Title: Development of Camelina Lines Resistant to Group 2 Herbicides

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Co-PI: Ian Burke

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Graduate Students: Dusty Walsh was originally supported by the project through 2010. Ebrahiem Babiker was then partially supported by the project during 2011.

Technical Support: Ron Sloot

Background: Because camelina is a new crop in North America, farmers and researchers have not optimized its agronomy and many questions remain about how to best produce it economically and sustainably. Weed infestation is currently the greatest limitation for the effective production of camelina. Broadleaf weeds, such as prickly lettuce, kochia, Russian thistle, mayweed chamomile, redroot pigweed, and common lambsquarters, and grasses such as Italian ryegrass, wild oat, and downy brome, are particularly troublesome. At present, there is only one herbicide registered for use in camelina and that herbicide only controls some grass weed species. To successfully integrate industrial oil seed crops into existing rotations in the Northwest, inputs need to be discovered and evaluated not only to protect the new crop, but also to mitigate impacts on other crops grown in rotation. Camelina is highly sensitive to soil residues of Group 2 herbicides, which are widely used in the region, and this has led to herbicide damage in many fields during first attempts to grow camelina. We have therefore identified herbicide insensitive camelina mutants including one that is resistant to both imidazolinones and sulfonyl ureas as described below.

Although camelina has been cultivated in Europe for over 2,000 years for oil and livestock fodder (1), its production in the last century was almost completely displaced by that of canola and oilseed rape. Accordingly, camelina breeding research has been limited. The current varieties are simply a small number of selections from the world camelina germplasm collections chosen for seed yield, seed weight, fatty acid profile, and agronomic characteristics. There is considerable variation in camelina germplasm (2, 3). Plants grow to a height of 30 to 120 cm; plants produce seeds with an average 1000-seed mass between 0.7 and 1.6 g; seed yields range from 2 to 3 t/ha; and oil content ranges from 28% to 42%. This relatively high variation indicates that rapid genetic gains could be made with intensive breeding. In addition, camelina is adapted to a wide range of environmental conditions, especially in semi-arid regions and in low-fertility or saline soils (4), making it a potential crop to grow on marginal land.

Much of the genetic variation that underlies agronomically important traits like oil content and seed yield is complex and is governed by quantitative trait loci (QTL; 5). The integration of DNA markers with conventional plant breeding has enormous potential to improve the efficiency and precision of crop breeding (6, 7). Although genetic and genomic knowledge of camelina is limited (8) the very close relationship between camelina and the model plant Arabidopsis thaliana (9,10) should make it possible to infer candidate genes and markers for beneficial agronomic traits. Rates of crop improvement through genetics are
difficult to predict in a crop like camelina where little public or private effort has been focused, but the considerable variation between lines indicates progress can be made quickly.

**Objectives:** The main objective is to identify lines that are less sensitive to imidazolinone herbicides like Pursuit and Beyond. Pursuit is used widely in legume rotations and Beyond is used increasingly in Clearfield wheat production. A secondary objective is to find lines with increased resistance to sulfonylureas, like Maverick. The ultimate objective is to release these lines to camelina breeders and possibly release a WSU variety so that the herbicide resistant traits are incorporated into commercial cultivars.

**Methods:** To identify mutations in camelina that confer tolerance to group 2 herbicides, we constructed EMS mutagenized populations of the camelina cultivars Calena and Cheyenne. The mutagenized seed was advanced by self-fertilization one generation without selection to allow mutant alleles to become homozygous to better reveal recessive or codominant alleles. Resistant individuals were identified and verified from mutant screens that examined very large populations (roughly $10^7$ M$_2$ plants) in the field with herbicide application. Progeny of potential mutants were then re-examined by re-application of the same herbicide on greenhouse grown seedlings. Lines with verified resistance were intercrossed and crossed to wild-type camelina to examine inheritance of the resistance. The mutant with the highest level of resistance, designated SM4, was originally identified in the Cheyenne background. This was crossed to the cultivar Calena and F$_2$ families from multiple F$_1$ plants were screened in the field for resistance to Pursuit at a 1 oz. active ingredient per acre rate. Seed from approximately 500 individual plants were selected to make F$_3$ families, which were subsequently planted in small plots at Lind in Spring 2011. Approximately 400 individual plants were selected from these plots to make F4-derived families which will be tested in replicated yield plots at Davenport spring 2012.

**Results and Discussion:** Our initial field experiment identified several putative mutants resistant to the herbicide imazethapyr (Pursuit). The putative mutants were transplanted to the greenhouse and seed was recovered from most. We tested the progeny with imazethapyr by spraying recommended field rates (4 oz/acre) to determine which of the families are derived from true herbicide resistant mutations. We also tested progeny seedlings of the putative mutants for tolerance to imazamox (Beyond herbicide). Four of the lines, designated IM1-IM4, showed significant levels of resistance to both herbicides and no morphological abnormalities. One of the mutants (IM1) was from the Cheyenne population and three were from the Calena population. All of the progeny from each mutant appeared to have similar levels of increased resistance to both herbicides indicating the original selection was homozygous for the mutation and that the mutation caused cross resistance. Fertility, seed set and morphology were similar to the parental lines.

We also screened the mutagenized populations to identify resistance to the sulfonylureas herbicide sulfosulfuron (Maverick). Seed from several possible mutants were collected and tested in the greenhouse for resistance. One of the lines, designated SM4, showed increased resistance. As with the IM1-IM4 mutants, seed from the SM4 mutant appeared true breeding for resistance indicating the original mutant plant selected was homozygous for the resistance gene.

The mutants were intercrossed to each other and to Calena. F$_2$ populations of resistant x susceptible crosses were made for all but IM3, where no F$_2$ seed were obtained. Examination of segregation in the F$_2$ populations indicated that the resistances of the IM1, IM2, IM4 and SM4 were caused by single dominant or codominant genes (Table 1). The levels of damage varied among the segregating F$_2$ seedlings so they were initially scored as resistant, susceptible and
intermediate. When the intermediate and resistant classes were combined, all of the mutants segregated 3:1 as expected for a single dominant gene. When the frequencies of the three classes were tested against a 1:2:1 ratio, the IM1 and SM1 progeny showed excesses of seedlings scored as intermediate. Testing progeny of some of these plants scored as intermediate indicated some bred true for resistance, indicating the F₂ plants were actually homozygous. Taken together, the data indicate that resistance segregates as a co-dominant gene, but the homozygous resistant and heterozygous plants are difficult to distinguish.

*Table 1:* Segregation of resistance to imazethapyr in F₂ families derived from resistant by susceptible crosses.

<table>
<thead>
<tr>
<th></th>
<th>Number of Plants</th>
<th></th>
<th></th>
<th></th>
<th>Chi-sq 1:2:1</th>
<th>Chi-sq 3:1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F₂</td>
<td>Total</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Susceptible</td>
<td></td>
</tr>
<tr>
<td>Calena/IM1</td>
<td>809</td>
<td>149</td>
<td>450</td>
<td>210</td>
<td>19.4351*</td>
<td>0.39596</td>
</tr>
<tr>
<td>Cheyenne/IM2</td>
<td>551</td>
<td>117</td>
<td>279</td>
<td>155</td>
<td>5.3303</td>
<td>2.8802</td>
</tr>
<tr>
<td>Cheyenne/IM4</td>
<td>646</td>
<td>145</td>
<td>354</td>
<td>147</td>
<td>5.9628</td>
<td>1.7358</td>
</tr>
<tr>
<td>Calena/SM4</td>
<td>821</td>
<td>173</td>
<td>452</td>
<td>196</td>
<td>9.6796*</td>
<td>0.5558</td>
</tr>
</tbody>
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* indicate Chi-square values that deviate significantly (P<.01) from expected ratios.

F₂ populations from crosses between the different mutants did not segregate for susceptibility or levels of resistance that were noticeably higher than the parent. This indicates that all of the resistances occurred at the same locus.

To better characterize their resistances, lines derived all five mutants were examined with group 2 herbicides from three different chemical classes (Fig. 1). All of the lines showed increased resistance to Imazethapyr and Flucarbazone, but SM4 showed higher levels of resistance. In addition, SM4 showed increased resistance to the sulfosuluron, while the others appeared similarly sensitive to the control plants.
To determine the nature of the mutation in the SM4 line, ALS encoding genes were PCR amplified and sequenced using degenerate primers. Four different genomic clones and 15 different cDNAs were sequenced. Surprisingly, 11 different sequences were identified among the 19 clones (Figure 2). Only one of the genes showed a nucleotide substitution that had been previously observed in yeast and tobacco which gained resistance to group 2 herbicides. This was the SM4-5-cDNA (Fig. 2) which showed a T to C substitution. The substitution caused a phenylalanine to leucine replacement at corresponding amino acid 578 (Figure 3). To determine if this change was unique to the SM4 variant, PCR primers were generated that were specific to the nucleotide substitution. The primers efficiently amplified DNA fragments of the expected size from the SM4 mutant but not from the parental line Cheyenne.

**DNA sequence**

<table>
<thead>
<tr>
<th>Cheyenne</th>
<th>SM4</th>
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<tbody>
<tr>
<td>TTGGCATGTTATGCAATGGGAGGATCGGTCTCTACAAAGCTAACCAGA</td>
<td>TTGGCATGTTATGCAATGGGAGGATCGGTCTCTACAAAGCTAACCAGA</td>
</tr>
</tbody>
</table>

**Amino acid sequence**

<table>
<thead>
<tr>
<th>Cheyenne</th>
<th>SM4</th>
</tr>
</thead>
<tbody>
<tr>
<td>LATIRVENLPVKILILNQHLMVQWMEDRFYKANRAHTYLGPNAAE</td>
<td>LATIRVENLPVKILILNQHLMVQWMEDRLYKANRAHTYLGPNAAE</td>
</tr>
</tbody>
</table>

![Figure 2. Alignment of polymorphic nucleotides from genomic and cDNA clones of the ALS encoding genes from the SM4 mutant.](image)

![Figure 3. Nucleotide substitution in the ALS encoding gene causing the increased resistance in the SM4 mutant. The resulting phenylalanine to leucine substitution is also shown.](image)

In conclusion, four of five mutants were very similar or identical in their responses to the herbicides tested, while the SM4 mutation appeared different and superior for practical purposes. SM4 showed higher levels of resistance to all three chemical classes tested and was the only mutant that showed increased resistance to sulfosuluron. Even the SM4 mutant, however, was not completely resistant to the herbicides when they are sprayed directly on the foliage because the seedlings typically are stunted at least temporarily. The incomplete resistance is probably due in part to the copy number of the ALS gene family in camelina. Eleven different sequences were identified in the SM4 line. Although this line was derived from a single M2 plant, it is possible it is heterozygous for some family members. The numbers of sequences generated, however, indicates there are at least six different ALS genes in camelina. Since most of the genes were identified as cDNA sequences, it appears that most of these genes are actively transcribed.

Since the SM4 mutant is partially resistant to three subclasses of the group 2 herbicides this is now the favorite mutant and is being bred for release. This mutant occurred in the
Cheyenne background and we have crossed it to Calena. Several large F2 families were planted in the field in June and sprayed with Pursuit. Seed from 470 vigorous plants were harvested and planted in duplicate plots at Lind in late winter (2011). Seeds from single plants were again selected and will be planted in yield plots this spring.

**Impact/Potential Outcomes:** Camelina varieties resistant to group 2 herbicides will help adoption of this crop in all rainfall regions. We hope to release a WSU cultivar in 2013 and we have already sent seed of the original mutant in the Cheyenne background to two different commercial breeding programs. We expect the SM4 mutation to be incorporated into several widely grown cultivars in the future, and expect this to reduce risks associated with camelina production in most regions.

**Affiliated projects and funding:** We have recently submitted a proposal as a subcontract to Colorado State University to the USDA BRDI program ($7M total) that will fund continuation of this work. In addition, we are currently cooperating with the University of Idaho on writing a Biofuel Feedstock Coordinated Agricultural Project for approximately $10M.

**Publications:**


Posters were presented in 2010 and 2011 at ASA-SSSA-CSSA meetings, the 2009 PNDSA meeting, the 2010 Clean Energy meeting.

A formal germplasm release publication is being planned for submission in late 2012.

Research agreements have been agreed upon with two companies and a licensing agreement is being drafted.

**Proposed Future Research/Extension:** Our main objective is to get the ALS-SM4 mutant allele homozygous into a good genetic background that can be released to breeders or grown as a variety. We will:

1) Select good yielding homozygous lines from our nursery in Lind.
2) Write and submit a germplasm release paper.
3) Write and submit a paper describing the mutants and their inheritance.
4) Amplify sufficient seed to begin field herbicide trials to test relative resistance to levels of group 2 herbicide carryover. These will be conducted in two locations and will begin with fall 2011 soil treatments and spring 2011 plantings at Davenport and Pullman. The cultivar Cheyenne will be compared to favorite ALS-SM4 lines.

**References:**


