Analysis of the Accase Mutation Profile of Italian Ryegrass (Lolium Perenne SSP. Multiflorum) Accessions Resistant to Accase Inhibitors

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ANALYSIS OF THE ACCASE MUTATION PROFILE OF ITALIAN RYEGRASS (*LOLIUM PERENNE* SSP. *MULTIFLORUM)* ACCESSIONS RESISTANT TO ACCASE INHIBITORS

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Abstract

Lolium perenne ssp. multiflorum (Italian ryegrass) resistant to ACCase inhibiting herbicides has been reported in many wheat producing counties across Arkansas. Resistance is believed to be the result of point mutations creating amino acid substitutions in the CT domain of the plastidic ACCase gene. This study explores the occurrence of mutations in the ACCase gene of ryegrass populations. Plant material was collected and DNA was extracted from 10 Arkansas ryegrass populations. Six of the populations were known to be resistant to the ACCase inhibitor diclofop-methyl, while the remaining four populations were known to be susceptible to diclofop-methyl. Two highly conserved regions of the plastidic ACCase gene known to contain mutations that confer resistance to ACCase inhibiting herbicides were then amplified and sequenced. Analysis of the sequences revealed that only 41% of the resistant populations expressed a mutation known to confer resistance. Several resistant populations of ryegrass did not contain any of the known mutations in their plastidic ACCase gene. This result means that either a mutation in a different region of the CT domain affects the affinity to ACCase inhibiting herbicides or the plants harbor a different mechanism of resistance. Further, in some resistant populations, not all plants within that population possessed a mutation known to cause resistance to ACCase inhibitors. This suggests that within a population, multiple mechanisms of resistance may exist. Further research is needed to determine the mechanism of resistance in diclofop-resistant plants that do not harbor mutations in the tested ACCase herbicide-binding domains.

Introduction

The use of herbicides is the most effective and economical weed control method for large farms and is the principle method of weed control adopted by crop producers. Herbicides disrupt essential biological processes in plants, causing plant death. Plants that possess the heritable ability to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type are classified as resistant (Heap 2005). Currently there are 121 herbicide-resistant weed species in the United States, and this number is growing rapidly with the continued reliance on herbicides (Heap 2008). One class of herbicide-resistant weeds is those that are resistant to acetyl-CoA carboxylase (ACCase) inhibitors.

The ACCase enyme. ACCase is a key enzyme in fatty acid biosynthesis and is one of the common targets of herbicide action. Higher plant ACCase is a multifunctional, biotinylated enzyme that catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, the precursor to fatty acids as well as to secondary metabolites such as flavonoids (Evenson et al. 1994). In plants there are two isoforms of ACCase; plastidic and cytosolic. The plastidic ACCase is essential in the biosynthesis of primary fatty acids and the cytosolic ACCase is involved in the biosynthesis of long-chain fatty acids (Yu et al. 2007). The homomeric ACCase in the cytosol of nearly all plant species and the heteromeric ACCase in the chloroplasts of dicots are insensitive to ACCase inhibitors. In contrast, the plastidic homomeric ACCase in nearly all grass species is herbicide sensitive, and this is the basis for selective control of grass weeds by ACCase herbicides. In grasses, like *Lolium multiflorum* (Italian ryegrass), 80% of enzymatic activity is associated with the plastidic form of the enzyme (De Prado et al. 2000). All ACCase isoforms contain three catalytic domains: the biotin carboxyl carrier, the biotin carboxylase, and the carboxyl transferase (CT) domains. The CT domain of the plastidic homomeric ACCase is the primary target site for ACCase inhibitor herbicides (Yu et al. 2007).

ACCase inhibitors. Herbicides that inhibit ACCase are often termed graminicides as they only affect grass species with virtually no effect on dicotyledonous species (De Prado et al. 2000). Three chemically distinct classes of herbicides known to inhibit ACCase are aryloxyphenoxypropionates (AOPPs), cyclohexanediones (CHDs), and a phenylpyrazolin class of herbicide called pinoxaden (Yu et al. 2007). It has long been reported that both AOPPs and CHDs inhibit the transfer of carbon dioxide to acetyl-CoA that is catalyzed by the CT subunit (Délye et al. 2002). This effectively kills sensitive plants by shutting down fatty acid biosynthesis (Zhang et al. 2003). Previous studies have shown that graminicides are reversible, linear, noncompetitive inhibitors of ACCase in susceptible grasses and that the carboxytransferase reaction is more sensitive to herbicide inhibition than is the biotin carboxylase reaction (Evenson et al. 1994).

Evolution of resistance. Today there are more than 200 herbicides used worldwide with 28 different modes of action (Vencill et al. 2002). The use of herbicides over time has resulted in the selection of weeds resistant to many

different classes of herbicides, with detrimental effects on weed management practices (Kaundun and Windass 2006). Extremely rare genetic mutations occur in nature; these impart tolerance to an herbicide. Repeated and widespread application of the same herbicide exerts a large-scale, high selection pressure on the weed population, selecting for those rare mutation events. Once one or a few individuals are selected and favored to reproduce in this system, tolerant individuals proliferate, giving rise to a resistant population. Thus, the widespread adoption of herbicides for grass weed control throughout the world has resulted in the appearance of numerous resistant weed populations.

The first report of resistance to ACCase-inhibiting herbicides came from Australia in 1982 and involved a *Lolium rigidum* (annual ryegrass) population resistant to diclofop-methyl (Bravin et al. 2001). *Lolium* is a genus composed of multiple species, mostly annual and mostly cross-pollinated. *Lolium multiflorum* is an annual, outcrossing species. Thus, genetic variability within and among populations is high, and the probability of selecting rare mutation events that could impart resistance to herbicides is also higher relative to self-pollinated species. Today, resistance to herbicides and other pesticides has become the number one concern in crop production.

Mechanisms of resistance. Resistance to ACCase-inhibiting herbicides may be conferred by one or more of five mechanisms. The first of these is a membrane mutation resulting in the ability to repolarize the membrane after herbicide-induced depolarization. In the SLR 31 biotype of *Lolium rigidum*, resistance to diclofop has been correlated to the ability of plants to recover from membrane depolarization events (Holtum et al. 1991). The second is a difference in the absorption and translocation of the herbicide between resistant and susceptible species. Biotypes of *Lolium rigidum* in Chile and Mississipi have expressed resistance to glyphosate partly due to reduced absorption and translocation of glyphosate (Michitte et al. 2007; Nandula et al. 2008). While glyphosate is not an ACCase-inhibiting herbicide, the same mechanism of resistance could confer resistance to ACCase inhibitors.

The third mechanism is the ability of resistant plants to metabolize or detoxify the herbicide. Populations of *Lolium multiflorum* in the United Kingdom have been found to confer resistance to ACCase-inhibiting herbicides through an enhanced rate of metabolism (Cocker et al. 2001). The fourth mechanism is an induction of ACCase to compensate for the inactivated enzyme. Biotypes of *Sorghum halepense* (johnsongrass) resistant to sethoxydim have been reported to have an increased production of ACCase (Bradley et al. 2001). Finally, resistance to ACCase-inhibiting herbicides may be due to an alteration in the target site. Reduced enzyme sensitivity to the inhibitor is an indication of this (Kuk et al. 2000).

Documented cases of resistance to ACCase inhibitors due to enhanced metabolism, absorption, and translocation are few. These mechanisms are believed to confer generally low

resistance levels (Kuk et al. 2000). Most cases of resistance to ACCase inhibitors are due to target site mutations. Thus far, in the CT domain of the plastidic *ACCase* gene, six distinct amino acid substitutions that individually endow resistance to certain ACCase herbicides have been characterized in different *Lolium* spp. These mutations (Ile_{1781}, Trp_{2027}, Ile_{2041}, Asp_{2078}, and Gly_{2096}) confer resistance to one or more of nine selective grass herbicides (Délye et al. 2002; Délye 2008). Cross resistance to multiple herbicides is common in herbicide-resistant species. However, as of 2007, experiments showed that ryegrass biotypes resistant to ACCase-inhibiting herbicides in Arkansas were not resistant to other herbicides with different modes of action (Kuk et al. 2000; Kuk et al. 2008).

Significance of this research. Ryegrass is a major weed problem in wheat production. Ryegrass competes with wheat for nutrients, inhibits tillering, causes lodging, and interferes with harvesting, causing up to 90% yield losses for growers (Crooks et al. 2003). The evolution of resistant ryegrass makes weed management in wheat production difficult and expensive (Fig. 1). The most predominant species of *Lolium* in Arkansas is *L. perenne* ssp. *multiflorum*. Widespread ryegrass resistance to ACCase-inhibiting herbicides has been documented in Arkansas (Fig. 2). As of 2007, there were about 100 cases of confirmed diclofop-resistant ryegrass populations in Arkansas and the adjacent wheat producing areas of Missouri and Louisiana (Kuk et al. 2008). It is hypothesized that resistance to ACCase inhibitors in Arkansas ryegrass populations is due to mutations in the herbicide-binding domains of ACCase. Thus, research on the occurrence of mutations in the *ACCase* gene of ryegrass populations was conducted. Sequencing the *ACCase* gene of herbicide-resistant ryegrass exhibiting different resistance patterns to various graminicides can potentially reveal specific mutations that underlie the whole-plant response to different herbicides. Researchers and extension agents could then use this information in determining which alternative herbicides are still effective for certain ryegrass populations. However, this would be a short-term cure. In the long term, sustainable integrated management practices will have to be implemented to curb the evolution of resistant populations.

Goals and Objectives. This research was designed to: (1) sequence the herbicide-binding domains of the carboxytransferase (CT) subunit of the ACCase enzyme; (2) assemble and analyze gene sequences for nucleotide polymorphisms; and (3) translate nucleotide sequences into amino acid sequences to identify amino acid mutations and correlate this with whole-plant responses to specific herbicides. The overall goals of the project were to relate mutation patterns in the herbicide-binding site of the ACCase enzyme of Italian ryegrass to its resistance pattern to various ACCase inhibitors and to identify possible molecular markers useful for predicting herbicide cross-resistance behavior in Italian ryegrass populations.
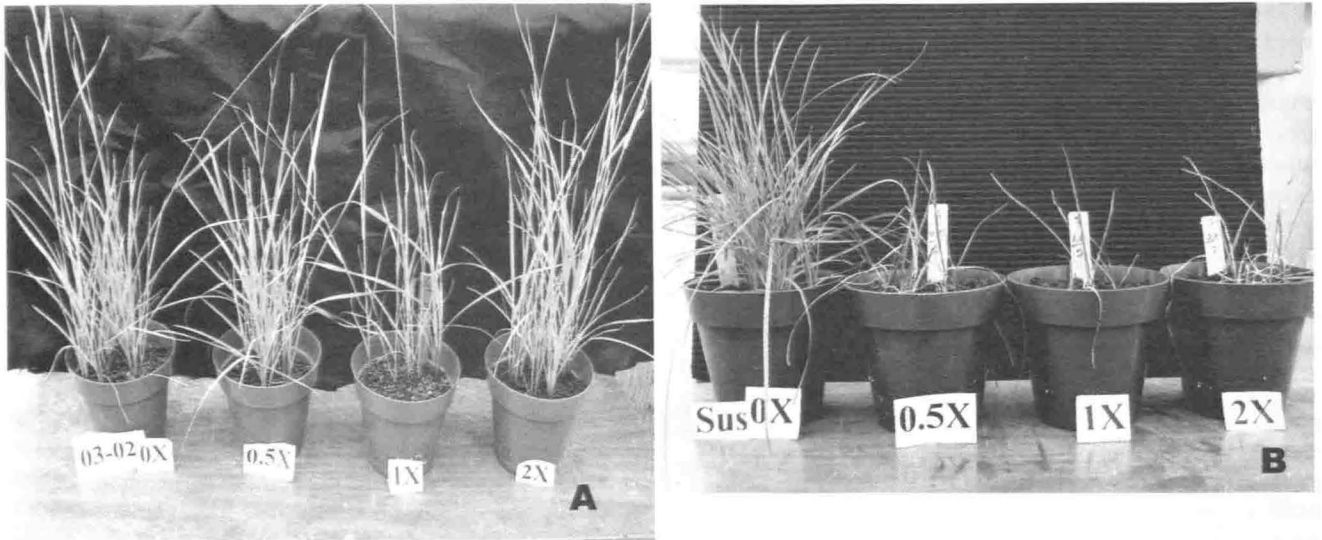
Figure 1. Ryegrass resistant to ACCase-inhibiting herbicides represents a major problem for wheat farmers. (A) shows the growth of resistant Lolium multiflorum plants at 0 to 2X rates of diclofop. (B) shows the growth of susceptible Lolium multiflorum plants at the same rates of diclofop. 1X is the recommended rate of application and is equal to 1.12 kilograms per hectare of active ingredient.

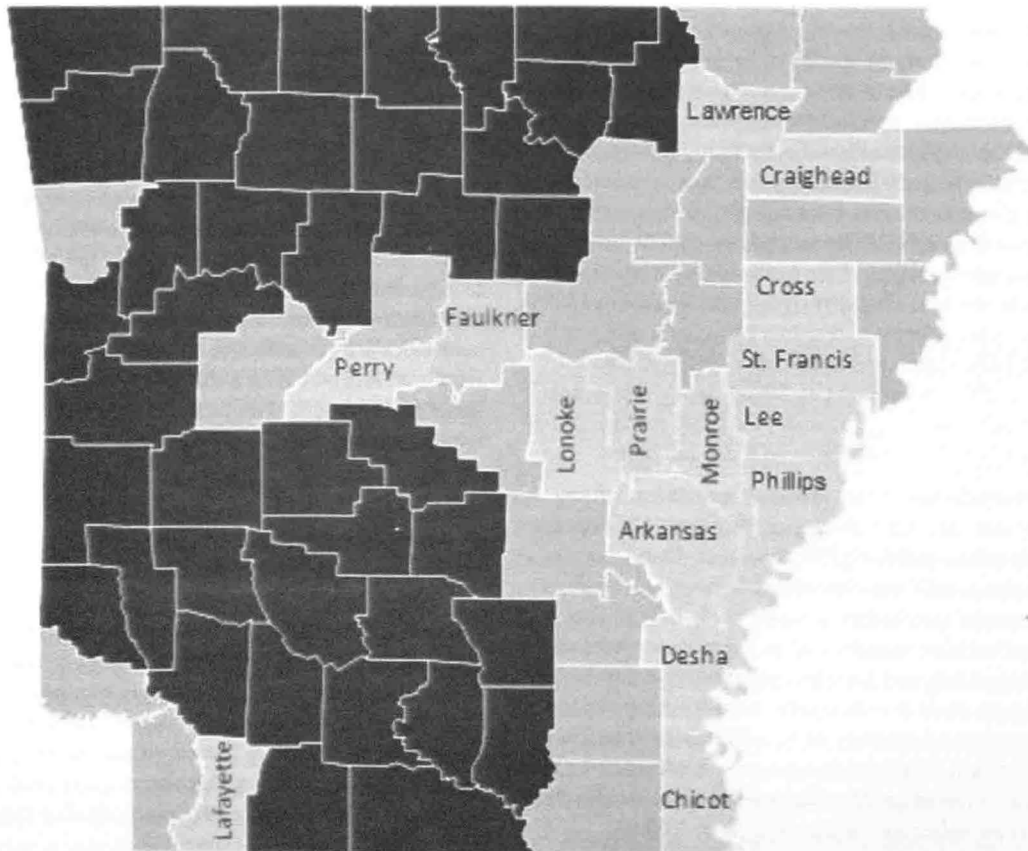**Figure 2.** Map of wheat-producing counties in Arkansas. Wheat-producing counties with identified cases of resistant ryegrass are in cyan. Wheat producing counties with no known cases of resistance are in salmon. Counties in black do not produce

Materials and Methods

Plant material. Ten ryegrass populations were used in this experiment. Six of the populations, 01-1, 04-2, 04-3, 05-7, 98-2, and 98-18, were known to be resistant to the ACCase inhibitor diclofop. The other four, 05-1, 05-2, Missouri (MO), and Texas (TX), were known to be susceptible. Seedlings were grown in the greenhouse and leaves from 4 seedlings per population were harvested. The tissues from each plant were then wrapped separately in aluminum foil and stored at -80°C until processed.

Extraction of genomic DNA. Genomic DNA was extracted using a hexadecyltrimethyl-ammonium bromide (CTAB) protocol by Doyle and Doyle (1987) with slight modifications. Briefly, 0.1 g of leaf tissue from each plant was ground to a fine powder in liquid nitrogen, transferred to 1.5-mL micro-centrifuge tubes and suspended in 500 μl of CTAB extraction buffer (100 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 2 M NaCl, 2% CTAB, 2% PVP-40, 1 mM phenanthroline, and 0.3% β-mercaptoethanol). The aqueous extracts were extracted once with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Total nucleic acids were precipitated from the supernatant by addition of an equal volume of isopropanol. The DNA pellet was then washed with absolute alcohol, air-dried, and resuspended in 30 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The genomic DNA was quantified by fluorometry, diluted to 50 ng μl⁻¹ with sterile deionized water, and immediately used as a template for the amplification of the *ACCase* gene by polymerase chain reaction (PCR).

Amplification of the CT domain of ACCase. Two primer pairs, ACcp1/ACcp1R and ACcp4/ACcp2R, were used to amplify two regions of the plastidic CT subunit of the *ACCase* gene where known mutations causing resistance to ACCase inhibitors occur (Délye and Michel 2005). These are universal primers that have been tested on 20+ grass species. These primers produce fragments of the plastidic ACCase without contamination from the cytosolic ACCase. There are two obstacles to molecular studies of grass plastidic ACCase. First, the gene itself is large (>12,000 bp) with a complex intron-exon structure. Second, there are two highly similar homomeric ACCase isoforms, and thus two highly similar *ACCase* genes in grasses. While one gene encodes the plastidic ACCase isoform that is highly sensitive to AOPPs and CHDs, the other encodes a cytosolic ACCase isoform that is tolerant to ACCase inhibitors (Délye and Michael 2005).

PCR amplifications were done in 20-μL reactions containing 4μL 5x buffer, 1μL 2 mM MgCl₂, 1μL 0.8 mM dNTPs, 2μL of each 0.2μM primer, 0.2μL of Promega Taq DNA Polymerase, and 50ng of DNA. The final volume was then adjusted to 20μL using sterile deionized water (Zhang and Powles 2006). The cycling program consisted of one initial denaturation step of 30 s at 95 C, followed by 37 cycles of 10 s at 95 C, 15 s at the specific annealing temperature and 45 s at 72 C, followed by a final extension step of 10 min at

72 C. The amplicons produced for fragment 1 were purified and sequenced on both strands using the ACcp1 and ACCp1R primers. The amplicons produced for fragment 2 were purified and sequenced on both strands using the ACcp4 and ACcp2R primers (Delye and Michel 2005).

Sequence analysis. Sequences obtained were aligned and analyzed for polymorphisms using BioEdit and Sequencher software. Multiple alignments were then done using ClustalW. Finally, amino acid mutations were summarized in relation to the whole-plant response of each population to ACCase herbicides.

Results and Discussion

The primer pair ACcp1/ACcp1R amplified the ryegrass nucleotide sequence creating fragment 1, while ACcp4/ACcp2R created fragment 2. Using *Alopecurus myosuroides* (blackgrass) as a reference sequence, fragment 1 amplified a region from base 5100 to 5660, while fragment 2 amplified a region from 5940 to 6360 (Fig. 3). Variation was present in the nucleotide sequences of both fragments within and across populations. The average percent nucleotide identity of fragment 1 between all populations was 99.467%. Fragment 2 had an average percent nucleotide identity of 98.7% (Table 1). The translated sequences fell between amino acid positions 1708 to 1886 and 1985 to 2118, respectively. The amino acid alignment revealed several mutations that are known to confer resistance in *Lolium* spp. Fragment 1 from all 10 populations did not contain any amino acid mutations known to lead to resistance, but mutations conferring resistance were found in fragment 2 in 41% of resistant populations sequenced.

At position 2027, all plants from the resistant 04-2 population encoded a cysteine rather than tryptophan (Fig. 5). This is a mutation that is known to cause resistance in *Alopecurus myosuroides* to clodinafop, diclofop, and haloxyfop. While this is not a mutation known to confer resistance to ACCase inhibitors in *Lolium* spp., some mutations in blackgrass also confer resistance in ryegrass, leading to the possibility that this mutation could cause resistance in *Lolium* spp. as well. Further experiments are needed to verify this. At position 2041, plants from resistant populations 98-18-8, 04-3-7, and 04-3-9 contained the mutation isoleucine to asparagine (Fig. 5). This mutation is known to confer broad resistance in *Lolium* spp. to the ACCase herbicides clodinafop, diclofop, and haloxyfop (Delye 2008). Also at position 2041, two plants in the diclofop-resistant population 98-2 contained an isoleucine to valine mutation (Fig. 5). This mutation also causes resistance to haloxyfop, but unlike the Ile_{2041}Asn mutation, an Ile_{2041}Val mutation is not known to cause resistance to diclofop or clodinafop (Delye 2008). Two of the resistant populations of ryegrass examined did not contain any mutations in their plastidic *ACCase* gene known to confer resistance to ACCase inhibitors. This means that either the plants contain a mutation in a different region of the CT subunit, which affected affinity to ACCase herbicides, or these plants harbor a different mechanism of resistance as discussed previously.
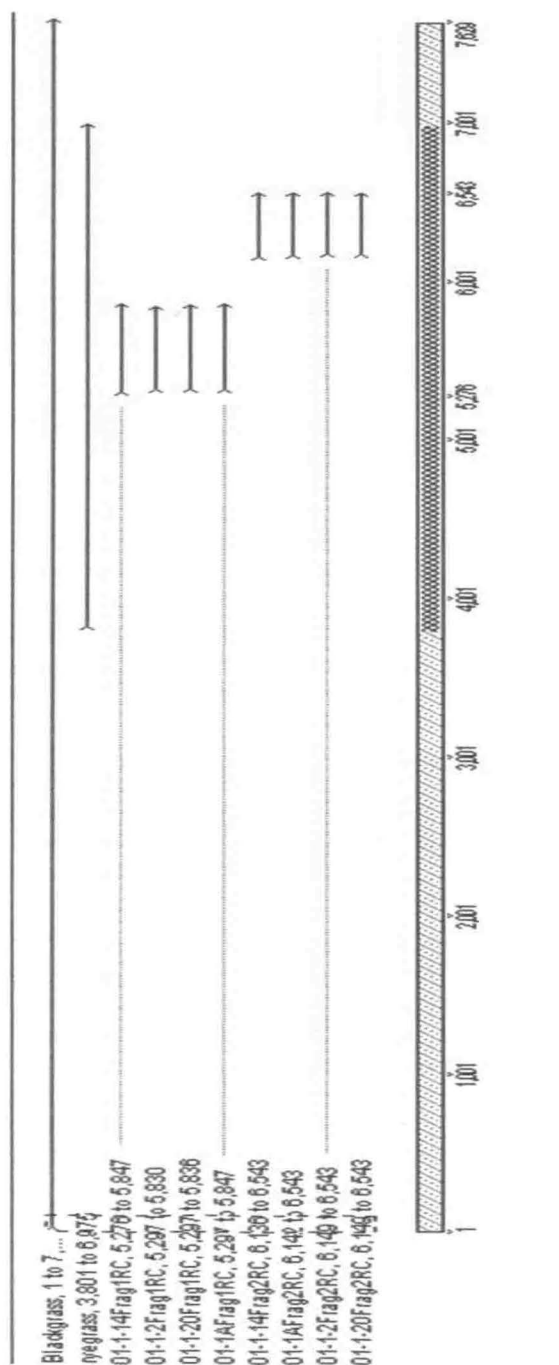
Figure 3. *Alignment of the amplified Lolium multiflorum fragments 1 and 2 versus the Alopecurus myosuroides ACCase gene (AJ310767.1) and the partial Lolium rigidum ACCase gene (AY995233.1). Fragment 1 aligned with Alopecurus myosuroides from base 5100 to 5660, while fragment 2 aligned from base 5940 to 6360*

| | % Nucleotide Identity | |
Accession Code	Fragment 1	Fragment 2
01-1	99.2	97.7
04-2	99.5	99.5
04-3	99.3	98.7
05-1	100.0	98.2
05-2	99.5	98.3
05-7	99.3	98.3
98-2	99.3	99.2
98-18	99.5	98.7
MO	99.5	99.0
TX	99.5	99.5
Overall Mean	**99.5**	**98.7**

The occurrence of multiple mechanisms of resistance within a population has been documented in *Lolium rigidum* biotypes from Australia (Tardif and Powles 1994). This could be due to the mating behavior of *Lolium* spp. and its intrinsic high genetic diversity. Herbicide treatment of large populations results in the survival of individuals that express any resistance mechanism conferring the ability to withstand herbicide at the rate used. With obligate cross-pollinated species, there is gene flow among the survivors resulting in exchange of different resistance genes and their accumulation in the next generation (Tardif and Powles 1994). It appears that the same event is occurring in ryegrass populations in Arkansas. Further research is needed to determine the mechanism of resistance in the diclofop-resistant plants tested that did not harbor mutations in their ACCase herbicide-binding domains.

It is concluded that in Arkansas, at least for some ryegrass populations, target site mutation is causing broad resistance to various ACCase inhibitors. These mutations, however, do not confer resistance to acetolactate synthase (ALS) inhibitors, which are also used for ryegrass management in wheat. Therefore, a short-term management strategy for ACCase-resistant ryegrass could still utilize an alternative herbicide mode of action such as the ALS inhibitors. The potential presence of multiple mechanisms of resistance within populations or different mechanisms of resistance between populations entails diversification of cultural management practices so that weed management and wheat production will become sustainable.

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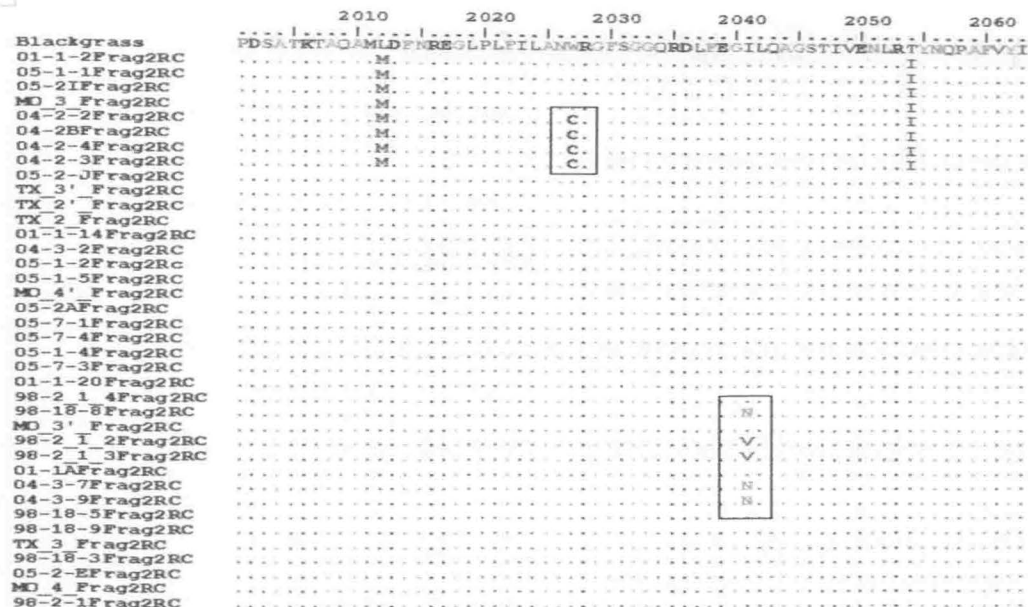Further, the fact that in several resistant populations, at least one but not all plants harbored a mutation known to cause resistance to ACCase inhibitors also suggests that within one population, multiple mechanisms of resistance may exist.

Figure 5. (sequence alignment of Blackgrass ACCase fragments, positions ~2010–2060)

Figure 5. Ile$_{2041}$Asn and Ile$_{2041}$Val are known to confer resistance in ryegrass to various ACCase inhibitors. Trp$_{2027}$Cys is known to cause resistance in *Alopecurus myosuroides* to ACCase inhibitors and will likely confer resistance in *Lolium* spp.

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Mentor Comments:

Nilda R. Burgos highlights how Thomas Stark's work provides a foundation for building a decision tool for future work with herbicides and other chemicals that develop resistance over time.. She writes:

I am writing this letter in support of the publication of the honors thesis of Thomas Stark entitled "Analysis of the ACCase mutation profile of Italian ryegrass (Lolium perenne ssp. multiflorum) accessions resistant to ACCase inhibitors" in the Inquiry Journal. Thomas was one of seven undergraduate honors students who applied for research mentorship in my laboratory in the fall of 2007, and one of two students I chose. He selected this research out of several alternative topics, all of which fit into the general area of my research program in weed physiology, biology, ecology, and molecular biology. Thomas received a full undergraduate research grant from the honors college for his thesis. My Ph.D. graduate students and I trained Thomas so he can conduct this research by himself. Beyond the initiation part, and assistance in sequence analysis, he did the rest of the work. Thomas' research is of great importance because it provides information about a critical issue in crop production, which is resistance to herbicides. The evolution of resistance to chemicals is not unique to agriculture; it happened first, and continues to be an issue, in the field of medicine. So this research has broader application for Thomas' education. In agriculture, pesticides (including herbicides) are widely used because it allows crop production in huge tracts of land to meet our needs for food,

fuel, and fiber. Extensive use of herbicides has resulted in the evolution of resistance in some weed species and now there are more than 200 herbicide-resistant weed species worldwide, with about 130 cases in the US. As for ryegrass, it is a major weed problem in wheat-producing states and has evolved resistance to ALS, ACCase, and EPSPS inhibitor herbicides. Knowing the resistance pattern of ryegrass is important because growers need this information to determine which herbicides are still effective for the population in his field. This is a critical planning tool. In Arkansas, resistance to ACCase inhibitors is widespread. Most cases of resistance are due to genetic mutations in the herbicide target site and the resistance pattern corresponds to the type of mutations; therefore, Thomas' research was done to determine which mutations exist in the resistant ryegrass populations. ACCase mutation profiles in ryegrass are not found in the literature. Thomas' research provides basic information for future research on ACCase mutation profiling of this economically important weed, which will eventually be a decision tool for wheat growers, researchers, and the chemical industry in developing alternative, sustainable management strategies for ryegrass in wheat.