Middle East. Warwick and Gugel (2003) compared genetic relationships among *C. abyssinica*, *C. hispanica* and *C. glabrata* and attempted a taxonomic separation of them using traditional morphological traits, agronomic and seed quality data, chromosome number, and various molecular data sets including RAPD data, chloroplast (cpDNA) restriction site data and ITS sequence data for the internal transcribed spacer region of the nuclear ribosomal DNA. The three species could be distinguished most reliably by a chromosome number. cpDNA restriction site data and ITS sequence data, two relatively conserved DNA data sets, supported the recognition of *C. glabrata* as a distinct species separate from the *C. hispanica*/*C. abyssinica* accessions. Both RAPD data and field evaluation data revealed greater amounts of genetic variation in *C. hispanica* compared with accessions of *C. abyssinica.* They found that *C. glabrata* was genetically distinct for all data sets and warrants separate species status.

7.3.4 Genomics

7.3.4.1 Organeller Genomics

Southern blot hybridization techniques used to examine the chloroplast DNA (cpDNA) sequences present in the mitochondrial DNAs (mtDNAs) of *C. abyssinica* and other related species of crucifer family led to the conclusion that DNA

has been transferred sequentially from the chloroplast to the mitochondrion during crucifer evolution and there cpDNA sequences can persist in the mitochondrial genome over long periods of evolutionary time (Nugent and Palmer 1988).

7.3.4.2 Organ-Specific Expression of Highly Divergent Thionin Variants

Most thionins of higher plants are toxic to various bacteria, fungi, and animal and plant cells. The only known exception is the seed-specific thionin 'crambin' of the crucifer *C. abyssinica*. Crambin has no net charge, is very hydrophobic and exhibits no toxicity. The existence of a large number of novel and highly variable thionin variants in Crambe has been deduced from cDNA sequences that were amplified by the polymerase chain reaction (PCR) from RNA of seeds, leaves and cotyledons. While the deduced amino acid sequences of the thionin domains of most of these thionin precursor molecules are highly divergent, the two other domains are conserved. Most of the predicted thionin variants are positively charged. The presence of positively charged residues in the thionin domains consistently correlates with the presence of a negatively charged residue in the C-terminal amino acid extension of the various thionin precursors. The different thionin variants are encoded by distinct sets of genes and are expressed in an organ-specific manner (Schrader-Fischer and Apel 1994).

7.3.4.3 Functional Characterization of the Fatty Acid Elongase Gene

A genomic fatty acid elongation 1 (FAE1) clone corresponding to a 1521-bp open reading frame, which encodes a protein of 507 amino acids was isolated from *C. abyssinica*. In yeast cells expression of CrFAE led to production of new very long chain monounsaturated fatty acids such as eicosenoic and erucic acids. Seed-specific expression in Arabidopsis thaliana resulted in up to a 12-fold increase in the proportion of erucic acid. On the other hand, in transgenic high-erucic *B. carinata* plants, the proportion of erucic acid was as high as 51.9 % in the best transgenic line, a net increase of 40 % compared to wild type. These results indicate that the CrFAE gene encodes a condensing enzyme involved in the biosynthesis of very long-chain fatty acids utilizing monounsaturated and saturated acyl substrates, with a strong capability for improving the erucic acid content (Mietkiewska et al. 2007).

7.3.4.4 Genomics for Phytoremediation

Arsenic contamination is widespread throughout the world and this toxic metalloid is known to cause cancers of organs such as liver, kidneys, skin, and lungs in human. Similarly, chromium pollution is a serious environmental problem with few cost-effective remediation strategies available. *C. abyssinica* accumulates significantly higher levels of arsenic and chromium as compared to other species of the Brassicaceae family. Thus crambe, a fast growing high biomass

crop, is an ideal candidate for phytoremediation of heavy metals contaminated soils. In order to understand the pathways involved in heavy metals metabolism and detoxification in plants, a PCR-Select Suppression Subtraction Hybridization (SSH) approach was employed to identify the differentially expressed transcripts in crambe plants under arsenate stress (Paulose et al. 2010) and chromium stress (Zulfiqar et al. 2011). A total of 105 differentially expressed subtracted cDNAs were sequenced from plants under the arsenate stress, which were found to represent 38 genes. These genes encode proteins functioning as antioxidants, metal transporters, reductases, enzymes involved in the protein degradation pathway, and several novel uncharacterized proteins. Upon chromium exposure, plants revealed a total of 72 differentially expressed subtracted cDNAs representing 43 genes. The subtracted cDNAs suggest that Cr stress significantly affects pathways related to stress/defense, ion transporters, sulfur assimilation, cell signaling, protein degradation, photosynthesis and cell metabolism. The transcripts corresponding to the subtracted cDNAs showed strong upregulation by arsenate and chromium stress as confirmed by the semi-quantitative RT-PCR. These studies revealed novel insights into the plant defense mechanisms and the regulation of genes and gene networks in response to heavy metal toxicity and further characterization of differentially expressed genes may enable the engineering of non-food high-biomass plants for phytoremediation of heavy metal contaminated soils and sediments.

7.4 *Lesquerella fendleri* L.

Lesquerella fendleri L. (Gray) S. Wats (commonly known as "Fendler's bladderpod" or "yellowtop" and desert mustard) is a member of the Brassicaceae and is an important plant producing seed oil high in hydroxy fatty acids (Carlson et al. 1990; Skarjinskaia et al. 2003). The plant is native to Arizona, New Mexico, Colorado, Utah, Texas, and Mexico. It is cultivated for the seed which yields up to 28 % oil rich in hydroxy fatty acids and 22 % protein used as supplement for livestock. Oils high in hydroxy fatty acids can replace castor oil, which is used extensively in industrial applications including cosmetics, plastics and coatings (Reed et al. 1997; Dykinga 1999). Within this genus, *Lesquerella fendleri* L. is a good candidate for domestication because it has the highest agronomic potential, low seed dormancy and low fruit dehiscence (Thompson and Dierig 1994; Ploschuk et al. 2003).

7.4.1 Plant Tissue Culture

Most tissue culture efforts made with *L. fendleri* were directed towards the establishment of an efficient transformation protocol. Wang et al. (2008) developed a protocol for regeneration and *Agrobacterium*-mediated genetic transformation of

L. fendleri. Initially they induced calli from hypocotyls and cotyledons on MS fortified with 0.5 mg l-1 BA, 1 mg l-1 NAA and 1 mg l-1 2,4-D, followed by co-cultivated for 2–3 days in darkness on MS supplemented with 0.5 mg l-1 BA, 0.2 mg l-1 NAA and 100 μmol l-1 As together with *Agrobacterium tumefaciens* strain EHA 105/pCAMBIA1301 harboring gene construct. Following co-cultivation, calli transfected by *A. tumefaciens* were transferred to MS with 0.5 mg l-1 BA, 0.2 mg l-1 NAA, 500 mg l-1 Cef and 10 mg l-1 hygromycin. After 4 weeks the resistant regenerants were transferred to MS with 0.5 mg l-1BA, 0.2 mg l-1 NAA, 500 mg l-1 Cef and 25 mg l-1 hygromycin for further selections. With this approach, they obtained 22.70 % the average regeneration frequency from transfected calli, and 6–13 regenerated shoots per callus.

7.4.1.1 Ovule Culture

Germplasm evaluations revealed several other *Lesquerella* species having significantly elevated hydroxy fatty acid contents or different types of hydroxy fatty acids other than that found primarily in *L. fendleri.* These wild species may be suitable donors for possible novel gene introgression by interspecific hybridization. Several barriers to interspecific hybridization exist. Saprophytic self-incompatibility prevents fertilization and silique development following pollination. Tomasi et al. (2002) obtained interspecific hybrids of *L. fendleri* with *L. auriculata*, *L. pallida* and *L. lindheimeri* utilizing ovule culture, producing sufficient F1 hybrid plants for further breeding purposes.

7.4.1.2 Protoplast Culture and Fusion

Intertribal *Brassica napus* (+) *Lesquerella fendleri* hybrids were produced by Skarzhinskaya et al. (1996) through polyethylene glycol-induced fusions of *B. napus* hypocotyl and *L. fendleri* mesophyll protoplasts. In the symmetric fusion experiments, protoplasts from the two materials were fused without any pretreatments. While in asymmetric fusion experiments, X-ray irradiation at doses of 180 and 200 Gy were used to limit the transfer of the *L. fendleri* genome to the hybrids and significantly decrease growth and differentiation of non-fused *L. fendleri* protoplasts. In total, 128 regenerated plants were identified as intertribal somatic hybrids on the basis of morphological criteria. Nuclear DNA analysis performed on 80 plants, using species specific sequences, demonstrated that 33 plants from the symmetric fusions and 43 plants from the asymmetric fusions were hybrids. X-ray irradiation of *L. fendleri* protoplasts increased the possibility of obtaining mature somatic hybrid plants with improved fertility. From the symmetric fusions 2 plants could be fertilised and set seeds after cross-pollination with *B. napus*. From the asymmetric fusions, 9 plants could be selfed as well as fertilised when backcrossed with *B. napus*. A total of 6 plants were found to have different chromosome numbers.

Transferring of *L. fendleri* genetically transformed plastids to *Brassica napus* plants has been achieved with the somatic hybridization method in which the protoplasts of *B. napus* chlorophyll-deficient plants were fused with gamma-irradiated protoplasts of *L. fendleri* transplastomic plants (Nitovs'ka et al. 2006). A total of 59 green hybrid colonies were isolated while shoot regeneration was observed in two cell lines and only one yielded morphologically normal plants. PCR and isozyme analyses showed that the plants were transplastomic cybrids containing *B. napus* nuclei and *L. fendleri* transformed chloroplasts. Recently, Asymmetric intergeneric hybrid plants were obtained through the protoplast fusion between *Orychophragmus violaceus* and *L. fendleri* (Ovcharenko et al. 2011). *L. fendleri* carried chloroplasts transformed with the fused aadA16gfp gene construct, conferring streptomycin–spectinomycin resistance and UV-induced green fluorescence. The somatic hybrids were selected using the properties of spectinomycin-induced plastid defects in "albino" *O. violaceus* plants (chloroplast recipient) combined with the γ-irradiation-induced inactivation of nuclei in plastid donor *L. fendleri.* The morphology and esterase isozyme pattern of the hybrid plant as well as the results of the PCR analysis of internal transcribed spacer of nuclear ribosomal DNA proved that the regenerated hybrids carried *O. violaceus* nuclei, while PCR amplification of the atpB-rbcL spacer and aadA16gfp gene fragments confirmed the presence of the transformed *L. fendleri* chloroplasts in these plants. Expression of the fused aadA16gfp gene construct was further confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis and the resistance of the obtained plants to both streptomycin and spectinomycin.

7.4.1.3 Cell Suspension Culture

In the context of plant molecular studies, model systems are useful for circumventing the lengthy time frames associated with plant development and the limited availability of working materials for analysis. Rapid growth and easy maintenance of suspension cell cultures can provide a constant supply of relevant fresh material for analysis (Biesaga-Koscielniak et al. 2008). *L. fendleri* suspension culture could be useful for studying the effects of different culture conditions, including exogenously applied phytohormones or metabolic inhibitors, as well as mutagenic agents, on lipid metabolic pathways.

In a protocol described for suspension culture system in *L. fendleri* by Kharenko et al. (2011), the hypocotyl segments from 12-day-old seedlings were incubated for 7 weeks on a medium for callus initiation (MS basal medium with B5 vitamins, 3.0 % sucrose, 1.0 mg/l 2,4-D and 0.1 mg/l kinetin at a pH of 5.8. For initiating suspension culture, 50 ml MS basal medium with 1.0 mg/l 2,4-D, 0.1 mg/l kinetin, B5 vitamins and 3.0 % sucrose was inoculated with 350 mg of 28-day-old callus and the flasks placed on an orbital shaker at 145 rpm were maintained at 24 °C with a photoperiod of 16 h at a light intensity of 30 $\mu E\ m^{-2}\ s^{-1}$. The suspension was sub-cultured every 14 days by transferring 10 ml of the suspension to 40 ml of fresh medium of the same composition. Characterization of

the lipid content in cell suspension culture showed a different range of fatty acids accumulating in the cells and predominant in the culture medium. Subsequently, the effect of application of abscisic acid (ABA), which modulates lipid accumulation, was assessed. Exogenously applied ABA was taken up by the cells and metabolized via the conjugation pathway, resulting in the accumulation of ABA-glucose ester. The cell line was responsive to exogenous ABA, resulting in increased cellular lipid content and increased accumulation of lipids in the culture medium. This novel *L. fendleri* suspension culture offers a valuable model system for efficient characterization of mechanisms associated with ABA-induced accumulation of lipids.

7.4.2 Molecular Markers

In an AFLP analysis performed by Du et al. (2008) to characterize 27 intertribal sexual F1 hybrids of *Brassica napus* (2n = 38) cultivars and *L. fendleri* (2n = 12) and their progenies obtained through the crossing between as a pollen parent. Analysis revealed that bands absent in *B. napus*, novel for two parents and specific for *L. fendleri* appeared in all F1 plants and their progenies. Some progenies showed the modified fatty acid profiles with higher levels of linoleic, linolenic, eicosanoic and erucic acids than those of *B. napus* parents. The occurrence of these partial hybrids with phenotypes, genomic and fatty acid alterations possibly resulted from the chromosome elimination and doubling accompanied by the introgression of *L. fendleri* DNA segments and genomic reorganization.

7.4.3 Genetic Transformation

Theoretically, the oil and lesquerolic acid content in *L. fendleri* can be increased through genetic engineering. The efforts made for improving the oil content and the quality have been described below.

7.4.3.1 Biolistic Approach for Plastid Transformation

Plastid transformation is an alternative to nuclear gene transformation. It is possible to achieve biological containment of agronomic traits such as resistance to herbicides and insects, to obtain high-value products through modification of the cellular metabolism and for the expression of recombinant proteins. The challenge of plastid transformation is that the plastid genome is present in many copy numbers and each genome should be altered in order to obtain a genetically stable line.

Skarjinskaia et al. (2003) developed a plastid transformation protocol for *L. fendleri,* a species with a high capacity for plant regeneration in tissue culture.

Transformation vector pZS391B carried an aadA16gfp marker gene conferring streptomycin–spectinomycin resistance and green fluorescence under UV light. Biolistic transformation of 51 *Lesquerella* leaf samples, followed by spectinomycin selection, yielded two transplastomic clones. The AAD–GFP fusion protein, the marker gene product, was localized to chloroplasts by confocal laser microscopy. Fertile plants and seed progeny were obtained in line Lf-pZS391B-1. In the 51 samples, a large number (108) of spontaneous mutants were identified. In five of the lines spectinomycin resistance was localized to a conserved stem structure by sequencing 16S rRNA genes. Success in *L. fendleri*, a wild oilseed species, extends plastid transformation beyond *Arabidopsis thaliana* in the Brassicaceae family.

7.4.3.2 Agrobacterium Mediated Transformation

A protocol for *Agrobacterium*-mediated genetic transformation of *L. fendleri* was developed by Wang et al. (2008). Calli were first induced from hypocotyls and cotyledons and then co-cultivated with *A. tumefaciens* strain EHA105/pCAMBIA1301 that harbored genes for uidA (GUS) and hygromycin resistance. They confirmed transgenic plants by polymerase chain reaction analysis, GUS histochemical assay and genomic Southern blot hybridization.

7.4.4 Genomics

An oleate 12-hydroxylase gene LFAH12, from *L. fendleri* was isolated on the basis of nucleotide sequence similarity to an oleate hydroxylase gene from *Ricinus communis* (Broun et al. 1998). Transgenic studies showed its expression restricted to seeds, but not in leaves or roots. However, hydroxylase activity was detectable in crude extracts of vegetative tissues. The discrepancy between the presence of activity and the lack of hydroxy fatty acids suggests selective removal and breakdown of hydroxy fatty acids in vegetative organs. High levels of LFAH12 mRNA accumulation did not lead to correspondingly high levels of protein accumulation, suggesting that accumulation of the hydroxylase may be controlled post-transcriptionally. Expression of the *L. fendleri* gene in transgenic plants of a fad2 mutant of Arabidopsis, which is deficient in cytoplasmic oleate delta 12 desaturase activity, resulted in partial suppression of the mutant phenotype in roots. Thus, unlike the hydroxylase from *R. communis*, the *L. fendleri* enzyme had both hydroxylase and desaturase activities.

L. fendleri seed oil contains up to 60 % hydroxy fatty acids, nearly all of which is the 20-carbon hydroxy fatty acid lesquerolic acid. However, lesquerolic acid is formed by the elongation of the 18-carbon hydroxy fatty acid, ricinoleic acid. To identify a gene encoding the enzyme involved in hydroxy fatty acid elongation, an *L. fendleri* genomic DNA library was screened using the coding region

of the Arabidopsis Fatty Acid Elongation1 gene as a probe (Moon et al. 2001). A gene LfKCS3 with a high sequence similarity to a known very long-chain fatty acid condensing enzymes, was isolated. LfKCS3 transcripts accumulated only in the embryos of *L. fendleri* and first appeared in the early stages of development. Transgenic expression studies in *Arabidopsis* confirmed that LfKCS3 condensing enzyme specifically catalyzes elongation of 18-carbon hydroxy fatty acids. In an another experiment, the LfKCS45 gene encoding a root-specific condensing enzyme with a high sequence similarity to known 3-ketoacyl-CoA synthases of the membrane-bound fatty acid elongase was isolated from *L. fendleri* (Moon et al. 2004). Reverse transcription-PCR experiments showed that the LfKCS45 gene is expressed only in root tips.

Seed oil is stored mostly as triacylglycerol (TAG). Although, the pathways and key genes involved in the hydroxyl fatty acids (HFA) synthesis have been elucidated, how they contribute to the accumulation of HFA in TAG is poorly understood. In order to understand how HFA synthesis is regulated in *L. fendleri*, Chen et al. (2011) examined the changes in fatty acid composition and gene expression during seed development from 7 days after pollination (DAP) to desiccation (49 DAP). They examined the expression patterns of three key genes involved in fatty acid synthesis during seed development. Using real-time polymerase chain reaction, the transcript level of the three lipid genes, LFAH12 (bifunctional oleate 12-hydroxylase: desaturase), LfKCS3 (3-ketoacyl-CoA synthase) and LfFen1 (oleate 12-desaturase) were quantified. While all of these genes displayed a bell-shaped expression pattern with a peak at 35 DAP and a sharp decline at 42–49 DAP, they had different expression levels during early seed development and maximum inductions. The results will advance our understanding of regulatory mechanisms underlying synthesis and accumulation of HFAs, which is useful to developing and implementing the effective genetic approaches for enhancing HFA production in *L. fendleri* and other oilseeds.

7.5 *Guizotia abyssinica* (L.f.) Cass.

Niger or Noug is an oil-seed crop, indigenous to Ethiopia and holds significant promise for improving rural livelihoods in Sub-Saharan Africa. It is of economic significance not only for domestic consumption in the countries where it is grown, but also as an export commodity to North America and Europe, where it is mainly sold as bird-feed under its English name Nigerseed (or “Thistle seed”). The species is used in intercropping systems, grows on poor but also extremely wet soils, and contributes to soil conservation. While not fully domesticated, and suffering from low yields and susceptibility to insect herbivores, it contributes up to 50 % of the Ethiopian oil-seed crop. Niger belongs to the Compositae/Asteraceae family and is closely related to sunflower. It differs from domesticated sunflower mainly due to its high level of branching, numerous flower heads and small seeds. The oil content of niger seed varies from 30 to 50 %. The fatty acid composition is

typical for seed oils of the compositae family with linoleic acid being a dominant component. Niger has been categorized as a 'Neglected and Underutilized Species (NUS)' in an effort to draw attention to this crop and highlight the importance of research on NUS (http://www.botany.ubc.ca/noug/).

Niger is also grown as a minor oil crop in India, Kenya, Uganda, Sudan, Malawi and some other African countries. Besides edible purposes, niger seed oil is utilized for the manufacture of soaps, paints and lubricants. The protein rich meal obtained after oil extraction is used as feed or for manure. Niger plant has an extremely low harvest index. Some problems hampering the realization of the full potential of niger are the low-yielding capacity of its cultivars and a susceptibility to diseases. In addition, self-incompatibility causes serious difficulty for inbred line development and maintenance which has impeded the improvement of the crop by conventional plant breeding techniques (Getinet and Sharma 1996).

7.5.1 Plant Tissue Culture

In vitro technology could serve as an alternative means for genetic upgrading and its application largely depends on the reliable plant regeneration system. Niger has been the subject of numerous cell/tissue culture studies. Regeneration has been reported from cotyledons and hypocotyl (Ganapathi and Nataraja 1993; Nikam and Shitole 1993; Adda et al. 1993, 1994), leaves (Sujata 1997; Jadimath et al. 1998; Kumar et al. 2000) and seedling explants (Nikam and Shitole 1997).

Naik and Murthy (2010) obtained regeneration from suspension cultures via somatic embryogenesis. Establishment of embryogenic suspension cultures has a great potential to aid crop improvement and is also suitable for in vitro selection of variants especially selection of salt tolerant, disease/toxin resistant and cold tolerant lines in crop plants.

Homozygous lines obtained through anther and microspore culture can be used for hybridization and crop improvement. Induction of embryogenesis from cultured anthers (Adda et al. 1993; Murthy et al. 2000; Hema and Murthy 2008) and plant regeneration from unpollinated ovule cultures (Bhat and Murthy 2007, 2008) has been reported in niger. The effects of amino acids (arginine, aspargine, cysteine, glutamine, glycine and proline) and polyamines (putrescine and spermidine) to enhance embryogenesis and plant regeneration from cultured anthers of niger cv. Ootacamund was also reported (Hema and Murthy 2008). Sujata (1997) was successful in effective maintenance of male sterile plants through culture of leaves from a male sterile plant developed through gamma irradiation.

7.5.2 Genetic Transformation

The introduction of specific desirable genes into niger can be achieved by genetic engineering. The prerequisite for a successful gene transfer of desirable traits is the establishment of an efficient transformation protocol. Murthy et al. (2003)

developed an efficient protocol for *Agrobacterium*-mediated genetic transformation of niger using hypocotyl and cotyledon explants from in vitro grown seedlings. They found that cotyledon was a better explant for transformation with a transformation frequency of 15 % while it was only 3 % in case of hypocotyls.

7.5.3 Molecular Techniques

Crop improvement through breeding depends on the magnitude of the genetic diversity and the extent to which this diversity is utilized. Characterization and evaluation are important to enhance the inherent value to conserved germplasm. The accessible collections of diverse cultivated as well as wild germplasm accessions have made great contribution to crop improvement. Molecular markers and their excellent attributes prove to be extremely useful for the assessment of genetic diversity as well as for identification and maintenance of germplasm collections. AFLP and RAPD markers were used to provide the estimates of the comparative genetic variation within and among populations of various *Guizotia* taxa with the goal of conserving and utilizing their genetic diversity (Geleta et al. 2007a). The genetic diversity analysis for Ethiopian niger was reported for the first time by Geleta et al. (2007b). Using RAPD analysis, they revealed the extent of genetic diversity among 70 populations of niger collected from 11 regions of Ethiopia, representing all its growing regions of the country. Ninety-seven percent of the loci studied were revealed to be polymorphic for the whole data set. UPGMA cluster analysis showed that most of the populations were clustered according to their place of origin. However, some populations were distant from the majority and seem to have unique genetic properties. They concluded that the crop has a wide genetic basis that may be used for the improvement of the species through the conventional breeding and/or the marker assisted selection. In a similar approach, Nagella et al. (2008) reported genetic diversity of selected Indian niger germplasm accessions of different origin and pedigree background through the use of RAPD markers. It was the first attempt to estimate genetic variability among the Indian niger cultivars using molecular markers. An ISSR marker analysis with merely five primers for genetic diversity within and among the population of 37 accessions from Ethiopia (Petros et al. 2007) amplified a total of 118 genomic DNA fragments of which 106 were polymorphic (89.83 %). Among ISSR markers UBC 888 was found most informative. The total genetic diversity and the coefficient of genetic differentiation suggested more variability within the populations than among them.

Fluorescence in situ hybridization (FISH) on somatic chromosome preparations of niger using a DNA probe of the 18S-5.8S-26S rRNA genes including the transcribed and non-transcribed spacer sequences revealed a maximum of six major and two minor signal sites of rDNA. The positions of the FISH signals coincided with the sites of the nucleolar organizer regions and their adjacent C-banded heterochromatin when present (Dagne et al. 2000).

Molecular techniques are being widely used in systematic and phylogenetic studies to measure the genetic relatedness based on DNA sequences variation (Soltis et al. 1998). The internal transcribed spacers (ITS) of the nuclear ribosomal RNA genes have been among the most widely used sequences for DNA sequence variation studies. However, in spite of the small number of species in the genus *Guizotia*, there are no DNA sequences available for systematic purposes. Bekele et al. (2007) drew phylogenetic inferences using the information from ITS sequence generated for five species of Guizotia and related this to the previous understanding and unresolved problems of the genus. They found that *G. scabra,* ssp. *Scabra*, *G. scabra* ssp. *schimperi* and *G. villosa* have contributed to the origin of *G. abyssinica,* the cultivated species of the genus. Based on their findings they suggested that the present composition of the species of genus *Guizotia* and the subtribe the genus presently placed in should be redefined.

7.5.4 Genomics

In order to develop genomic tools and resources for population genetic studies, phylogeographic and evolutionary analyses, research on mating systems, studies of gene flow between the crop and its wild relatives, as well as to aid modern breeding efforts in a non-model organism like niger, generation of a library of ESTs is often the first step. EST databases can be used for many different purposes, including genome wide studies of gene expression and selection, the study of gene family evolution or simply for providing sequence data for molecular marker development (Bouck and Vision 2007). The development of Simple Sequence Repeat markers (SSRs) from ESTs has become the method of choice for many researchers, as it is a more time- and cost-efficient alternative to more traditional approaches, such as library construction, enrichment, and screening.

Recently, Dempewolf et al. (2010) developed a library of the expressed sequence tags, the microsatellite markers using EST sequences, and the chloroplast genome sequence aimed to establish genomic tools and resources for niger. The EST library consisted of 25 711 Sanger reads, assembled into 17 538 contigs and singletons, of which 4781 were functionally annotated using the *Arabidopsis* Information Resource (TAIR). From the EST library, they selected 43 microsatellites and then designed and tested primers for their amplification. These microsatellite markers can be utilized to study the level and partitioning of the genetic diversity in niger more closely, and arrive at a better understanding of phylogeographic patterns. There is a wealth of genomic resources available in niger's closest crop relative, sunflower (*Helianthus annuus* L.), owing to its global importance as an oilseed crop and its status as model species for research on speciation. By comparing sequences of chloroplast genome of niger with the plastid genomes of sunflower and lettuce, they were able to assess the level of Compositae chloroplast genome divergence at a finer scale as compared to previous analyses. This further enhanced the understanding of Compositae plastid genome evolution.

As the chloroplast is an important source of markers for phylogenetic and phylogeographic analyses, making the full chloroplast genome sequence of niger available empowers researchers to assess the usefulness of a wide range of chloroplast DNA markers for such studies in niger, and the family Compositae as a whole. Based on chloroplast sequence comparison, they did not find large rearrangements between the niger and the sunflower chloroplast genomes and only 1.8 % sites showed a sequence divergence between the two species. They also identified 34 tRNAs, 4 rRNA sequences and 80 coding sequences including one region (trnH-psbA) with 15 % sequence divergence between niger and sunflower. This divergence may prove to be particularly useful for phylogeographic studies in niger and its wild relatives.

7.6 *Camelina sativa* (L.) Crantz

The crucifer oilseed species *Camelina sativa* (L.) Crantz variously known as camelina, false flax, gold of pleasure, German sesame or Siberian oilseed is of a particular interest amongst all the under-exploited oilseed crops of the family Brassicaceae. It is native to Northern Europe and to Central Asian areas (www.underutilized-species.org). It is one of a number of species subject to the renewed interest as a possible alternative oil crop. It has agronomic low-input features with 35–40 % oil content in seed and an unusual fatty acid composition with high levels of alpha-linolenic acid vis-à-vis unusually high cholesterol and brassicasterol content (188 and 133 ppm) as compared to other vegetable oils. Although high cholesterol and presence of eicosenoic acid (15 %) pose a hurdle for its approval as food oil, the presence of omega-3-fatty acids makes its oil unique and nutritionally rich. A cold-pressed meal of *Camelina* after oil extraction contains 10–14 % oil by weight and 40 % protein with lower glucosinolate levels, making it a desirable animal feed. With a variety of non-food usages of the oil as drying oil and in environmentally safe painting and coating applications, minimal agronomic input requirement for cultivation makes it a potential crop for use as bio-fuel without interfering with the edible oil trade and competition for available resources (Agarwal et al. 2010).

7.6.1 Plant Tissue Culture

There have been very few published in vitro studies on *Camelina*. This species has previously been used as a fusion partner in somatic hybridization studies with other Brassica species (Narasimhalu et al. 1994; Hansen 1998; Sigareva and Earle 1999; Jiang et al. 2009). The first report of its culture establishment and regeneration in vitro came from Tattersall and Millam (1999). They successfully established in vitro systems for the regeneration of shoots from leaf explants which

were more efficient for the regeneration of a root and shoots than hypocotyls. It was found that for regeneration from leaf tissue the use of auxin (NAA) alone in the medium above a level of 0.54 μM resulted in root or callus growth. Cytokinin, in the form of BA alone failed to induce regeneration, but a combination of 4.44 μM BA and 0.54 μM NAA induced shoot regeneration at the rates over 10.0 shoots per explant. The rooting response of explants was increased from a control level of 26.4 to 46.7 % by the addition of 5.4 μM NAA. Regenerated shoots were successfully transplanted to soil and flowered and set seeds normally.

7.6.1.1 Protoplast Fusion

Protoplasts of *Camelina* have been used in somatic hybridization with *Brassica* species; however, in all cases, the focus of these studies was to improve the *Brassica* species. Black spot, caused by *Alternaria brassicae* and *A. brassicicola,* is an important disease in all the *Brassica oleracea* vegetables. Sufficient resistance to the pathogen is not found within the species or in the species that readily cross to *B. oleracea*. *Camelina* is highly resistant to *Alternaria spp*. and has, in addition, other desirable characters for the improvement of *B. oleracea*. Protoplast fusion was performed for the production of intertribal somatic hybrids between rapid cycling *B. oleracea* (tribe Brassiceae), which has good regenerability and *C. sativa* (tribe Sisymbrieae) by polyethylene glycol (PEG) treatment, as a step towards the transfer of resistance to this disease into *Brassica* vegetable crops (Hansen 1998). The *B. oleracea* fusion partner was inactivated by treatment with iodoacetate. *C. sativa* has poor regenerability hence, no pretreatment was needed for this species. The protoplasts were cultured using a feeder layer system. A total of 2903 calli were isolated from the fusions. Fourteen of these initiated shoots, i.e., 0.5 % regeneration frequency. Approximately 110 shoots were excised from 6 of these calli and transferred to rooting medium. Rooted plantlets grew vigorously in vitro and flowering was frequently observed. However, establishment of rooted shoots in soil was unsuccessful. Hybrid identity was confirmed by intermediate shoot morphology, RAPD marker analysis, and flow cytometric estimation of nuclear DNA content. In a similar experiment carried out by Sigareva and Earle (1999) rooted plants grew in soil up to 4–5 weeks, and some produced sterile flowers. Two of three hybrids tested showed a high level of resistance to *A. brassicicola.* Resistance was correlated with the induction of high levels of the phytoalexin 'camalexin' 48 h after inoculation, as in the resistant Camelina fusion partner. In contrast, susceptible somatic hybrids produced much lower levels of camalexin.

Intertribal somatic hybrids between *Brassica napus* and *Camelina sativa* were also developed by protoplast electrofusion (Jiang et al. 2009). Hybrid identity of the regenerants was determined using flow cytometric analysis of nuclear DNA content and simple sequence repeat (SSR) marker analysis. Three hybrids exhibited specific bands for *B. napus* and *C. sativa*. These hybrids showed intermediate leaf, flower and seed morphology compared with the two parental species.

The seeds of these three hybrids had a modified fatty acid profile, indicating higher levels of linolenic and eicosanoic acids than those of *B. napus*. Their results suggest that somatic hybridization offers opportunities for transferring the entire genomes between *B. napus* and *C. sativa* in improving rapeseed breeding.

7.6.1.2 Microspore Culture

Doubled haploidy (DH) protocols have been used in breeding programs for many years to develop improved crop varieties. In order for doubled haploidy to be effective in a breeding program, an efficient microspore culture protocol is required. The conditions leading to the induction and development of microspore-derived embryos vary depending on the species, and therefore doubled haploidy methods have to be determined for each species. A number of factors influence microspore embryogenesis including genotype, stage of microspore development, donor plant growing conditions, media composition, and culture conditions. Microspore-derived embryos have been produced from *C. sativa* (Ferrie and Bethune 2011). The microspores from buds of 1–3 mm in length were isolated and purified in full-strength B5 extraction medium and cultured in NLN medium with 12.5 % sucrose and 12.5 % polyethylene glycol 4000 (PEG) without glutamine, at a density of 10,000 microspores per milliliter. Glutamine was added to the cultures 72 h after extraction to give a final concentration of 0.8 g/L. The microspore cultures were maintained at 24 °C in dark. After 28 days the generated embryos were transferred to light for continued development and to allow the embryos to become green. After 5–10 days, the embryos were plated on solid medium (½ strength B5, 1 % sucrose, 0.8 % agar, pH 5.8) and kept at 22 °C under 16 h photoperiod. The highest embryogenic frequency achieved was 38 microspore-derived embryos from 100,000 microspores. After approximately 6–8 weeks, regenerated plantlets with well developed roots and shoots were transferred to greenhouse for development.

7.6.2 Genetic Transformation

The high percentage of polyunsaturated fatty acids makes camelina oil more susceptible to oxidation and thus is undesirable for fuel and other industrial applications. Therefore, it is necessary to modify camelina oils to find a role for this potential crop in the world oilseed market. *Camelina* has great potential to become a biotechnological platform for genetically engineered products. There have been limited research activities on camelina biotechnology. However, like many cruciferous oil producing plants such as *Arabidopsis thaliana* and *Brassica napus*, *Camelina* is also amenable to transformation. Efficient methods for *Camelina* genetic transformation would facilitate a series of experiments designed to enhance the fatty acid profile of this species.

7.6.2.1 Transformation via Tissue Regeneration

In vitro *Agrobacterium*-mediated gene transfer involves the introduction of a transgene into appropriate plant tissue and regeneration of the tissue into a whole plant. This method has been widely and successfully used with many dicot and monocot crops. However, transformation by tissue culture can be time-consuming and generally very particular to the skills of the researcher performing the transformation. Kuvshinov et al. (2002, 2004) were pioneers to demonstrate efficient transformation in this crop using hypocotyls, cotyledon and leaf explants through the *Agrobacterium* method. Among explants leaf segments showed highest transformation efficiency. They selected and regenerated the transformed plants on MS medium supplemented with variable concentrations of auxins and cytokinins.

7.6.2.2 In Planta Transformation

In order to improve oil quality and other agronomic characters, Lu and Kang (2008) developed an efficient and simple in planta transformation method to generate transgenic camelina plants. The method included *Agrobacterium*-mediated inoculation of plants at early flowering stage along with a vacuum infiltration procedure. They used a fluorescent protein (DsRed) as a visual selection marker, which allowed them to conveniently screen mature transgenic seeds from a large number of untransformed seeds. Using this method, over 1 % of transgenic seeds could be obtained. Genetic analysis revealed that most of transgenic plants contain a single copy of transgene. In addition, to demonstrate that camelina can be effectively used to produce genetically engineered products, they transformed camelina seeds with a castor fatty acid hydroxylase (FAH12) gene. Fatty acid methyl ester analyses by gas chromatography indicated that all red seeds analyzed accumulated novel fatty acids, which had been previously identified in transgenic FAH 12 Arabidopsis as ricinoleic acid, the major component of castor oil, and three other hydroxy fatty acids: densipolic acid; lesquerolic acid; and auricolic acid. Red fluorescent seeds confirmed the transformation successfully expressing the castor FAH12 gene. They concluded that this low-cost oilseed crop, *C. sativa*, has great utility as an economical platform for a plethora of genetically engineered industrial and pharmaceutical products.

7.6.2.3 Floral Dip Method

Nguyen et al. (2011) described successful transformation using floral dip method resulting into greater than 1 % transformation efficiency. They transformed *Camelina* plants through contacting the plants to a dipping solution comprising Agrobacterium, a sugar, and a nonionic surfactant. Their method does not require a vacuum infiltration step.

Among above three methods in planta *Agrobacterium* -mediated gene transfer has advantages over tissue culture intensive methods. For example, in planta methods do not require performance by a specialist, and less equipment, labor and reagents are needed to obtain transformed plants. Thus, there is minimal somaclonal variation as compared to that typically encountered with tissue culture.

7.6.3 Molecular Markers

Camelina is an alternative oilseed crop species with limited information about the origin and diversity of available germplasm. The first study involving the use of molecular marker in this crop was reported by Vollmann et al. (2005). They evaluated a representative set of 41 accessions selected based on oil content, protein content and phenotypic data from a set of 130 *Camelina* germplasms, using random amplified polymorphic DNA (RAPD) analysis. Of 24 primers, 15 were polymorphic producing a total of 30 marker loci. Genetic distance estimates between the 41 accessions were calculated, based both on RAPD polymorphism and on seed quality characteristics, and dendrograms were generated for comparison. Similarities were found between the two different clustering approaches and grouping was partly in agreement with pedigree information or geographic origin.

During construction of a genetic linkage map of camelina, Gehringer et al. (2006) screened a total of 256 amplified fragment length polymorphism (AFLP) primer combinations for polymorphisms between the two mapping parents 'Lindo' and 'Licalla'. Among those, 44 primer combinations with the highest rate of polymorphism were used to genotype the 181 single seed descent lines. In addition, they also screened a set of 400 publicly available *Brassica* SSR primers in the parental lines. The majority of the SSR primers did not amplify loci in *C. sativa*; however eight polymorphic SSR markers were identified of which four could be integrated into the genetic map.

In an another study, amplified fragment length polymorphism (AFLP) fingerprinting revealed high levels of diversity within the 53 accessions of camelina in order to investigate the role of geographical origin in genetic variation and fatty acid content and a link between ecogeography and both origin and key oil traits (Ghamkhar et al. 2010). The accessions were categorized by principal coordinate analysis using molecular marker data, enabling identification of links between geographical distribution and these categories. Their results clearly confirmed that camelina oil quality characteristics are strongly influenced by environmental factors. The unprecedented high genetic diversity in this group of accessions offers an excellent opportunity to investigate valuable genes for successful adaptation of camelina to specific ecogeographical conditions such as drought.

7.6.4 Quantitative Trait Loci Analysis

Notwithstanding its potential for oil production, there is limited molecular and genomic information on this crop. In addition, a limited amount of molecular and

sequence information is available for *C. sativa.* In contrast to the vegetable and oilseed *Brassica* species, almost no information was available prior to the studies of Gehringer et al. (2006) with regard to the genomic make up of *C. sativa* and the genetic control of complex agronomic traits in this species. They constructed a genetic linkage map and used this map for Quantitative Trait Loci (QTL) studies. Gehringer et al. (2006) constructed a genetic map for *C. sativa* using amplified fragment length polymorphism (AFLP) and *Brassica* simple sequence repeat (SSR) markers, in a population of recombinant inbred lines that were developed, through single-seed descent, from a cross between 'Lindo' and 'Licalla', two phenotypically distinct parental varieties. At first, a chromosome number of $2n = 40$ was confirmed in all ten mitotic metaphases from each of the parental genotypes. Accordingly, the linkage map was constructed, which contained 157 AFLP markers and 3 *Brassica* SSR markers, on a total of 20 linkage groups, corresponding to $n = 20$. The map covered a total length of 1385.6 cm, with an average marker interval of 8.6 cm. A moderate level of DNA sequence conservation between *C. sativa* and the *Brassica* A, B and C genomes was demonstrated by the ability of 55 out of 406 tested *Brassica* SSR primer combinations to amplify microsatellite loci in *C. sativa* showing monomorphic amplification products, indicating partial genome homoeology with the *Brassica* species. A genetic map of Camelina will prove to be a valuable tool for future genomics-assisted improvement of this crop.

In *C. sativa* QTL studies were restricted due to the absence of a genetic map for this species which is a prerequisite for QTL detection. Gehringer et al. (2006) used the constructed genetic map with the data from field trials with different fertilization treatments (0 and 80 kg N/ha) at multiple locations over 3 years, to localize QTLs for agronomic characters including seed yield, oil content, 1000-seed weight (TSW), and plant height. QTLs were localized in the genetic map by composite interval mapping (CIM). They detected a total of eight significant QTLs for oil content; four for seed yield; two each for TSW, linoleic acid, linolenic acid and eicosenic acid; one each for plant height, oleic acid and erucic acid, on 12 different linkage groups. The major QTL for oil content was detected on LG4, which also co-localizes with a QTL for seed yield, may be a promising target for simultaneous marker assisted improvement of seed yield and oil content. A *Brassica* SSR marker that in oilseed rape is linked to a QTL for erucic acid biosynthesis and oil content was the nearest marker to a QTL for oleic acid, linoleic acid, eicosenic acid and oil content in C. sativa. Some yield QTLs were found only with the N0 treatment, and might represent loci contributing to the competitiveness of false flax in low-nutrient soils. Their results represent a starting point for future marker-assisted breeding.

Understanding the *Camelina sativa* genome is essential if agronomic properties are to be improved through molecular assisted breeding, mutation breeding, and/or genetic manipulation. For example, modification of the oil composition for superior biodiesel is a natural goal for this oilseed crop. Target genes for modification could therefore include Fatty acid desaturase 2 (FAD2), a membrane bound delta-12-desaturase which converts oleic acid to linoleic acid, and Fatty acid elongase 1 (FAE1) which sequentially adds 2 carbon units to 18 carbon fatty acid CoA conjugates, resulting in very long chain fatty acids.

7.6.5 Genomics

C. sativa is a member of the family Brassicaceae, and thus is a relative of both the genetic model organism Arabidopsis thaliana and the oilseed crop *Brassica napus*. The close relationship between *C. sativa* and *Arabidopsis* makes the *Arabidopsis* genome an ideal reference point for the development of genetic and genomic tools in *C. sativa*.

Manipulation of genes affecting traits of interest requires knowledge of their duplication status. Whole genome duplication is particularly relevant because it is common in plants, and because in the case of allopolyploidy it results in two or three independent copies of each gene. Based on their experiments involving FAD2 and FAE1 genes, Hutcheon et al. (2010) reported that *C. sativa* is a hexaploid, whose oil composition is likely influenced by more than one functional copy of both genes. As a first step to characterize genes involved in fatty acid biosynthesis, they determined the copy number of FAD2 and FAE1 by Southern blot analysis. PCR amplified fragments, consisting of conserved regions of FAD2 and FAE1 obtained using designed primers based on *Arabidopsis* genomic sequence, were used as probe. Results of the Southern blots revealed three bands in *C. sativa* for both FAD2 and FAE1, whereas hybridization revealed only a single band in Arabidopsis for both genes. These results suggest that FAD2 and FAE1 occur in at least three copies in *C. sativa*, while they are single copy in *Arabidopsis*. In many species fatty acid genes have been found to be multi-copy; therefore Hutcheon et al. (2010) verified their result using blot hybridization of the gene LEAFY (LFY), which is known to be single copy in a wide variety of species from several plant families. Three bands were observed following hybridization with the LFY probe of the same blot as was used for FAD2 and FAE1, suggesting LFY also exists as three copies in *C. sativa*. Transcript studies resulted that all three copies of both CsFAD2 and CsFAE1 were expressed in developing seeds, and sequence alignments showed the presence of previously described conserved sites, suggesting that all three copies of both genes could be functional. The regions downstream of CsFAD2 and upstream of CsFAE1 demonstrated colinearity with the *Arabidopsis* genome. In addition, the three expressed haplotypes were observed for six predicted single-copy genes in 454 sequencing analysis and results from flow cytometry indicated that the DNA content of *C. sativa* is approximately three-fold that of diploid *Camelina* relatives. Based on their results, they proposed that *C. sativa* be considered an allohexaploid.

In order to characterize the largely unexplored genome of *C. sativa*, Galasso et al. (2010) developed a new version of the cTBP (combinatorial tubulin-based polymorphism) method based on intron-length polymorphism (ILP), to rapidly characterize the β-tubulin gene family. *C. sativa* β-tubulin gene family of is composed of at least 20 different β-tubulin isotypes, named CsTUB1 through CsTUB20. The method, named h-TBP, allows the rapid cloning of the β-tubulin genomic sequences that encompass the two introns, invariantly present at fixed positions within the coding region of the vast majority of the plant species. The β-tubulin sequences cloned by h-TBP also comprised part of exon1 and exon3

and the whole sequence of exon2. Comparison of the β-tubulin exon sequences of *C. sativa* with those of *Arabidopsis* thaliana, the closest relative among crucifers, defines distinct groups of putative orthologous genes. Analysis of the *C. sativa* β-tubulin intron sequences reveals some molecular features that can provide the first hints for the understanding of intron plasticity and evolution.

It is desirable to increase the monounsaturated oleic acid (18:1), and to decrease polyunsaturated fatty acids (PUFA), linoleic (18:2) and α-linolenic (18:3) acids, in camelina oils to improve oxidative stability. 18:1 desaturation is mainly controlled by the microsomal oleate desaturase encoded by the FAD2 gene. Three FAD2 genes, designated CsFAD2-1 to 3, were identified in *Camelina* by Kang et al. (2011). Functional expression of these genes in yeast confirmed that they all encode microsomal oleate desaturases. Although the three CsFAD2 genes share very high sequence similarity, they showed different expression patterns. Expression of CsFAD2-1 was detected in all the tissues examined, including developing seed, flower, as well as in vegetable tissues such as leaf, root, and stem. Transcripts of CsFAD2-2 and CsFAD2-3 were mainly detected in developing seeds, suggesting their major roles in storage oil desaturation in seed. The introns of the three CsFAD2 genes, which showed greater sequence variations, may provide additional resources for designing molecular markers in breeding. They also demonstrated the roles of CsFAD2 in PUFA synthesis by the mutant analysis and antisense gene expression in camelina seed.

7.7 *Brassica carinata* A. Braun

Brassica carinata A. Braun (Ethiopian mustard), considered to have originated in Ethiopia, is among the oldest oil crops cultivated in Ethiopia, however, hardly cultivated in other parts of the world. It is used both as a vegetable and as an oil seed crop. Oil from the wild species is high in erucic acid, which is toxic, though there are some cultivars that contain very little erucic acid and can be used as food. The seed can also be crushed and used as a condiment. It is an amphidiploid species (2n = 34, BBCC) derived from the diploid species *Brassica nigra* (2n = 16, BB) and *Brassica oleracea* (2n = 18, CC). Owing to its drought and heat tolerance, the crop is now being considered as an alternative to *B. napus* and *B. juncea* in drier areas and has been evaluated as a potential oilseed crop in the United States, Canada, India, Italy and Spain (Teklewold and Becker 2006a, b; Warwick et al. 2006). It is not only an important source of edible oil, but also known to be tolerant to heavy metals and is a potential candidate for phytoremediation. Cultivars of yellow-seeded *B. carinata* are currently being developed in North America for the biodiesel and fish feed biorefinery markets (Li et al. 2009).

7.7.1 Tissue Culture

Protocol of plant regeneration in *B. carinata* was developed by Narasimhulu and Chopra (1987) aimed to creating somaclonal variation for plant type and adaptability, so that this species can fit into cropping systems in Indian agriculture. They

assessed the response of cotyledonary and stem explants for callus induction and shoot regeneration on MS and B5 basal media containing different combinations of auxin and cytokinin concentrations. MS medium supplemented with BA and NAA favoured callus induction. Supplementing MS with combinations of BA and IAA, as also with BA alone, regenerated shoots from the explants with a high frequency. The frequency of shoot regeneration and the mean number of shoots per explant were higher in cotyledons than in stem explants on identical growth regulator combinations. On B5 medium, supplemented with BA (2 mg/l) and IBA (0.4 mg/l), compact callus was produced which regenerated shoots on transfer to medium containing BA (0.8 mg/l). Jain et al. (1988) found MS medium with zeatin (1.0 mg 1-1) and IAA (0.1 mg l-1) to be best for shoot organogenesis on which the cotyledonary explants invariably underwent callusing followed by multiple shoot formation, which could be separated and subcultured for further propagation. Number of shoots per cotyledon explant cultured varied from 0 to as many as 50. Shoot organogenesis also declined with the reduction in photoperiod from continuous light to 16 h. The shoots were easily rooted during prolonged incubation on the same medium and whole plants could be regenerated and grown to maturity. Genotypic differences among carinata accessions for regeneration were common. Further, species-specific responses for in vitro shoot regeneration from cotyledon explants of three basic diploid species of *Brassica*, *B. campestris* (AA), *B. nigra* (BB), *B. oleracea* (CC) and their amphidiploids *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC) have also been observed; in particular *B. carinata* showed less regeneration frequencies than the parental diploid species (Narasimhulu and Chopra 1988). In an another study, immature stem segments of seven different genotypes of *B. carinata* produced shoots with variable frequencies when cultured in MS medium with BAP and picloram at 0.2 mg/l each (Narasimhulu et al. 1992b). They observed that ‘Line 171’ produced shoots with 100 % efficiency from both cut ends of the explant.

Hypocotyl explants from 6 to 7-day-old (but not younger or older) seedlings cultured on medium containing combinations of 2 mg l-1 BA and 0.01 mg l-1 NAA or 4 mg l-1 kinetin and 0.01 mg l-1 2,4-D regenerated shoots at 100 % frequency (Yang et al. 1991). Explants showed higher regeneration capacity at the distal end than the proximal end, and the upper segment of hypocotyl was more regenerative than the lower one. Regenerants were successfully rooted on half-strength growth regulator-free medium, acclimatized and developed into normal, fertile plants.

7.7.1.1 Anther and Microspore Culture

Isolated microspores from a cultivar of *B. carinata* was cultured in modified Nitsch and Nitsch (NN) medium supplemented with 13 % (W/V) sucrose, 0.05 mg/l benzyladenine (BA) and 1.00 mg/l naphthaleneacetic acid (NAA). Embryogenic responses were observed at cultured temperatures ranging from 22 to 32 °C. The highest frequency of embryos occurred at 30 °C and 7–54

embryos per anther (approx. 17,000 microspores per anther) developed (Chuong and Beversdorf 1985). A split temperature culture regime of incubation at 32 °C for 3 days followed by incubation at 25 °C resulted in both high embryo yields and a high percentage of normal embryos. Plantlet development from microspore-derived embryos appeared to be influenced by both medium and culture conditions.

Pollen embryogenesis occurred in the anther cultures of two genotypes of *B. carinata* following pretreatment of anthers at 35 °C for 3 or 6 days that was essential for the induction of androgenesis on growth regulator-free culture medium. A combination of sucrose and glucose was found better than sucrose alone. However, none of the pollen embryos germinated normally. Complete plants were raised through adventitious bud differentiation from their hypocotyls (Arora and Bhojwani 1988).

7.7.1.2 Protoplast Culture and Fusion

Protoplasts isolated from hypocotyls of three-day-old seedlings of *B. carinata* cv R-2128 were cultured in a modified Nitsch and Nitsch liquid medium containing 13 % sucrose, 0.4 % Ficoll, 0.25 mg/l BA, 0.5 mg/l NAA and 0.5 mg/l 2,4-D (Chuong et al. 1987). After 4–6 weeks developing microcalli were approximately 0.5 mm in diameter were transferred onto MS medium containing 3 % sucrose, 0.4 % agarose, 200 mg/l casein hydrolysate, 5 mg/l BA and 0.5 mg/l NAA, pH 5.7. Approximately 20 % of the calli transferred to this medium produced plantlets. Narasimhulu et al. (1992a) reported rapid and efficient plant regeneration in protoplasts isolated from hypocotyls of 7-d-old seedlings of three genotypes of *B. carinata* after enzymatic digestion in cellulase R-10 (0.5 %) and pectolyase Y-23 (0.025 %). The protoplasts were stabilized with 0.4 M mannitol used as osmoticum, and were cultured in darkness in Kao's liquid medium containing 0.4 M glucose and the growth regulators 2,4-D (1.0 mg/l), NAA (0.1 mg/l) and zeatin riboside (0.5 mg/l). These were transferred to 16 h photoperiod conditions after 3 days of dark culture, and the medium was diluted to reduce the osmoticum on the seventh and tenth days of culture. Developed microcolonies, upon transfer to MS agarose medium with 2,4-D (0.1 mg/l), BAP (1 mg/l) and 0.1 M sucrose, proliferated further to produce callus clumps. The plating efficiency of the three genotypes varied from 1 to 2 %. Calli 2–3 mm in diameter were further transferred to MS agarose plates with zeatin (2 mg/l) where they produced shoot buds and shoots with frequencies ranging from 22.5 to 74.2 % for the three genotypes. The shoots were rooted in medium with IBA (1 mg/l) and were then established in soil. The total time required for the protoplast-to-plant development was 8–10 weeks.

Protoplasts isolated from cotyledons of *B. carinata*, underwent sustained division when cultured at 5.0 × 104 ml-1 in modified 8p medium (KM8P) with 1.0 % (w/v) Seaplaque agarose. Cell colonies produced callus when agarose droplets, in which the protoplasts had been embedded, were transferred to K8 medium with 0.6 % (w/v) Type I agarose on day 16, giving a plating efficiency

of 1.6 %. It was found that 70 % of the protoplast derived-tissues produced shoot buds after subculture to MS medium containing 3.0 % (w/v) sucrose, 1.125 mgl-1 BAP, 0.035 mgl-1 GA and 0.6 % (w/v) Type I agarose, resulting in shoot formation from 1.1 % of the protoplasts originally plated. Regenerated shoots developed prolific root systems when placed on hormone-free MS medium with 1.0 % (w/v) sucrose and 0.6 % (w/v) Type I agarose (Jaiswal et al. 1990).

Jourdan and Salazar (1993) obtained 64 hybrid plants in two fusion experiments in an attempt to resynthesize *Brassica carinata* (BBCC) by the protoplast fusion between *B. nigra* (BB) and *B. oleracea* (CC) and identified them to be true hybrids by isoenzyme analysis, nuclear DNA content, chromosome number, and intermediate morphology. Of these plants 56 % were normal amphidiploids with $2n = 34$ chromosomes and a DNA content equivalent to that of natural *B. carinata*. The remaining plants were polyploid, morphologically abnormal, and infertile. The majority of the hybrids contained both chloroplasts and mitochondria from *B. nigra*, but some plants combined chloroplast and mitochondria from the different progenitors. Hybrids with a DNA content equivalent to that of *B. carinata* had a wide range of male fertility (4–98 %), but consistently low female fertility. However, only a few selfed seed could be produced, but these germinated and grew into vigorous plants.

Intergeneric protoplast fusion has also been attempted to transfer Alternaria blight resistance from *Camelina sativa* into *B. carinata* (Narasimhulu et al. 1994). Polyethylene glycol mediated fusion between protoplasts from etiolated hypocotyls of *B. carinata* and mesophyll protoplasts of *C. sativa* resulted to 6.8 % mean frequency of heterokaryons. Three hybrid shoots were regenerated, each from a single fusion derived callus but these shoots failed to produce roots capable of withstanding transplantation. Confirmation of hybridity was obtained from the morphology of in vitro produced leaves, somatic chromosome number in leaf tips, and restriction fragment length polymorphism for a nuclear rDNA probe. Analysis for organelle constitution using RFLPs indicated that the hybrid contained chrloroplasts derived from *Camelina* and mitochondria from the cultivated *Brassica* species.

7.7.2 Genetic Transformation

Narasimhulu et al. (1992b) selected immature stem segments of 'Line 171' for genetic transformation using a non-oncogenic *Agrobacterium tumefaciens* containing plasmid PCV 730, binary vector carrying resistance genes for kanamycin and hygromycin. A co-cultivation period of 4 days with a bacterial concentration of approximately 2.5×10 cells/ml, followed by a recovery period of 2 days, produced transformed shoots that could be selected and rooted in the presence of kanamycin at 15 mg/l. Transformation was confirmed by neomycin phosphotransferase assay and Southern blot analysis. Seed analysis of transformed plants indicated that kanamycin resistance was inherited in the progeny.

Cotyledonary petioles and hypocotyl explants were used for *Agrobacterium*-mediated transformation with a construct containing the selectable marker genes, neomycin phosphotransferase II, phosphinothricin acetyl transferase and the reporter gene β-glucuronidase, under the control of a tandem 35S promoter (Babic et al. 1998). Although transformation was achieved with both cotyledonary petioles and hypocotyls, cotyledonary petioles responded best, with 30–50 % of the explants producing GUS-positive shoots after selection on 25 mg/l kanamycin. Direct selection on L-phosphinothricin also produced resistant shoots at a lower frequency (1–2 %).

B. carinata offers an attractive alternative for the production of recombinant proteins using oleosin technology. Hirudin, a blood anticoagulant protein from leeches was produced in *B. carinata* seeds using oleosin as a carrier (Chaudhary et al. 1998). Cotyledonary petioles were infected with *Agrobacterium* strains containing oleosin-glucuronidase (pCGNOBPGUS-A) or oleosin-hirudin (pCGN-OBHIRT) constructs. Polymerase chain reaction and neomycin phosphotransferase II enzyme assays confirmed the presence of the fusion genes in plants regenerating under selection. The fusion polypeptides were correctly expressed and targeted to the oil-bodies of the seeds with high fidelity (ca. 90 %). Recombinant protein was purified from all other cellular protein by a simple flotation process and cleaved from oil-bodies using the endoprotease, factor Xa. Hirudin activity was measured using a colorimetric thrombin inhibition assay and an activity in the range of 0.2–0.4 antithrombin units per milligram of oil-body protein was detected.

Eicosapentaenoic acid (EPA) plays an important role in many aspects of human health. Recently, Cheng et al. (2009) successfully expressed two novel genes, an 18-carbon ω3 desaturase (CpDesX) from *Claviceps purpurea* and a 20-carbon ω3 desaturase (Pir-ω3) from *Pythium irregulare* in zero-erucic acid B. carinata with EPA levels in transgenic seed of this line reaching up to 25 %. They also reported that conlinin1 promoter from flax functioned reasonably well in *B. carinata,* and can serve as an alternative to the napin promoter from *B. napus*.

7.7.3 Molecular Markers

As PCR techniques have developed over the last 15 years, the wealth of new DNA marker technologies have arisen which have facilitated the analysis of genetic relationships in crops species as an important component of crop improvement. It helps to analyze genetic variability of cultivars, select parental materials for hybridization for making new genetic recombination select inbred parents or tester for maximizing heterotic response and identify materials that should be maintained to preserve maximum genetic diversity in germplasm sources. However, Information on genetic diversity and genetic relationships among genotypes of *B. carinata* and marker assisted selection is currently limited.

7.7.3.1 Random Amplified Polymorphic DNA

Geographic diversity is a potent source of allelic diversity. The extent of genetic diversity among Forty-three germplasm accessions of Ethiopian mustard from five different countries, comprising 29 accessions from eight different geographic regions of Ethiopia and 14 exotic accessions from Australia, Pakistan, Spain, and Zambia were analyzed using random amplified polymorphic DNA (RAPD) technique (Teklewold and Becker 2006a). A set of 50 primers yielded a total of 275 polymorphic bands allowing an unequivocal separation of every Ethiopian mustard accession. The usefulness of the 50 RAPD primers in measuring heterozygosity and distinguishing accessions was variable such that polymorphic information content (PIC) varied from 0.05 to 0.40, band informativeness (BI) from 0.05 to 0.65 and primer resolving power (RP) from 0.15 to 6.83. Jaccard's similarity coefficients ranged from 0.44 to 0.87 indicating the presence of a high level of genetic diversity. On the average, Australian and Ethiopian accessions were the most similar while, Spanish and Zambian accessions were the most distant ones. In another investigation the extent and structure of genetic variation in sixty-one accessions of *B. carinata* from 49 collection sites in Tanzania using RAPD markers resulted in 88 % variation among accessions, 4 % among regions and 8 % within accessions (Volis et al. 2009).

RAPD has also been applied to investigate heterosis in 36 F1 individuals, generated from crosses among nine inbred lines representing seven different geographic regions of Ethiopia. The nine parents along with their 36 F1s were evaluated using 14 phenotypic traits and 182 RAPD markers. The analysis depicted low correlation between phenotypic and molecular distances and it was concluded that parental distances estimated from phenotypic traits better predicted heterosis, F1 performance and GCA than distances estimated from RAPD markers (Teklewold and Becker 2006b).

7.7.3.2 Simple Sequence Repeats Markers

The presence of high levels of sinigrin in the seeds represents a serious constraint for the commercial utilization of Ethiopian mustard meal. Introgression of genes for low glucosinolate content from *B. juncea* into *B. carinata* was attempted through back crossing (Marquez-Lema et al. 2008). BC1F1 seed from crosses between double zero *B. juncea* line Heera and *B. carinata* line N2-142 was produced and further back cross generations were advanced. Forty-three BC1F4 derived lines were selected and subjected to a detailed phenotypic and molecular evaluation to identify lines with low glucosinolate content and genetic proximity to *B. carinata* using sixteen phenotypic traits and 80 Simple Sequence Repeats (SSR) markers. Eight BC1F4 derived lines were very close to N2-142 both at the phenotypic and molecular level. Three of them, with average glucosinolate contents from 52 to 61 micromoles $g - 1$, compared to 35 micromoles $g - 1$ for Heera and 86 micromoles $g - 1$ for N2-142 were selected.

Marquez-Lema et al. (2010) first time reported on the transferability and amplification quality of microsatellite (SSR) markers of the public domain in *B. carinata*. They studied the amplification of a set of 73 SSRs from *B. nigra* and *B. napus* in *B. carinata*, and compared the results with those obtained in the amplification of the same markers in other *Brassica* species. This set of SSRs from *B. nigra* (B genome) and *B. napus* (AC genome) led to the identification of the 3 basic genomes of the *Brassica* species tested. 94.3 % of the SSR markers from *B. nigra* and 97.4 % of those from *B. napus* amplified SSR-specific products in *B. carinata*. Very high-quality amplification with a strong signal and easy scoring in *B. carinata* was recorded for 52.8 % of the specific loci from *B. nigra* SSRs and 59.3 % of the specific loci from *B. napus* SSRs, compared to 66.7 % in *B. nigra* and 62.8 % in *B. napus*. These high-quality transferable SSR markers may provide an efficient and cost-effective platform to advance in molecular research in *B. carinata*.

7.7.3.3 Amplified Fragment Length Polymorphisms

Amplified Fragment Length Polymorphisms (AFLP) has been used to evaluate patterns and levels of genetic diversity in sixty six *B. carinata* germplasms in western Canada with comparison to twenty *B. juncea* and seven *B. nigra* accessions (Warwick et al. 2006). A total of 296 AFLP bands were generated from four primer pair combinations and scored for presence/absence in all the accessions of three species. Based on the analysis, *B. carinata* was found less genetically diverse than the other two species. The differences in diversity were evident in the proportion of polymorphic loci within each species: 23, 35 and 50 % for *B. carinata*, *B. nigra* and *B. juncea*, respectively. AFLPs proved to be useful for fingerprinting cultivars as two primer pair combinations were sufficient to uniquely identify all the accessions of *B. carinata*.

7.7.4 Genomics

Since *Brassica* species represent the closest crop plant relatives to the model plant *Arabidopsis thaliana*, significant progress will be achieved in the coming years through integration of candidate gene approaches in crop brassicas, using the detailed information now available for the *Arabidopsis* genome. The integration of information from the model plant with the increasing supply of data from physical mapping and sequencing of the diploid *Brassica* genomes will undoubtedly give great insight into the genetics underlying both simple and complex traits in oilseed crops of *Brassica*.

7.7.4.1 Genome Studies

Studies involving chloroplast (ct) DNA fragment patterns generated by digestion with fifteen restriction endonucleases from the three elementary *Brassica* species (*B. nigra*, *B. oleracea* and *B. campestris*) and the three amphiploid *Brassica*

species (*B. carinata*, *B. napus* and *B. juncea*) showed that in all species restriction sites for enzymes with GC-rich recognition sequences were less frequent and not as variable as for those with AT-rich sequences. The ct DNA fragment patterns of *B. carinata* were virtually identical to those of *B. nigra* indicating its origin and little alteration since the origin of this amphiploid (Erickson et al. 1983). Flow cytometry has been used to estimate 2C nuclear DNA content in parents and interspecific F1 hybrids from the crosses between *Brassica campestris*, *B. carinata*, *B. juncea* and *B. napus* obtained through in vitro ovary and ovule culture (Sabharwal and Dolezel 1993). It was found that in comparison with the A genome, the B and the C genomes of *Brassica* contained 26.9 and 43.9 % more DNA, respectively. The Flow cytometric analysis of nuclear DNA content might be a useful tool in *Brassica* breeding to distinguish interspecific hybrids containing various genome combinations.

7.7.4.2 Seed Coat and Seedling Leaf Pigmentation

Flavonoid differences between near-isogenic lines of yellow- and brown-seeded *B. carinata* were used to identify a genetic block in seed coat and seedling leaf pigment biosynthesis (Marles et al. 2003). Seed coat pigment in the brown-seeded line consisted of proanthocyanidins (condensed tannins), while anthocyanin was absent. Where as dihydroquercetin, dihydrokaempferol, quercetin and kaempferol accumulated only in the mature seed coat of the yellow-seeded line, indicating dihydroflavonol reductase (DFR) as an element of genetic control in pigment biosynthesis. The DFR transcripts from the developing seed coat in the yellow-seeded line were absent or less abundant at 5–30 days after pollination compared to transcript levels in the brown-seeded line. When grown at 25/20 °C (day/night) temperature, seedling leaves of the yellow-seeded line also exhibited a reduced expression of DFR and contained less anthocyanin compared to the respective tissues from the plants of the brown-seeded line. Cooler (18/15 °C) growing temperatures affected seedling leaf pigmentation, mature seed coat colouration and DFR expression in the yellow-seeded line while, the brown-seeded line tissues were unaffected by these temperature changes. The results were suggestive of a temperature-sensitive regulator of DFR in the yellow-seeded line of *B. carinata* which ultimately affects the formation of pigments in the seedling leaves and in the mature seed coats.

7.7.4.3 Antisense Repression and Silencing of the Endogenous FAD2 Gene

Erucic acid and its derivatives represent important industrial feedstock compounds, and, in this regard, there is an increasing demand for the production of high erucate oils. With a goal to develop high erucic acid *B. carinata* lines with the increased proportions of erucic acid and very long-chain fatty acids, the

expression of the endogenous FAD2 gene was manipulated using co-suppression and antisense approaches. Both methods resulted in transgenic lines exhibiting decreased proportions of polyunsaturated C18 fatty acids (18:2 + 18:3) and concomitant and significantly increased proportions of 18:1, 22:1 and total very long-chain fatty acids (Jadhav et al. 2005).

The 3′-UTR of the FAD2 gene was cloned by PCR and used to prepare an intron-spliced hairpin RNA (ihpRNA) construct. Compared to that of the wild type (control) background, this construct, when expressed in *B. carinata*, resulted in a high degree of FAD2 gene silencing accompanied by strong increases of up to 16 and 10 % in oleic acid and erucic acid proportions, respectively (Mietkiewska et al. 2008). The increase in 18:1 was accompanied by a concomitant proportional reduction in 18:2. Further experiment involving transformation of *B. carinata* with combination of ihpRNA with *Crambe abyssinica* FAE gene under the control of seed specific napin promoter resulted in an even greater increase in erucic acid proportions, by up to 16 % in T1 segregating seeds as compared to that of the control.

7.7.4.4 Genomics for Phytoremediation by *B. carinata*

Metal salt contamination of soils is a serious environmental problem with potential harmful consequences to agriculture and human health. About 20 % of the world's cultivated land and nearly half of all irrigated lands are affected by salinity. In nature, plant species possess a range of mechanisms involved in the detoxification of metals, allowing some to survive better than others under metal stress. Understanding these mechanisms will impact strongly on the success of developing salt tolerant crops.

Recently, *B. carinata* has been reported as a promising phytoextractor for Zn, Cu, Ni and Pb for the purpose of phytoremediation in multiply metal contaminated soils without suffering a significant biomass reduction (Purakayastha et al. 2008). Isolation and characterization of novel defence-related genes induced by copper, salicylic acid, methyl jasmonate, abscisic acid and pathogen infection have been reported in this species (Zheng et al. 2001).

Exposure of *B. carinata* seedlings to the increasing concentrations of a non-physiological ion, lithium, showed significant effects on the germination rate, root length, chlorophyll content and fresh weight in brown-seeded and yellow-seeded near-isogenic lines (Li et al. 2009). The lipid and phenolic composition dramatically changed in brown-seeded seedlings after lithium exposure. In contrast, the yellow-seeded plants maintained the same phenolic and lipid composition before and after exposure to lithium and did not tolerate the high metal concentrations tolerated by the brown-seeded line. Microarray analysis using *B. napus* 15000 expressed sequence tags (EST) array indicated a total of 89 genes in the brown-seeded line and 95 genes in the yellow-seeded line. These genes differentially expressed more than 20-fold and 1083 genes with more than 2-fold after the treatment of *B. carinata* seedlings with lithium chloride. The putative functions of

the differentially expressed genes included proteins involved in defense, primary metabolism, transcription, transportation, secondary metabolism, cytochrome P450, as well as proteins with unknown functions. From the results of this study, *B. carinata* brown-seeded germplasm showed an ability to survive under moderately high concentrations of lithium chloride (>150 mM) and has some potential in phytoremediation of lithium-contaminated water and soil.

7.7.5 Proteomics

In order to understand the biochemical basis for the observed resistance against fungal pathogen *Leptosphaeria maculans* in the plants generated by an interspecific cross between the highly susceptible *B. napus* and the highly resistant *B. carinata,* changes in the leaf protein profiles of hybrid lines were investigated (Subramanian et al. 2005). Two-dimensional electrophoresis followed by tandem mass spectrometry led to the identification of proteins unique to the susceptible (five proteins) and resistant genotypes (seven proteins) as well as those that were differentially expressed in the resistant genotype 48 h after a challenge with the pathogen (twenty eight proteins). The proteins identified as being unique in the resistant plant material included superoxide dismutase, nitrate reductase, and carbonic anhydrase. Photosynthetic enzymes (fructose bisphosphate aldolase, triose phosphate isomerase and sedoheptulose bisphosphatase), dehydroascorbate reductase, peroxiredoxin, malate dehydrogenase, glutamine synthetase, N-glyceraldehyde-2-phosphotransferase, and peptidyl-prolyl cis–trans isomerase were observed to be elevated in the resistant genotype upon pathogen challenge. They further validated the increased levels of the antioxidant enzyme superoxide dismutase by spectrophotometric and in-gel activity assays.

7.8 *Sesamum indicum* L.

Sesame has been described as the most ancient oilseed crop in the world and is regarded as queen of oilseeds, perhaps for its resistance to oxidation and rancidity, even when stored at ordinary ambient air temperatures (Bedigian and Harlan 1986). The importance of sesame lies in the quality of the oil, the presence of antioxidants sesamin and sesmolin, its antiquity and use in religious rituals in India, Egypt and Persian region. The world production is estimated at 3.66 million tones with Asia and Africa producing 2.55 and 0.95 million tons, respectively (Anonymous 2008). The major sesame growing countries are India, China, Myanmar and Sudan. Unfortunately, average world yield of sesame is still low at 0.46 ton ha^{-1} (FAO 2005). The area and production of this crop is declining in the traditional areas. Despite the potential for increasing the production and

productivity of sesame, there are a number of challenges inhibiting sesame production and productivity. Among many production constraints, the most important include a lack of improved cultivars and a poor seed supply system. In addition, there are severe biotic stresses, such as bacterial blight (*Xanthomonas campestris* pv. sesami), phyllody (Mycoplasma-like organism), Fusarium wilt (*Fusarium oxysporum*), Powdery mildew (*Oidium erysiphoides*), Alternaria leaf spot (*Alternaria sesame*) and Cercospora leaf spot (*Cercospora sesame*) (Daniel 2008). The above mentioned constraints to the productivity of sesame pose the need of concerte efforts for sesame crop improvement.

7.8.1 Plant Tissue Culture

Plant tissue culture technology has been available to plant breeders for nearly four decades and has been extensively employed for the crop improvement in several oil seed crops. However, very little information is available on sesame. It is found to be highly recalcitrant in nature. The first reported study on tissue culture in Sesame was that of Lee et al. (1985) from shoot tip culture and George et al. (1987) from different parts of sesame. Effects of explants and hormone combinations on callus induction were studied by Kim et al. (1987) in order to in vitro selection of herbicide tolerant lines of sesame. However, successful plant regeneration from herbicide tolerant calli was achieved by Chae et al. (1987). The effect of growth regulators on organ cultures (Kim and Byeon 1991) and its combination with cold pretreatment and genotype in anther culture (Lee et al. 1988) of sesame was investigated. Micropropagation has been achieved from shoot tip (Rao and Vaidyanath 1997a), nodal explants (Gangopadhyay et al. 1998) and leaf (Sharma and Pareek 1998) cultures. Somatic embryos were obtained from zygotic embryos (Ram et al. 1990) and seedling-derived callus (Mary and Jayabalan 1997; Xu et al. 1997) with low conversion frequencies in callus cultures. Indirect adventitious shoot regeneration from hypocotyl and/or cotyledon explants has also been reported but at low frequencies (Rao and Vaidyanath 1997b; Takin and Turgut 1997; Younghee 2001). Bhaskaran and Jayabalan (2006) reported standardization of a reproducible morphogenesis, micropropagation and callus induction protocol in cultivated varieties of sesame. Influence of macronutrients, plant growth hormones and genotype on adventitious shoot regeneration from cotyledon explants in sesame was reported by Were et al. (2006). High-frequency plant regeneration through direct adventitious shoot formation from de-embryonated cotyledon segments of sesame was achieved by Seo et al. (2007). Chattopadhyaya et al. (2010) established an efficient protocol for shoot regeneration from sesame internodes using the transverse thin cell layer (tTCL) culture method. Abdellatef et al. (2010) evaluated the in vitro regeneration capacity of sesame cultivar exposed to culture media containing ethylene inhibitors such as cobalt chloride and silver nitrate and found growth promotive effects due to reduction in ethylene concentration or inhibition of ethylene action.

A simple and efficient protocol for producing an inter-specific hybrid between *Sesamum alatum* and *S. indicum* through ovule culture has been optimized (Rajeswari et al. 2010). Direct organogenesis was successfully achieved when the ovules, excised from 7-day-old capsules from the cross *S. alatum* × *S. indicum*, were cultured on MS medium containing 8.8 μM benzylaminopurine (BAP), 2.8 μM indole acetic acid (IAA) and 1712.3 μM glutamine. The regenerants produced roots on half strength MS medium supplemented with 0.27 μM NAA. Phenotypically, the hybrid plants were intermediate to those of parents for majority of the traits. Peroxidase and esterase isozymes were found to be useful in the identification of hybrid plants. Further, screening against phyllody disease under greenhouse conditions revealed that the hybrids were moderately resistant.

7.8.2 Genetic Transformation

The yield potential of this crop is very low when compared with major oil seed crops due to early senescence and extreme susceptibility to biotic and abiotic stress factors including photosensitivity (Rao et al. 2002). Wild species of sesame possess genes for resistance to biotic and abiotic stresses (Joshi 1961; Kolte 1985; Brar and Ahuja 1979; Weiss 1971). However, introgression of useful genes from wild species into cultivars via conventional breeding has not been successful due to post-fertilization barriers. The only option left for improvement of sesame is to transfer genes from other sources through genetic transformation techniques. However, the main obstacle to genetic transformation is the recalcitrant nature of sesame to in vitro regeneration (Baskaran and Jayabalan 2006). There are very few reports on shoot regeneration, with low frequencies in a few genotypes from cotyledon and/or hypocotyl explants (Rao and Vaidyanath 1997a; Taskin and Turgut 1997; Younghee 2001; Were et al. 2006; Seo et al. 2007). Somatic embryos have also been induced from hypocotyl-derived calluses, but no plant regeneration was achieved (Mary and Jayabalan 1997). Although sesame has been shown to be susceptible to *Agrobacterium tumefaciens*, but no transformed shoot/plant was recovered (Taskin et al. 1999). For the first time, Yadav et al. (2010) reported conditions for establishing an *A. tumefaciens*-mediated transformation protocol for generation of fertile transgenic sesame plants. This was achieved through the development of an efficient method of plant regeneration through the direct multiple shoot organogenesis from cotyledon explants and the establishment of an optimal selection system.

Hairy root cultures using *Agrobacterium rhizogenes* have been successfully established (Ogasawara et al. 1993; Jin et al. 2005). In order to investigate the possible quinone derivative intermediate of Anthraquinones in sesame, hairy root culture was induced by Furumoto et al. (2007) through direct infection of axenic seedlings with *Agrobacterium rhizogenes* ATCC 15834. The established hairy root clone (SI-16) was subcultured in a phytohormone-free B5 liquid medium containing 2 % sucrose at 25 °C on a rotary shaker at 80 rpm in the dark at intervals of 14–18 days. After 4 weeks of inoculation, the secreted pigments were separated

from the medium by gravity filtration. The same hairy root culture was further used to explain the biosynthetic origin of 2-geranyl-1,4-naphthoquinone and its biogenetically related anthraquinone administering 13C labeled glucose to the hairy root culture (Furumoto and Hoshikuma 2011). Using hypocotyl and cotyledon explants from sesame seedlings, hairy root cultures were established and cDNA coding for Dehydroascorbate reductase (DHAR) (Chun et al. 2007) and peroxidase (Chun et al. 2009) were characterized and cloned from the roots. The frequency of sesame hairy root formation was higher from hypocotyl than cotyledon explant. It was also found that DHAR and peroxidase genes were differentially expressed in distinct tissues of sesame plant.

7.8.3 Molecular Techniques

DNA markers provide a powerful tool for genetic evaluation and marker-assisted breeding of crops, and especially for cultivar identification. Among the different types of molecular markers, random amplified polymorphic DNA (RAPD) markers are particularly useful for the assessment of genetic diversity because of their simplicity, speed and relatively low cost (Nybom 2004). RAPD markers have been used extensively in several crops including cucumber (Horejsi and Staub 1999), potato (Demeke et al. 1996) and pepper (Prince et al. 1995). Abdellatef et al. (2008) used RAPD markers to characterize 10 germplasm collections from Sudan to investigate the genetic diversity of selected sesame germplasm accessions from different origin and pedigree background.

Diversity estimates in cultivated plants provide a rationale for conservation strategies and support the selection of starting material for breeding programs. The diversity measures applied to crops usually have been limited to the assessment of genome polymorphism at the DNA level. Occasionally, selected morphological features are recorded and the content of key chemical constituents is determined, but unbiased and comprehensive chemical phenotypes have not been included systematically in diversity surveys. Laurentin et al. (2008) assessed metabolic diversity in sesame by non-targeted metabolic profiling and elucidated the relationship between metabolic and genome diversity in sesame and observed different patterns of diversity at the genomic and metabolic levels, which indicates that selection plays a significant role in the evolution of metabolic diversity in sesame. Earlier Laurentin and Karlovsky (2007) and Ali et al. (2007) successfully used AFLP to distinguish cultivars of sesame to elucidate the genetic relationship among genotypes.

The determination of genetic differences among crop genotypes has become the primary need to grant patent and the protection of Plant Breeder's Rights (PBR). Sharma et al. (2009) characterized 16 sesame genotypes by employing RAPD and ISSR markers and suggested that putative variety specific RAPD and ISSR markers could be converted to Co-dominant Sequence Characterized Amplified Region/Sequence Tagged Site (SCAR/STS) markers to develop robust variety specific markers. The comparative analysis for the genetic diversity of sesame has

been carried out using agro-morphological and molecular markers such as RAPD among twelve sesame populations collected from three regions in Cambodia and Vietnam (Pham et al. 2011) and ISSR among eighteen genotypes of sesame collected from various agro-climatic regions of Iran along with six exotic genotypes from the Asian countries (Parsaeian et al. 2011). ISSR analyses with merely 13 ISSR primers for genetic variation among them revealed 170 loci, of which 130 (76.47 %) were polymorphic. A high genetic variation was revealed both by agro-morphological and molecular markers within and among the sesame populations. Although both agro-morphological and RAPD markers were found to be useful in genetic diversity analysis in sesame, their combined use would give superior results. Further, the parental lines for hybridization should be selected on the basis of genetic diversity rather than the geographical distribution.

In another approach, sequence-related amplified polymorphism (SRAP) was used by Zhang et al. (2011) for the analysis of 67 sesame cultivars from the major sesame producing areas of China. A total of 561 bands were amplified using 21 SRAP random primer pairs, with 265 of them were polymorphic, resulting in a polymorphism ratio of 47.2 %. The average genetic similarity coefficient and the genetic distance of the 67 cultivars were 0.9104 and 0.0706, respectively, indicating limited genetic diversity and narrow genetic basis. The genetic basis of landraces was found wider than that of bred cultivars.

7.8.4 Marker Assisted Selection

Uzun et al. (2003) were first to identify a molecular marker linked to an agronomically important trait in sesame. They identified an AFLP marker linked to the closed capsule mutant trait in sesame using bulked segregant analysis (BSA) approach on segregating progenies of a cross between the closed capsule mutant line ‘cc3’, and the Turkish variety ‘Muganli-57’. They tested a total of 72 primer combinations to screen for linkage to the trait, but only one closely linked AFLP marker was identified. The linkage was confirmed by analysing the AFLP profile from single plants. They suggested that this marker had a potential to accelerate breeding programmes aimed at modifying unwanted side-effects of the closed capsule mutation through marker-assisted selection.

The first report on molecular tagging of the dt gene regulating determinate growth habit in sesame came from Uzun and Cagirgan (2009). The development of determinate cultivars has become an objective of high priority in sesame breeding programmes. They investigated RAPD and inter simple sequence repeat (ISSR) techniques for the development of molecular markers for this induced mutant character. Using the F2 segregating population and bulked segregant approach, they were able to detect two ISSR marker loci originated from a (CT)8AGC primer. They proposed that this marker would be potentially useful for assisting sesame breeding programmes through marker assisted selection and can facilitate the integration of determinate growth habit into new genetic backgrounds.

7.8.5 Genomics

In spite of extensive efforts to develop new sesame varieties by conventional and mutational breeding, the lack of a non-shattering sesame variety is one of the major barriers to obtaining high yield of sesame seeds (Yermanos et al. 1972; Ashri 1987). In addition, after oil extraction, the remaining meal corresponding to 50 % of seed dry weight is wasted or used for feeding poultry. Therefore, identification of novel genes involved in biosynthesis of sesame-specific flavor or lignans and understanding the metabolic pathways from photosynthates toward storage oil are desirable as an aid to improve the quality and quantity of oil in sesame cultivars. Sesame is an important oil crop, but limited transcriptomic and genomic data are currently available. In addition, a shortage of sesame molecular markers limits the efficiency and accuracy of genetic breeding. High-throughput transcriptomic sequencing is essential to generate a large transcriptome sequence dataset for gene discovery and molecular marker development. Expressed Sequence Tags (ESTs) generated by large-scale single-pass cDNA sequencing have proven valuable for the identification of novel genes in specific metabolic pathways. cDNA clones encoding seed-specific stearoyl-acyl carrier protein desaturase (Yukawa et al. 1996) and metallothionein-like protein (Chyan et al. 2005) have been isolated and their expression analysis revealed the maximum accumulation of RNA transcripts in the seeds.

Suh et al. (2003) in order to elucidate the metabolic pathways for lignans in developing sesame seeds and to identify genes involved in accumulation of storage products and in the biosynthesis of antioxidant lignans, obtained 3328 Expressed Sequence Tags (ESTs) from a cDNA library of 5–25 days old immature sesame seeds. ESTs were clustered and analyzed by the BLASTX or FASTAX program against the GenBank NR and Arabidopsis proteome databases. They carried out a comparative analysis between developing sesame and Arabidopsis seed ESTs for gene expression profiles during development of green and non-green seeds. Analyses of these two seed EST sets helped to identify similar and differential gene expression profiles during seed development, and to identify a large number of sesame seed-specific genes. Seed-specific expression of several candidate genes was confirmed by northern blot analysis. They identified EST candidates for genes possibly involved in biosynthesis of sesame lignans, sesamin and sesamolin, and suggested a possible metabolic pathway for the generation of cofactors required for synthesis of storage lipid in non-green oilseeds.

Sesame seed has been recognized as a nutritional protein source owing to its richness in methionine. Storage proteins have been implicated in allergenic responses to sesame consumption. Two abundant storage proteins, 11S globulin and 2S albumin, constitute 60–70 and 15–25 % of total sesame proteins, respectively. Two gene families separately encoding four 11S globulin and three 2S albumin isoforms were identified in a database search of 3328 expressed sequence tag (EST) sequences from maturing sesame seeds (Hsiao et al. 2006). Full-length cDNA sequences derived from these two gene families were completed by PCR using a

maturing sesame cDNA library as the template. The amino acid compositions of these deduced storage proteins revealed that the richness in methionine is attributed mainly to two 2S albumin isoforms and partly to one 11S globulin isoform.

Leaves of *Sesamum* spp. are used as leafy vegetables in Nigeria and many tropical areas around the world. Sesame leaves are also being used in Japan as a new food material containing functional components. Sesamin, a major lignan constituent of sesame seed, is considered responsible for a number of beneficial health effects in humans. Hata et al. (2010) scrutinized genotypic differences in sesamin content of two Japanese sesame varieties that differ in seed sesamin content (Higher in 'Gomazou' and lower in 'Kin-goma'). The expression of the sesamin biosynthetic gene CYP81Q1 was analysed through Quantitative RT-PCR analysis of stem and leaf samples performed with the Real-time PCR system relative to the expression of the reference gene 18S rRNA. The gene expression was found to be considerably higher in 'Gomazou' than in 'Kin-goma', indicating that genotypic difference of CYP81Q1 gene expression is one of the important factors affecting leaf sesamin contents. They further reported that the CYP81Q1 gene expression and sesamin content in leaves are photoperiod dependent (Hata et al. 2011) and concluded that cultivation of sesame under continuous light enables high-yield production of sesame leaves containing distinctively high levels of sesamin.

Recently introduced technique, Illumina paired-end sequencing is a fast and cost-effective approach to gene discovery and molecular marker development in non-model organisms. Sesame transcriptomes from five tissues were sequenced using this technology (Wei et al. 2011) leading to generation of 86,222 unigenes with an average length of 629 bp. Of the unigenes, 46,585 had significant similarity with proteins in the NCBI nonredundant protein database and Swiss-Prot database. In total, 22,003 unigenes were mapped onto 119 pathways using the Kyoto Encyclopedia of Genes and Genomes Pathway database (KEGG). Furthermore, 44,750 unigenes showed homology to 15,460 Arabidopsis genes based on BLASTx analysis against The Arabidopsis Information Resource (TAIR). Among these, 7,702 unigenes were converted into SSR markers (EST-SSR) in which dinucleotide SSRs were the dominant repeat motif (5,166). Randomly selected forty EST-SSR primer pairs successfully amplified DNA fragments and detected significant amounts of polymorphism among 24 sesame accessions.

7.9 Conclusions

Neglected crops are essential to the livelihoods of millions of poor farmers throughout the world. Newer technologies will certainly play their part in the process of improvement, conservation and use strategies. There are already a number of examples which show how useful neglected crops can be, but often only in small research scale activities which needs to be scaled up. Perhaps there is a need for some deliberate determination of the way in which these powerful tools can be best used for such crops. As implied above, there is also a lot of work to be done

on the development of sustainable linkages between various organizations, farmers and consumers. It will always be unlikely that any one organization will have the resources to support work on the scale needed for the individual neglected oilseed crops. Thus a major challenge will be to make sustainable networks and filieres. Strengthened community involvement in the management of underutilized crops and deliberate attention to resourcing their needs for new materials (and securing access to existing ones) will provide a basis for some more work on key production issues. The first of these is obviously that of the development of improved materials. Participatory plant breeding approaches including transgenics and molecular approaches may not only be an important element of the work on these crops; it will be the only feasible approach to obtaining improved materials.

Ultimately, we have to recognize that neglected oilseed crops present their own range of problems and opportunities. These are important to many farmers in the ways that are complementary to and are different from their concerns for the major crops. Attempting to copy large crop solutions across these species will help neither in the improved conservation and use of the crops nor the interests of the farmers who grow them. Developing an agenda specific to the crops will have to be recognized as an important and continuing need.

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