GENETIC INVESTIGATION OF WILD OAT WITH ACETYL-COA CARBOXYLASE GENE SEQUENCE VARIATION

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Genetic Investigation of Wild Oat with Acetyl-CoA Carboxylase Gene Sequence Variation

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ABSTRACT

Wild oat (Avena fatua) is a grass weed species that infests cropland. Common post-emergent herbicides for controlling wild oat are those that inhibit acetyl-CoA carboxylase (ACCase) and acetolactate synthase (ALS). Variation among plastidic ACCase gene sequences of herbicide-susceptible wild oat biotypes USDA96 and KYN119 revealed ACCase gene diversity consistent with possible separate diploid ancestry, with KYN119 more likely to share diploid ancestry with herbicide-resistant UM1. USDA96 wild oat shows low-level tolerance to the ALS-inhibiting herbicide flucarbazone, and the inheritance of this tolerance was studied among F3 families generated from KYN119 and USDA96 reciprocal crosses. Quantitative inheritance was observed at the below-label flucarbazone rate of 1.81 g ai/ha. Some F3 families had higher post-treatment main shoot dry weights than either parent, which may be due to heterosis and/or genetic contributions from both parents. No evidence for association between the Acc1;1 ACCase gene and low-level tolerance to flucarbazone was observed.

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CHAPTER 1. INTRODUCTION

Weeds are unwanted plants that compete with agriculturally important crops and reduce their yield and quality (Rao 2000). Several methods have been utilized to control weed growth including chemical, organic, mechanical and physical tools. Chemical control with herbicides has been the most popular method to control weeds due to lower labor demand compared to other methods. Herbicides are chemicals that kill or suppress unwanted weedy plants. Many herbicides are selective and kill the weedy plants leaving the crop unharmed. Some herbicides are known to imitate plant hormones and interfere with weed growth whereas others inhibit important enzymes. Herbicides can be classified according to their activity, use, mechanism of action and chemical family. Depending on activity, herbicides are classified as contact or systemic. Contact herbicides destroy plant tissues contacted by the chemical, whereas systemic herbicides are translocated through the plant and can be more effective than contact herbicides. According to their use, herbicides are classified as pre-emergent or post-emergent. Pre-emergent herbicides are applied to the soil before the growth of weedy plants to prevent their germination or emergence. Post-emergent herbicides are applied after the crops or weeds have emerged. Herbicides are classified according to their mechanism of action, which indicates the enzyme or the biochemical step affected in the plant. Finally, herbicides are categorized into chemical families based on similar chemical properties and mechanism of action. Many weeds have evolved resistance to various herbicides due to the persistent use of herbicides, which has resulted in selection pressure towards herbicide-resistant populations.

Avena fatua L. (wild oat) is a weedy plant species of the Aveneae (oat) tribe in the Poaceae (grass) family. It was introduced into North America as an impurity in food and feed by

Europeans. It is a major weed that infests some of our major food crops such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and oat (*Avena sativa* L.) and also interferes with several other crops such as soybean (*Glycine max* L.) and flax (*Linum usitatissimum* L.). The intensity of competition varies with the type of crop. Wild oat is highly competitive with wheat and may reduce wheat yield significantly with a few plants (Anonymous 2011).

To control the growth of wild oat, several pre-emergent and post-emergent herbicides with different mechanisms of action have been utilized. Some of the most common wild oat herbicides are acetyl-CoA carboxylase (ACCase)-inhibiting herbicides and acetolactate synthase (ALS)-inhibiting herbicides. Acetyl-CoA carboxylase-inhibiting herbicides act by inhibiting the essential enzyme ACCase involved in fatty acid synthesis, which leads to harmful effects within plants (Burton et al. 1991). Christoffers et al. (2002) indicated that a major cause of resistance to ACCase-inhibitors is a non-synonymous point mutation in the ACCase target site. Other herbicides inhibit the enzyme ALS, which is involved in the synthesis of essential amino acids valine, leucine and isoleucine, leading to plant starvation (Durner et al. 1990). Much of the resistance to ALS inhibitors in present day wild oat is suspected to be due to a non-target-site resistance mechanism, herbicide metabolism, where plants are better able to metabolize herbicide to non-toxic forms compared to susceptible wild oat (Nandula and Messersmith 2001). The efficacy of ACCase and ALS inhibitors has decreased due to the evolution of resistance in some populations. The persistent use of these herbicides has resulted in the selection of populations with resistance. It has also been noted that the use of low rates of these herbicides has resulted in the evolution of herbicide resistance (Busi and Powles 2009).

UM1 is a well-studied wild oat biotype (Heap et al. 1993), which was demonstrated to carry a modified form of ACCase that is resistant to herbicide inhibition, indicating that

resistance was associated with an ACCase gene mutation (Shukla et al. 1997). Later, it was determined that an isoleucine to leucine mutation in ACCase confers herbicide resistance in UM1 wild oat (Christoffers et al. 2002). USDA96 and KYN119 biotypes are confirmed to be susceptible to ACCase-inhibiting herbicides, while USDA96 also exhibits low-level tolerance to the ALS-inhibitor flucarbazone (M. J. Christoffers, personal communication). The research described herein focused on determining the inheritance of low-level flucarbazone tolerance by studying differences in herbicide response among parents and F_2-derived F_3 families of USDA96 × KYN119 and reciprocal crosses. Also in this study, the first sequence information for the carboxyl transferase (CT) domain of KYN119 plastidic ACCases and additional sequence information of USDA96 plastidic ACCases was obtained. The long term goal of this study is to identify factors that contribute to genetic diversity in wild oat and to characterize genes that may influence optimum management of wild oat populations.

CHAPTER 2. LITERATURE REVIEW

Wild oat is a weedy plant species of the oat tribe in the grass family. Wild oat was introduced into North America by European settlers as an impurity in seed and feed. It prefers cool temperatures and moist soil (Sharma and Vanden Born 1978). Wild oat can germinate at relatively low soil temperatures (5 C), which gives it a temporal advantage to grow and establish in field populations early in the growing season. In 1953, it was rated as the most troublesome weed for almost the entire cultivated region of the Northern Plains of North America including the prairie provinces of Canada, the northern half of South Dakota, the northwest fringe of Minnesota, and North Dakota and Montana (Wood 1953). In 1970, the total area estimated to be infested with wild oat in Canada and the U.S. was over 25 million ha (Nalewaja 1970). It is a major weed of farming systems and is especially a problem among grass crops such as wheat and cultivated oat, where it competes for resources and reduces crop yield and quality. Wild oat competition varies with the type of crop, and in wheat, it is highly competitive and can dramatically affect yield, even with small infestations (Anonymous 2011). Based on yield losses due to wild oat competition, competitive abilities of various crops have been reported (Dew 1972, Dew and Keys 1976). Among studied crops, flax is known as a poor competitor, rapeseed (*Brassica napus* L.) as an intermediate and barley as a strong competitor. A number of factors determine the intensity of competition between the crop and the weed such as the relative date of emergence, relative rate of crop/weed growth, density of wild oat plants and the influence of the crop on competition. Competition is more intense leading to greater yield losses when the weed emerges before the crop. Intense competition begins at the two- to three-leaf stage of wild oat (Chancellor and Peters 1976). It has been found that due to competition from wild oat, the

protein content of wheat and barley decreases (Friesen et al. 1960), and the oil content of flax seed is reduced (Bell and Nalewaja 1968).

2.1. Genomics and Evolution of Wild Oat

To develop optimum control methods for weeds, it is useful to have a good knowledge of their genomic structure, origin and evolution. Relatively little research has focused on the genomics of wild oat in comparison with other *Avena* species. Wild oat is an allohexaploid (2n=6x=42) and consists of A, C and D genomes similar to other hexaploid *Avena* species. The chromosomes of wild oat have been found to have several inter- and intra-genomic translocations, some of which make it unique from other *Avena* species (Yang et al. 1998). Five intergenomic translocations have been found in allotetraploid oat (*Avena maroccana* Gdgr.) and 18 intergenomic rearrangements have been found in allohexaploid oat (*Avena sativa* L.) (Leitch and Bennett 1997).

There are various diploid, tetraploid and hexaploid species within the genus *Avena*. The formation of present-day wild oat and other hexaploid *Avena* species is hypothesized to have involved hybridization of two diploids (A genome and C genome), followed by doubling of the chromosome number to form a tetraploid (AC genomes) (Figure 1) (Rajhathy and Thomas 1974). The identity of the tetraploid progenitor of hexaploid oat and wild oat is not known, but has been proposed to be *Avena insularis* Ladiz., *Avena maroccana*, or *Avena murphyi* Ladiz. (Nikoloudakis and Katsiotis 2008). This tetraploid then likely hybridized with a third diploid (D genome) to form an initial ACD triploid, followed by doubling of the chromosome number resulting in hexaploid wild oat or its progenitor (AACCDD).

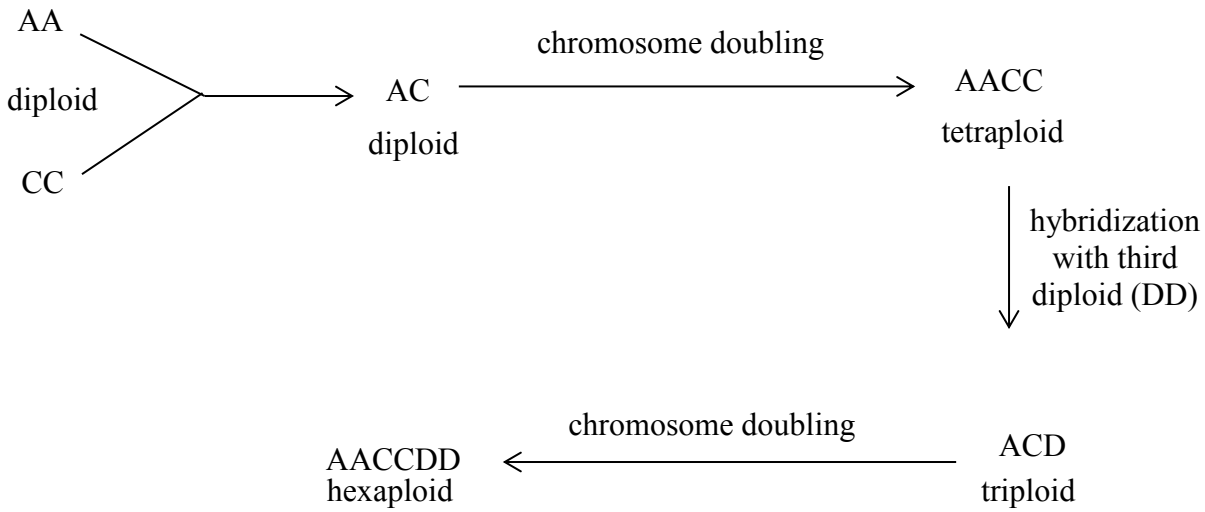

Figure 1. Proposed formation of the wild oat allohexaploid. This is one possibility but it is not clear if AACC or DDCC formed first.

Despite the proposal of AACC tetraploid involvement in the evolution of hexaploid *Avena* species by Rajhathy and Thomas (1974), it has not been clearly proven as to which genome (A or D) was involved in formation of the tetraploid progenitor and which resulted in the formation of the hexaploid. Because there are no known D-genome *Avena* diploids and because the A and D genomes are very similar (Linares et al. 1998), the A and D genomes are often considered together as A/D genomes. In *Avena* species, A and D are more similar to each other than to the C genome (Leggett and Markland 1995a, 1995b). Hybridization was not seen on A- and D-genome chromosomes when probed with DNA from C-genome diploid species, whereas these chromosomes probed with DNA from A-genome diploid species produced hybridization to all 14 A/D chromosomes.

2.1.1. Polyploidy and multiple-polyploidization

Polyploidy is a common speciation mechanism in the plant kingdom and is characterized by the presence of more than one nuclear genome per cell (Leitch and Bennett 1997). There are two major categories of polyploids; autopolyploids and allopolyploids. Autopolyploids are species with multiple chromosome sets from the same species. Allopolyploids result from an interspecific hybridization of two fully differentiated genomes (Stebbins 1947). Polyploidization results in duplication of genetic loci, and allopolyploidy results in the creation of homeologous loci contributed by different donor taxa at the time of polyploid formation (Caldwell et al. 2004). Polyploidization events are important sources of genetic diversity that enable polyploids to adapt to new ecological niches and to enhance their agricultural importance (Levin 1980; Osborn et al. 2003). Polyploids exhibit higher levels of genomic diversity than their progenitors and genome reconstruction events such as intergenomic translocations are a characteristic trait.

Multiple polyploidization events and genome origins may be common in allopolyploid evolution (Soltis and Soltis 1995). More than one origin of hexaploid wheat was indicated by α-amylase isozyme patterns in wheat relative to *Triticum tauschii* (Coss.) Schmal., its D-genome progenitor (Nishikawa et al. 1980). More polymorphism has been observed among *T. tauschii* accessions than among wheat accessions (Kam-Morgan et al. 1989), suggesting that the progenitors of wheat contain more genetic variability than hexaploid wheat itself (Talbert et al. 1998). It has been concluded by Caldwell et al. (2004) that both hexaploid wheat and jointed goatgrass (*Aegilops cylindrical* Host) originated repeatedly with at least two distinct progenitors contributing to the formation of the D genome in both species.

2.2. Herbicides and Their Resistance Mechanisms

Herbicides are chemicals utilized to control weeds. They kill weedy plants or inhibit their normal growth. They are distinguished by activity, use, chemical family and their mechanism of action. Several pre-emergent and post-emergent herbicides with different mechanisms of action are used to control the growth of wild oat. Some of the most common include the ACCase-inhibiting herbicides and ALS-inhibiting herbicides. Due to the persistent use of herbicides, the frequency of herbicide resistance in weed populations has increased. Herbicide resistance is the capacity of a plant species to survive and reproduce after exposure to a dose of herbicide otherwise lethal to the wild type and this resistance might be naturally occurring or induced by techniques such as genetic engineering or selection (Anonymous 1998). Herbicide resistance is broadly classified as target-site or non-target-site resistance.

2.2.1. Target-site resistance

Target sites are enzymes, proteins and other places in plants where herbicides interact and bind. When there is a disruption in the herbicide binding site, the herbicide may not be able to bind to the target's active site. The disruptions are usually due to a non-synonymous point mutation occurring at the binding site. These mutations change the amino acid sequence of the protein thereby destroying the interaction between herbicide and the protein.

2.2.1.1. ACCase inhibitors

The essential enzyme ACCase is required for fatty acid synthesis in plants and its inhibition leads to plant death. Plants have ACCase in both their cytosol and plastids, with grasses typically having multifunctional eukaryotic plastidic ACCases coded by nuclear genes

distinct from the genes of cytosolic ACCases (Konishi et al. 1996). Plastidic and cytosolic ACCases are biotinylated enzymes that catalyze the carboxylation of acetyl-CoA to produce malonyl-CoA. This reaction is the initial step of fatty acid synthesis in plastids whereas cytosolic ACCases produce malonyl-CoA for the elongation of fatty acids (Sasaki et al. 1995). The carboxylation of acetyl-CoA is a two-step process involving the carboxylation of biotin in the first step and the transfer of the carboxyl group from biotin to acetyl-CoA in the second step:

(a) enzyme-biotin + HCO_3^- + ATP → enzyme-biotin-CO_2^- + ADP + Pi

(b) enzyme-biotin-CO_2 + acetyl-CoA → malonyl-CoA + enzyme-biotin

where (a) is the reaction at the carboxylation site, and (b) is the reaction at the carboxyl transferase (CT) site.

The ACCase enzyme complex has three functional domains: biotin carboxylase (BC), biotin carboxylase carrier protein (BCCP) and carboxyl transferase (CT). Step (a) takes place at the BC domain and step (b) takes place at the CT domain. The BCCP carries the biotin cofactor and is involved with the movement of carboxylated biotin between the BC and CT site. Herbicidal inhibitors of ACCase block the transfer of the carboxyl group to acetyl-CoA, thereby limiting malonyl-CoA production and perhaps inhibiting the formation of cell membranes (Burton et al. 1991; Rendina et al. 1990). Efficacy of these herbicides has decreased due to the development of herbicide resistance in some populations mainly because of the persistent use of herbicides. It is known that widespread use of herbicides has selected for resistant populations in more than 200 weed species (Heap 2012).

Herbicides are known to target specific enzymes, and random mutations provide resistance due to amino acid substitutions that reduce herbicide binding to the target site. Herbicide resistance is a result of evolution and its consequences depend on certain factors that

are genetic, biological, herbicidal or operational. Genetic factors include the inheritance, frequency, number, dominance and fitness cost of resistance genes. Important biological factors include whether the species is cross-pollinated or self-pollinated and also seed production tendency, seed longevity in the seed bank, and seed or pollen movement capacity. Herbicidal factors are dependent on the herbicide chemical structure, site of action and residual activity. Operational factors include the dosage of herbicide and environmental factors (Powles and Yu 2010).

Experiments were conducted to study pollen mediated gene flow in wild oat in order to determine the effect of pollen movement on the spread of herbicide resistance genes in this species. Murray et al. (2002) used the ACCase-inhibitor-resistant biotype UM1 as a pollen donor and the ACCase-inhibitor-susceptible biotype UM5 as a pollen acceptor. Hybrid progeny were identified utilizing the herbicide resistance trait as a marker. When in spring wheat, the mean wild oat outcrossing rate was 0.08% at low and 0.05% at high wild oat densities, respectively. In less competitive flax, the mean wild oat outcrossing rate was 0.07% at low and 0.16% at high wild oat densities, respectively. It was concluded that the contribution of pollen movement driving resistance evolution and the spread of resistance in wild oat populations would be relatively negligible compared with resistant seed production and dispersal from resistant plants. Herbicide resistance in the well-studied UM1 wild oat biotype (Heap et al. 1993) was demonstrated to be the result of a modified form of ACCase that was resistant to herbicide inhibition, indicating that resistance was associated with an ACCase gene mutation (Shukla et al. 1997). Later, it was determined that an isoleucine to leucine mutation in ACCase confers herbicide resistance in UM1 wild oat (Christoffers et al. 2002). This substitution has resulted in resistance to many ACCase herbicides within aryloxyphenoxypropionate (APP),

cyclohexanedione (CHD) and phenylpyrazoline (PPZ) chemical groups (Powles and Yu 2010). Plastidic ACCase sequences of a confirmed susceptible wild oat, USDA96 (Jacobsohn and Andersen 1968), were compared to those of UM1 wild oat to identify the resistance mutation. In addition, Christoffers et al. (2002) identified more ACCase sequence differences between USDA96 and UM1 than just the point mutation conferring herbicide resistance in UM1.

2.2.1.2. ALS inhibitors

Acetolactate synthase is the first enzyme in the biosynthetic pathway for synthesis of the branched-chain amino acids valine, leucine and isoleucine (Durner et al. 1990). Acetolactate synthase leads to the formation of acetohydroxybutyrate by catalyzing the conjugation of ketobutyrate with pyruvate and also the conjugation of two molecules of pyruvate to form acetolactate. It is a target of many herbicides including those of the sulfonylurea (SU) (Chaleff and Mauvais 1984), imidazolinone (IMI) (Shaner et al. 1984), sulfonylaminocarbonyltriazolinone (SACT) (Santel et al. 1998), triazolopyrimidine (TP) (Jabusch and Tjeerdema 2008) and pyrimidinyl-thiobenzoate (PTB) chemical families. Interference with the synthesis of the important amino acids mentioned above leads to starvation in plants and ultimately stops DNA synthesis. This also hampers phloem transport in plants (Hall and Devine 1993). There are likely other toxic effects associated with ALS inhibition that, along with the lack of branched chain amino acids, cause plant death. The exact cause of toxicity remains unclear. These herbicides are known to control many weed species and have low mammalian toxicity. As a result, they are used extensively worldwide (Powles and Yu 2010).

A recent study by Beckie et al. (2012) provided the first report of ALS target-site mutations in *Avena* species and four previously undocumented *Acc1* (plastidic ACCase) mutations. The study was performed to investigate target- and non-target-site resistance

mechanisms in four ACCase-inhibitor resistant, four ALS-inhibitor resistant, and eight ACCase- and ALS-inhibitor cross-resistant wild oat populations from western Canada. An *Acc1* mutation was found in eight of the twelve ACCase-inhibitor resistant populations. Two of the twelve ALS-inhibitor resistant populations showed an ALS target-site mutation, with substitution of serine 653 to threonine being the identified cause of resistance. The study also included the treatment of seedlings of one *Acc1* target-site mutant population and four populations with no ACCase or ALS mutation, with the cytochrome P450 monooxygenase (P450) inhibitor malathion followed by application of one of four ALS- or ACCase-inhibiting herbicides. The results of the study revealed control or suppression of the populations, suggesting the involvement of the non-target-site cytochrome P450 enzyme system in causing resistance to both ACCase and ALS inhibitors.

2.2.2. Non-target-site resistance mechanism

Besides target-site alterations, herbicide resistance can be the result of one or more mechanisms that minimize the amount of active herbicide reaching the target site. These non-target-site mechanisms involve decreased herbicide penetration, decreased herbicide translocation and increased herbicide metabolism. Non-target-site resistance to ALS-inhibiting herbicides in wild oat could be due to herbicide metabolism (Nandula and Messersmith 2001), which is a powerful and widely occurring resistance mechanism.

2.2.2.1. Herbicide metabolism by cytochrome P450 monooxygenase

Detoxification by enzymes of the P450 family is one of the resistance mechanisms relating to herbicide metabolism. Plants have a high number of P450 genes and they have P450s bound to the endoplasmic reticulum (Powles and Yu 2010). These P450 enzymes catalyze many

plant metabolic reactions and their role in herbicide conversion is dealkylation or dehydroxylation. They metabolize certain herbicides into products with reduced toxicity. Wheat and maize (*Zea mays* L.) are popular crops with P450-mediated herbicide metabolism capacity (Siminszky 2006). Herbicide resistance based on P450 metabolism is a very problematic mechanism because these enzymes can metabolize herbicides with different mechanisms of action (Powles and Yu 2010). Grasses (monocots) have more P450 genes than dicots and many grass weed species such as rigid ryegrass (*Lolium rigidum* Gaudin), have evolved resistance to different herbicides through increased rates of P450-mediated herbicide metabolism (Heap and Knight 1986). While many resistant plants may be better able to metabolize herbicides to non-toxic forms compared to susceptible plants, there has not been much research on P450 herbicide metabolism in weeds (Powles and Yu 2010).

2.2.2.2. Other non-target-site herbicide resistance mechanisms

Glutathione S-transferases (GSTs) are a family of enzymes that are known to catalyze the conjugation of glutathione to a large number of electrophilic and hydrophobic substrates to generate non-toxic peptide derivatives. They are involved in stress response as well as herbicide detoxification by glutathione conjugation with herbicides. They prevent oxidative stress by their interaction with active oxygen species (Dixon et al. 1998). The GSTs are as diverse as P450s and their dynamic evolution allows them to detoxify a range of chemicals. The first evidence for the implication of GSTs with herbicide resistance in weeds came in 1970 when the relationship between glutathione (GSH) conjugation and atrazine resistance was interpreted in several grass species (Jensen et al. 1977).

Glycosyltranferases are another family of enzymes that can detoxify herbicides and catalyze the conjugation of herbicides directly. They consist of a large gene family where

proteins catalyze the conjugation of a sugar molecule to a range of lipophilic small molecule acceptors including plant hormones, secondary metabolites and herbicides (Bowles et al. 2005). Diversity is an important factor for glycosyltransferase-mediated non-target-site herbicide resistance enabling enzymes to utilize a wide range of sugar acceptors including herbicides. The primary evidence of glycosyltransferases having a role in non-target-site herbicide resistance in weeds has come through induced glycosyltransferase activity in multiple herbicide-resistant blackgrass (*Alopecurus myosuroides* Huds.) (Brazier et al. 2002).

Another class of enzymes, ATP-binding cassette (ABC) transporters, provide herbicide resistance by compartmentalizing herbicides and their metabolites. Little research has emphasized the link between ABC transporters and non-target-site herbicide resistance in weeds but their activity in model plant and crop species is well established. *AtMRP1* was characterized as the first ABC transporter gene in mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.], which was able to transport the glutathione-conjugated herbicide metolachlor (Lu et al. 1997). The ABC transporters are also characterized as one of the most diverse gene families. Their various roles in plants include excretion of toxic compounds, translocation of fatty acids and phospholipids, and transportation of chlorophyll catabolites, auxins and heavy metals to maintain cell homeostasis (Schulz and Kolukisaglu 2006).

2.3. Low-Level Tolerance and Selection with Low Herbicide Rates

Herbicide tolerance is significantly different from herbicide resistance. Herbicide tolerance is the tendency of a plant species to survive and reproduce after herbicide exposure. Tolerance is a wild-type state and is not induced by techniques of genetic manipulation nor evolved by herbicide selection (Anonymous 1998). While plant species may be tolerant to

herbicides at field use rates, low-level tolerance is defined here as tolerance to herbicides at below labeled field rates.

Some studies have revealed that low rates of herbicides can result in the evolution of herbicide resistance. Studies with herbicide-susceptible ryegrass (*Lolium* sp.) selected with low rates of diclofop resulted in rapid evolution of resistance (Neve and Powles 2005a, 2005b). In a similar study, the selection of a ryegrass population with low rates of glyphosate resulted in the evolution of a modest level of glyphosate resistance (Busi and Powles 2009). Manalil et al. (2011) stated that using herbicides at rates that can cause very high target weed mortality may be advantageous by avoiding rapid evolution of herbicide resistance and cross-resistance in genetically variable ryegrass.

2.4. Wild Oat and ACCase Genes

Three plastidic ACCase gene sequences (*Acc1;1*, *Acc1;2*, and *Acc1;3*), representing three different gene loci, have been found in wild oat (Christoffers et al. 2002). The number of identified plastidic ACCase gene loci is consistent with the hexaploid nature of wild oat. Polymorphism exists among *Acc1;1* alleles, with USDA96 and UM1 wild oat carrying the herbicide susceptibility allele *Acc1;1-1* and the herbicide resistance allele *Acc1;1-2*, respectively (Christoffers et al. 2002). The previously unsequenced allele of another herbicide-susceptible wild oat, KYN119 (Mengistu et al. 2003), has been designated *Acc1;1-6*. Sequencing of *Acc1;2* and *Acc1;3* did not identify allelic differences between the USDA96 and UM1 wild oat biotypes (Christoffers et al. 2002).

Alleles *Acc1;1-1* (USDA96) and *Acc1;1-2* (UM1) differ in six synonymous single nucleotide polymorphisms (SNPs) and two non-synonymous SNPs along a 2039 bp region of the

ACCase CT domain (Christoffers et al. 2002). One of the non-synonymous SNPs represents an isoleucine to leucine mutation that confers resistance to ACCase-inhibiting herbicides in UM1 wild oat, but the other SNPs have no known association with resistance. The neutral sequence differences between *Acc1;1-1* and *Acc1;1-2* may possibly be due to different diploid ancestry (M. J. Christoffers, personal communication). Based on previous cleaved amplified product (CAP) marker analysis, the *Acc1;1-6* allele of herbicide-susceptible KYN119 was preliminarily identified as being more similar to *Acc1;1-2* than *Acc1;1-1*.

We hypothesized that *Acc1;2* and *Acc1;3* originated from two diploids involved in the formation of the tetraploid ancestor of wild oat (Figure 2). This tetraploid, in separate events, may have hybridized with diploids carrying alleles similar to either *Acc1;1-1* or *Acc1;1-6*, resulting in the formation of originally susceptible hexaploids (represented by USDA96 and KYN119, respectively). The *Acc1;1-2* resistance allele of UM1 wild oat is likely a mutated form of *Acc1;1-6*.

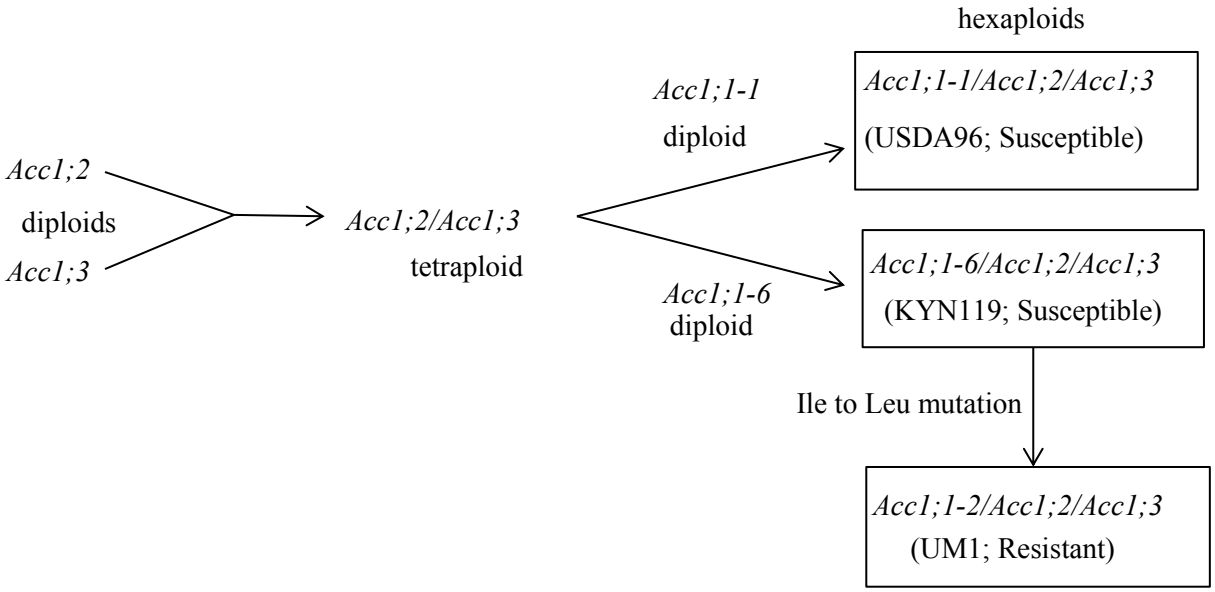

Figure 2. Hypothesized phylogeny of plastidic ACCase genes involved in the formation of allohexaploid wild oat. Resistance/susceptibility refers to ACCase-inhibiting herbicides. Ile is isoleucine and Leu is leucine.

If KYN119 and USDA96 wild oat indeed have different diploid ancestors, then additional genetic differences between the biotypes would be expected. It has been observed that there is a difference in the response of KYN119 and USDA96 to below-label rates of the ALS-inhibiting herbicide flucarbazone (M. J. Christoffers, personal communication).

Flucarbazone is commonly formulated as flucarbazone-sodium, trade name Everest, and belongs to the SACT chemical family (Nandula and Messersmith 2001; Santel et al. 1999). While both KYN119 and USDA96 biotypes are considered susceptible to flucarbazone, growth of USDA96 shows less inhibition to the herbicide (low-level tolerance), than KYN119 wild oat at below-label rates (M. J. Christoffers, unpublished data). The low-level flucarbazone tolerance response for UM1 wild oat has not been studied (Table 1).

Table 1. Plastidic ACCase alleles and responses to ACCase- and ALS-inhibiting herbicides of wild oat biotypes.

Biotype	**ACCase alleles**	**Response to ACCase-inhibiting herbicides**	**Response to ALS-inhibiting herbicide (flucarbazone)**
USDA96	*Acc1;1-1* *Acc1;2* *Acc1;3*	Susceptible	Low-level tolerance
KYN119	*Acc1;1-6* *Acc1;2* *Acc1;3*	Susceptible	Susceptible
UM1	*Acc1;1-2* *Acc1;2* *Acc1;3*	Resistant	Not determined

CHAPTER 3. RESEARCH OBJECTIVES

The long-term goals associated with this project are to identify factors that contribute to genetic diversity in wild oat and to characterize genes that may influence optimum management of wild oat populations. The current project investigated genetic differences between two herbicide-susceptible wild oat biotypes differing in plastidic ACCase sequence. Specific research objectives were:

1. Obtain DNA sequence information for the CT domain of KYN119 plastidic ACCases and additional CT domain sequence information of USDA96 plastidic ACCases, as was previously obtained for UM1 wild oat (Varanasi 2008). It was proposed that differences among ACCase alleles and loci would be characterized by comparing sequencing results obtained from herbicide-susceptible KYN119 and USDA96 wild oat and sequences previously obtained from UM1 wild oat. It was hypothesized that the *Acc1;1* gene of KYN119 would be dissimilar to that of USDA96 but similar to UM1, except for lack of the isoleucine to leucine herbicide resistance mutation.

2. Determine the inheritance of low-level flucarbazone tolerance by studying differences in herbicide response among parents and F_2 progeny of USDA96 × KYN119 and reciprocal crosses. The possibility of association between low-level flucarbazone tolerance and *Acc1;1* alleles was also to be determined. We hypothesized that low-level flucarbazone tolerance is an inherited trait, possibly due to a single gene. Association between *Acc1;1* and the tolerance gene(s) was not expected.

CHAPTER 4. ACETYL-COA CARBOXYLASE GENE SEQUENCING AND ANALYSIS

4.1. Introduction

Some DNA sequencing information for the plastidic ACCase gene loci *Acc1;1*, *Acc1;2* and *Acc1;3*, from ACCase-inhibitor resistant wild oat biotype UM1 and from ACCase-inhibitor susceptible biotype USDA96 had previously been obtained (Christoffers et al. 2002). Comparison of these three plastidic ACCase loci for UM1 and USDA96 biotypes revealed the isoleucine to leucine mutation responsible for causing herbicide resistance in UM1. The full CT domain sequences of UM1 ACCase gene loci were subsequently obtained by Varanasi (2008). However, another susceptible biotype, KYN119, had not yet been sequenced.

The objective of this study was to investigate genetic differences between herbicide-susceptible biotypes USDA96 and KYN119 by obtaining complete CT domain sequences. It was hypothesized that the *Acc1;1* gene of KYN119 would be dissimilar to that of USDA96 but similar to UM1 except for lack of the isoleucine to leucine mutation at amino acid position 1781. The *Acc1;1-2* allele in UM1 is hypothesized to be a mutated form of *Acc1;1-6*, which is in KYN119. Confirmation of this hypothesis would also support USDA96 and KYN119 having different diploid ancestors.

4.2. Materials and Methods

4.2.1. Experimental material

USDA96, a biotype susceptible to APP and CHD herbicides, was originally collected in the Red River Valley of Minnesota and North Dakota in 1964 (Jacobsohn and Andersen 1968). KYN119 is also known to be susceptible to APP and CHD herbicides and was collected during a Red River Valley survey in 2000 (Mengistu et al. 2003). Seeds of each biotype were dehulled and placed uniformly on moistened filter paper in 100 × 15 mm petri dishes. The seeds were then placed in the dark at 4 C overnight. The next day, the seeds were pierced with a needle to break dormancy and to stimulate germination. The seeds were then kept in the dark at 4 C for an additional two days prior to being planted separately in clay pots (15 × 17 cm) with Sunshine Mix #1 potting soil (Sun Gro Horticultural Distribution, Inc., Bellevue, WA) in the greenhouse.

4.2.2. Genomic DNA extraction

Genomic DNA of USDA96 and KYN119 wild oat inbred lines was extracted using the protocol of Stein et al. (2001). Plant tissue was harvested about two weeks after emergence. Between 200 and 300 mg of tissue was harvested, weighed and placed in pre-made polyethylene bags. Each bag received 1.2 ml of cetyltrimethylammonium bromide (CTAB) extraction buffer prior to closure with a bag sealer (Kapak Corporation, Minneapolis, MN). The tissue was then hand homogenized and incubated in a water bath at 65 C for one hour. The bag contents were then transferred to 15 ml centrifuge tubes and 800 µl of cold dichloromethane:isoamyl alcohol (24:1 v/v) was added. Tubes were placed horizontally in ice on an orbital shaker at 30 rpm for 15 min. The tubes were then centrifuged at 3184 × g for 20 min. The supernatant was transferred

into 2 ml centrifuge tubes and treated with 5 µl RNase A (10 mg/ml) (Sigma-Aldrich R4875) for 15 min in a 37 C water bath. The DNA was precipitated with 0.7 volume of isopropanol and the tubes were inverted end to end several times to clump DNA. The clumped DNA was collected with a glass hook, transferred to 1.5 ml capless tubes containing 0.7 ml of Wash 1 [76% ethanol (EtOH) and 200 mM sodium acetate (NaOAc)], and incubated for 5 min at room temperature. The glass hook with DNA was then transferred to another 1.5 ml tube containing 0.7 ml of Wash 2 [76% EtOH and 10 mM ammonium acetate (NH_4OAc)] and incubated again for 5 min at room temperature. The glass hook with DNA was then transferred to a new 2 ml tube and allowed to air dry. The hook was broken off and the DNA was resuspended in 200 µl of filter-sterile water.

4.2.3. PCR amplification and purification

Plastidic ACCase gene sequences were amplified by PCR from genomic template DNA of USDA96 and KYN119 using *PfuTurbo* DNA polymerase (Agilent Technologies, Santa Clara, CA). This polymerase provides high-fidelity, blunt-ended PCR products compatible with the pPCR-Script Amp SK(+) vector (Agilent technologies, Santa Clara, CA), which was used for cloning.

Gene-specific forward and reverse PCR primers utilized were AFACCF16 and AFACCR29, respectively (Table 1). They are specific for plastidic ACCase but do not discriminate among homeologous loci. These primers were previously used to amplify the CT domain of plastidic ACCase and were able to amplify all three loci in UM1 wild oat (Varanasi 2008). The expected PCR product size was 2136 bp.

Table 2. Primers utilized in this study and their sequences.

Primer name	Primer sequences
AFACCF16	5' AACCCTGAACGTGGATTTAAGTAC 3'
AFACCR29	5' ACTTACACTCCTCAACCAGGC 3'
T3	5’ ATTAACCCTCACTAAAG 3’
T7	5’ CGCGTAATACGACTCACTATAG 3’
AFACC1DNSTRM	5’ TGTGGGTGTTATAGCTGTGG 3’
AFACCR19	5’ CCCTAAGCTCTTTGATATGCTCC 3’
AFACCF2	5’ GTACAGCTCCCACATGCAG 3’
AFACCR32	5’ CTGCCTCAGAAGCCATGTACC 3’
AFACCF38	5’ GCATTGATGACAGCCAAGGG 3’
AFACCR5A	5’ CCTGCAACATCTGAGAGCAAC 3’
AFACCF23	5’ GAGGACGTTCTTGCAAAGGAG 3’
AFACCR44	5’ GTGCCTTGAATGACATAGACCACC 3’
AFACCF32	5’ CTCTTCTGTTATAGCGCACAAGAC 3’
AFACCR45	5’ GCTCTAGCCCACTTACACTCCTCA 3’
AFACCF51	5' CGGTCTGTTCCTCGTGCA 3'
AFACCR46	5' TTGAATGACATAGACCACCAACAA 3'
AFACCR48(dCAPS)	5' CTGAGCCACCTCAATATATTAGAAACACTT3'

PCR samples of 100 µl each were prepared with final concentrations of 250 ng genomic DNA, 100 µM of each dNTP, 1X manufacturer's buffer, 5 units of *PfuTurbo* DNA polymerase and 500 nM of each primer. PCR samples were incubated at 94 C for 1 min; followed by 25 cycles of 94 C for 1 min, 55 C for 1 min, and 72 C for 3 min; and a final cycle of 72 C for 10 min. The PCR was limited to 25 cycles of amplification and 100 µM each dNTP for increased polymerase fidelity. The PCR products were electrophoresed on 1.2% agarose gels in order to confirm amplification from genomic DNA. After confirmation, the PCR products were ethanol precipitated and quantitated using a Turner Quantech digital filter fluorometer (Barnstead Thermolyne Corporation, Dubuque, IA) utilizing PicoGreen (Invitrogen P7581) as the fluorescent dye. The samples were prepared as 1:500 dilutions with 1X Tris-EDTA (TE). Lambda DNA was utilized to prepare DNA standards with concentrations of 1.0, 0.8, 0.5, 0.2, and 0.01 ng/µl, along with a 0.0 ng/µl blank to calibrate the fluorometer. The PCR was repeated when necessary to obtain the desired amount of product required for ligation into the plasmid vector. Prior to cloning, the final product was purified with the Strataprep PCR Purification Kit (Agilent Technologies, Santa Clara, CA).

4.2.4. Cloning and plasmid DNA isolation

PCR fragments were cloned according to the manufacturer's protocol using the PCR-Script Amp Cloning Kit (Agilent Technologies, Santa Clara, CA). The purified PCR product was ligated into the pPCR-Script Amp SK(+) vector with a 40:1 insert to vector ratio. Heat shock was performed in a 42 C water bath for 30 sec with the ligated vector and XL10-Gold Kan ultracompetent cells of *Escherichia coli*. The transformed cells were grown on lysogeny broth (LB) (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) petri plates with 15 mg/ml bacto agar

and 50 µg/ml ampicillin. To facilitate color screening, 100 µl of 2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 100 µl of 10 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) were spread on the LB-ampicillin agar plates. Color screening was used to identify and select white colonies with putative plasmid inserts from the plates, which were subsequently restreaked on new plates.

Single white colonies were suspended in 5 ml of terrific broth (TB) (12 g/L peptone, 24 g/L yeast extract, 5 g/L glycerol) with 10 µl of 25 mg/ml ampicillin and were allowed to grow for 16 hours in a 37 C shaker incubator at 225 rpm. The plasmid DNA was then isolated using the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Madison, WI) and was stored at 4 C. The generation of recombinant DNA in this cloning project was approved by the NDSU Institutional Biosafety Committee (IBC), protocol B11006.

4.2.5. Sequencing and analysis

Restriction enzyme digestions were performed on plasmid DNA (see section 2.4) in order to verify that the plasmid inserts were ACCase. The restriction enzyme *Pst*I (Roche Applied Science, Indianapolis, IN), with a six-base recognition sequence (5' CTGCA$^{\downarrow}$G 3') and known recognition sites within previously sequenced wild oat ACCases, was used to verify insert identity and orientation among the clones. Four clones containing putative ACCase inserts were sent for sequencing to Northwoods DNA, Inc. (Solway, MN). Initial sequences were obtained from one insert end by using primers T3 or T7 (Table 1). An NCBI (National Center for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) search, as described by Johnson et al. (2008), was used to verify ACCase homology. Alignment of insert sequences with known wild oat ACCase sequences was used to identify specific gene loci and was done

using the Multalin computer program (Corpet 1988). Additional sequencing was performed using the alternative plasmid-specific primer (T3 or T7) for each clone, and internal primers were used to further complete the sequence of each insert, i.e., internally sequence the portions not reached by T3 or T7 sequencing reactions. These primers included AFACC1DNSTRM, AFACCR19, AFACCF2, AFACCR32, AFACCF38, and AFACCR5A (Table 1). Not all internal primers were used for all clones.

4.2.6. Confirmation of polymorphism discovered in *Acc1;1-6* (KYN119)

Repetition of amplification, cloning, and sequencing of ACCases from each wild oat biotype was necessary in order to confirm polymorphisms and identify sequence artifacts. Due to poor amplification when PCR was repeated using the original AFACCF16 and AFACCR29 primers, PCR with other primer sets was necessary in order to validate a SNP discovered in *Acc1;1-6*. Some primers utilized were AFACCF23, AFACCR44, AFACCF32, and AFACCR45 (Table 1). Unfortunately, PCR performed with these primers gave non-specific amplification. Modification of PCR annealing temperatures and extension times did not improve results with these primers.

PCR performed with another set of primers, AFACCF51 and AFACCR46 (Table 1), gave good amplification results. PCR and purification, cloning, plasmid DNA isolation, sequencing and analysis were performed using the same parameters as mentioned in sections 2.3 to 2.5. The restriction enzyme *Eco*RV (5' GAT$^{\downarrow}$ATC 3') (Roche Applied Science, Indianapolis, IN) was utilized for restriction enzyme digestion of plasmid DNA in order to identify clones with ACCase inserts.

4.2.7. Sequence information for *Acc1;3*

Additional research was done in order to specifically obtain sequence information for *Acc1;3* of USDA96 and KYN119. Genomic DNA of these two biotypes was digested with the restriction enzyme *Acu*I [5' CTGAAG$(N)_{16}^{\downarrow}$ 3'] (New England Biolabs, Ipswich, MA) followed by ethanol precipitation. Based on previous information, *Acu*I cuts the PCR target region of *Acc1;1* and *Acc1;2*, but not *Acc1;3*. PCR and purification were then performed on this ethanol-precipitated product using the same parameters as mentioned in section 2.3. Cloning and plasmid DNA isolation was also performed using similar techniques as mentioned in section 2.4. Clones were sequenced with T3 or T7 primers in addition to the PCR forward primer AFACCF16.

4.3. Results and Discussion

Sequence information for the *Acc1;1-6* allele of KYN119 was obtained. The resulting sequence was 2091 bp long (excluding primers). Alignment of this sequence with *Acc1;1-2* of UM1 confirmed that *Acc1;1-6* lacks the isoleucine to leucine herbicide resistance mutation. No additional differences within the CT domains of the two alleles were revealed. However, a SNP was discovered in the aligned intron at nucleotide position 2197, where *Acc1;1-6* has adenine and *Acc1;1-2* has guanine (Figures 3 and 4A). This SNP does not represent an amino acid polymorphism because it is present in the intron of the gene.

New sequencing information of *Acc1;1-1* of USDA96 was obtained in addition to what was already available from the study of Christoffers et al. (2002) (Figure 3). The sequencing result of the current study covered a total of 2091 bp (excluding primers). This extended the previous sequence of *Acc1;1-1* (GenBank accession AF231335) an additional 457 bp to a total of 2496 bp. Multiple sequence alignment indicated the presence of four new SNPs in addition to six

previously identified SNPs between the clone representing the *Acc1;1-1* allele of USDA96 and the *Acc1;1-6* allele of KYN119. Among these four new SNPs, three were unique to the *Acc1;1-1* clone and the fourth SNP was similar to *Acc1;2* sequences but different than *Acc1;1-6* and *Acc1;1-2*. All four SNPs were present in the intron of the gene. Unique SNPs in the sequence of the *Acc1;1-1* clone were cytosine instead of thymine at nucleotide position 2135, guanine instead of cytosine at nucleotide position 2138, and adenine instead of cytosine at nucleotide position 2204. The fourth SNP was at nucleotide position 2199, where *Acc1;1-6* and *Acc1;1-2* had adenine instead of the guanine of *Acc1;1-1* and *Acc1;2* sequences (Figure 4A). The SNPs present in the *Acc1;1-1* clone have not yet been confirmed by sequencing another *Acc1;1-1* clone(s) from a separate PCR reaction.

Sequencing information for *Acc1;2* genetic loci of USDA96 and KYN119 was also obtained. Sequencing of *Acc1;2* for USDA96 covered a total of 2091 bp. This extended GenBank accession AF231337 an additional 457 bp to a total of 2496 bp. The sequencing of *Acc1;2* for KYN119 also gave a total sequence of 2091 bp. The multiple sequence alignment of these new sequences of *Acc1;2* did not reveal any difference between the USDA96 and KYN119 biotypes (Figure 3). The cloning experiments in the current study did not reveal clones representing *Acc1;3* genetic loci for USDA96 and KYN119, perhaps due to insufficient clone screening or non-amplification of *Acc1;3*.

In a previous study by Christoffers et al. (2002), several SNPs were revealed among the plastidic ACCase sequences of USDA96 and UM1. This previous study identified the isoleucine to leucine mutation at amino acid position 1781 (amino acid positions standardized to blackgrass ACCase) in *Acc1;1-2* of UM1 wild oat. The ability of this mutation to confer herbicide resistance has been confirmed among several grass weed species such as blackgrass, ryegrass (reviewed by

Powles and Yu 2010). Varanasi (2008) obtained additional sequence information for *Acc1;1-2*, *Acc1;2*, and *Acc1;3* of UM1 wild oat, the results of which supported *Acc1;1* and *Acc1;2* being more similar to each other than to *Acc1;3*.

The unrooted phylogenetic tree in Figure 4B and the homology observed between the sequences of *Acc1;1-6* of KYN119 and *Acc1;1-2* of UM1 supports our hypothesis that *Acc1;1-6* and *Acc1;1-2* are more similar to each other than either is to *Acc1;2* or *Acc1;1-1*, and that *Acc1;1-2* may be a mutated form of *Acc1;1-6*. These results support the possibility that UM1 and KYN119 may have shared diploid ancestry. The SNPs observed among *Acc1;1-1* of USDA96 and *Acc1;1-6* of KYN119 are consistent with the possibility that these biotypes may have had different diploid ancestors.

Figure 3. Multiple sequence alignment generated with MultAlin. Acc1;1-1 is *Acc1;1-1* (GenBank accession AF231335) of USDA96, Acc1;2USDA is *Acc1;2* (GenBank accession AF231337) of USDA96, c_Acc1;1-1 is the sequence of the *Acc1;1-1* clone of USDA96, Acc1;1-2 is the *Acc1;1-2* allele (GenBank accession HQ244398) of UM1, Acc1;2UM1 is *Acc1;2* (GenBank accession HQ244399) of UM1, c_Acc1;2US is the *Acc1;2* clone of USDA96, Acc1;1-6 is the *Acc1;1-6* allele of KYN119, and Acc1;2kyn is the *Acc1;2* sequence of KYN119. Nucleotide position numbers are standardized to GenBank accessions AF231335 and AF231337.

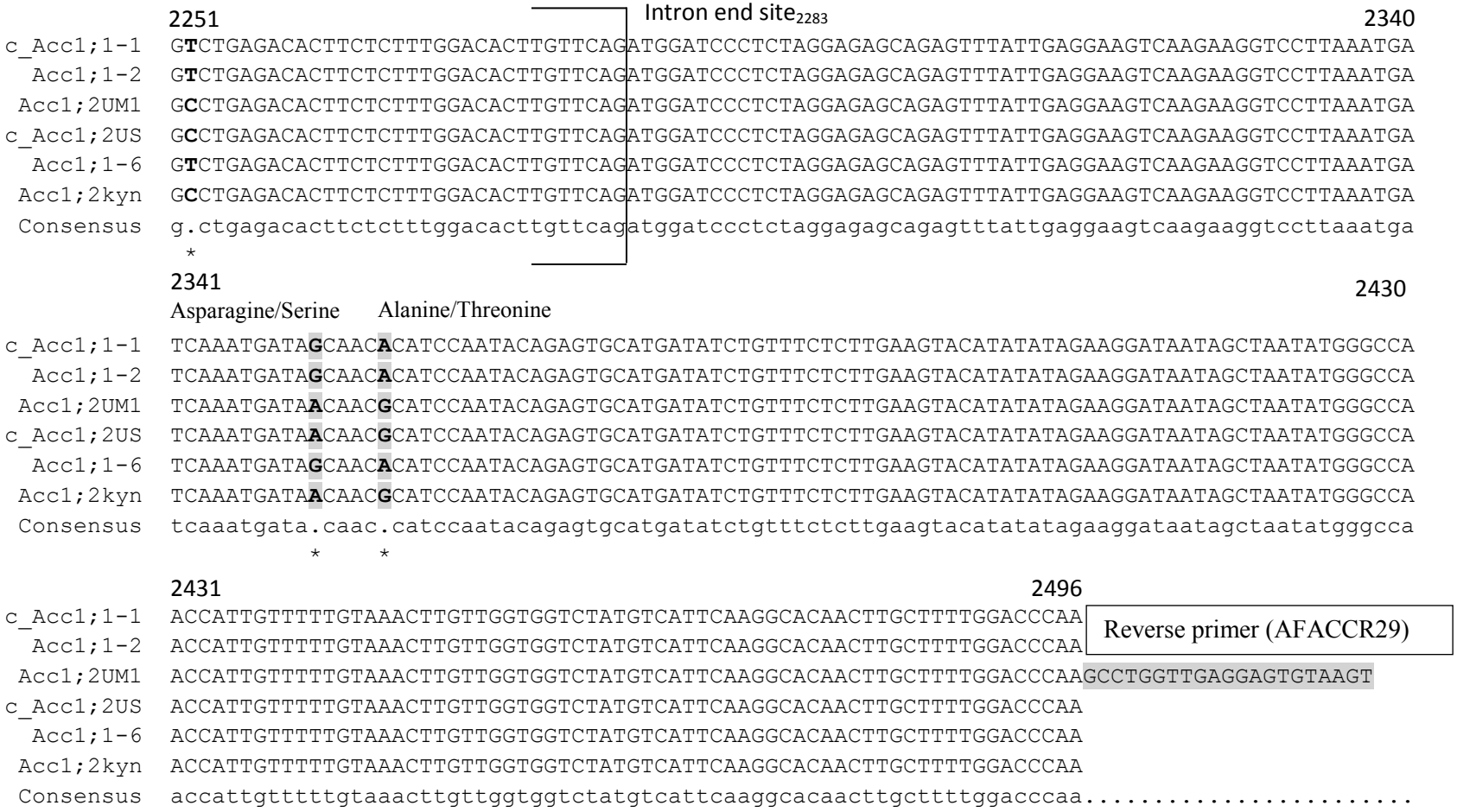

Figure 3. Multiple sequence alignment generated with MultAlin. (continued)

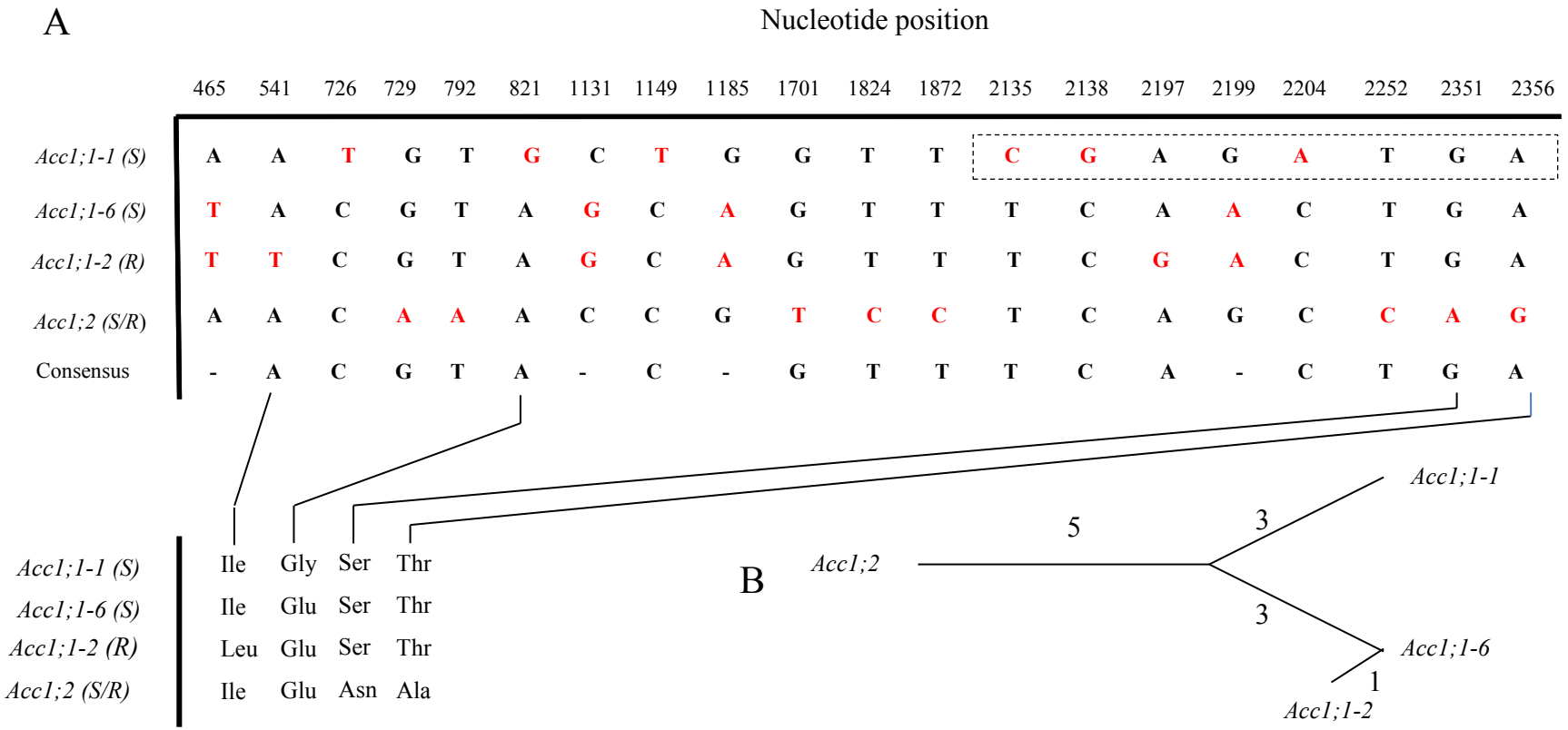

Figure 4. (A) Single nucleotide polymorphisms (SNPs) with associated amino acid polymorphisms and their positions in wild oat ACCase. *Acc1;1-2* information is from GenBank accession HQ244398. The SNPs fall in the region amplified by AFACCF16 and AFACCR29 primers. The dotted box indicates unconfirmed SNPs. (B) Unrooted phylogenetic tree based on sequence comparison of *Acc1;1-6*, *Acc1;1-2*, *Acc1;1-1* and *Acc1;2*. The numbers correspond to the number of SNPs through position 1872 (not including SNPs corresponding to the boxed region of unconfirmed *Acc1;1-1* sequence).

CHAPTER 5. INHERITANCE OF LOW-LEVEL TOLERANCE TO ALS-INHIBITOR FLUCARBAZONE IN WILD OAT BIOTYPES; USDA96 AND KYN119

5.1. Introduction

Many wild oat biotypes have shown resistance to herbicides with different mechanisms of action, most popularly ACCase and ALS inhibitors (Powles and Yu 2010). Resistance to different mechanisms of action might be the result of target-site or non-target-site resistance mechanism(s). Target-site resistance is mainly due to genetic mutations affecting target enzymes or other target proteins; whereas non-target-site resistance is suspected to be due to processes such as increased herbicide metabolism or decreased herbicide translocation. For example, target-site resistance in the wild oat biotype UM1 has been found to be a result of an isoleucine to leucine mutation in the ACCase target enzyme (Christoffers et al. 2002). This mutation indeed results in resistance across the ACCase-inhibiting APP, CHD and PPZ herbicide chemical groups (Powles and Yu 2010).

Non-target-site resistance to ALS inhibitors in wild oat may be due to increased herbicide metabolism (Nandula and Messersmith 2001). Herbicide metabolism with P450 enzymes is a problematic mechanism because these P450 genes are abundant in grass weed species and they can metabolize herbicides with different mechanisms of action. By this mechanism, resistant plants are better able to metabolize herbicides to non-toxic forms compared to susceptible plants (Powles and Yu 2010). A report of target-site resistance to ALS-inhibitors in wild oat by Beckie

et al. (2012), stated that P450 enzymes are also likely involved in causing resistance to both ACCase and ALS inhibitors among Canadian wild oat.

From past studies, we know that usage of below-label rates of certain herbicides has caused evolution of herbicide resistance in weed populations. Studies with herbicide-susceptible ryegrass (*Lolium* sp.) selected with low rates of diclofop resulted in rapid evolution of resistance (Neve and Powles 2005a, 2005b). In a similar study, the selection of a ryegrass population with low rates of glyphosate resulted in the evolution of a modest level of glyphosate resistance (Busi and Powles 2009). Our unpublished lab data also suggested a difference in the response of KYN119 and USDA96 wild oat to below-label rates of the ALS-inhibiting herbicide flucarbazone (M. J. Christoffers, personal communication). While both KYN119 and USDA96 biotypes are considered susceptible to flucarbazone, growth of USDA96 showed less inhibition to the herbicide (low-level tolerance) than KYN119 wild oat at below-label herbicide rates. The objective of the current study was to determine the inheritance of low-level flucarbazone tolerance by studying differences in herbicide response among parents and F_2 progeny of USDA96 × KYN119 and reciprocal crosses.

5.2. Materials and Methods

5.2.1. Generation of F_2 hybrid seed

Seeds from inbred lines of USDA96 and KYN119 were dehulled and soaked in distilled water in the dark at 4 C overnight in petri plates. Seed dormancy was broken by poking the seeds with a pin after one day followed by incubation in the dark at 4 C for an additional two days. Seeds were then planted in individual clay pots with Sunshine Mix #1 potting soil in the greenhouse and were watered and fertilized as needed. After approximately two months, florets

of female plants were emasculated and pollinated two days later with pollen from the alternative biotype. Reciprocal crosses were performed. Approximately three weeks after pollination, mature F_1 seeds were harvested. Dormancy of F_1 seeds were broken as above, and F_2 seeds of individual self-pollinated F_1 plants were harvested at maturity.

5.2.2. Preliminary herbicide-response study

A preliminary study was performed to determine the best herbicide treatment rate for qualitatively categorizing plant response to flucarbazone, trade name Everest WDG (Arysta LifeScience, Cary, NC). Seeds of USDA96 and KYN119 were treated as above to break dormancy and achieve uniform growth. Seeds were then planted in 21 × 4.5 cm plastic cones. Seven plants each of USDA96 and KYN119 were then sprayed with seven different rates of flucarbazone at the 3-leaf stage. The rates used were 1x (29 g ai/ha), 1/2x (14.5 g ai/ha), 1/4x (7.25 g ai/ha), 1/8x (3.625 g ai/ha), 1/16x (1.81 g ai/ha), 1/32x (0.9 g ai/ha) and 0x. Non-ionic surfactant (NIS) adjuvant Activator 90 (Loveland Products Inc., Greeley, CO), at a rate of 0.25% v/v, was added to each treatment. A cabinet sprayer (De Vries Inc., Minneapolis, MN) was utilized for spraying with a 650067 TeeJet tip at a pressure of 275.79 kPa and a speed of 3.38 km/hr. Calibration was performed before spraying so as to ensure proper equipment settings. The plants were located 47 cm below the nozzle, as measured half way into the plant canopy. Plants were visually evaluated two and a half weeks after treatment.

5.2.3. Preliminary analysis of F_2 herbicide response

Plants of the F_2 generation (reciprocal crosses) were grown in the greenhouse as above and at the two- to three-leaf stage were sprayed with 1/8x flucarbazone (parameters as in section

2.2). Flucarbazone response was assessed by scoring main shoot survival/death at three weeks after treatment. Results were compared to Mendelian segregation ratios and statistically tested for goodness-of-fit using chi-square analysis. The main shoot of a single plant of the susceptible biotype KYN119 died, while a majority of KYN119 plants had alive main shoots. The qualitative analysis of main shoot survival/death thus proved unreliable at the 1/8x flucarbazone rate, necessitating additional experiments to determine the best flucarbazone rate for segregation analysis.

5.2.4. Repeated dose-response experiments

Three dose-response experiments were performed to determine the best flucarbazone treatment rate for distinguishing between USDA96 and KYN119 herbicide response phenotypes. These experiments focused on above-ground dry weight three weeks after flucarbazone treatment.

5.2.4.1. Experiment 1

In the first dose-response experiment, eight plants of each biotype (USDA96 and KYN119), were sprayed with four different rates of flucarbazone at about the 2- to 3-leaf stage. The rates used were 0x (water + adjuvant), 1/8x, 1/4x and 1/2x flucarbazone. Non-ionic surfactant (NIS) adjuvant (Activator 90) at a rate of 0.25% v/v was added to each treatment as in the preliminary study above. The plants were harvested three weeks after treatment. The main shoot, including all leaves of the main shoot, was harvested and placed in one envelope. The tillers, including all tiller leaves, were harvested and placed in another envelope. The envelopes were oven-dried at 80 C for 48 hours to dehydrate the tissue. The samples were then weighed on a balance and dry weight data was analyzed by obtaining p-values from the T-test function of

Microsoft Excel (Microsoft Corporation, Redmond, WA) for each treatment rate. A USDA96/KYN119 (U/K) means ratio, which is the mean above-ground dry weight of USDA96 divided by that of KYN119, was also computed for each treatment rate.

5.2.4.2. Experiment 2 and 3

In the second experiment, eight plants of each biotype (USDA96 and KYN119), were sprayed with six different rates of flucarbazone at the 2.5- to 3-leaf stage. The rates were 1/2x, 1/4x, 1/8x, 1/16x, 1/32x and 0x. Non-ionic surfactant (NIS) adjuvant (Activator 90) at a rate of 0.25% v/v was added to each treatment as in the previous preliminary study. Eight untreated plants of each biotype were harvested on the day of treatment with main shoot and tiller tissue placed in separate envelopes as mentioned above and dried at 80 C for 48 hours. Three weeks after treatment plants were harvested and dried, as above. The tissue was then weighed. The third experiment was a repetition of the second experiment with the only difference being that plants were mostly at the 2- to 2.5- leaf stage, with some at the 3-leaf stage. For both the second and third experiments, significance was again tested by obtaining p-values from T-tests, and U/K means ratios were again computed for each treatment rate.

5.2.5. Analysis of F_3 herbicide response

Untreated F_2 plants were grown and self-pollinated to obtain progeny. Individual panicles were covered with glassine bags to eliminate cross-pollination. The bagged panicles were allowed to grow until the seeds were mature, after which seeds were harvested as F_3 families from individual F_2 plants. The F_3 family seeds obtained were then grown in the greenhouse in cones to study herbicide response.

Eighty F_3 families from the KYN119 and USDA96 cross and 80 F_3 families from the reciprocal cross were treated with herbicide. The 80 families of each cross were treated in two separate rounds of spraying (40 F_3 families per round, and a total of four rounds). Twenty plants from each of the 80 F_3 families (total of 1600 plants of KYN119 × USDA96 and another 1600 plants of USDA96 × KYN119) were treated with 1/16x flucarbazone at the 2.5- to 3- leaf stage (a few plants were younger than the 2.5-leaf stage). Twenty plants of each parent (USDA96 and KYN119) were also included in each spraying run. When K × U set 1 was sprayed, the temperature was 30 C and the relative humidity was 45%. When U × K set 1 was sprayed, the temperature was 24 C and the relative humidity was 38%. When K × U set 2 was sprayed, the temperature was 24 C and the relative humidity was 41%. When U × K set 2 was sprayed, the temperature was 27 C and the relative humidity was 24%.

The above-ground plant tissue was harvested three weeks after treatment, keeping main shoot and tiller tissue separate as before, and dried in an oven at 80 C for 48 hours. The dried main shoot tissue was then weighed and subsequently analyzed using the single-factor Analysis of Variance (ANOVA) tool in Microsoft Excel (Microsoft Coorporation, Redmond, WA). In the interest of time, tiller tissue was not weighed nor analyzed. Dry weight data were $\log_{10}$-transformed prior to analysis and were grouped by family. For each of the four data sets, the calculated F value was compared to the critical F value to test the null hypothesis. For sets where the calculated F value was greater than the critical F value, the null hypothesis was rejected indicating that there was a significant difference among families. Differences among the means of all F_3 families and the two parents (USDA96 and KYN119) were calculated for each set. The absolute values of the differences were utilized. Significant differences were tested with the least significant difference (LSD) formula:

$$LSD = t_{0.05} \sqrt{(MS \times 2/n)}$$

where MS is the mean square value within groups and n is the number of samples in a group. The absolute values of differences between means were then checked to see if they were greater than the calculated LSD, which divided the families into four categories: (A) families with mean similar to KYN119, (B) families with mean similar to USDA96, (C) families with mean similar to both USDA96 and KYN119, and (D) families with mean not similar to either parent. Least Significant Ratios (LSRs) were also generated by back-transforming LSDs ($LSR = 10^{LSD}$).

5.2.6. Genotyping of F_2 plants

A sample of leaf tissue was harvested from each F_2 plant that was grown and self-pollinated to obtain F_3 seeds. The tissue (150-300 mg) was frozen for future genomic DNA extraction. The extraction was done as before, but clumping of DNA was not seen after adding isopropanol for DNA precipitation. This was perhaps due to the fresh tissue being fairly mature at harvest. The DNA was therefore precipitated by centrifugation and resuspended in 100 or 200 µl of filter-sterile water. PCR was performed on the genomic DNA using the forward primer AFACCF16 and a reverse dCAPS (derived cleaved amplified polymorphic sequence) primer AFACCR48 (Table 2). The PCR samples were prepared as 20 µl reactions with final concentrations of 50 ng genomic DNA, 200 µM of each dNTP, 1X GoTaq buffer, 0.5 units of GoTaq DNA polymerase (Promega, Madison, WI), and 500 nM of each primer. PCR samples were incubated at 94 C for 3 min; followed by 30 cycles of 94 C for 45 sec, 58 C for 30 sec, and 72 C for 1 min 30 sec; and a final cycle of 72 C for 5 min. The dCAPS primer was utilized due to the lack of an *Acc1;1-1* allele-specific restriction enzyme site. The dCAPS primer incorporated the recognition site for restriction enzyme *Xmn*I (5' GAANN$^{\downarrow}$NNTTC 3') (NEB, Ipswich, MA),

into the PCR product of the *Acc1;1-1* allele of USDA96. The PCR product size was 470 bp, which included the recognition site for another restriction enzyme, *Ple*I (5' GAGTC$(N)_4^{\downarrow}$ 3') (NEB, Ipswich, MA), which was specific for the *Acc1;1-6* allele of KYN119. The PCR products were electrophoresed on a 1.2% agarose gel to confirm amplification and then ethanol precipitated. The precipitated DNA was resuspended in 20 or 40 µl of filter-sterile water depending on the initial estimated PCR concentration. This product was then digested with *Ple*I and *Xmn*I in separate 15 µl reactions. The digested product was electrophoresed on a 3.5% agarose SFR (super fine resolution) gel to obtain better resolution than could be obtained with standard agarose.

5.3. Results and Discussion

5.3.1. Preliminary herbicide-response study

Results obtained at two and a half weeks after treatment showed that the 1/8x rate gave the best visible phenotype distinction between USDA96 and KYN119 wild oat. At the 1/8x rate, plants of both biotypes continued to grow and tiller; however, the main shoots of KYN119 died (full susceptibility), while those of USDA96 survived (low-level tolerance).

5.3.2. Analysis of F_2 herbicide response

The main shoot of a single plant of susceptible biotype KYN119 died, while a majority of KYN119 plants had main shoots that remained alive (Table 3). This inconsistency in herbicide response among plants of the KYN119 parental line indicated that F_2 segregation results in this experiment were not reliable.

Table 3. Main shoot survival or death among F_2 progeny of USDA96 × KYN119 and reciprocal crosses after treatment with 1/8x (3.625 g/ha) flucarbazone.[a]

	Main shoot		
Biotype	**Sprayed**	**Survived**	**Dead**
USDA96	8	8	0
KYN119	9	8	1
	F_2 generation data		
U × K (A)[b]	20	20	0
U × K (D)	16	15	1
K × U (B)	20	20	0
K × U (C)	19	19	0

[a] Abbreviations: U, USDA96; K, KYN119.[b] A, B, C, and D were derived from separate F_1 plants.

5.3.3. Dose-response experiments

In the first dose-response experiment, the p-value for the 1/8x flucarbazone rate did not show significant differentiation between USDA96 and KYN119 (Figure 5). Moreover, the U/K ratio for all the treatment rates was less than 1.0, most likely due to faster growth of KYN119 compared to USDA96 prior to herbicide treatment. So, the first dose-response experiment did not show the expected low-level tolerance of USDA96. When lower rates were included in the second dose-response experiment, the 1/16x treatment rate showed a difference between USDA96 and KYN119 that was closest to significance while having a U/K ratio greater than 1.0. USDA96, having a significantly higher main shoot dry weight than KYN119 (U/K > 1.0 with $p \leq 0.05$), was only observed at the 1/8x rate of the third experiment where p was 0.008. However, the dose-response experiments as a whole suggested that low-level tolerance to flucarbazone in USDA96 might be best expressed at the 1/16x rate because it was in the middle of the range (1/32x to 1/8x) that generally gave the highest U/K ratio.

These dose-response results were obtained using eight plants of each wild oat biotype for each herbicide rate. The overall lack of significantly higher USDA96 main shoot dry weight compared to KYN119 suggested that more than eight plants would be necessary for subsequent experiments. Alternatively, it is likely that analyzing these dose-response data as a percentage of untreated control for each biotype would have more clearly revealed low-level tolerance in USDA96 by minimizing the effects of the apparent faster growth rate of KYN119. However, the purpose of these dose-response experiments was to determine the best herbicide rate for screening F_2 and/or F_3 plants having both USDA96 and KYN119 as parents.

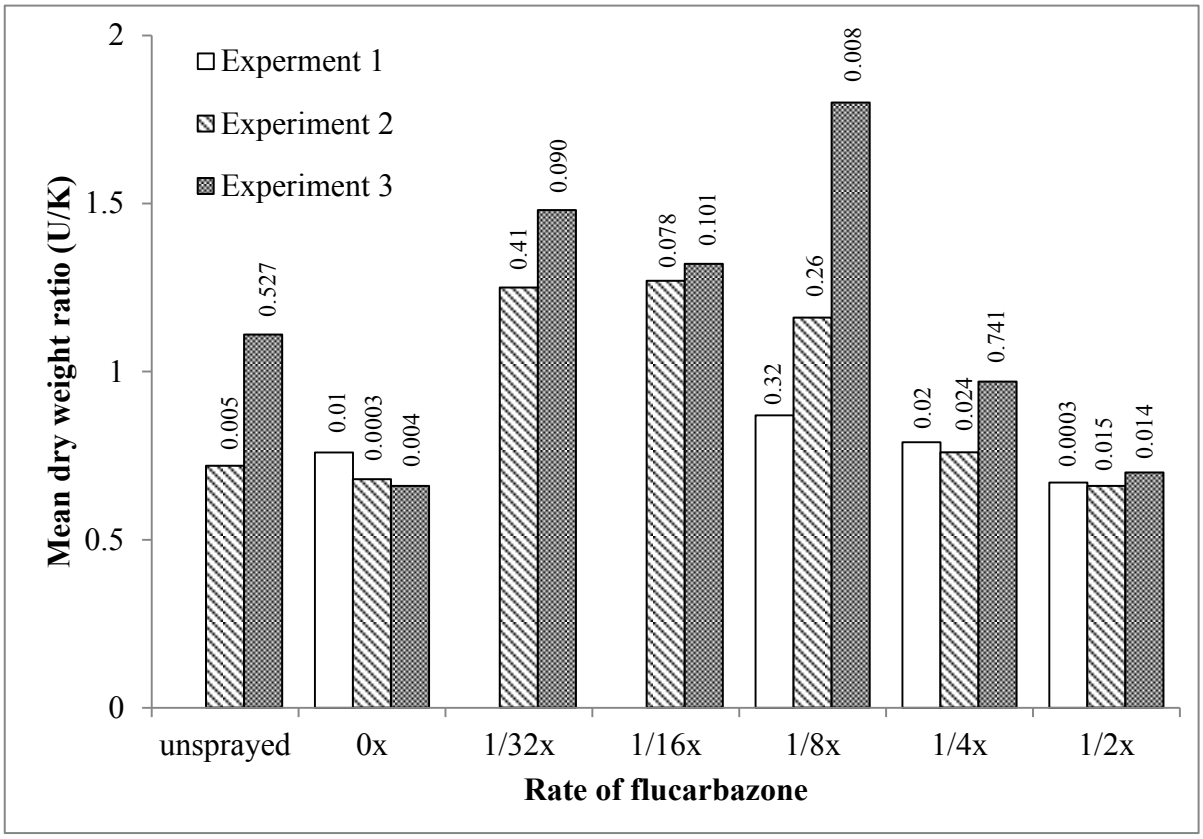

Figure 5. Ratio of the mean dry weight (above-ground main shoot) of USDA96 (U) to that of KYN119 (K), where 1x flucarbazone = 29 g ai/ha. Values above bars are p-values where $p \leq 0.05$ indicates that U is significantly different from K.

5.3.4. Analysis of F_3 herbicide response

5.3.4.1. Analysis of USDA96 × KYN119 set 1

In this data set, the average main shoot dry weight of USDA96 (170.4 mg) (back-transformed average = 164.1 mg) was significantly greater than the average dry weight of KYN119 (128.6 mg) (back-transformed average = 125.5 mg) (LSR = 1.183) (Table 4). Single factor ANOVA analysis was conducted on 40 F_3 families along with the two parental biotypes. The F value generated from ANOVA was greater than the F critical value, so the null hypothesis was rejected (Table 5). This data set had 17 F_3 families that were similar to the USDA96 parent and 11 families similar to the KYN119 parent. Eight families were not significantly different from either parent and 4 families were significantly different from both parents. Three F_3 families that were not similar to either parent had an average dry weight higher than either parent. Family 4 (209.9 mg), family 11 (249.3 mg) and family 41 (203.0 mg) were the families with higher average dry weights than KYN119 (128.6 mg) and USDA96 (170.4 mg). The F_3 families were categorized into four groups. Group A contained families with similar average dry weight to KYN119, group B contained families with similar average dry weight to USDA96, group C contained families with similar average dry weight to both KYN119 and USDA96, and Group D contained families with average dry weight significantly different from both KYN119 and USDA96. A histogram generated by plotting the average dry weights of the families appeared to approximate a normal distribution (Figure 6).

Table 4. Analysis of U × K set 1 F_3 families after treatment with 1/16x (1.81 g ai/ha) flucarbazone. Families are sorted based on average dry weight (mg) of above-ground main shoots. Group A and B represent families having similar average dry weights to KYN119 (K) and USDA96 (U), respectively. Group C represents families with similar average dry weight to both KYN119 and USDA96. Group D represents families with average dry weights significantly different from KYN119 and significantly different from USDA96.

Family	Count	Average	Group
family 11	20	249.3	D
family 4	20	209.9	D
family 41	20	203.0	D
family 16	20	195.7	B
family 44	20	190.2	B
family 60	20	180.5	B
family 22	20	177.6	B
family 80	20	177.1	B
family 53	20	175.5	B
USDA96	20	170.4	
family 6	20	169.6	B
family 34	20	168.7	B
family 49	20	167.1	B
family 26	20	166.9	B
family 8	20	165.1	B
family 57	20	164.2	B
family 39	20	161.0	B
family 33	20	159.5	B
family 43	20	158.0	B
family 115	20	156.1	B
family 24	20	153.6	C
family 32	20	153.2	B
family 5	20	151.4	C
family 77	20	151.1	C
family 51	20	150.2	C
family 28	20	149.7	C
family 36	20	145.9	C
family 19	20	145.5	C
family 46	20	144.9	C
family 10	20	140.8	A
family 17	20	137.3	A
family 58	20	135.4	A
family 62	20	134.1	A
family 55	20	132.2	A
family 121	20	130.4	A
family 38	20	129.4	A
KYN119	20	128.6	
family 1	20	125.4	A
family 3	20	121.9	A
family 7	20	120.5	A
family 70	20	111.9	A
family 63	20	107.0	D

Table 5. ANOVA single-factor analysis of main shoot dry weights (mg) of U × K set 1 F_3 families treated with 1/16x (1.81 g ai/ha) flucarbazone.

ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	4.50215	41	0.10981	7.94975	2.66E-37	1.40439
Within Groups	11.02265	798	0.01381			
Total	15.52480	839				

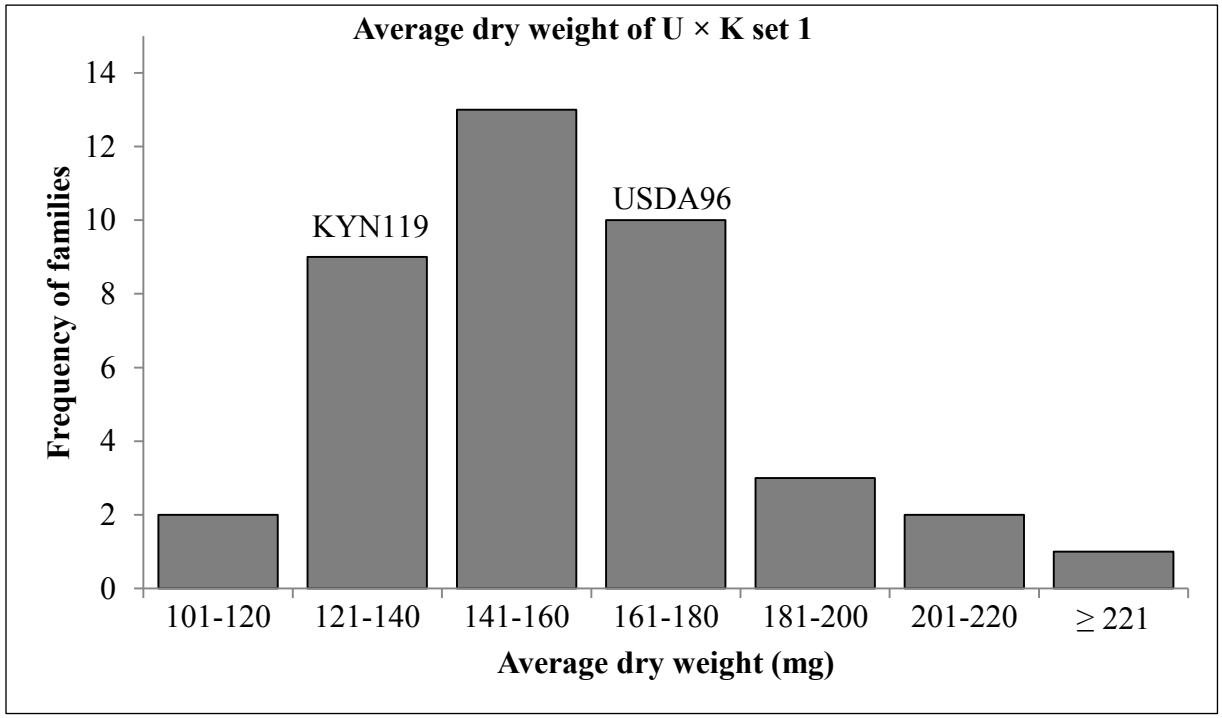

Figure 6. Histogram showing the distribution of average main shoot dry weights of 40 F_3 families of U × K set 1. Designation of USDA96 (U) and KYN119 (K) above bars indicates the dry weight of the respective parent.

5.3.4.2. Analysis of USDA96 × KYN119 set 2

In this data set, the average dry weight of USDA96 (141.4 mg) (back-transformed average = 133.2 mg) was not significantly higher than the average dry weight of KYN119 (129.2 mg) (back-transformed average = 126.1 mg) (LSR = 1.2078) (Table 6). Compared with USDA96 × KYN119 set 1, there were more families in this set that had an average dry weight higher than either parent. The F value generated by ANOVA on parents and F_3 families was greater than the F critical value, and the null hypothesis of means being equal was rejected (Table 7). There were 6 families that were statistically similar to the USDA96 parent, 3 families that were similar to the KYN119 parent, 15 families that were similar to both parents, and 16 families that were not statistically similar to either parent. Table 7 shows the results of single-factor ANOVA analysis. A histogram generated by plotting the average dry weights of the families visually approximates a normal distribution (Figure 7).

Table 6. Analysis of U × K set 2 F_3 families after treatment with 1/16x (1.81 g ai/ha) flucarbazone. Families are sorted based on average dry weight (mg) of above-ground main shoots. Group A and B represent families having similar average dry weights to KYN119 (K) and USDA96 (U), respectively. Group C represents families with similar average dry weight to both KYN119 and USDA96. Group D represents families with average dry weights significantly different from KYN119 and significantly different from USDA96.

Family	Count	Average	Group	Family	Count	Average	Group
family 20	20	210.6	D	family 117	20	157.6	B
family 59	20	209.0	D	family 104	20	153.6	C
family 23	20	207.3	D	family 56	20	150.2	C
family 31	20	206.4	D	family 75	20	148.9	C
family 40	20	198.5	D	family 97	20	147.3	C
family 25	20	196.0	D	family 84	20	146.5	C
family 100	20	195.0	D	family 15	20	146.3	C
family 102	20	190.4	D	family 82	20	145.9	C
family 2	20	183.5	D	family 95	20	145.8	C
family 69	20	182.3	D	family 9	20	145.1	C
family 79	20	180.8	D	family 30	20	143.1	C
family 112	20	177.6	D	USDA96	20	141.4	
family 29	20	175.5	D	family 111	20	139.8	C
family 71	20	170.2	D	family 65	20	138.9	C
family 68	21	169.3	D	family 119	20	138.4	C
family 27	20	168.4	D	family 108	20	134.3	C
family 67	19	163.1	B	KYN119	20	129.2	
family 45	19	162.6	B	family 91	20	116.4	A
family 35	20	160.4	B	family 86	20	115.6	C
family 106	20	159.7	B	family 14	20	113.6	A
family 72	20	158.7	B	family 93	20	110.6	A

Table 7. ANOVA single-factor analysis of main shoot dry weights (mg) of U × K set 2 F_3 families treated with 1/16x (1.81 g ai/ha) flucarbazone.

ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	4.70058	41	0.11465	6.54156	2.38E-29	1.40441
Within Groups	13.96836	797	0.01753			
Total	18.66894	838				

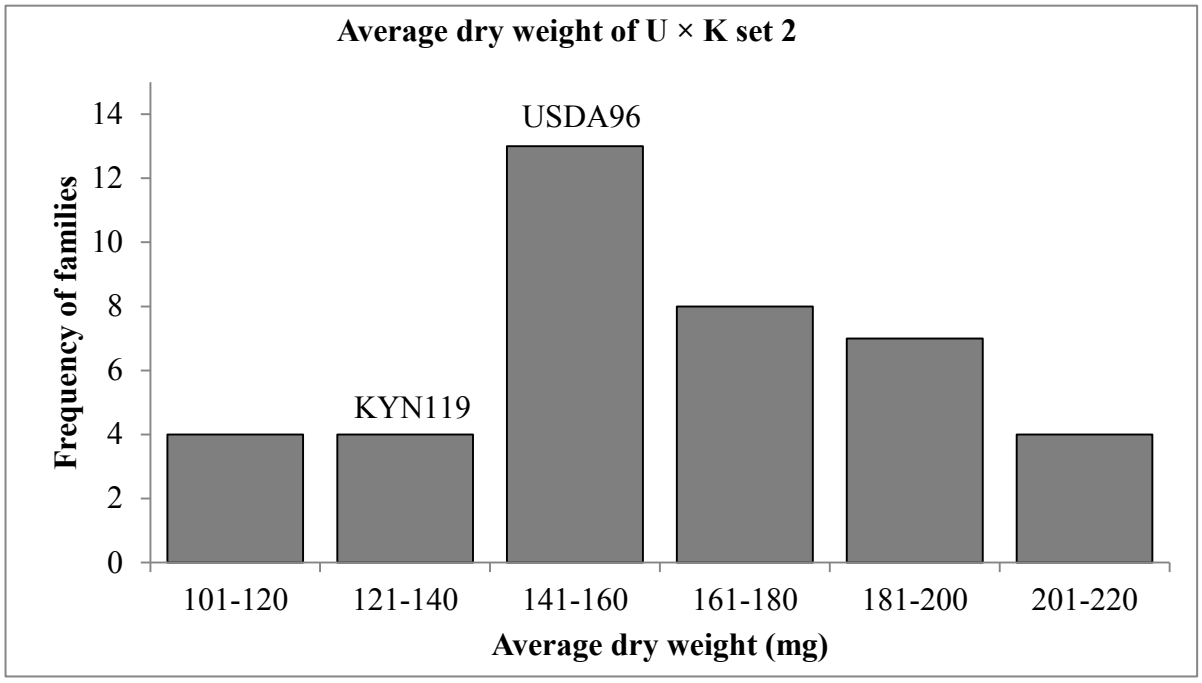

Figure 7. Histogram showing the distribution of average main shoot dry weights of 40 F_3 families of U × K set 2. Designation of USDA96 (U) and KYN119 (K) above bars indicates the dry weight of the respective parent.

5.3.4.3. Analysis of KYN119 × USDA96 set 1

In K × U set 1, the average main shoot dry weights of parents were not significantly different. The average dry weight of KYN119 was 89.4 mg (back-transformed average = 87.7 mg) and that of USDA96 was 87.1 mg (back-transformed average = 82.9 mg) (LSR = 1.219) (Table 8). The F value of parents and F_3 families was again greater than the F critical value so the null hypothesis was rejected (Table 9). There were 7 families that were statistically similar to the KYN119 parent, 2 that were similar to the USDA96 parent, 15 that were similar to both parents and 16 that were not similar to either parent. Again, there were many families that had a higher average dry weight than either parent. A histogram generated by plotting the average dry weights of the families appeared to approximate a normal distribution (Figure 8).

Table 8. Analysis of K × U set 1 F_3 families after treatment with 1/16x (1.81 g ai/ha) flucarbazone. Families are sorted based on average dry weight (mg) of above-ground main shoots. Group A and B represent families having similar average dry weights to KYN119 (K) and USDA96 (U), respectively. Group C represents families with similar average dry weight to both KYN119 and USDA96. Group D represents families with average dry weights significantly different from KYN119 and significantly different from USDA96.

Family	Count	Average	Group
family 4F	20	170.0	D
family 2V	20	162.1	D
family 3D	20	140.8	D
family 3X	20	137.8	D
family 3H	20	127.1	D
family 5T	20	127.1	D
family 3M	20	126.3	D
family 5U	20	126.0	D
family 4N	20	122.2	D
family 3R	20	119.7	D
family 4Y	20	119.1	D
family 3A	20	118.5	D
family 3K	20	117.1	D
family AA	20	115.9	D
family 4G	20	115.3	D
family OO	20	114.7	D
family 5L	20	112.0	A
family P	20	109.3	A
family N	20	108.8	A
family W	20	108.5	A
family 3J	20	107.0	A
family FF	20	106.9	A
family CC	20	106.2	C
family 5Z	20	105.2	C
family 4C	20	105.0	C
family 5E	20	99.9	C
family KK	20	95.9	C
family 5-O	20	95.8	C
family 5D	20	94.7	C
family E	20	93.6	C
family B	20	93.4	C
family G	20	90.9	C
family 5I	20	90.0	C
KYN119	20	89.4	
USDA96	20	87.1	
family HH	20	85.9	C
family MM	20	84.9	C
family J	20	79.6	A
family L	20	77.2	C
family 4S	20	73.9	C
family QQ	20	72.3	B
family 3B	20	71.7	B

Table 9. ANOVA single-factor analysis of main shoot dry weights (mg) of K × U set 1 F_3 families treated with 1/16x (1.81 g ai/ha) flucarbazone.

ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	5.64822	41	0.13776	7.20498	4.01E-33	1.40439
Within Groups	15.25800	798	0.01912			
Total	20.90622	839				

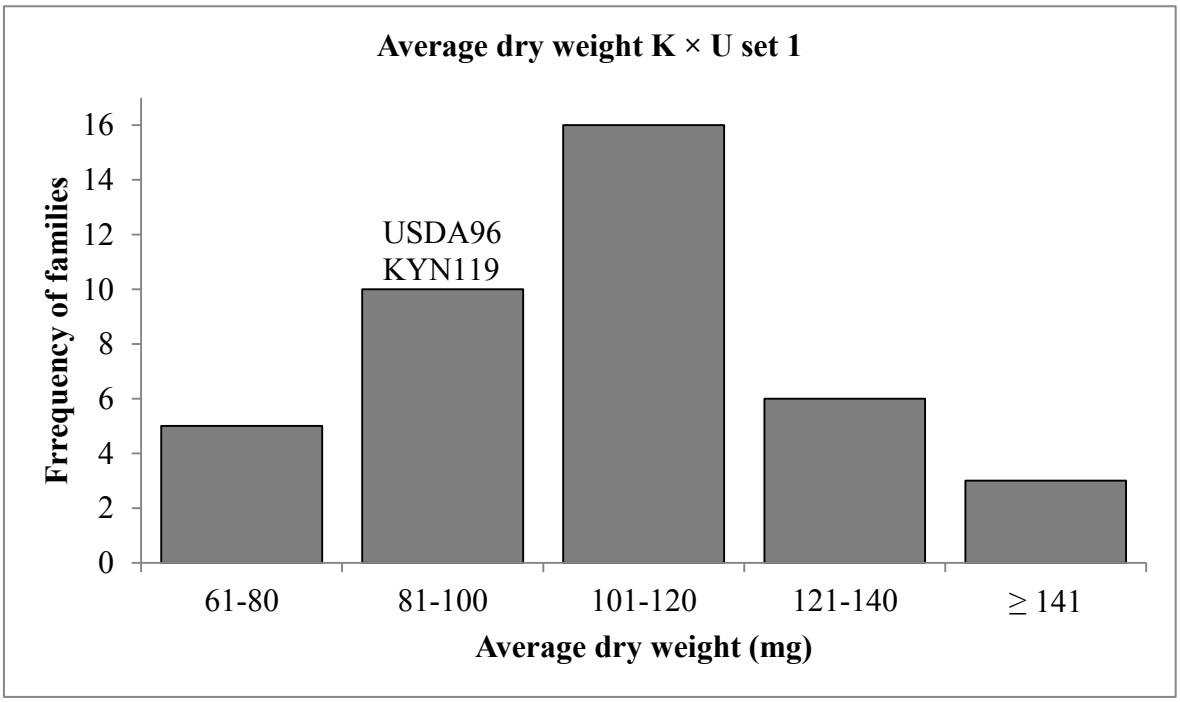

Figure 8. Histogram showing the distribution of average main shoot dry weights of 40 F_3 families of K × U set 1. Designation of USDA96 (U) and KYN119 (K) above bars indicates the dry weight of the respective parent.

5.3.4.4. Analysis KYN119 × USDA96 set 2

In K × U set 2, the average dry weights of parents were significantly different. The average dry weight of KYN119 was 158.6 mg (back-transformed average = 149.8 mg) and the average dry weight of USDA96 was 254.8 mg (back-transformed average = 252.8 mg) (LSR = 1.2218) (Table 10). The ANOVA results of the 40 F_3 families and parents gave an F value higher than the F critical value, which rejected the null hypothesis (Table 11). There were 3 families that were statistically similar to the USDA96 parent, 30 families that were similar to the KYN119 parent, and 7 families that were not similar to either parent. Visual inspection of a histogram generated by plotting the average dry weights of the families appeared to approximate a normal distribution (Figure 9).

Table 10. Analysis of K × U set 2 F_3 families after treatment with 1/16x (1.81 g ai/ha) flucarbazone. Families are sorted based on average dry weight (mg) of above-ground main shoots. Group A and B represent families having similar average dry weights to KYN119 (K) and USDA96 (U), respectively. Group D represents families with average dry weights significantly different from KYN119 and significantly different from USDA96.

Family	Count	Average	Group
USDA96	20	254.8	
family rr	20	225.7	D
family 5k	20	223.5	B
family ss	20	222.6	B
family pp	20	218.3	B
famly 4t	20	217.7	A
family ee	20	217.2	D
family 5q	20	215.8	D
family 3n	20	202.9	D
family x	20	202.8	D
family 5n	20	193.4	D
family 5g	20	192.7	A
family d	20	190.5	A
family ll	20	185.9	A
family m	20	183.2	A
family o	20	182.6	A
family 4m	20	182.3	A
family bb	20	181.5	A
family 5b	20	180.3	A
family q	20	179.8	A
family 3p	20	174.9	A
family 3s	20	172.8	A
family 3L	20	171.7	A
family c	20	168.7	A
family r	20	168.4	A
family 3F	20	168.2	A
family 3v	20	168.0	A
family 2w	20	165.2	A
family 5a	20	164.2	A
family 3c	20	162.7	A
family dd	20	158.7	A
KYN119	20	158.6	
family 3e	20	158.3	A
family gg	20	156.1	A
family 4j	20	154.3	A
family jj	20	149.8	A
family ii	20	147.7	A
family 5y	20	145.2	A
family 4h	20	141.5	A
family 4a	20	134.4	A
family 4z	20	134.0	A
family f	20	125.8	D

Table 11. ANOVA single-factor analysis of main shoot dry weights (mg) of K × U set 2 F_3 families treated with 1/16x (1.81 g ai/ha) flucarbazone.

ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	3.77929	41	0.09218	4.66286	1.45E-18	1.40439
Within Groups	15.77527	798	0.01977			
Total	19.55456	839				

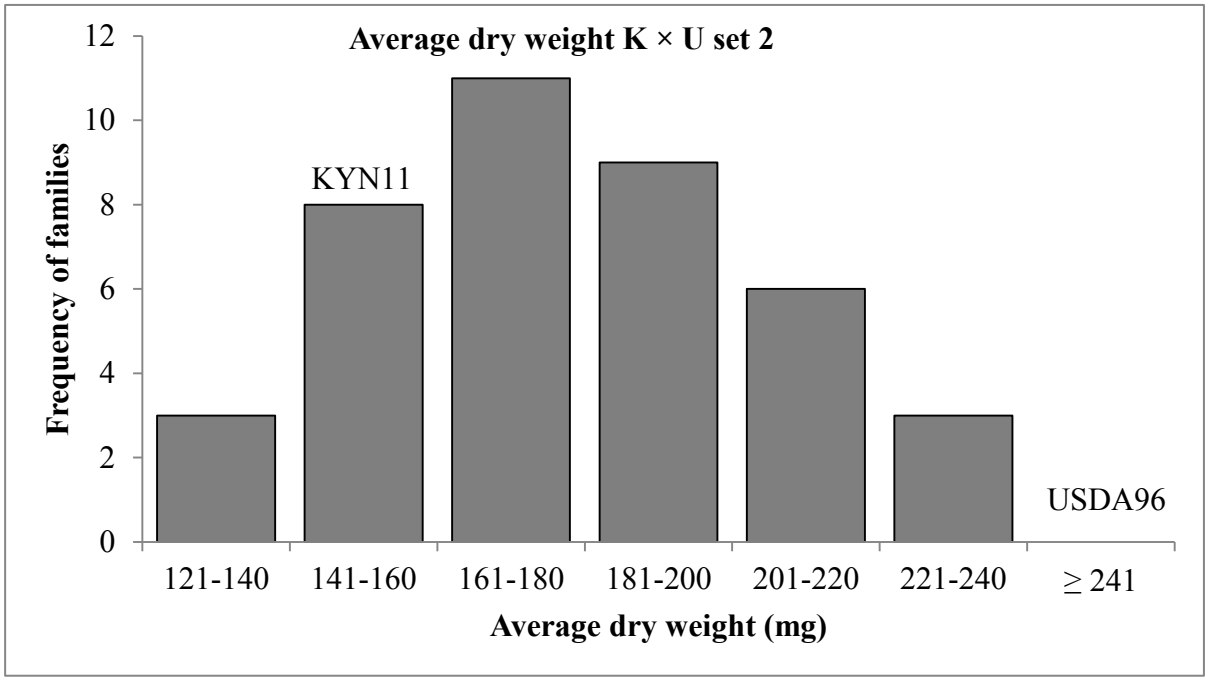

Figure 9. Histogram showing the distribution of average main shoot dry weights of 40 F_3 families of K × U set 2. Designation of USDA96 (U) and KYN119 (K) above bars indicates the dry weight of the respective parent.

The reason that some families had an average main shoot dry weight that was higher than either parent could possibly be due to KYN119 contributing gene(s) for relatively fast growth and USDA96 contributing gene(s) for low-level tolerance to flucarbazone. A combination of these genes from both parents may have contributed to both a fast growth rate and low-level tolerance for some of the families. Heterosis could be another possible reason for the relatively high main shoot dry weight of some families compared to the KYN119 and USDA96 parents. The continuous variation seen across F_3 families is a strong indication that in this study, low-level tolerance to flucarbazone, as influenced by the faster growth rate of KYN119 compared to USDA96, was inherited as a quantitative trait.

5.3.5. Genotyping of F_2 plants

The F_2 parents of all 80 F_3 families of U × K sets 1 and 2 and all 80 families of K × U sets 1 and 2 were genotyped for the ACCase alleles *Acc1;1-1* and *Acc1;1-6*. Genomic DNA from each F_2 parent was used to PCR-amplify plastidic ACCase followed by genotyping with the restriction enzymes *Ple*I (CAPs marker) and *Xmn*I (dCAPs marker) in two separate 15 µl reactions. The *Ple*I enzyme only cuts the *Acc1;1-6* allele of KYN119 and *Xmn*I only cuts the *Acc1;1-1* allele of USDA96. Both enzymes have a single restriction site for their respective alleles in the amplified product. USDA96 digested with *Xmn*I gives bands of 470 bp, 444 bp and 26 bp. The KYN119 PCR product, when digested with *Ple*I, gives bands of 470 bp, 395 bp and 75 bp. We were not able to detect bands <100 bp. Parental DNA was amplified and digested to serve as a control in each gel. Homogeneity chi-square tests were conducted to determine if the results from the two sets of each cross could be combined.

Homogeneity chi-square results for U × K sets 1 and 2 (Table 12) and K × U sets 1 and 2 (Table 13) were p = 0.17 and p = 0.39, respectively. Therefore, no statistical differences were detected between the sets of either cross, and genotype counts were combined within reciprocal crosses (Tables 14 and 15). The F_2 results of each cross were then tested for goodness-of-fit to a 1:2:1 ratio using chi-square analysis. Neither reciprocal cross was significantly different from a 1:2:1 ratio, with p = 0.35 for U × K (Table 14), and p = 0.53 for K × U (Table 15). These results were consistent with *Acc1;1-1* and *Acc1;1-6* being allelic and confirmed that the *Ple*I CAPs marker and the *Xmn*I dCAPs marker are codominant, as expected.

Table 12. Genotyping results for F_2 plants of K × U sets 1 and 2, where 1-6/1-6 plants are homozygous for the *Acc1;1-6* allele of KYN119 (K), 1-1/1-1 plants are homozygous for the *Acc1;1-1* allele of USDA96 (U), and 1-6/1-1 plants are heterozygous.

Set	**1-6/1-1**	**1-6/1-6**	**1-1/1-1**	**Total**
K × U set 1	21	13	6	40
K × U set 2	18	11	11	40
Total	39	24	17	80

Table 13. Genotyping results for F_2 plants of U × K sets 1 and 2, where 1-6/1-6 plants are homozygous for the *Acc1;1-6* allele of KYN119 (K), 1-1/1-1 plants are homozygous for the *Acc1;1-1* allele of USDA96 (U), and 1-6/1-1 plants are heterozygous.

Set	**1-6/1-1**	**1-6/1-6**	**1-1/1-1**	**Total**
U × K set 1	16	11	13	40
U × K set 2	23	5	12	40
Total	39	16	25	80

Table 14. Genotypes observed among F_2 progeny of U × K sets 1 and 2 combined, where 1-6/1-6 plants are homozygous for the *Acc1;1-6* allele of KYN119 (K), 1-1/1-1 plants are homozygous for the *Acc1;1-1* allele of USDA96 (U), and 1-6/1-1 plants are heterozygous. Expected numbers are based on a 1:2:1 genotypic ratio.

Genotype	**Observed**	**Expected**
1-6/1-6	16	20
1-6/1-1	39	40
1-1/1-1	25	20
Total	80	80

Table 15. Genotypes observed among F_2 progeny of K × U sets 1 and 2 combined, where 1-6/1-6 plants are homozygous for the *Acc1;1-6* allele of KYN119 (K), 1-1/1-1 plants are homozygous for the *Acc1;1-1* allele of USDA96 (U), and 1-6/1-1 plants are heterozygous. Expected numbers are based on a 1:2:1 genotypic ratio.

Genotype	**Observed**	**Expected**
1-6/1-6	24	20
1-6/1-1	39	40
1-1/1-1	17	20
Total	80	80

5.3.6. Genotype/Phenotype association

The average main shoot dry weights for F_3 families were compared with the plastidic ACCase genotype of their F_2 parents to detect the possibility of association between the *Acc1;1* plastidic ACCase gene and low-level flucarbazone tolerance. The average dry weights for F_3 families were compared across F_2 ACCase genotype groups (homozygous *Acc1;1-1*, heterozygous *Acc1;1-1/Acc1;1-6*, and homozygous *Acc1;1-6*). Both sets of U × K and K × U crosses were analyzed separately. No resulting F values were greater than their corresponding F critical values which indicates no significant F_3 flucarbazone response differences among F_2 ACCase genotypes (Tables 16, 17, 18, and 19). Results are further summarized in Table 20.

We previously concluded that low-level tolerance to flucarbazone might be a quantitative trait, which may decrease the possibility of observing a correlation between the average dry weight of F_3 families after flucarbazone treatment and the ACCase genotype of F_2 parents. This is because both USDA96 and KYN119 parents may be contributing gene(s) for increased main shoot dry weights among flucarbazone-treated F_3 progeny plants. However, association between flucarbazone tolerance and the *Acc1;1* gene of wild oat was not expected, and our results are consistent with this hypothesis. Note, also, that some F_2 genotypes show higher average F_3 dry weight compared to the USDA96 parent (Table 20). This might also be because of a gene(s) for faster growth from the KYN119 parent and a gene(s) for low-level flucarbazone tolerance from the USDA96 parent, which may have resulted in overall higher growth rate in some of the families.

Table 16. ANOVA single-factor analysis of average F_3 dry weights across three F_2 genotypes (groups) in U × K set 1.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	801.9459	2	400.973	0.494266	0.613989	3.251924
Within Groups	30016.22	37	811.2492			
Total	30818.17	39				

Table 17. ANOVA single-factor analysis of average F_3 dry weights across three F_2 genotypes (groups) in U × K set 2.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1182.857	2	591.4287	0.798061	0.457798	3.251924
Within Groups	27420.02	37	741.0816			
Total	28602.88	39				

Table 18. ANOVA single-factor analysis of average F_3 dry weights across three F_2 genotypes (groups) in K × U set 1.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	367.3587	2	183.6793	0.363285	0.697841	3.251924
Within Groups	18707.47	37	505.6072			
Total	19074.82	39				

Table 19. ANOVA single-factor analysis of average F_3 dry weights across three F_2 genotypes (groups) in K × U set 2.

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	143.784	2	71.89202	0.095512	0.909131	3.251924
Within Groups	27849.92	37	752.7005			
Total	27993.7	39				

Table 20. Average main shoot dry weights of parents USDA96 (U) and KYN119 (K), and F_3 families grouped by homozygous *Acc1;1-1*, heterozygous (*Acc1;1-1*/*Acc1;1-6*), and homozygous *Acc1;1-6* F_2 genotype for two sets each of U × K and K × U crosses.[ab]

Set	**USDA96**	**KYN119**	***Acc1;1-1/Acc1;1-1***	***Acc1;1-1/Acc1;1-6***	***Acc1;1-6/Acc1;1-6***
	(mg)	(mg)	(mg)	(mg)	(mg)
U × K set 1	170.4	128.6	157.6_{ns} (13)	151.8_{ns} (16)	162.7_{ns} (11)
U × K set 2	141.4	129.2	166.5_{ns} (12)	157.1_{ns} (23)	170.8_{ns} (5)
K × U set 1	87.1	89.4	102.4_{ns} (6)	107.7_{ns} (21)	111.7_{ns} (13)
K × U set 2	254.8	158.6	176.1_{ns} (11)	179.3_{ns} (18)	175.0_{ns} (11)

[a] ns, non-significant.

[b] Numbers in parentheses are the number of samples in each category.

CHAPTER 6. SUMMARY

Sequencing information of wild oat plastidic ACCase was obtained for *Acc1;1-6* (KYN119), *Acc1;1-1* (USDA96) and *Acc1;2* (KYN119 and USDA96). A multiple sequence alignment revealed that within the sequenced portion, the only non-synonymous substitution between *Acc1;1-6* (KYN119) and *Acc1;1-2* (UM1) is the isoleucine to leucine herbicide resistance mutation in UM1 wild oat. An additional SNP between *Acc1;1-6* and *Acc1;1-2* was found within an intron. In contrast, several SNPs were observed among *Acc1;1-6* (KYN119) and *Acc1;1-1* (USDA96), indicating that they might be from different diploid ancestors. These results were consistent with the hypothesis that *Acc1;1-2* is a mutated form of *Acc1;1-6*, rather than *Acc1;1-1*, and further support the possibility that KYN119 and UM1 might have shared diploid ancestry that is different from USDA96. Different diploid ancestry of USDA96 and KYN119 is a possible reason for low-level flucarbazone tolerance in USDA96, compared to full susceptibility for KYN119.

Treatment of USDA96 and KYN119 wild oat with flucarbazone showed variability in herbicide response. In the preliminary flucarbazone-response study, we were able to observe a phenotypic distinction in the main shoots of plants of the KYN119 and USDA96 biotypes at the 1/8x treatment rate. Thus, the 1/8x rate was utilized to screen F_2 families, but the results did not show a qualitative distinction between the two biotypes and reliable qualitative scoring was not possible. Herbicide response was alternatively evaluated in subsequent experiments by analyzing dry weights of above-ground main shoots. Dose-response experiments were performed to obtain USDA96/KYN119 (U/K) means ratios, which were calculated by dividing the mean above-ground dry weight of USDA96 by that of KYN119 for different treatment rates. The results

indicated that the 1/16x rate provided the best separation between the two biotypes. Thus, the 1/16x treatment rate was utilized to evaluate the flucarbazone response of F_3 wild oat families.

Continuous variation in flucarbazone response was observed among F_2-derived F_3 families generated from reciprocal crosses of USDA96 and KYN119. The results indicated that low-level flucarbazone tolerance might be inherited as a quantitative trait, perhaps due to environmental factors or multiple genes involved with flucarbazone response. Interpretation of the results was confounded by possible contribution of gene(s) from the KYN119 parent that may promote main shoot dry weight accumulation.

Some F_3 families had higher average main shoot dry weights than either parent, suggesting better flucarbazone tolerance. This might be due to a combination of both KYN119 gene(s) for relatively fast growth and USDA96 gene(s) for flucarbazone tolerance, resulting in increased average main shoot dry weights for some F_3 families. Hybrid vigor (heterosis) could be another possible reason for a higher average dry weight of some F_3 families compared to parents.

Genotyping the F_2 parents of the F_3 families with allele-specific restriction enzymes detecting *Acc1;1-1* and *Acc1;1-6* confirmed that these ACCase genes are allelic. No association between F_2 ACCase genotype and F_3 family response to flucarbazone was detected, suggesting that *Acc1;1-1* and low-level flucarbazone tolerance in USDA96 are not genetically linked.

Future research needs to be conducted to sequence additional genes other than ACCase in order to provide further evidence that USDA96 and KYN119 have different diploid ancestry. Further studies should be done on the F_3 families showing better tolerance to flucarbazone than parents, including the production of purified, inbred lines from these families. A comparative study should then be performed to compare dry weights of these purified lines with USDA96 under untreated and treated conditions.

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