Cultivated meadowfoam (Limnanthes alba Benth.) is an annual oil seed crop native to southern Oregon, California, and British Columbia. The genus Limnanthes is composed of nine species and divided into two sections, Inflexae and Reflexae. The seed oil of meadowfoam is a rich source of erucic acid and several novel very long-chain fatty acids (VLCs). The former has been linked to increased risk of heart disease. The safe limit of erucic acid for human consumption is up to 5% of total fatty acids. Because the erucic acid concentrations of wildtype lines typically range from ~9 to 23% and low erucic acid variants have not been discovered, chemical mutagenesis was used to develop a mutant line (LE76) with greatly reduced erucic acid (~3%). The phenotypic distributions of F₂ progeny from crosses between wildtype and mutant lines were continuous and differed across genetic backgrounds. Quantitative trait loci (QTL) affecting erucic and dienoic acid were mapped using F₂₃ progeny from a cross between LE76 and
Wheeler (a wildtype line) and a simple sequence repeat (SSR) map spanning the meadowfoam genome. The domestication of meadowfoam was based on *L. alba*, belonging to section Inflexae. The secondary and tertiary gene pools have not been important to the domestication process and have not supplied diversity for meadowfoam breeding. With the objectives of introgressing genes from wild relatives and also producing cytoplasmic male sterile lines by inserting the nuclear genome of *L. alba* into wild cytoplasm, inter-sectional crosses involving *L. alba* and three subspecies of *L. douglasii* and intra-sectional crosses involving *L. alba* and two subspecies of *L. floccosa* were carried out. The isolation mechanisms involved in keeping species apart from each other were found to be different within and between sections. The study of partially fertile intra-sectional hybrids showed that the reduced pollen viability (30-33%) was not due to structural differences between the chromosomes of the two species, as normal meiotic behavior was observed in PMCs. The inter-sectional crosses were found to be incompatible and various abnormalities during pollen tube growth were observed.
Genetics of Low Erucic Acid and Cytological Analyses of Wide Hybrids in Meadowfoam

by

Sonali Dilip Gandhi

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APPROVED:

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Dr. Steven J. Knapp initiated, advised and supervised all aspects of the projects. He substantially assisted in formulating the hypotheses and revising the manuscripts. Jimmie Crane in association with Dr. Steven J. Knapp developed the lower erucic mutant line (LE76), Wheeler, OMF64, OMF40-11, OMF109-3, and OMF156. He also helped in fatty acid profiling. Dr. Venkatakrishnakishore developed the SSR markers used in this study for construction of the genetic map. He also helped in collection of flowers and further processing for pollen tube observation. Dr. Oscar Riera-Lizarazu provided the facilities for microscopy and also guided in making cytological preparations for pollen tube observation as well as meiotic analysis of the pollen mother cells.
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Genetics of Low Erucic Acid and Cytological Analyses of Wide Hybrids in Meadowfoam

CHAPTER 1

INTRODUCTION

Cultivated meadowfoam (*Limnanthes alba* Benth.) is an annual oil seed crop native to southern Oregon, California and British Columbia (Mason, 1952; Kalin, 1971; Jain, 1986). The genus *Limnanthes* comprises of nine species. The genus has been divided into two sections, Inflexae and Reflexae based on the morphological characters of petals folding inward or outward during seed maturation (Mason, 1952). The entire genus *Limnanthes* is diploid (2n=10), hermaphroditic and self-compatible (Mason, 1952).

The extent of genetic variation available in a crop is a prerequisite for its improvement due to the fact that efficiency of selection mainly depends upon it. The two major approaches employed by plant breeders in order to widen the genetic base of a crop are i) accessing the genetic diversity present in wild relatives of crop plants by distant hybridization and, ii) inducing mutations using chemical or physical agents. We used the approach of induced chemical mutagenesis for development of low erucic meadowfoam due to the lack of natural variation for erucic acid in meadowfoam. We also tried to make inter and intra-sectional hybrids
to expand the gene pool by introducing alien genes carried by wild species in meadowfoam.

Meadowfoam seed oil is very unique in many aspects. It contains substantial amounts of unsaturated very long chain (C20 and C22) fatty acids – VLCFs (Earl et al., 1959; Bagby et al., 1961; Smith et al., 1960; Miller et al., 1964; Higgins et al., 1971). The concentration of VLCFs in meadowfoam seed oil ranges from 94 to 96 % compared to 45 to 56 % in rapeseed. Meadowfoam seed oil has less than 2 % saturated fatty acids (Knapp and Crane, 1995), which is significantly less than rapeseed (6 to 8 %) and other widely consumed seed oils (Weiss, 1983). The principal fatty acids found in meadowfoam seed oil are erucic acid (22:1Δ13), cis-5-eicosenoic acid (20:1Δ5), cis-5-docosenoic acid (22:1Δ5) and cis-5, cis-13-eicosenoic acid (22:2 Δ5Δ13) or dienoic acid (Earl et al., 1959; Smith et al., 1960; Bagby et al., 1961). The seed oil of meadowfoam also has very high oxidative stability (Muuse et al., 1992; Isbell, 1997; Mund and Isbell, 1999). This, together coupled with a low general toxicity has made meadowfoam seed oil important for a number of non-food applications (Isbell, 1997, 1999), in particular the cosmetic industry (Dworak, 1994).

One of the major factors that may prevent the entry of meadowfoam oil into food, medical, nutritional, and pharmaceutical markets is the presence of high levels (8 to 23.2 %) of erucic acid in generic (wild type) meadowfoam. High levels of erucic acid in the diet have been associated with health problems (Borg, 1975; McCutcheon et al., 1975). The United States Food and Drug Administration
currently permits dietary oils containing no more than 2% of erucic acid for human consumption (Federal register, 1985). Whereas, European union food commission allows 5% erucic acid of total fatty acids in rapeseed oil meant for human consumption (Official Journal of the European Community, 2001). The development of low erucic acid meadowfoam cultivars may open the doors of the edible, medicinal and pharmaceutical markets for meadowfoam seed oil.

Meadowfoam is a recently domesticated oilseed crop. *Limnanthes alba* was identified by Gentry and Miller (1965) as the most promising species for domestication. The domestication process, which began more than thirty years ago (Crane and Calhoun, 1974) has been entirely based on *L. alba*. The secondary and tertiary gene pools have not been important to the domestication process and have not supplied diversity for meadowfoam breeding.

*Limnanthes alba* belongs to section Inflexae. The section Inflexae is comprised of four species: *L. alba*, *L. floccosa*, *L. gracilis* and *L. montana*. The two subspecies of *L. alba* ssp. alba and *L. alba* ssp versicolor form the primary gene pool of *Limnanthes* whereas the secondary gene pool is composed of *L. floccosa*, *L. montana*, and *L. gracilis*. All the species belonging to section Reflexae (*L. bakery*, *L. douglasii*, *L. macounii*, *L. striata*, and *L. maculans*), are classified as the tertiary gene pool of *Limnanthes* based on the fertility of inter sub-specific and interspecific hybridization with *L. alba* (reviewed by Knapp and Crane, 1999).

The wild relatives of crop plants have been important sources of genetic variability for economically important traits such as disease/insect resistance and
male sterility. Similarly wild species of meadowfoam possess genes with potential utility in breeding.

Besides combining useful genes from different species, interspecific hybridization is an important tool for developing cytoplasmic male sterility (CMS) by inserting the nuclear genome of a cultivated species into wild cytoplasm. This approach has resulted in the identification of CMS-inducing cytoplasms in a number of genera (Schable and Wise, 1998). However very few attempts have been made previously to make distant hybrids in meadowfoam. Mason (1952) made limited attempts to make inter-sectional crosses. He crossed *L. alba* ssp. *versicolor* with *L. douglasii* ssp. *douglasii* in both directions and *L. alba* ssp. *alba* with *L. douglasii* ssp. *rosea* in one direction. Crosses with *L. douglasii* ssp. *nivea* were never attempted. All crosses in his study failed to set seed. Also, intra-sectional, interspecific hybridization involving *L. alba* and two sub-species of *L. floccosa*, *pumila* and *bellingiana*, has never been reported.

The two long range goals of this research are to i) open the doors to food, pharmaceutical, and nutritional markets for meadowfoam seed oil and, ii) access the genetic diversity in wild species belonging to section Inflexae and Reflexae and develop cytoplasmic male-sterile lines by substituting the nuclear genome of *L. alba* into alien cytoplasm. In order to build the basic framework to achieve the above long range goals the present study with the following specific objectives was undertaken in meadowfoam.

1) To develop low erucic acid meadowfoam lines using mutagenesis.
ii) Study the performance of the low erucic mutant in different genetic backgrounds.

iii) Map the genetic factors underlying fatty acid composition differences in mutant and wild type meadowfoam.


v) Understand the nature of barriers involved in making the above mentioned inter- and intra-sectional hybrids.
CHAPTER 2

MAPPING GENETIC FACTORS UNDERLYING LOW ERUCIC ACID CONCENTRATION IN MEADOWFOAM

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Abstract

The seed oil of meadowfoam (*Limnanthes alba* Benth.) is very unique as it contains substantial amounts of unsaturated very long chain (C₂₀ and C₂₂) fatty acids—VLCFs. Meadowfoam seed oil has less than 2% saturated fatty acids. The principal fatty acids of meadowfoam seed oil are erucic acid (22:1Δ13), cis-5-eicosenoic acid (20:1Δ5), cis-5-docosenoic acid (22:1Δ5) and cis-5, cis-13-eicosenoic acid (22:2 Δ5Δ13) or dienoic acid. This fatty acid composition provides the oil with unusual properties such as high oxidative stability, which has made meadowfoam seed oil valuable for a number of non-food applications. The development of low erucic cultivars is essential for penetrating pharmaceutical and edible oil markets. High levels of erucic acid in the diet have been associated with health problems. The safe limit of erucic acid for human consumption is up to 5% of total fatty acids. Because the erucic acid concentrations of wildtype lines typically range from ~9 to 23% and low erucic acid variants have not been discovered, chemical mutagenesis was used to develop a mutant line (LE76) with greatly reduced erucic acid. LE76 produced three-fold less erucic acid than the wildtype (~3 as opposed to ~9%). We postulated that the erucic acid phenotype of LE76 was caused by a single mutation; however, the phenotypic distributions of F₂ progeny from crosses between wildtype and mutant lines were continuous and differed across genetic backgrounds. Quantitative trait loci (QTL) affecting erucic acid and dienoic acid were mapped using F₂₃ progeny from a cross between LE76...
and Wheeler (a wildtype line) and a simple sequence repeat (SSR) map spanning
the meadowfoam genome.

**Keywords:** QTL, erucic acid, dienoic acid, chemical mutagenesis
Introduction

The seeds of meadowfoam (*Limnanthes alba* Benth.) are rich source of oil and novel fatty acids (Earl et al., 1959; Bagby et al., 1961; Smith et al., 1960; Miller et al., 1964). Meadowfoam is a distant relative of *Arabidopsis thaliana* (Wheeler et al., 2000). The seed oils of taxa from both families (Limnanthaceae and Brassicaceae) are rich sources of erucic (22:1Δ13) and other very long chain fatty acids (VLCFs), primarily C20 and C22 compounds. Although VLCFs are produced by rapeseed (*Brassica napus* L.), crambe (*Crambe anthelminica* L.) (Downey and Craig, 1964; Harvey and Downey, 1964) and several other crucifers, the VLCFs of the Limnanthaceae and Brassicaceae differ in a few important ways. First, meadowfoam is a richer source of VLCFs (Earl et al., 1959; Bagby et al., 1961; Smith et al., 1960; Miller et al., 1964; Higgins et al., 1971) than the Brassicaceae. VLCF content in meadowfoam oil ranges from 94 to 96 % compared to 45 to 56 % in rapeseed.

Second, meadowfoam is a source of “Δ5 unsaturated” fatty acids not found in the Brassicaceae. The primary Δ5 fatty acids produced by meadowfoam are cis-5-eicosenoic acid (20:1Δ5), cis-5-docosenoic acid (22:1Δ5), and cis-5, cis-13-eicosenoic acid (22:2 Δ5Δ13) or dienoic acid (Earl et al., 1959; Smith et al., 1960; Bagby et al., 1961). The unique physical and chemical properties of meadowfoam oil can primarily be traced to the unique fatty acid composition (Isbell, 1997, 1999). One of the unique properties of meadowfoam oil is extraordinary oxidative

Third, less than 2 % of the fatty acids in meadowfoam oil are saturated (Knapp and Crane, 1995), which is significantly less than rapeseed (6 to 8 %). The saturated fat content of meadowfoam oil is the lowest reported thus far among locally and globally important vegetable oils (Weiss, 1983). Because saturated fatty acids comprise less than 2 % of the total fatty acids, meadowfoam oil seems to be an excellent candidate for nutritional and medical applications and markets. Virtually no research has been done on the specific nutritional and medical effects and properties of meadowfoam oil.

One of the major factors that is preventing the entry of meadowfoam oil into food, medical, nutritional, and pharmaceutical markets is the presence of high levels (8 to 23.2 %) of erucic acid in generic (wild type) meadowfoam. High levels of erucic acid in the diet have been associated with health problems (Borg, 1975; McCutcheon et al., 1975). Diets containing rapeseed oils with greater than 10% erucic acid resulted in accumulation of erucic acid in tissue lipids, reduced growth, myocardial lipidosis and necrosis in rats. The maximal safe intake of erucic acid that does not lead to lipidosis in rat heart has been estimated to be 2 to 10% dietary fatty acids (Beare et al., 1972; Abdellatif and Vles, 1973; Kramer, 1973; Christophersen et al., 1982). The United States Food and Drug Administration currently permits
dietary oils containing no more than 2% of erucic acid for human consumption (Federal register, 1985). Whereas, European Union Food Commission allows 5% erucic acid of total fatty acids in rapeseed oil meant for human consumption (Official Journal of the European Community, 2001). The development of low erucic acid cultivars may open the doors of the edible, medicinal and pharmaceutical markets for meadowfoam seed oil. An understanding of the biosynthesis and genetics of erucic acid in meadowfoam would provide a major boost to the efficient development of low erucic acid meadowfoam cultivars.

In most seed oils the end product of *de novo* fatty acid synthesis are 16- and 18-carbon fatty acids. The synthesis of fatty acids is carried out in the chloroplast and other plastids. Palmitic (16:0), stearic (18:0), and oleic (18:1Δ5) acids are produced through sequential condensation of two carbon units into $C_{16}$ and $C_{18}$ fatty acyl chains. These fatty acids are released from acyl carrier protein through the activities of thioesterases. In some higher plants such as *Arabidopsis thaliana* L., rapeseed, and many other species, longer chains are produced through the activities of elongases (Cassagne et al., 1994; Bao et al., 1998). In these species, the acyl residues freed from the carrier protein are carried to the cytoplasm and converted to the acyl-CoA esters by acyl-CoA synthetases, which serve as substrates for further elongation into very long chain fatty acids (VLCFs) and desaturation in species producing polyunsaturated fatty acids. These processes and assembly of triacylglycerols are carried out in the endoplasmic reticulum (Bao et al., 1998).
In *Arabidopsis thaliana* L. and rapeseed 18:1 substrate is elongated to 20:1Δ11 and 20:1Δ11 to erucic acid through the activity of elongases (Bao et al., 1998; Harwood, 1996; Kunst et al., 1992; Cassagne et al., 1994). However Pollard and Stumpf (1980) proposed a variant of this pathway for VLCF synthesis in meadowfoam. They suggested the two branches in pathway where one branch produces 20:0 and 22:0 by elongating 16:0. The elongated fatty acids are further desaturated by Δ5 desaturase, thereby yielding 20:1Δ5 and 22:1Δ5. The other branch produces 20:1Δ11, erucic acid (20:1Δ13) and dienoic acid (20:2Δ5Δ13). Sandager and Styme (2000) suggested the involvement of two different thioesterases *FatA* and *FatB* in VLCF synthesis of developing seeds of *L. douglasii*. *FatA* is primarily active on 18:0 and 18:1Δ9 and *FatB* is primarily active on 14:0 and 16:0.

Knapp and Crane (1998) have showed that erucic acid concentration is controlled by a dominant gene (E-locus), which decreases erucic acid content and increases dienoic acid content in meadowfoam. The E-locus has been recently mapped using a population derived from a cross between *L. alba* ssp. *versicolor* and *L. alba* ssp. *alba* (Katengam et al. 2001). *L. alba* ssp. *versicolor* typically produces more erucic and less dienoic acid than *L. alba* ssp. *alba*.

The lowest erucic acid concentration in meadowfoam reported from nature is 6.5% by Knapp and Crane (1995). The understanding of the basic genetics and biosynthesis of erucic acid in meadowfoam coupled with the lack of natural variation for low erucic acid encouraged us to develop low erucic acid lines in
meadowfoam using mutagenesis. The major objective of this study was to develop a low erucic meadowfoam using chemical mutagenesis and to map the genetic factors underlying the differences in the erucic acid concentrations in wild type and mutant meadowfoam lines.
Material and Methods

Fatty acid profiling

The chemical analysis of seeds using destructive or non-destructive (half-seed) method was carried out as described by Knapp and Crane (1998). The method described by Knapp and Crane (1995) was used for extraction of fatty acid methyl esters from seed samples and measurement of fatty acid concentrations using gas chromatography. Half seed (non-destructive method) to whole seeds (destructive method) were placed in a test tube having 0.5 ml hexane and crushed with Teflon rod. The sample was incubated for 15 min and evaporated to dryness with a stream of N₂ at 50°C. 0.1 ml of ethyl ether and 0.1M KOH were added to the sample. After incubating the sample for 5 min at 50°C, 0.1 ml of 0.15 M HCL and 0.5 ml of hexane were added and mixed. The top layer was used to measure the fatty acid concentrations using a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Inc., Avondale, CA, USA). Standard samples with known 18:1Δ5, 18:1Δ9, 18:3 (linolenic acid), 20:0, 20:1Δ11, 20:1Δ5, 22:1Δ5, erucic and dienoic acid concentrations were used to identify peaks and check measurements (the standards were obtained from Terry Isbell, USDA-ARS, NCAUR, Peoria, IL).
Low erucic acid mutant development

A low erucic acid mutant (LE76) of meadowfoam was developed by exposing seeds of the open pollinated cultivar Mermaid to methanesulfonic acid ethyl ester (EMS). Two hundred seeds ($M_0$) of Mermaid were soaked in distilled water (300 g seeds/liter of water) for 18 hrs at 4°C. The imbibed seeds were then soaked in 0.04 M EMS (300 g seeds/liter of solution, pH adjusted to 7.0 using 0.1 M phosphate buffer) for 8 h at 4°C. The chemically treated seeds were rinsed four times with distilled water and soaked for 18 h in distilled water at 4°C. Finally the seeds were germinated at 4°C on a moist blotter paper in covered 11 x 11 x 3 cm plastic boxes. Seedlings were transplanted to potting soil (pumice: peat moss: sandy loam) in 7.5 x 7.5 cm² plastic pots and grown in a growth chamber (Model CEL 37-14, Sherar-Gillette Co., Marshall, MI) for 25 to 28 days at 15°C with 18 h of fluorescent light. These plants were subsequently transplanted in bigger pots and transferred in a greenhouse at 22°C with 16 h of fluorescent light. $M_1$ plants were grown in greenhouse and manually self-pollinated. $M_2$ seeds were collected and fatty acid content was measured non-destructively (as mentioned above). Only $M_2$ seeds selected for low erucic acid were germinated and $M_2$ plants were grown in the greenhouse. Selfing and selection were repeated through the $M_5$ generation.
Crosses of the mutant with wild type lines

The low erucic acid mutant of meadowfoam (LE76) was crossed with three different meadowfoam lines having wild type erucic acid concentrations (Wheeler, OMF40-11, and OMF64). OMF40-11 is an S₅ inbred developed from Mermaid and Wheeler is an open pollinated cultivar (Knapp and Crane, 1999). They both belong to *L. alba* ssp. *alba*. OMF64 is a self-pollinated S₅ inbred belonging to *L. alba* ssp. *versicolor* (Crane and Knapp, 2000). F₁ plants were grown in greenhouse and selfed to produce F₂ seeds. F₂ seeds from the crosses were non-destructively analyzed for fatty acid content. In case of Wheeler x LE76, all F₂ plants were selfed and F₂₃ seed were collected. Bulk samples of twenty seeds (F₂₃) from each F₂ plant of the cross Wheeler x LE76, LE76, Wheeler, OMF40-11 and OMF64 were analyzed destructively for their fatty acid content.

Genetic mapping

The genetic map was constructed using 94 (Wheeler x LE76) F₂ progeny derived from a single F₁ seed. The young leaves from LE76, Wheeler and 94 F₂ plants were harvested and frozen at −80°C prior to DNA extraction. Genomic DNA from the frozen leaf tissue was extracted according to Lodhi et al. (1994) with some modifications. One to two grams of frozen tissue was ground in the presence of liquid nitrogen and incubated with 2% CTAB (cetyltrimethylammonium bromide)
extraction buffer for 1 h at 65°C. Chloroform extraction was carried out and the aqueous phase was transferred and mixed with 0.5 volume of 5M NaCl. The DNA was precipitated with 2 volumes of cold 95% ethanol and refrigerated at 4°C overnight. The DNA pellets were dissolved in TE (10 mM Tris-HCL and 0.1 mM EDTA, pH 8.0) buffer. Finally the dissolved DNA samples were treated with RNase (10 μg/μl) for 1 h at 37°C.

Total of 369 simple sequence repeat (SSR) markers (Kishore, 2002) were screened using the genomic DNA of LE76 and Wheeler to identify polymorphic SSRs. Only the polymorphic SSRs were assayed using the genomic DNA of 94 F₂ progeny as described by Kishore (2002), using polyacrylamide gels and fluorescently labeled amplicons on an ABI Prism 377 DNA sequencer (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA). Filter set C and the genescan 500 TAMRA internal standard were used for assays performed with 6FAM, TET, and HEX labeled amplicons. The amplicons for each SSR marker were separately produced. They were pooled post-PCR, and loaded into each lane: each amplicon in each pool lane was labeled with a different fluorophore (e.g. 6FAM, HEX and TET). Three-color multiplexes were used for unambiguous identification of the DNA fragments produced by each SSR primer pair. GeneScan ver. 2.1 and Genotyper ver.2.0 (Applied Biosystem, Perkin-Elmer, Foster City, CA, USA) software were used for recording allele lengths, which were also checked manually. The individual loci amplified by multilocus SSR markers were labeled with consecutive letters such as A, B, C etc.
Genetic maps were constructed using G-Mendel (Holloway and Knapp, 1993). Tests for segregation distortion were performed for each locus using log-likelihood ratio (G) tests. The observed ratio was significantly different from the expected ratios of 1:2:1 for co-dominant and 3:1 for dominant markers when \( G > \chi^2_{(1, 0.01)} \), where \( G \) is a log likelihood test statistic and \( \chi^2 \) is a random variable from the \( \chi^2 \) distribution with one degree of freedom. The markers were assigned to linkage groups with the criteria of likelihood odds (LOD) threshold of 5.0 and a recombination frequency threshold of 0.25. In case of \( F_2 \) population, it is often difficult to order dominant marker loci linked in repulsion phase (Knapp et al., 1995). Hence only dominant markers linked in coupling phase along with co-dominant markers were used for construction of maps. Loci were ordered using ORDER function of G-MENDEL. Map distances (cM) were calculated using Kosambi (1944) mapping function.

Mapping of QTLs

Trait means and correlations were calculated using SAS (SAS institute, Cary, NC). The broad sense heritability of the traits was calculated on the basis of parent-offspring correlation (Frey and Horner, 1957). The erucic and dienoic acid concentrations measured from the bulk samples of 20 \( F_2 \) seeds of the 94 \( F_2 \) plants along with the linkage map were used to perform composite interval mapping. This
was done using the ZmapQTL mapping method in QTLCartographer version 1.3 (Wang et al., 2002). The null hypothesis of no QTL was tested for positions throughout the genome by comparing LOD scores to an empirical genome-wide significance threshold calculated from 1000 permutations for $P=0.05$ (Doerge and Churchill, 1996). Interactions between significant QTL for each trait were tested, as the interactions between the closest loci for two different QTL. This was performed using PROC ANOVA in SAS.
Results

The low erucic acid mutant

Three $M_2$ lines with altered fatty acid profile were identified among 200 $M_2$ lines produced by EMS mutagenesis. The $M_2$ line LE76 produced less erucic acid than the wild type (WT) (Table 2.1). The fatty acid composition of LE76 $M_2$

Table 2.1. Fatty acid concentrations for wildtype (WT) and low erucic acid (LE76 $M_2$, $M_3$, $M_4$, and $M_5$) meadowfoam lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>Fatty acid ± SE* (%)</th>
<th>20:1Δ5</th>
<th>22:1Δ5</th>
<th>22:1Δ13</th>
<th>22:2Δ5Δ13</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>65.5±0.46</td>
<td>3.4±0.16</td>
<td>9.4±0.38</td>
<td>17.3±0.62</td>
</tr>
<tr>
<td>LE76-1M2</td>
<td></td>
<td>62.2</td>
<td>2.5</td>
<td>2.7</td>
<td>28.2</td>
</tr>
<tr>
<td>LE76-1M3</td>
<td></td>
<td>55.8±0.51</td>
<td>2.9±0.09</td>
<td>4.9±0.21</td>
<td>30.6±0.45</td>
</tr>
<tr>
<td>LE76-1-11M3</td>
<td></td>
<td>43.3</td>
<td>3.0</td>
<td>1.3</td>
<td>30.6</td>
</tr>
<tr>
<td>LE76-1-11M4</td>
<td></td>
<td>54.6±0.42</td>
<td>3.0±0.09</td>
<td>3.3±0.16</td>
<td>34.8±0.49</td>
</tr>
<tr>
<td>LE76-1-11-1M4</td>
<td></td>
<td>51.24</td>
<td>2.34</td>
<td>2.65</td>
<td>39.88</td>
</tr>
<tr>
<td>LE76-1-11-1M5</td>
<td></td>
<td>56.8±0.52</td>
<td>2.8±0.1</td>
<td>2.9±0.19</td>
<td>32.8±0.52</td>
</tr>
</tbody>
</table>

SE* standard errors are not given for phenotypes of selected single individuals.
individuals were screened using half seed samples. One $M_2$ individual (LE76-1) from the low end of the erucic acid distribution was selected, germinated, and selfed to produce an $M_3$ line. The erucic acid concentration of LE76-1M$_2$ was 2.7 %. One hundred and five LE76 M$_3$ individuals were non-destructively assayed using half seed samples (Fig. 2.1). The erucic acid concentration of the $M_3$ progeny ranged from 0 to 10.8 %. One $M_3$ individual (LE76-1-11) from the low end of the erucic acid concentration was selected and selfed to produce an $M_4$ line. The erucic acid concentration of the selected $M_3$ individual was 1.3 % The erucic range among
twenty eight M₄ individuals was much narrower than among M₃ individuals. One M₄ individual was selected (LE76-1-11-1) and selfed to produce the M₅ line. The erucic acid concentration of selected individual (LE76-1-11-1) was 2.65 %. The erucic acid concentrations of forty M₅ individuals fell in the same range (0 to 5.5 %) as M₄ individuals. The erucic phenotypes of the selected M₄ and M₅ lines were not significantly different and the erucic acid phenotypes of M₅ individuals fell in a narrow range (0 to 5 %) (Fig. 2.1); thus, M₅ progeny and beyond were bulked. The M₅ line produced ~3 % erucic acid, three-fold less than the wildtype, and ~30 % dienoic acid, two-fold more than the wildtype (Table 2.1 and Fig. 2.1). Because of the lack of obvious segregation among M₅ individuals, we concluded that the M₅ line was homozygous for induced mutations affecting fatty acid composition.

**Segregation of the Low Erucic Phenotype in Three Genetic Backgrounds**

The genetic basis of the low erucic acid phenotype of LE76-1-11-1 M₅ (hereafter LE76) was assessed by screening F₂ intercross progeny from crosses between LE76 and three genetically diverse wildtypes (Wheeler, OMF40-11, and OMF64). The phenotypes of the parent lines are shown in Table 2. Wheeler is an open-pollinated L. alba ssp. alba cultivar, OMF40-11 is an inbred line isolated from the L. alba ssp. alba cultivar Mermaid, and OMF64 is an inbred line isolated from a wild L. albasp. versicolor population. The 20:1Δ5, erucic, and dienoic acid
Table 2.2. Mean fatty acid concentrations for Wheeler, OMF40-11, OMF64 and minimum, maximum and mean fatty acid concentrations for \( F_2 \) progeny of the three crosses.

<table>
<thead>
<tr>
<th></th>
<th>Mean fatty acid ±SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20:1Δ5</td>
</tr>
<tr>
<td>LE76</td>
<td>53.74±3.6</td>
</tr>
<tr>
<td>Wheeler</td>
<td>61.34±4.6</td>
</tr>
<tr>
<td>OMF40-11</td>
<td>63.34±1.45</td>
</tr>
<tr>
<td>OMF64</td>
<td>57.97±2.4</td>
</tr>
<tr>
<td>Wheeler x LE76</td>
<td>64.67±2.0</td>
</tr>
<tr>
<td>OMF40-11 x LE76</td>
<td>66.34±1.96</td>
</tr>
<tr>
<td>OMF64 x LE76</td>
<td>64.41±2.3</td>
</tr>
</tbody>
</table>
concentrations of LE76 and the wildtype parents were significantly different ($p < 0.0001$). 22:1Δ5 concentration of LE76 was only significantly different ($p < 0.0001$) from Wheeler (Table 2.2). Fatty acid profiles of 135, 127 and 201 $F_2$ individuals derived from the crosses Wheeler x LE76, OMF40-11 x LE76 and OMF64 x LE76 respectively were analyzed to study the performance of the mutant in different genetic backgrounds. The erucic acid distributions for the three crosses were continuous, normal, and spanned different phenotypic ranges (Fig. 2.2). Crosses to

![Graphs showing erucic and dienoic acid concentrations for F2 progeny of three crosses.](image)

Figure 2.2. Erucic and dienoic acid concentrations for F2 progeny of three crosses.
L. alba ssp. alba wildtypes (Wheeler and OMF40-11) produced F₂ progeny from the lower end of the distribution with erucic acid phenotypes (3.6 and 2.9 %) close to the low erucic acid parent (~3 %), whereas the cross to L. alba ssp. versicolor (OMF64) produced F₂ progeny from the lower end of the distribution with a minimum of 5.8 % erucic acid; thus, the OMF64 x LE76 cross did not produce any erucic acid segregates in the low erucic acid range (3 % or less). The upper ends of the erucic acid distributions for the OMF40-1 x LE76 and OMF64 x LE76 crosses surpassed the erucic acid concentrations of the respective wildtype parents. The OMF40-11 x LE76 F₂ cross produced progeny with a maximum of 9.6 % erucic acid, whereas the OMF64 x LE76 cross produced F₂ progeny with a maximum of 24.8 % erucic acid. The upper end of the erucic acid distribution for the Wheeler x LE76 cross (13.4 %) did not surpass the wildtype parent (13.2 %). Thus, the lower and upper ends of the OMF64 x LE76 erucic acid distribution were ~3 % greater than the phenotypes of the parents.

Similarly, the dienoic acid distributions for the three crosses were continuous, normal, and spanned different phenotypic ranges (Fig. 2.2). The maximum dienoic acid concentrations of F₂ progeny from each cross were less than LE76. The dienoic acid minimums and maximums were lowest for F₂ progeny from the L. alba ssp. versicolor cross. Several OMF64 x LE76 F₂ progeny from the upper end of the erucic acid distribution had dienoic acid concentrations in the 6.3 to 8.0 % range, significantly less than wildtype germplasm from both subspecies (Knapp and Crane, 1995).
While no low erucic acid progeny were produced by the OMF64 x LE76 cross, 
20 ‘high erucic acid’ progeny were produced. Three out of 135 Wheeler x LE76 
F2 progeny and four out of 127 OMF40-11 x LE76 F2 progeny had low erucic acid 
(~3 % or less). None of the crosses produced transgressive segregates for low 
erucic acid. Conversely, two crosses (OMF40-11 x LE76 and OMF64 x LE76) 
produced transgressive segregates for high erucic acid. Finally, each cross 
produced transgressive segregates with dienoic acid concentrations less than the 
wildtype parents (Fig. 2.2).

F2 progeny from the three crosses had 20:1Δ5 concentrations greater than or 
equal to the upper (wildtype) parent, e.g., OMF64 produced 58.0 % of 20:1Δ5, 
whereas F2 progeny produced 57.1 to 70.1 % 20:1Δ5 (Table 2.2 and Fig. 2.3).

Erucic acid concentrations were significantly negatively correlated with 
dienoic acid concentrations in all the three crosses, but most strongly in the cross to 
*L. alba* ssp. *versicolor* (Table 2.3). Erucic acid also showed significant negative 
association with 20:1Δ5 concentration in the progeny of OMF40-11 x LE76 and 
OMF64 x LE76. However, erucic acid was positively associated with 22:1Δ5 
concentration in the progeny of OMF40-11 x LE76 and OMF64 x LE76. Dienoic 
acid showed a significantly negative association with 20:1Δ5 among the progeny of 
Wheeler x LE76 and OMF40-11 x LE76. However, dienoic acid showed a 
marginal positive correlation with 20:1Δ5 concentration in the progeny of OMF64 
x LE76. Dienoic acid was also significantly negatively associated with 22:1Δ5 
concentration in all three crosses.
Figure 2.3. Frequency distribution of 20:1Δ5 concentration for F₂ progeny of three crosses.
Table 2.3. Phenotypic correlations between major fatty acids of meadowfoam in F2 progeny of crosses Wheeler x LE76, OMF40-11 x LE76 and OMF64 x LE76

<table>
<thead>
<tr>
<th>Cross</th>
<th>Trait</th>
<th>20:1Δ5</th>
<th>22:1Δ5</th>
<th>22:1Δ13</th>
<th>22:2Δ5Δ13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheeler x LE76</td>
<td>20:1Δ5</td>
<td>1.00</td>
<td>0.35**</td>
<td>-0.05</td>
<td>-0.63**</td>
</tr>
<tr>
<td></td>
<td>22:1Δ5</td>
<td></td>
<td>1.00</td>
<td>0.14</td>
<td>-0.59**</td>
</tr>
<tr>
<td></td>
<td>22:1Δ13</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>-0.65**</td>
</tr>
<tr>
<td></td>
<td>22:2Δ5Δ13</td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>OMF40-11 x LE76</td>
<td>20:1Δ5</td>
<td>1.00</td>
<td>0.12</td>
<td>-0.36**</td>
<td>-0.69**</td>
</tr>
<tr>
<td></td>
<td>22:1Δ5</td>
<td></td>
<td>1.00</td>
<td>0.21*</td>
<td>-0.45**</td>
</tr>
<tr>
<td></td>
<td>22:1Δ13</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>-0.33**</td>
</tr>
<tr>
<td></td>
<td>22:2Δ5Δ13</td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>OMF64 x LE76</td>
<td>20:1Δ5</td>
<td>1.00</td>
<td>-0.13</td>
<td>-0.63**</td>
<td>0.19*</td>
</tr>
<tr>
<td></td>
<td>22:1Δ5</td>
<td></td>
<td>1.00</td>
<td>0.31**</td>
<td>-0.51**</td>
</tr>
<tr>
<td></td>
<td>22:1Δ13</td>
<td></td>
<td>1.00</td>
<td></td>
<td>-0.85**</td>
</tr>
<tr>
<td></td>
<td>22:2Δ5Δ13</td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
</tbody>
</table>

** significant at P<0.0001
* significant at P=0.05

Linkage map

Since a continuous phenotypic distribution for erucic and dienoic acid concentrations was observed in all three crosses, we decided to map the genetic factors underlying the erucic and dienoic acid concentration differences between the mutant and wild type meadowfoam by using a quantitative trait loci (QTL) mapping approach. The F2 progeny derived from the cross Wheeler x LE76 was
used as the mapping population to make a linkage map and the fatty acid profiles of $F_{2.3}$ seeds were used for the QTL analysis. Out of 369 SSR markers screened on genomic DNA samples of the mapping parents (Wheeler and LE76), 74 loci were polymorphic. Of the polymorphic SSR loci, 48 were co-dominant and 26 loci were dominant. The codominant SSR markers (48) and dominant SSR markers lacking null alleles in Wheeler (11) were used to construct the genetic linkage map. Of the 59 segregating SSR marker loci, 54 coalesced into five linkage groups. The map was 370.9 cM long and had a mean density of 7.1 cM per SSR marker locus (Table 2.4). Five (ORM 343, ORM544, ORM585, ORM604, ORM613) SSR loci were unlinked. Presence of unlinked loci can be explained theoretically as either due to Type II errors or are located ~27.5 cM distal to terminal SSR marker loci on the genetic linkage map. As all the unlinked markers were singletons, we predicted that at least ~137.5 cM of the genetic linkage map is missing. The actual genetic linkage map of meadowfoam based on this mapping population should be 508.4 cM long or longer. Hence, we estimated that the genetic linkage map in present study spans 72.9% of the meadowfoam genome.
Table 2.4. Summary of the meadowfoam simple sequence repeat map.

<table>
<thead>
<tr>
<th>Linkage group</th>
<th>Number of markers</th>
<th>Number of loci</th>
<th>Distance between loci</th>
<th>Length of Linkage group (cM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Min. (cM)</td>
<td>Mean (cM)</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>12</td>
<td>1.6</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>12</td>
<td>1.6</td>
<td>6.9</td>
</tr>
<tr>
<td>3</td>
<td>09</td>
<td>10</td>
<td>1.1</td>
<td>8.8</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>13</td>
<td>1.7</td>
<td>6.3</td>
</tr>
<tr>
<td>5</td>
<td>07</td>
<td>07</td>
<td>0.5</td>
<td>9.1</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>54</td>
<td>1.1</td>
<td>7.1</td>
</tr>
</tbody>
</table>

The SSR markers were randomly distributed across the genome (Fig. 2.4). The widest gap was 22.4 cM on linkage group 3. Of the 54 mapped SSR marker loci, only four showed significant segregation distortion. ORM371 and ORM281 had an excess of LE76 alleles, whereas ORM466 and ORM614 had an excess of Wheeler alleles.

We mapped 34 new SSR loci as 20 out of 54 mapped SSR loci have also been previously mapped by Kishore (2002) in inter-subspecific mapping population. Based on the common markers we could confirm the grouping and ordering of loci between the two maps.
Mapping QTL Underlying Fatty Acid Composition Differences

Broad-sense heritabilities were 0.43 for erucic and 0.27 for dienoic acid concentration among F3 families. Erucic and dienoic acid were significantly negatively correlated (\( p = 0.001 \) (\( r = -0.63 \)) in F2;3 families. The erucic acid
concentrations for F2:3 families ranged from 2.9 to 12.6 % with a mean of 6.6 % (Table 2.5). The distribution of the F2:3 progeny for erucic acid concentration was

Table 2.5. Mean fatty acid concentration for Wheeler and LE76 and minimum, maximum fatty acid concentrations for 94 F2:3 derived families.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean±SD (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wheeler</td>
<td>LE76</td>
<td>F2:3 families</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
</tr>
<tr>
<td>20:1Δ5</td>
<td>61.35±4.6</td>
<td>53.75±3.6</td>
<td>61.50±3.0</td>
</tr>
<tr>
<td>22:1Δ5</td>
<td>4.62±1.0</td>
<td>3.49±0.6</td>
<td>5.32±0.8</td>
</tr>
<tr>
<td>22:1Δ13</td>
<td>13.28±3.7</td>
<td>3.28±1.8</td>
<td>6.59±2.0</td>
</tr>
<tr>
<td>22:2Δ5Δ13</td>
<td>14.70±5.5</td>
<td>33.60±3.4</td>
<td>25.14±3.8</td>
</tr>
</tbody>
</table>

normal (Fig. 2.5A). A single transgressive family was observed for low erucic acid phenotype. The distribution of the F2:3 families for dienoic acid concentration was also normal (Fig. 2.5B). The dienoic acid concentration ranged from 16.0 to 37.9 % with mean of 25.1 % (Table 2.5).
Figure 2.5. Frequency distribution of erucic and dienoic acid concentration for 94 F$_{2.3}$ families of the cross Wheeler X LE76.

Composite interval mapping identified a total of four QTL (Fig. 2.6) underlying the differences in the concentrations of the erucic and dienoic acid in the seed oil of the mutant and wild type meadowfoam.
Figure 2.6. LOD plots of the QTL identified for erucic and dienoic acid concentrations.
Table 2.6. Summary of the QTL detected for erucic and dienoic acid concentrations.

<table>
<thead>
<tr>
<th>Trait</th>
<th>QTL</th>
<th>Linkage group</th>
<th>NML*</th>
<th>LOD</th>
<th>PVE (%)</th>
<th>a</th>
<th>d</th>
<th>d/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erucic Acid (er)</td>
<td>er2.1</td>
<td>2</td>
<td>ORM427</td>
<td>5.22</td>
<td>16.9</td>
<td>1.46</td>
<td>0.54</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>er2.2</td>
<td>2</td>
<td>ORM515</td>
<td>3.04</td>
<td>8.3</td>
<td>-0.83</td>
<td>0.92</td>
<td>-1.11</td>
</tr>
<tr>
<td></td>
<td>er3.1</td>
<td>3</td>
<td>ORM333</td>
<td>7.45</td>
<td>24.0</td>
<td>1.50</td>
<td>-0.48</td>
<td>-0.32</td>
</tr>
<tr>
<td>Dienoic Acid (dien)</td>
<td>dien3.1</td>
<td>3</td>
<td>ORM187</td>
<td>7.78</td>
<td>29.6</td>
<td>-2.95</td>
<td>-1.89</td>
<td>0.63</td>
</tr>
</tbody>
</table>

*NML* = nearest marker locus to the QTL
%PVE = percentage of phenotypic variation explained
a = additive gene action
d = dominant gene action
Erucic acid (er): A total of three QTL were detected underlying the erucic acid concentration differences between LE76 and Wheeler (Fig. 2.6). Two of these QTL (er2.1 and er2.2) were located on linkage group 2 whereas one QTL (er 3.1) was located on linkage group 3. A total of 49% of the phenotypic variance was explained (PVE) by the three QTL detected. The maximum amount of phenotypic variance explained by a single QTL (er 3.1) was 24% of phenotypic variance (Table 2.6). As expected the alleles from the low erucic parent (LE76) were found to be associated with two QTL (er 2.1 and er 3.1) having larger PVE, for decrease in erucic acid content. Additive effects were observed for alleles at these QTL for the differences in the erucic acid concentration. The allele from the high erucic parent (Wheeler) was responsible for decreasing the erucic acid concentration at QTL er 2.2.

Dienoic acid (dien): One QTL (dien 3.1) was identified on linkage group 3 (Fig. 2.6) to account for the differences in the concentration of dienoic acid in the seed oil of Wheeler and LE76. The amount of phenotypic variance explained by dien 3.1 was 29.6% (Table 2.6). The allele from the low erucic parent (LE76) contributed towards an increase in the dienoic acid at this QTL with additive gene action. None of the interactions between the QTL for either single trait or two traits was significant.
The genetic variation available in a crop is a prerequisite for its improvement due to the fact that efficiency of selection mainly depends upon it. Lack of natural variation in a crop system leads the plant breeders to induce mutations in the crop of interest and exploit useful mutants in the plant breeding programs. Plant breeders for various agronomic as well as qualitative traits have exploited induced mutagenesis using chemical or physical agents for crop improvement. We discuss here the development of low erucic acid meadowfoam line through mutation breeding, its performance in different genetic backgrounds and mapping the genetic factors underlying the differences in the fatty acid concentrations from wild type meadowfoam.

Induced mutagenesis to manipulate the fatty acid profiles of seed oil have been used in Brassica species (Rakow, 1973; Robbelen and Nitsch, 1975; Auld et al., 1992; Kott et al., 1996; Velasco et al., 1995, 1998), soybean (Erickson et al., 1988; Bubeck et al., 1989; Wilcox and Cavins, 1990; Burton et al., 1994), Arabidopsis (Browse et al., 1986) and many other oilseed crops. A chemical mutagen EMS is usually a preferred agent in these studies, because of the chromosomal damage caused by radiations in the target organism (Medrano et al., 1986). Most of the fatty acid mutations developed by chemical mutagenesis were recessive and obtained after screening several thousands of M2 and M3 progeny in Brassica species. Hence, very few mutants in fatty acid biosynthesis of Brassica species have been
identified (Auld et al., 1992). However, the frequency of mutation seemed higher in meadowfoam than that reported for other plants. We could identify three individuals out of 200 M2 with different fatty acid profile in this study. In another study in our lab we identified two mutants showing decreased erucic acid content out of 205 M2 plants (data not shown). Thus confirming the higher frequency of fatty acid mutations in meadowfoam compared to Brassica species.

The development of seed oils having a low or high percentage of erucic acid for various edible and industrial applications is currently a topic of research (Barro et al., 2001). The low erucic acid mutant identified in our study showed a three-fold decrease in erucic acid content when compared with the wild type meadowfoam. Similar results were shown in Brassica carinata where they recorded two-fold decrease in erucic acid content (Velasco et al., 1995; Barro et al., 2001) in mutants identified through chemical mutagenesis. In Brassica species, cultivars virtually free of erucic acid have also been developed as a result of coordinated research using chemical mutagenesis, recurrent selection and hybridization (Fernandez-Escobar et al., 1988; Velasco et al., 1995; Getinet et al., 1997). The near or total absence of erucic acid in rapeseed is caused due to a mutation in the fatty acid elongation 1 gene (Roscoe et al., 2001). This mutation is characterized by a great decrease in quantities of VLCFAs. A large decrease in the quantity of 20:1Δ11 and concomitant increase in C18 lipid species along with absence of erucic acid was observed for erucic acid free cultivars in rapeseed. This mutation also resembles the seed fatty acid elongation (FAE1) mutants of A. thaliana (James and Dooner, 1990;
Lemieux et al., 1990 Kunst et al., 1992). However, in this study as the total concentration of VLCFs was not reduced we assume that the low erucic acid mutant was not caused by elongase lesions as that of rapeseed and Arabidopsis.

The erucic and dienoic acid are sequentially produced in one branch of the pathway from elongated 18:1 substrate from oleate (18:1Δ9) as per the pathway proposed for very long chain fatty acid synthesis in meadowfoam (Pollard and Stumpf, 1980). Erucic acid is further desaturated by Δ5 desaturase to produce dienoic acid. While 20:1Δ5 and 22:1Δ5 are sequentially produced in another branch of the pathway from elongated 20:0 substrate from palmitate (16:0). Sandager and Stymne (2000) suggested the involvement of two different thioesterases FatA and FatB type in long chain fatty acid synthesis in developing seeds of meadowfoam. Where FatA type enzyme has a principle activity on 18:0 and 18:1Δ9 and FatB type enzyme primarily acts on 14:0 and 16:0. The low erucic mutant in our study showed significantly increased dienoic acid with reduction in 20:1Δ5 and 22:1Δ5 than wild type meadowfoam. Hence we speculate that the mutation might be affecting the activity of enzyme FatB that is responsible for hydrolyzing the acyl chains of palmitate (16:0) in the process of producing 20:1Δ5 and 22:1Δ5. Consequently, increased activity of elongase and especially Δ5 desaturase due to lack of proportionate substrates leads to increased dienoic acid with reduction in erucic acid. However this speculation is based on the assumption that the same Δ5-desaturase enzyme is involved in both branches of pathway. The
discovery and cloning of a Δ5 desaturase from *L. douglasii* (Cahoon et al., 2000) opens the way to study this possibility.

As the mutant identified in this study was developed by chemical mutagenesis using EMS, it was expected that the mutant phenotype would be caused due to a single gene mutation and we could map this mutation as a Mendelian factor. However, a continuous phenotypic distribution for erucic acid was observed in the F$_2$ progeny of all the three crosses studied. The cross between LE76 and OMF64, an *L. alba* ssp. *versicolor* line, failed to produce low erucic acid progeny. Conversely, crosses between LE76 and wild type *L. alba* ssp. *alba* lines (e.g., Wheeler and OMF40-11) produced progeny with low erucic acid (≈3.5%). Hence we speculate that OMF64 and other *L. alba* ssp. *versicolor* lines carry a linked mutation (E locus – Knapp and Crane, 1998) that interacts with the low erucic acid mutation(s) in LE76. The strong selection against mutant locus/loci in this genetic background is also possible. However the occurrence of low erucic individuals in segregating population of other two crosses where LE76 was crossed with individuals belonging to *L. alba* ssp. *alba* substantiates the former possibility.

Extreme transgressive segregants were observed for higher concentration of 20:1Δ5 in all three crosses studied. Presence of such outliers can be explained on the basis of epistasis or overdominance. Also when the parental lines fixed for set of alleles having opposite effects are crossed, extreme phenotypes appear (Lynch and Walsh, 1997). Phenomenon of overdominance seems logical for 20:1Δ5 as extreme phenotypes only towards higher concentration were observed.
The initial strategy was to use F$_2$ progeny of the cross between OMF64 and LE76 for mapping the genetic factors underlying the differences in the erucic acid concentrations of the mutant and wild type meadowfoam, as they had the highest difference for erucic acid concentration. However, the failure to recover low erucic parental type in the segregating progeny of this cross led us not to use the F$_2$ progeny from OMF64 x LE76 as the mapping population. The erucic acid concentration difference between OMF40-11 and LE76 was lower compared to the difference between Wheeler and LE76. The polymorphism at DNA level was also lower between OMF40-11 and LE76 as both of them were derived from cultivar Mermaid by selection and mutagenesis respectively (data not shown). Hence the F$_2$ progeny of Wheeler x LE76 was used for mapping the genetic factors underlying the erucic acid concentration differences between the mutant and wild type meadowfoam.

SSR markers have become the marker system of choice in many plant species because of the high level of polymorphism (Wang et al., 1994). They are PCR (polymerase chain reaction) based and suitable for high throughput genotyping. Genetic maps based on SSRs have been developed for wheat (Roder et al., 1998), rice (Temnykykh et al., 2000), barley (Ramsay et al., 2000), sunflower (Tang et al., 2002) and meadowfoam (Kishore, 2002). We used the SSR markers developed by Kishore (2002) to construct the linkage map of meadowfoam. A stringent LOD score (5.0) was used to construct the linkage map so as to increase the efficiency of detecting true linkage and minimizing false positives in assigning markers to
linkage groups (Type I errors). All the 54 SSR loci that coalesced into five linkage groups were randomly distributed across the genome of meadowfoam, which is in agreement with the results of Kishore (2002). There was no clustering of the SSR markers in our linkage map unlike the SSR maps in several other crops such as barley and wheat (Ramsay et al., 2000; Roder et al., 1998) that showed considerable clustering of SSR markers especially near centromeric regions. With an average inter locus spacing of 7.1 cM the linkage map developed in this study is an excellent framework map for mapping genetic factors underlying the differences in the fatty acid concentrations of the mutant and wild type meadowfoam. The genetic linkage map constructed in this study was second genetic linkage map for meadowfoam comprised of SSRs. Hence, it gave us a powerful tool for comparing and confirming the grouping and ordering of loci based on common markers between this and previous map.

A total of three QTL were identified for the difference in the erucic acid concentration, whereas one QTL was identified for the difference in dienoic acid concentration between the mutant (LE76) and wild type (Wheeler) meadowfoam. Alleles from LE76 contributed for decreasing the erucic acid concentration at two (er 2.1 and er 3.1) out of three QTL. Whereas, an allele from Wheeler was responsible for decreasing the erucic acid concentration at er 2.2. Hence we speculate that the QTL detected in our study for erucic acid concentration differences in the mutant and wild type meadowfoam include the mutated locus by EMS along with some other modifier loci, which may or may not be targeted by
EMS. The presence of modifier alleles for erucic and dienoic acid was also suggested by Knapp and Crane (1998) while studying the genetic basis of fatty acid profile differences between the two subspecies of meadowfoam. They observed the continuous variation for these two traits among E_ and ee progeny in segregating populations. Further Katengam et al., (1999) empirically tested for the presence of genetic effects other than the E locus by selecting and crossing E_ individuals from the tails of dienoic acid distribution from a backcross population. They found a continuous distribution for both erucic and dienoic acid in the 100 F_2 progeny derived from the cross between two E_ individuals suggesting the presence of modifier alleles. A total of 49 % of the phenotypic variance was explained by the QTL identified in this study for erucic acid concentration differences. The heritability of erucic acid calculated by the parent-offspring (F_2 – F_2;_3) correlation method was 0.43. Heritability of dienoic acid was low (0.27) which is also reflected in the low total phenotypic variance (29 %) explained by the QTL (dien 3.1) identified in this study.

The two fatty acids erucic and dienoic were moderately but significantly and negatively correlated (r =-0.63) in this study. The presence of two QTL er 3.1 and dien 3.1 for erucic and dienoic acid respectively in close proximity of each other suggesting the relatedness of these traits. Occurrence of QTL for related traits in close proximity is a common phenomenon. Diers and Shoemaker (1992) also mapped quantitative trait loci conditioning five major fatty acids mainly on two linkage groups in soybean.
Despite providing excellent information regarding the genetic factors underlying the fatty acid concentration differences between mutant and wild type meadowfoam there are some limitations to this study. The modest size of the F2 mapping population (n = 94) may have limited our ability to identify QTL and to estimate correctly the magnitude of their effects (reviewed in Lynch and Walsh, 1998). When interactions between significant QTL were tested it was found that none of the interactions were significant. Thus suggesting that epistasis was not involved in the QTL identified in this study. However we need to recognize that interactions may be present between the QTL and the loci without significant main effects (Knapp, personal communication), which was not tested.

Despite the limitations mentioned above, the development of near-isogenic lines (NILs) and low erucic acid cultivars should proceed more rapidly and efficiently by using flanking DNA markers to introgress the underlying mutation(s) once the QTL are validated. DNA fingerprinting of advanced backcrossed generations of lines selected for low erucic acid would provide a reliable and rapid tool for validation of QTL identified in this study. In conclusion, this study has elucidated the development of the first low erucic acid meadowfoam along with the genetic factors underlying the differences in the fatty acid concentration of the mutant and wild type meadowfoam.
References


CHAPTER 3

DIFFERENTIAL REPRODUCTIVE ISOLATION MECHANISMS WITHIN
AND BETWEEN SECTIONS OF GENUS *LIMNANTHES*

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Abstract

Cultivated meadowfoam (*Limnanthes alba* Benth.) is an annual oil seed crop native to southern Oregon, California and British Columbia. The genus *Limnanthes* is composed of nine species and divided into two sections, Inflexae and Reflexae. Meadowfoam has been domesticated since 1973. The domestication of meadowfoam was based on *L. alba*, belonging to section Inflexae. With the objectives of introgressing genes from wild relatives and also producing cytoplasmic male sterile lines by inserting the nuclear genome of *L. alba* into a wild cytoplasm, inter-sectional crosses involving *L. alba* and three subspecies of *L. douglasii* and intra-sectional crosses involving *L. alba* and two subspecies of *L. floccosa* were carried out. The isolation mechanisms involved in keeping species apart from each other were found to be different within and between sections. The intra-sectional, inter-specific hybrids could be made by making the involved species coincide in their flowering time. The study of partially fertile intra-sectional hybrids showed that the reduced pollen viability (30-33%) was not due to structural differences between the chromosomes of the two species, as normal meiotic behavior was observed in PMCs. The inter-sectional crosses were found to be non-compatible and various abnormalities during pollen tube growth were observed.

**Keywords:** Inter-specific hybrids, meiosis, Brassicales
Introduction

Cultivated meadowfoam (*Limnanthes alba* Benth.) is an annual oil seed crop native to southern Oregon, California and British Columbia (Mason, 1952; Kalin, 1971; Jain, 1986). The seed oil of meadowfoam contains unique unsaturated very long chain fatty acids (C_{20} and C_{22}) with outstanding oxidative stability (Smith et al., 1960; Bagby et al., 1961; Miller et al., 1964; Knapp and Crane, 1995, 1998; Isbell, 1997). *Limnanthes* (Order Brassicales) is a distant relative of *Arabidopsis* (Wheeler et al., 2000). The genus *Limnanthes* comprises of nine species. The genus has been divided into two sections, Inflexae and Reflexae based on the morphological characters of petals folding inward or outward during seed maturation (Mason, 1952). The entire genus *Limnanthes* is diploid (2n=10), hermaphroditic and self-compatible (Mason, 1952).

*Limnanthes alba* was identified by Gentry and Miller (1965) as the most promising species for domestication. The domestication process, which began more than thirty years ago (Crane and Calhoun, 1974) has been entirely based on *L. alba*. The secondary and tertiary gene pools have not been important to the domestication process and have not supplied diversity for meadowfoam breeding.

*Limnanthes alba* belongs to section Inflexae. This section is comprised of four species viz., *L. alba*, *L. floccosa*, *L. gracilis* and *L. montana*. The two subspecies of *L. alba* ie. *alba* and *versicolor* form the primary gene pool of *Limnanthes* whereas the secondary gene pool is composed of *L. floccosa*, *L. montana*, and *L. gracilis*. 
The species belonging to section Reflexae include *L. bakery; L. douglasii; L. macounii; L. striata* and *L. maculans*, and are classified as the tertiary gene pool of *Limnanthes* based on the fertility of intersubspecific and inter specific hybridization with *L. alba* (reviewed by Knapp and Crane, 1999).

The major reason for using interspecific hybridization in plant breeding is to expand the gene pool by introducing alien genes carried by wild species. The wild relatives of crop plants have been important sources of genetic variability for economically important traits eg., disease/insect resistance and male sterility. Similarly wild species of meadowfoam possess genes with potential utility in breeding.

Besides combining useful genes from different species, interspecific hybridization is an important tool for developing cytoplasmic male sterility (CMS) by inserting the nuclear genome of a cultivated species into wild cytoplasm. This approach has resulted in the identification of CMS-inducing cytoplasms in number of genera. The best characterized CMS-inducing cytoplasm of sunflower, PET1 and *ogu* cytoplasm of radish, which confers male sterility upon *B. napus* were derived from distant hybridization (Reviewed by Schnable and Wise, 1998).

Meadowfoam is naturally allogamous heterotic species. The heterotic potential of this crop can be best exploited by making single cross hybrids. The most efficient way of doing this will be having CMS system developed in this crop. As this is a new crop no hybrid seed production system has been developed yet for meadowfoam.
Very few attempts have been made previously to make distant hybrids in meadowfoam. Mason (1952) made limited attempts to make inter-sectional crosses. He crossed *L. alba* ssp. *versicolor* with *L. douglasii* ssp. *douglasii* in both directions and *L. alba* ssp. *alba* with *L. douglasii* ssp. *rosea* in one direction. Crosses with *L. douglasii* ssp. * nivea* were never attempted. All crosses in his study failed to set seed. Also, intra-sectional, interspecific hybridization involving *L. alba* and two sub-species of *L. floccosa* viz., *pumila* and *bellingeriana* has never been reported. The two long-range goals of this research are to access the genetic diversity in the secondary and tertiary gene pool of *Limnanthes* and to develop a cytoplasmic male sterility system for efficient hybrid seed production in meadowfoam. I order to tap the possibility of making hybrid seed and to understand the nature of barriers involved, the present study of interspecific hybridization was undertaken in meadowfoam.
Materials and Methods

Plant Material

This study was performed using two lines of *L. alba* viz., OMF109-3 and OMF156 to cross with randomly chosen plants of *L. floccosa* ssp. *pumila* (LFP, PI283721) and *L. floccosa* ssp. *bellingeriana* (LFB, PI283720), to produce intra-sectional hybrids, and with *L. douglasii* ssp. *douglasii* (LDD, PI278170), *L. douglasii* ssp. *nivea* (LDN, PI283713), *L. douglasii* ssp. *rosea* (LDR, PI283715) to produce inter-sectional hybrids. The seeds of LFP, LFB, LDD, LDN and LDR are held by the United State Department of Agriculture (USDA) National Plant Germplasm System (NPGS) and the Oregon State university (OSU) Centre for Oilseed Research (CORE) (Knapp and Crane, 1999). OMF109-3 is highly self-pollinating line, developed by hybridizing an *L. alba* ssp. *alba* individual (derived from a cross between a high oil line and open pollinated cultivar Mermaid) with OMF64 (a self pollinating *L. alba* ssp. *versicolor* inbred line, Knapp and Crane, 2000) and selecting for high autonomous seed set. OMF156 is an outcrossing line which was developed by inter-pollinating an open pollinated population based on a collection of *L. alba* ssp. *alba* and *L. alba* ssp. *versicolor*. Both sub-species of *L. floccosa* are highly self-pollinated due to partial cleistogamy, whereas, all three sub-species of *L. douglasii* are cross-pollinated due to protandry and heterostyly.
Pollination and crosses

Meadowfoam seeds were germinated at 4°C on moist blotter paper in covered 11- by 11 by 3 cm plastic boxes. Seedlings were transplanted to potting soil (pumice: peat moss: sandy loam) in 7.5 cm² plastic pots and grown in a growth chamber (Model CEL 37-14, Sherar-Gillette Co., Marshall, MI) for 25 to 28 days at 15°C with 18 h of fluorescent light. These plants were subsequently transplanted in bigger pots and grown in a greenhouse at 22°C with 16 h of fluorescent light.

The flowering times of three species were synchronized by transferring late flowering lines to warm temperatures first to induce flowering. Early flowering lines were transferred 10 to 15 days later. *L. alba* plants were transferred from low to high temperature 10 to 15 days earlier than *L. floccosa* plants. Similarly *L. douglasii* plants were transferred from low to high temperature 10 to 15 days earlier than *L. alba*.

Flowers were emasculated one to two days pre-anthesis and manually pollinated one to three days post-anthesis. Stigmas become receptive 12-36 h post-anthesis in *L. floccosa* and *L. alba* and 48-72 h post-anthesis in *L. douglasii*. Pollen from freshly dehisced anthers was used. *L. floccosa x L. alba* crosses were only produced using *L. floccosa* as the female. We crossed *L. alba* and *L. douglasii* reciprocally.
Meiotic analysis

Unopened flower buds of OMF109-3, LFP and LFP x OMF109-3 were collected and fixed in three parts absolute ethanol: one part 45% acetic acid fixative for analyses of pollen mother cells (PMCs). Anthers were isolated and squashed in 2% acetocarmine. Crystals of FeSO₄ were added to squashes to enhance staining. PMCs were observed at diakinesis, metaphase I, and anaphase I of meiosis to observe chromosome pairing and separation under light microscope (Zeiss Axioscope 2).

Bulk pollen samples were collected from parents and hybrids at anthesis, stained with I₂KI (Jensen, 1989) and scored for stainability under a light microscope. The percentage of stainable pollen was estimated from 1000 pollen grains per entry.

Pollen tube growth evaluation

Pollen tube growth was evaluated as described by Martin (1959) in all of the inter-sectional crosses and five controls OMF109-3 x OMF109-3; LDD x LDD; LDN x LDN; OMF156 x OMF109-3; LDD x LDN. Flowers were manually pollinated at 24 to 36 h post-anthesis in L. alba and 48 to 72 h post-anthesis in L. douglasii. Flowers were harvested at 6, 12, 18 and 24 hrs post pollination Twenty
flowers were emasculated and pollinated per cross combination per time point. Flowers were fixed in FAA (1 Formalin: 8 absolute ethanol: 1 glacial acetic acid) for 24 hrs. Styles were isolated, rinsed in tap water, cleared and softened in 8N NaOH for 14 h, and stained for callose with 0.1% solution of aniline blue dye in 0.1N K₃PO₄ for 4 h. Treated styles were mounted in a drop of Vectashield, an antifade (purchased from Vector Laboratories Inc. Burlingame, CA-94010) and covered with a cover slip with gentle squashing. The slides were observed using epifluorescence under Zeiss Axioscope 2. The styles of *L. douglasii* lines were divided into five sections, whereas the styles of OMF109-3 and OMF156 were divided into three and four sections respectively for the quantification of pollen tube presence. A total of 960 styles for 12 non-compatible and 400 styles for five compatible crosses were observed at 6, 12, 18 and 24 hrs after pollination for pollen germination, penetration, and pollen tube elongation.
Results

The inter and intra-sectional crosses carried out are listed in Table 3.1.

Table 3.1. Summary of crosses attempted.

<table>
<thead>
<tr>
<th>Cross</th>
<th>OMF156</th>
<th>OMF109-3</th>
<th>LDD</th>
<th>LDN</th>
<th>LDR</th>
<th>LFP</th>
<th>LFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMF156</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OMF109-3</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDD</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDN</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDR</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LFP</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LFB</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Section Inflexae hybrids

The use of *L. floccosa* plants as the male parent was impeded by difficulty in isolating minute pollen grains. The crosses carried out within section Inflexae
involving LFP, LFB and OMF156, OMF109-3 produced seed. From nine LFP flowers pollinated with OMF109-3 pollen five seeds were obtained. Eleven LFP flowers, pollinated with OMF156 produced 4 seeds. Eight and nine LFB flowers were pollinated with OMF109-3 and OMF156 respectively. Cross between LFB and OMF109-3 produced 3 seeds. LFB x OMF156 produced two seeds. Seeds from only LFP x OMF109-3 germinated.

The hybrid plants resembled to their pollen parent OMF109-3 in branching pattern and other characteristics such as hairy leaves and hairy sepals. The flower size of hybrids was intermediate to both the parents. The hybrid progeny was male and female fertile. This was tested by selfing the hybrid and backcrossing it to OMF109-3. Approximately 30-40 self-pollinations and backcrosses were sufficient to produce 70 and 50 seeds respectively. However the hybrid showed very low pollen viability (33%) (Fig. 3.1) as compared to both parents (97.4, 90.8 % in OMF109-3 and LFP, respectively).

**Meiotic behavior of F₁**

No meiotic abnormalities were observed in the parents or hybrids. Every cell observed at metaphase I and diakinesis of first meiotic division had five bivalents
Figure 3.1. Pollen grains of OMF109-3 and LFP x OMF109-3, stained with I$_2$KI at a magnification of 100X.

(Fig. 3.2). Ring bivalents were more frequent than rod bivalents in both parents (LFP and OMF109-3). The frequency of rod bivalents was greater in the hybrid

Figure 3.2. Diakinesis, Metaphase I and Anaphase I observed in the PMCs of OMF109-3 and LFP x OMF109-3.
than the parents (Table 3.2). Similarly, the chiasma frequency of the hybrid (6.86) was significantly (p<0.001) less than the parents. A total of 100 PMCs in anaphase I were observed for LFP, OMF109-3, and the hybrids. It was found that the chromosomes disjoined normally in parents as well as hybrids. No irregularities were observed in the distribution of the chromosomes (Fig.3.2).

### Table 3.2. Number of ring and rod bivalents and chiasma frequency observed for ‘n’ cells at diakinesis of meiosis I.

<table>
<thead>
<tr>
<th>Population</th>
<th>Number of cells</th>
<th>Rings (Mean)</th>
<th>Rods (Mean)</th>
<th>Chiasma frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFP</td>
<td>100</td>
<td>351 (3.51)</td>
<td>149 (1.49)</td>
<td>8.51</td>
</tr>
<tr>
<td>OMF109-3</td>
<td>100</td>
<td>396 (3.96)</td>
<td>104 (1.04)</td>
<td>8.96</td>
</tr>
<tr>
<td>LFP x OMF109-3</td>
<td>110</td>
<td>205 (1.87)</td>
<td>345 (3.13)</td>
<td>6.87</td>
</tr>
</tbody>
</table>

**Inter-sectional crosses**

Total of ~250 pollinations were done for 12 inter-sectional crosses. None of the inter-sectional crosses were successful. To dissect the reasons behind the failure, a
thorough examination of these crosses was done. The variation in length of the styles between different species is known to play a role in successful pollen tube elongation and fertilization of the ovule. Species belonging to section Inflexae differ in their style length from species belonging to section Reflexae. The average style length for OMF109-3 was 4.7 mm and for OMF156 was 5.8 mm. The styles of LDD, LDN and LDR are 7.9, 6.4 and 7.0 mm long, respectively (Fig. 3.3).

![Figure 3.3. Style morphology for OMF156 (L. alba), OMF109-3 (L. alba), L. douglasii ssp. nivea, L. douglasii ssp. rosea, L. douglasii ssp. douglasii](image_url)

Because of style length differences and the tendency of long styled species to fail as females, one would expect that the pollen from Reflexae individuals should easily reach the base of the short styles of Inflexae individuals. However, it was observed
that none of the pollen tubes reached the base of the style, irrespective of the
direction of the crosses, suggesting involvement of factors other than the length of
the style.

**Pollen germination and penetration**

Pollen germination for compatible crosses (Fig. 3.4) (the intra-specific crosses analyzed as controls, which produced seed) ranged from 77 to 92%. However, pollen germination in incompatible crosses (all the inter-sectional crosses which failed to produce seed) ranged from only 32 to 69%. The mean pollen germination for compatible crosses was 84%, which was significantly different (p<0.0001) from the mean pollen germination of 48% in incompatible crosses.

The pollen penetration for incompatible crosses varied from 7 to 23%, whereas for the compatible crosses it varied from 18 to 48% (Table 3.3). The mean pollen
Figure 3.4. Pollen germination, penetration, and pollen tube growth in compatible and incompatible crosses.
Table 3.3. Pollen germination, penetration and pollen tubes in compatible and non-compatible crosses

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of styles</th>
<th>Number of pollen grains</th>
<th>Germinated pollen grains (%)</th>
<th>Penetrated pollen grains (%)</th>
<th>Number of pollen tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compatible crosses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMF109-3 x OMF109-3</td>
<td>80</td>
<td>1575</td>
<td>91.93</td>
<td>39.49</td>
<td>453</td>
</tr>
<tr>
<td>LDD x LDD</td>
<td>80</td>
<td>2207</td>
<td>78.61</td>
<td>47.76</td>
<td>460</td>
</tr>
<tr>
<td>LDN x LDN</td>
<td>80</td>
<td>2171</td>
<td>87.93</td>
<td>32.01</td>
<td>512</td>
</tr>
<tr>
<td>OMF156 x OMF109-3</td>
<td>80</td>
<td>2502</td>
<td>85.57</td>
<td>38.88</td>
<td>595</td>
</tr>
<tr>
<td>LDD x LDN</td>
<td>80</td>
<td>4168</td>
<td>77.25</td>
<td>17.97</td>
<td>407</td>
</tr>
<tr>
<td><strong>In-compatible crosses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDD x OMF156</td>
<td>80</td>
<td>1124</td>
<td>43.5</td>
<td>8.27</td>
<td>22</td>
</tr>
<tr>
<td>LDD x OMF109-3</td>
<td>80</td>
<td>1018</td>
<td>32.02</td>
<td>15.60</td>
<td>23</td>
</tr>
<tr>
<td>LDN x OMF156</td>
<td>80</td>
<td>1111</td>
<td>38.70</td>
<td>13.23</td>
<td>56</td>
</tr>
<tr>
<td>LDN x OMF109-3</td>
<td>80</td>
<td>734</td>
<td>61.58</td>
<td>21.25</td>
<td>51</td>
</tr>
<tr>
<td>LDR x OMF156</td>
<td>80</td>
<td>946</td>
<td>33.80</td>
<td>11.80</td>
<td>23</td>
</tr>
<tr>
<td>LDR x OMF109-3</td>
<td>80</td>
<td>1049</td>
<td>45.09</td>
<td>12.20</td>
<td>16</td>
</tr>
<tr>
<td>OMF109-3 x LDD</td>
<td>80</td>
<td>1553</td>
<td>68.83</td>
<td>12.56</td>
<td>61</td>
</tr>
<tr>
<td>OMF109-3 x LDN</td>
<td>80</td>
<td>1056</td>
<td>56.62</td>
<td>7.38</td>
<td>7</td>
</tr>
<tr>
<td>OMF109-3 x LDR</td>
<td>80</td>
<td>1247</td>
<td>46.83</td>
<td>8.18</td>
<td>8</td>
</tr>
<tr>
<td>OMF156 x LDD</td>
<td>80</td>
<td>1679</td>
<td>56.99</td>
<td>22.57</td>
<td>76</td>
</tr>
<tr>
<td>OMF156 x LDN</td>
<td>80</td>
<td>545</td>
<td>54.31</td>
<td>17.60</td>
<td>28</td>
</tr>
<tr>
<td>OMF156 x LDR</td>
<td>80</td>
<td>889</td>
<td>42.96</td>
<td>15.07</td>
<td>50</td>
</tr>
</tbody>
</table>

$\psi =$ Percentages of total number of pollen grains

$\phi =$ Percentages of total number of pollen grains
penetration of non-compatible crosses was almost three times significantly (p<0.0001) lower than the mean pollen penetration of compatible crosses.

**Pollen tube elongation**

In in-compatible crosses a total of 45 pollen tubes were found in 240 styles, observed at 6 hrs after pollination. When the same number of styles were observed at 12, 18 and 24 hrs after pollination, they had 100, 127 and 150 pollen tubes, respectively. There was no significant difference (p = 0.31) between the number of pollen tubes observed at 18 hrs and 24 hrs after pollination. From the total 421 pollen tubes observed in 960 styles, not one pollen tube reached the base of the style. For compatible crosses, 476 pollen tubes were found in 100 styles observed at 6 hrs after pollination. The number of pollen tubes increased to 618 at 24 hrs after pollination. There was no significant difference (p = 0.69) between the number of pollen tubes observed at 18 and 24 hrs after pollination for compatible crosses. In compatible crosses, all the pollen tubes observed at 6 hrs after pollination had already reached the base of the style (Fig. 3.4). Thus suggesting that in regular cases it would not take longer than 6 hrs for the germinated pollen tube to reach the base of the style. Although not a single pollen tube was found at the base of the styles for all the in-compatible crosses, the combinations OMF156 x LDD and OMF109-3 x LDD were found to be most promising based on the total
number of pollen tubes (76 for OMF156 x LDD and 61 for OMF109-3 x LDD) observed in 80 styles.

Sites of inhibition

The mechanism of inhibition for pollen development in inter-sectional crosses was found to operate at two different sites, the stigma and the style. At the stigmatic level, pollen development was arrested either by preventing germination or preventing penetration of germinated pollen due to heavy callose deposition either on the stigmatic surface (Fig. 3.5A) or in growing pollen tubes of germinated pollen (Fig. 3.4). Out of the 12 incompatible crosses, six crosses viz., LDD x OMF156, LDD x OMF109-3, LDR x OMF109-3, OMF109-3 x LDN, OMF109-3 x LDR, OMF156 x LDN showed callose deposition on the stigmatic surface (Table 4). In case of the pollen, which germinated and penetrated the stigmatic tissue, most of the pollen tubes traveled successfully through the stylar neck. However they were arrested in the lower portion of the styles (Fig. 3.4). In L. douglasii individuals, the pollen tubes were arrested in the lower two fifth portion of the style, whereas for L. alba individuals the pollen tubes were arrested in the lower one third or one fourth region of the styles (Table 3.4). The only cross showing arrested pollen tubes in the first one fifth part of the style was LDN x OMF156. In the cross LDD x OMF156, 100% of the pollen tubes were arrested in the lower
Figure 3.5. Heavy callose deposition on the stigmatic surface in LDD x OMF156 (A), a pollen tube turning back in OMF156 x LDD (B), swollen tip of a pollen tube in LDR x OMF156 (C), a burst pollen tube in LDD x OMF156 (D), a forked pollen tube in OMF109-3 x LDD (E), and a coiling pollen tube in OMF156 x LDN (F).
Table 3.4. Inhibition sites for incompatible crosses.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of styles</th>
<th>Number of pollen tubes</th>
<th>Callose deposition on stigma (% styles)</th>
<th>% Pollen tube stopped*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1st section</td>
<td>2nd section</td>
</tr>
<tr>
<td>LDD x OMF156</td>
<td>80</td>
<td>22</td>
<td>6.25</td>
<td>0.0</td>
</tr>
<tr>
<td>LDD x OMF109-3</td>
<td>80</td>
<td>23</td>
<td>16.25</td>
<td>0.0</td>
</tr>
<tr>
<td>LDN x OMF156</td>
<td>80</td>
<td>56</td>
<td>0.0</td>
<td>1.85</td>
</tr>
<tr>
<td>LDN x OMF109-3</td>
<td>80</td>
<td>51</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>LDR x OMF156</td>
<td>80</td>
<td>23</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>LDR x OMF109-3</td>
<td>80</td>
<td>16</td>
<td>18.75</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF109-3 x LDD</td>
<td>80</td>
<td>61</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF109-3 x LDN</td>
<td>80</td>
<td>7</td>
<td>15.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF109-3 x LDR</td>
<td>80</td>
<td>8</td>
<td>12.5</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF156 x LDD</td>
<td>80</td>
<td>76</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF156 x LDN</td>
<td>80</td>
<td>28</td>
<td>11.25</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF156 x LDR</td>
<td>80</td>
<td>50</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* The styles of LDD, LDN, LDR were divided in five sections and the styles of OMF109-3 and OMF156 were divided in three and four sections respectively.
one-fifth (near base) portion of the style. LDN x OMF109-3 and LDN x OMF156 were other two crosses showing the maximum proportion of total pollen tubes (97.5 and 85.2\% respectively) arrested in the lower one fifth region of the style. In case of crosses where *L. alba* individuals were used as females, OMF109-3 x LDD and OMF156 x LDN showed the highest percentages (85.0 and 80.6) of pollen tubes arrested near the base.

**Pollen tube growth abnormalities**

The nomenclature for abnormalities in pollen tube growth observed in intersectional, and interspecific crosses in this study is based on the categories defined by Willliams et al. (1982) and Rego et al. (2000) with some modifications. The following five categories of abnormalities were observed:

1. Disoriented or loss of direction - pollen tube grows either backward or horizontally instead of growing towards the base of the style (Fig.5B)
2. Swollen tip pollen tube - the tip of pollen tube is swollen and ceased to grow (Fig.3.5C)
3. Burst pollen tube - the tip of the pollen tube burst after swelling (Fig.3.5D)
4. Forked pollen tube - the tip of the pollen tube is bifurcated (Fig.3.5E)
5. Coiling pollen tube – the pollen tube coils around itself (Fig.3.5F)
Among the five abnormalities listed above, swollen tip pollen tubes (ii) and loss of directional growth (i) were the most prevalent classes observed in all the incompatible crosses. OMF109-3 x LDD and OM156 x LDD showed the highest percentages (88.52 and 78.95, respectively) of pollen tubes with swollen tip (Table 3.5). Coiling pollen tubes were observed in only three crosses viz., LDD x OMF109-3, LDN x OMF156 and LDN x OMF109-3. Forked pollen tube (OMF109-3 x LDD) and burst pollen tubes (OMF156 x LDD) were observed in only one cross each.
Table 3.5. Abnormalities observed in pollen tube growth of non-compatible crosses.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of pollen tubes</th>
<th>Burst</th>
<th>Swollen tip</th>
<th>Disoriented</th>
<th>Forked</th>
<th>Coiling</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDD x OMF156</td>
<td>22</td>
<td>0.0</td>
<td>54.54</td>
<td>45.45</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>LDD x OMF109-3</td>
<td>23</td>
<td>0.0</td>
<td>43.48</td>
<td>39.13</td>
<td>0.0</td>
<td>17.39</td>
</tr>
<tr>
<td>LDN x OMF156</td>
<td>56</td>
<td>0.0</td>
<td>27.78</td>
<td>46.29</td>
<td>0.0</td>
<td>5.56</td>
</tr>
<tr>
<td>LDN x OMF109-3</td>
<td>51</td>
<td>0.0</td>
<td>54.90</td>
<td>37.25</td>
<td>0.0</td>
<td>7.84</td>
</tr>
<tr>
<td>LDR x OMF156</td>
<td>23</td>
<td>0.0</td>
<td>69.56</td>
<td>30.43</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>LDR x OMF109-3</td>
<td>16</td>
<td>0.0</td>
<td>62.50</td>
<td>37.50</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF109-3 x LDD</td>
<td>61</td>
<td>0.0</td>
<td>88.52</td>
<td>8.19</td>
<td>3.28</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF109-3 x LDN</td>
<td>7</td>
<td>0.0</td>
<td>71.42</td>
<td>28.57</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF109-3 x LDR</td>
<td>8</td>
<td>0.0</td>
<td>62.50</td>
<td>37.50</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF156 x LDD</td>
<td>76</td>
<td>1.32</td>
<td>78.95</td>
<td>19.73</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF156 x LDN</td>
<td>28</td>
<td>0.0</td>
<td>42.86</td>
<td>57.14</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OMF156 x LDR</td>
<td>50</td>
<td>0.0</td>
<td>70.00</td>
<td>30.00</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Gene transfer from one species to another is prevented by many isolation mechanisms in nature; these include spatial isolation, non-overlapping flowering time, and cleistogamy, which act as barriers only in nature but not in breeding programs (Hermsen, 1992). On the other hand, various pre-fertilization and post-fertilization barriers due to genetic differences hinder the process of bringing different species together even in breeding programs. It was found that successful crosses could be made between *L. floccosa* and *L. alba* individuals that belong to the same section Inflexae. The reproductive isolation mechanisms evolved to keep these two species apart in nature were non-overlapping flowering time and partial cleistogamy in *L. floccosa*. However, both these barriers in nature could be overcome in the breeding program. In crosses between the species *L. douglasii* and *L. alba* that belong to different sections, non-overlapping flowering time was the only obvious barrier in nature. However, detailed studies have shown that nature has ensured the separation of these two species by evolving pre-fertilization barriers such as lack of pollen-pistil recognition and defective pollen tube growth. Hence it is equally difficult even in breeding programs to bring these two species together.
Successful hybrid production between LFP and OMF109-3 has given us the hope of utilizing species from the secondary gene pool of Limnanthes for improvement of cultivated meadowfoam. However, failed germination of hybrid seeds from other intra-sectional crosses listed in Table 1 demonstrates that the seed set in inter-specific and inter-generic crosses is a product of interaction between both, species as well as the individual genotypes involved (Bozorgipour and Snape, 1990). Similar observations were reported by Choudhari and Joshi (2001) in Brassica where they found a marked difference in crossability of two genotypes (RBT63 and RBT58) of B. tournefortii with one B. rapa genotype (BRBS). RBT63 produced 3.7% hybrids when crossed with BRBS whereas only 0.9% hybrids were produced when RBT58 was crossed with BRBS. Rescuing embryos through embryo culture in intra-sectional crosses where seeds failed to germinate was beyond the scope of this study. However, the technique of embryo rescue for immature embryos is well documented in meadowfoam (Southworth and Kwiatkowski, 1995) and should be employed in order to recover these intra-sectional hybrids.

The presence of characteristics from both parental species in the hybrid indicates that the F₁ plants had genes of both parents combined. The hybrids in the present study showed considerably reduced pollen viability (30-33%) as compared to both parents. Many of the previous investigations involving interspecific or
distant hybrids attributed the phenomenon of reduced pollen viability or male sterility to various meiotic irregularities such as prevalence of univalents accompanied by erratic segregation in *Cuphea* (Ali and Knapp, 1995) and *Brassica* (Choudhari and Joshi, 2001); chromosomal bridges, fragments and sticky chromosomes in *Pisum* (Nirmala and Kaul, 1994); absent or defective cytokinesis in *Glycine* (Skorupska and Palmer, 1990), while cell fusion was considered to be one of the causes of male sterility in *Pennisetum* (Rao et al., 1989). Such meiotic abnormalities in inter-specific hybrids arise due to structural differences between the chromosomes of parental species leading to abnormal microspore development. Perfect chromosome pairing with five bivalents in each cell observed for the hybrid in this study suggests that the genome of *L. floccosa* and *L. alba* are homologous. Hence the low fertility of hybrids is not due to structural differences in the chromosomes of parental species as shown by Adayapalam et al. (1999) in *Lathyrus*. However, reduced homology between the chromosomes of the two different species was evident in present study by the reduced number of ring bivalents in hybrids as compared to both parents, leading to lower chiasma frequency.

There are a number of causes for male sterility in higher plants. The male sterility genes exhibit a wide array of action ranging from complete absence of the androecium to the production of inviable pollen grains due to abnormal microsporogenesis. They act with marked precision on various stages of microsporogenesis (pre-meiosis, meiosis, post-meiosis) and prevent the normal
development of sporogenic tissue, tapetum cells, pollen mother cells and microspores (Singh, 1993). As no meiotic irregularities were observed in this study, the mechanism leading to male sterility or pollen inviability must be operating in post-meiotic stages of microspore development. Post-meiotic degeneration of microspores was also observed by Chhabra et al. (1997) in Pennisetum while studying the effects of different cytoplasms on microsporogenesis and anther development using iso-nuclear A-lines. Degenerated pollen grains in a morphologically normal anther is considered as gametophytic male sterility (Kaul, 1988).

As both of the parental species involved in hybridization showed full male fertility and the hybrid showed 3:1 ratio of dead to viable pollen, we speculate that pollen death may be occurring due to the interaction between nuclear genes and the cytoplasm during pollen development. The nucleo-cytoplasmic interaction was also very well documented in genus Limnanthes by Kesseli and Jain (1984) to account for gynodioecy observed in natural populations of L. douglasii. The most plausible explanation for the 3:1 ratio for dead to viable for the hybrid (LFP x OMF109-3) in this study is the possible involvement of two unlinked loci (from L. floccosa), which are required for compatible (viable) development of a microgametophyte (pollen grain) in the cytoplasm of L. floccosa as in the case of maize T-cytoplasm, sunflower PET-cytoplasm and onion T-cytoplasm (Schnable and Wise, 1998). However, further backcrosses are necessary to substitute the nuclear genome of L.
floccosa with L. alba into L. floccosa cytoplasm to understand the exact genetic nature of the phenomenon observed in this study.

**Inter-sectional crosses**

The results of the present study suggest that the barriers to inter-sectional hybridization are bi-lateral where inhibition mechanism operates at four stages indicated in Fig. 3.6 with some degree of independent control. The stigmatic and stylar inhibition to normal pollen development reported in this study is in agreement with previous studies carried out to analyze the pre-fertilization barriers in non-compatible crosses such as Williams et al. (1982) in the Ericaceae family, Kandasamy et al. (1994) in interspecific crosses between Brassica and Arabidopsis, Rego et al. (2000) in yellow passion fruit, Tamari et al. (2001) in Turneracea family.

The presence of either germinated or un-germinated pollen on the stigmatic surface (Fig. 3.4, 3.5A) in all the non-compatible crosses in this study suggest that the wet stigma of Limnanthes (classified according to Heslop-Harrison and Shivanna (1977), does support pollen adhesion to some extent due to nutrient rich exudates that promotes pollen hydration in a somewhat indiscriminate manner (Wilhemi and Preuss, 1997).

The mechanism for inhibiting the penetration of the stigmatic surface by the pollen tube was more specific. The rejection response was manifested either by
Figure 3.6. Schematic representation of checkpoints to prohibit the growth of pollen tube in incompatible crosses.

calllose deposition on the stigmatic surface (Fig. 3.5A) adjacent to incompatible pollen or within the germinating pollen tubes, especially at the tip of these rejected pollen tubes (Fig. 3.4). This cell-to-cell reaction between incompatible pollen and papillar cells of the stigma was also observed in previous studies carried out in yellow passion fruit by Rego et al. (2000). They attributed this reaction to a protein produced by the S-locus and categorized the inhibition as homomorphic
sporophytic incompatibility. The observation of arrested pollen tube growth with abnormalities mostly in the lower part of the style in our study is also in agreement with previous study by Gaget et al. (1984). Gaget et al. (1984) reported abnormal pollen tube growth arrested in the lower part of styles while studying inter-sectional crosses in *Populus*. In the case of yellow passion fruit, stylar inhibition arrested pollen tubes in the upper one third of the style (Rego et al., 2000) for self-incompatible crosses. Gaget et al. (1984) and Rego et al. (2000) both speculated on the presence of an interaction between pollen tube and stylar components as in the classical gametophytic self-incompatibility system. All of the species in the present study are self-compatible and no evidence for presence of an S-gene controlled self-incompatibility system of either the sporophytic or gametophytic type has been presented yet for genus *Limnanthes*. However, inhibitory responses in the form of either callose deposition or arrested pollen tube growth suggest the presence of an S-allele variant that has lost the function of self-recognition. Considering the complex structure of the S-locus, Trognitz and Schmiediche (1993) suggested a model for a common basis of intra-specific self-incompatibility and inter-specific incompatibility. They suggested that the S-locus has two major components-style and pollen specific. Stylar protein inhibits the pollen tube growth, and vice versa. The process of inhibition is modified based on the incompatibility/compatibility system. In the case of self-compatible species, recognition of S-allele does not occur due to missing or non-expression of S in either style or pollen or both. Hence, pollen tube suppresses the stylar products and fertilize the ovule. However, for
incompatible, inter-specific crosses where S alleles are different, the process of recognition is meaningless. The interaction between stylar and pollen products is not affected by either matching or differing factors. In this case varying degrees of inhibition at various sites in the style is observed. The reaction depends upon the ability of the two components to inhibit each other. Although this model is based on the assumption of interaction between style and pollen genotypes, it seems to fit well for the phenomenon observed in our study. Speculation of presence of modified S alleles seems also justifiable because of the close relationship of genus Limnanthes with crucifers (Rodman et al., 1998). The crucifer family includes self-incompatible genera, such as Brassica, and self-fertile genera such as Arabidopsis. The self-incompatibility system is controlled genetically by the multifunctional gene complex of the S locus in Brassica is very well studied (Boyes et al., 1997). Conner et al. (1998) identified an Arabidopsis homeolog of the Brassica S locus region. However, no sequence similar to the Brassica S locus gene those are required for the self-incompatibility responses were detected in the Arabidopsis genome. Thus absence of self-recognition genes at the S locus in Arabidopsis is a very well documented phenomenon, which might also be true in the genus Limnanthes.

The species belonging to section Reflexae have considerably larger styles than those of species belonging to section Inflexae as mentioned earlier in this study. However, crosses in both directions i.e. involving short styled female parent (Inflexae) with long styled pollen parent (Reflexae) and vice versa failed. Also, in
all the non-compatible crosses studied, the major proportion of pollen tube arrested was in the lower part or near the base of the style. These two observations rule out the possibility of either lack of intrinsic ability of pollen tubes to grow beyond the length of their own pistils as suggested by Shivanna (1996) or pollen from long styled individuals can easily reach and fertilize the ovules of short styled individuals as shown in Lycopersicon (Liedl et al., 1996); Leucaena (Sorensson and Brewbaker, 1994) and Bromeliads (Vervaeke et al. 2001).

The various abnormalities such as lack of direction, arrested pollen tube growth or even burst pollen tubes observed in this study basically suggest the lack of signals or guidance cues necessary to direct the pollen tube into the transmitting tissue of the style and finally towards the ovule. Although the exact nature of these guidance cues is yet to be known, glycoproteins secreted by styles (Cheung et al., 1995); carbohydrate-rich molecules secreted by ovules (Herrero, 2000), and a high calcium ion concentration in synergid cells (Cheung and Wu, 2001) have all been suggested as possible guidance cues for pollen tubes as they approach the ovules. However, specificity must exist among guidance cues. As pollen tubes from one species do not usually target ovules of other or related species (Shimuzu and Okada, 2000). A single genetic mechanism cannot be used to explain the phenomenon observed in this study. It is equally possible that the pollen-pistil recognition in two groups i.e. long-styled individuals of section Reflexae and short-styled individuals of section Inflexae may have evolved separately in each floral morph. Thus both the long style and the short styled species may not share a
common molecular basis involving the recognition of the pollen by the style (Lloyd and Webb, 1992).

Pre-fertilization barriers can be overcome using methods such as bud or delayed pollination combined with embryo rescue techniques if necessary. There are equal numbers of successful and unsuccessful examples for overcoming the pre-fertilization barriers by bud pollination (Lewin, 1993; Westwood et al., 1997; Kandasamy et al., 1994). The success and failure largely varies from species to species. Hence, further attempts to access the diversity present in section Reflexae can be made using bud pollination for the promising crosses mentioned earlier in this study.

In conclusion, the mechanisms evolved for isolation of species in the two sections of genus *Limnanthes* are different. Hence the diversity present within the section Inflexae seems to be more accessible for enhancing meadowfoam improvement further by not only combining the best genes from different species but also recovering male sterile cytoplasm in future backcross generations. However, further attempts in terms of overcoming pre-fertilization barriers are necessary to access the diversity present in section Reflexae.
References


CHAPTER 4

CONCLUSIONS

Meadowfoam (*Limnanthes alba* Benth.) is an annual oil seed crop native to southern Oregon, California and British Columbia (Mason, 1952; Kalin, 1971; Jain, 1986). Meadowfoam was originally developed as a source of chemical feedstocks for industrial markets (Isbell, 1997). The major fatty acids found in meadowfoam seed oil are cis-5-eicosenoic (20:1 Δ 5), erucic (22:1 Δ 13), cis-5, cis-13-eicosenoic (22:2 Δ 5 Δ 13), and cis-5-docosenoic (22:1 Δ 5) acid (Earle et al., 1959; Bagby et al., 1961).

The consumption of erucic acid (22:1 Δ 13), a fatty acid found in the seed oils of meadowfoam, rapeseed (*Brassica napus* L.), and several Brassicaceae, has been linked to increased risk of heart disease (Borg, 1975; McCutcheon et al., 1975). The United States Food and Drug Administration currently permits dietary oils containing no more than 2% of erucic acid for human consumption (Federal Register, 1985). Whereas, European Union Food Commission allows 5% erucic acid of total fatty acids in rapeseed oil meant for human consumption (Official Journal of the European Community, 2001).

The development of low erucic acid meadowfoam cultivars may open the doors of the edible, medicinal and pharmaceutical markets for meadowfoam seed oil. We
used the approach of induced chemical mutagenesis using EMS for the
development of low erucic meadowfoam due to the lack of natural variation (the
lowest erucic acid concentration in meadowfoam reported from nature is 6.5% by
Knapp and Crane, 1995) for erucic acid in meadowfoam.

The low erucic acid mutant identified in our study showed a three-fold decrease
in erucic acid content (~3 % vs ~9 %) when compared with the wild type
meadowfoam. The mutant phenotype does not seem to be caused by elongase
lesions as that of Brassica and Arabidopsis (Roscoe et al., 2001; James and Dooner,
1990; Lemieux et al., 1990 Kunst et al., 1992) as the total concentration of VLCFs
was not reduced. The performance of the mutant differed in different genetic
backgrounds. F2 progeny of Wheeler x LE76 was used for mapping the genetic
factors underlying the erucic acid concentration differences between the mutant and
wild type meadowfoam. The linkage map comprised of 54 SSR loci was
constructed. A total of three QTL were identified for the difference in the erucic
acid concentration, whereas five QTL were identified for the difference in dienoic
acid concentration between the mutant (LE76) and wild type (Wheeler)
meadowfoam. The development of near-isogenic lines (NILs) and low erucic acid
cultivars should proceed more rapidly and efficiently using flanking DNA markers
to introgress the underlying mutation(s) once the QTL are validated.

Cultivated meadowfoam (Limnanthes alba Benth.) is one of eight species of
Limnanthes split between two sections: Inflexae (L. alba, L. gracilis, L. floccosa,
and L. montana) and Reflexae (L. douglasii, L. bakeri, L. striata, and L. macounii).
Various subspecies of *L. alba*, *L. gracilis*, *L. floccosa*, and *L. douglasii* have been described (Mason, 1952). The domestication of meadowfoam began around thirty years ago (Crane and Calhoun, 1974). The domestication process has been entirely based on *L. alba*. The secondary and tertiary gene pools have not been important to the domestication process and have not supplied diversity for meadowfoam breeding. Very few attempts have been made previously to make distant hybrids in meadowfoam. (Mason, 1952). No successful inter-sectional hybrid has been reported till date in meadowfoam. Also, intra-sectional, inter-specific hybridization involving *L. alba* and two sub-species of *L. floccosa* viz., *pumila* and *bellingeriana* has never been reported. The specific aim of this study was to assess the feasibility of producing hybrids between cultivated meadowfoam (*L. alba*) and *L. floccosa* ssp. *pumila*, *L. floccosa* ssp. *bellingeriana*, *L. douglasii* ssp. *douglasii*, *L. douglasii* ssp. *nivea*, and *L. douglasii* ssp. *rosea* for the purpose of accessing the genetic diversity in these species and developing cytoplasmic male-sterile lines by substituting the nuclear genome of *L. alba* into alien cytoplasms.

We found that the differential isolation mechanisms are involved within and between sections of *Limnanthes*. Where the barriers of partial cleistogamy and non-overlapping flowering could be overcome and hybrid seed was produced in intra-sectional crosses. Whereas non-overlapping time was found not to be the only barrier involved in making inter-sectional hybrids. Out of four intra-sectional crosses, seeds of only LFP x OMF109-3 geminated. The hybrid showed reduced pollen viability (~30-33 %). However the low fertility of the hybrid was not due to
structural differences in the chromosomes of parental species as the meiotic analysis of PMCs showed normal chromosome pairing and disjunction in metaphase I and anaphase I, respectively. As no meiotic irregularities were observed in this study, the mechanism leading to male sterility or pollen inviability must be operating in post-meiotic stages of microspore development. On the other hand, all the inter-sectional crosses studied showed abnormal pollen tube growth. The mechanism of inhibition of pollen development in inter-sectional crosses was found to operate at two different sites, the stigma and the style. At the stigmatic level, pollen development was arrested either by preventing germination or preventing penetration of germinated pollen due to heavy callose deposition on the stigmatic surface. Pollen tube growth was arrested mainly in lower part of the style due to various abnormalities. Abnormalities observed in this study were disoriented or loss of direction, swollen tip, burst, forked, coiling pollen tubes. Swollen tip pollen tube and loss of directional growth were the most prevalent classes observed in all the incompatible crosses.

Hence it seems that the diversity present within the section Inflexae is more accessible for enhancing meadowfoam breeding further by not only combining the best genes from different species but also recovering male sterile cytoplasm in future backcross generations for efficient hybrid seed production. However, further attempts in terms of overcoming pre-fertilization barriers are necessary to access the diversity present in section Reflexae.
We reported here

i) The development of the first low erucic acid meadowfoam.

ii) The genetics underlying the differences in the erucic and dienoic acid differences in low erucic mutant and wild type in meadowfoam.

iii) Fertility of intra-specific hybrids and pre-fertilization barriers involved in making the intra-sectional hybrids in meadowfoam.


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