DISSERTATION

DEVELOPMENT AND CHARACTERIZATION OF WHEAT MUTANTS RESISTANT TO ACETYL CO-ENZYME A CARBOXYLASE INHIBITORS

Submitted by

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ABSTRACT

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Weed management in bread wheat (*Triticum aestivum* L.) is currently limited when undesirable grass species are present due to limited options for herbicidal selectivity between the species. The incorporation of an acetyl co-enzyme A carboxylase (ACCase) inhibitor tolerance trait in wheat would allow growers to expand the arsenal and effectiveness available for managing troublesome grass species. To develop this new trait, ethyl methanesulfonate mutagenesis (EMS) was utilized to induce point mutations in the ACCase gene. Seeds mutagenized with 0.75% EMS for 2.5 hours were grown and seed was collected. Lethal applications of clethodim and quizalofop were applied to plants in the M2 generation. Approximately 200 surviving plants were collected out of the total of 2.5 million M2 seeds planted. M3 greenhouse screening revealed roughly half the plants selected with quizalofop in the M2 generation contained an increased level of resistance to the herbicide. Dose response studies confirmed a two- to- eight fold increase in resistance over wild-type wheat and limited cross-resistance across other ACCase inhibiting herbicides. DNA sequencing of the ACCase gene in quizalofop mutants uncovered a novel point mutation resulting in a Ala -> Val substitution at position 2004 from the *Alopecurus myosuroides* numbering scheme. A ^{14}C-based enzyme assay established a three- to- ten fold increase in ACCase activity in the presence of quizalofop from plants containing the new mutation. The experiment successfully generated quizalofop resistant mutant wheat and led to the

discovery of a previously unknown point mutation. This mutation has the potential for future implementation in a wheat cultivar resistant to quizalofop.

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Chapter 1. Current Status of ACCase Herbicides and Evaluation of their Potential in a Herbicide Resistant Wheat Cropping System

Summary

Graminicides have been used effectively in cereal and broadleaf crops since the late 1970s. Despite the recent increase in herbicide resistant crop cultivars, there has been very little work on ACCase resistance development in some of the world's leading food crops. The ACCase enzyme is now well characterized, creating the potential for research into developing herbicide resistant wheat. Dose response studies were conducted on wheat and weed species to determine sensitivity to ACCase-inhibiting herbicides. Clethodim and quizalofop were highly effective at low doses on all species. Their activity would make them useful for weed management in wheat and allow easy identification of herbicide resistant wheat phenotypes in a large population. Before ACCase herbicides can become the centerpiece of a herbicide-resistant cropping system, protections must be incorporated into the system due to the affinity of weedy species to rapidly acquire resistance to ACCase herbicides. An effective stewardship program should accompany the release of the resistant cultivar to avoid the development of new resistance alleles and prevent the spread of existing ones to ensure the longevity of the system.

Introduction

Acetyl Co-enzyme A Carboxylase (ACCase) inhibitors are among the most effective herbicides against grass species. These herbicides target the plastidic, homomeric ACCase of grass species. It is important to note that an ACCase isozyme

located in the cytosol is not affected by ACCase inhibitors. Similarly, broadleaf plants are unaffected by ACCase herbicides due to an insensitive, heteromeric version of ACCase in the chloroplast (Konishi and Sasaki 1994; Alban et al. 1994).

In the multi-subunit version, ACCase inhibitors function by binding to the carboxyltransferase domain, which changes the conformation of the protein enough to prevent acetyl co-enzyme A from interacting with the ACCase molecule. This creates nearly competitive inhibition (Zhang et al. 2004) of the first committed step of de novo fatty acid biosynthesis (Catanzaro et al. 1993, Rendina et al. 1988, Burton et al. 1987). This pathway is the only means by which a plant can produce malonyl co-enzyme A (Redina et al. 1988).

Symptomology of ACCase inhibition is first noticeable within a matter of days by chlorosis at the growing point in grass species. After about a week, the growing point becomes necrotic. At this point the newest leaves on the plant can be excised by lightly pulling on them. Older tissue appears unaffected by the herbicides until much later. The exact cause of plant death is still unclear. It has been postulated that a buildup of cytotoxic hydroperoxides are the cause of death after ACCase inhibition (Cummins et al. 1999).

Even though ACCase herbicides are used exclusively for grass weed control, some ACCase herbicides such as fenoxaprop, diclofop, and tralkoxydim, can be applied to grass crops. This tolerance is due to a differential rate of metabolism among grass species of these ACCase inhibitors (Shimabukuro et al. 1977, 1979). Tolerant grass species translocate the herbicide slower and detoxify the active herbicidal form faster than sensitive species.

The high selection pressure and the ability of some grass species to metabolize ACCase herbicides led to resistance problems soon after release. In 1982, less than five years after ACCase herbicides were commercialized, the first incidence of ACCase resistance was reported in Australia with annual ryegrass (*Lolium rigidum* L.) (Heap and Knight 1982). By 1987, just seven years after ACCase herbicide commercialization in the United States of America, Italian ryegrass (*Lolium multiflorum* L.) had developed ACCase resistance in Oregon (Stanger and Appleby 1989). This was after seven consecutive years of diclofop application (Gronwald et al. 1992).

ACCase resistance appears more often compared to other modes of action in susceptible species (Beckie et al 1999). This could be attributed to the high selection pressure applied by ACCase herbicides and the high use percentage in many broadleaf crops and some cereal grains (Legere et al 2000). Currently, there are three known mechanisms of ACCase resistance: target-site, metabolism, and protein over-expression. The first discovered and most frequent resistance mechanism is due to target-site mutations. Nomenclature is based on the blackgrass (*Alopecurus myosuroides* Huds.) ACCase which include at least eight resistance mutations: Ile-1781-Leu (the most common, Zagnitko et al 2001), Trp-1999-Cys (Liu et al 2007), Trp-2027-Cys (Delye et al 2005), Ile-2041-Asn (Delye et al 2003), Ile-2041-Val (Delye et al 2003), Asp-2078-Gly (Delye et al 2005), Cys-2088-Arg (Yu et al 2007), Gly-2096-Ala (Delye et al 2005). Likely there are more herbicide resistance mutations possible in ACCase but not yet reported.

Enhanced metabolism via increased cytochrome P450 activity is a common resistance mechanism as well. This mechanism was first reported in 1991 in annual

ryegrass (Holtum et al 1991). When enhanced metabolism occurs, it can lead to cross-resistance to other herbicides, including chemistries that have not been used before.

Over-expression of ACCase is a much rarer phenomenon than the previous two mechanisms. This was first achieved during selection of a sethoxydim resistant corn variety (Parker et al 1990), and again more recently (Dong et al 2010). There is limited evidence that weed species also can develop the ability to over-express ACCase (Bradley et al 2001).

Any one or combination of resistance mechanisms could be employed in wheat to develop a herbicide resistant phenotype. This would allow for increased weed control options in wheat. Chemical control options for grass weeds is limited in most cereal crops, primarily relaying on some acetolactate synthase (ALS) and ACCase inhibitors for post emergence applications. Imidazolinone-tolerant wheat has allowed the use of herbicides with greater activity on certain species than was achievable before its release. ACCase resistant wheat would expand the options to an even greater degree, enabling highly effective control of many species.

One complicated aspect of developing a herbicide resistant wheat cultivar comes from the hexaploid nature of wheat. Wheat has three copies of ACCase, one on each of its three genomes (Gornicki et al 1997). This presents a challenge in both the discovery and development of a cultivar with a resistant phenotype. In wild oat (*Avena fatua* L.), which is also hexaploid, only one resistant allele is required on one genome to produce a resistant phenotype (Christoffers et al 2002). While wheat is also hexaploid it is not known if a mutation on a single genome would produce ACCase resistance. With the known information regarding ACCase resistance development, and with recent advances

in knowledge of wheat ACCase (Chalupska et al 2008), it seems likely that mutagenesis could generate resistant alleles that could be rapidly discovered through whole plant screening. To determine the potential success of ACCase resistant wheat, the following studies were designed to evaluate their effectiveness on grass species problematic in wheat and including wheat.

Materials and methods

Weed dose response

A study was conducted to determine lethal doses of clethodim (Select 2 EC, Valent U.S.A. Corporation, Walnut Creek, CA, 94596) and quizalofop (Assure II, E.I. du Pont de Nemours and Company, Wilmington, DE, 19898) on common grass weed species in the western Great Plains. Weed species evaluated were downy brome (*Bromus tectorum* L.), feral rye (*Secale cereale* L.), green foxtail (*Setaria viridis* L.), Japanese brome (*Bromus japonica* L.), jointed goatgrass (*Aegilops cylindrica* L.), and wild oat. Clethodim and quizalofop were chosen due to their efficacy and because they are representatives from the two main ACCase herbicide families, cyclohexanediones (CHDs) and aryloxyphenoxypropionic acids (AOPPs).

Weed seeds were sown in rows with five seeds per row of each species in 54.5 by 28 by 6.3 cm flats with potting soil (Fafard #2 SV, Conrad Fafard, Inc, Agawam, MA 01001). Greenhouse conditions were set to 22 °C with a 14 hour daylength and natural lighting supplemented with 400 joule sec^{-1} sodium halide lamps. At the three leaf growth stage, seedlings were treated with herbicide. The low-end labeled use-rates were used as the 1x rate for the dose response; for clethodim this was 70 g ai ha^{-1} and for quizalofop

this was 31 g ai ha^{-1}. The experiment consisted of an untreated set of plants and five herbicide treatments. The herbicide rates were 2x, 1x, 0.5x, 0.25x, and 0.125x calculated from criteria above. Each herbicide solution was applied with 1% v/v methylated seed oil (MSO, Destiny, Winfield Solutions, St. Paul, MN 55164). Treatments were applied with an overhead track sprayer with a spray volume of 187 L ha^{-1} at 206 kPa. Plants were clipped above the newest growing point seven days after treatment (DAT). At 14 and 28 DAT, plants were evaluated categorically as either dead or alive. An LD_{90} was calculated as the herbicide dose in which 90% plant mortality was achieved. The study was conducted as a randomized complete block with six replications, and repeated once. Logistic analysis was performed using SAS 9.2 (SAS institute, Inc., Cary, NC, 27513) via the Probit procedure.

Wheat dose response

To efficiently identify resistant and susceptible wheat plants in the future, a dose response study was done to determine wild-type susceptibility to common ACCase herbicides that are normally lethal to wheat. The herbicides used were clethodim and sethoxydim (Poast, BASF Corporation, Research Triangle Park, NC, 27709) from the CHD family and fluazifop (Fusilade DX, Syngenta Crop Protection, Inc. Greensboro, NC, 27419) and quizalofop from the AOPP family. Hatcher winter wheat was the target variety for the study since it was used extensively in studying ACCase resistance development.

Rates of clethodim and quizalofop were the same as above. The 1x fluazifop rate was 87 g ai ha^{-1} and the 1x sethoxydim rate was 105 g ai ha^{-1}. This study was conducted and analyzed in a similar manner as above with the exception of rates above 1x. Instead

of using the 2x rate, the study included the upper labeled use-rates of 280 g ai ha^{-1} for clethodim, 210 g ai ha^{-1} for fluazifop, 92 g ai ha^{-1} for quizalofop, and 315 g ai ha^{-1} for sethoxydim. There was also an additional rate directly between the high and low-end use rates. All treatments contained 1% v/v MSO.

Results

Weed dose response

Of the species evaluated, all were controlled below the U.S. labeled field use-rates for quizalofop of 31 g ai ha^{-1} (Figure 1). Downy brome, green foxtail, and wild oat also were controlled by clethodim below 70 g ai ha^{-1}, the labeled use-rate for field application of clethodim, but required a slightly higher dose to control the remaining species.

This study demonstrates that both clethodim and quizalofop can achieve a high level of control in grassy weeds. Compared to clethodim, quizalofop provided better control at a lower relative use-rate. As such, in the absence of weed resistance, quizalofop would be a better chemistry to incorporate into an ACCase resistant wheat system. This would allow more diversified herbicide options for controlling many of these troublesome weeds, particularly acetolactate synthase (ALS) inhibitor resistant biotypes.

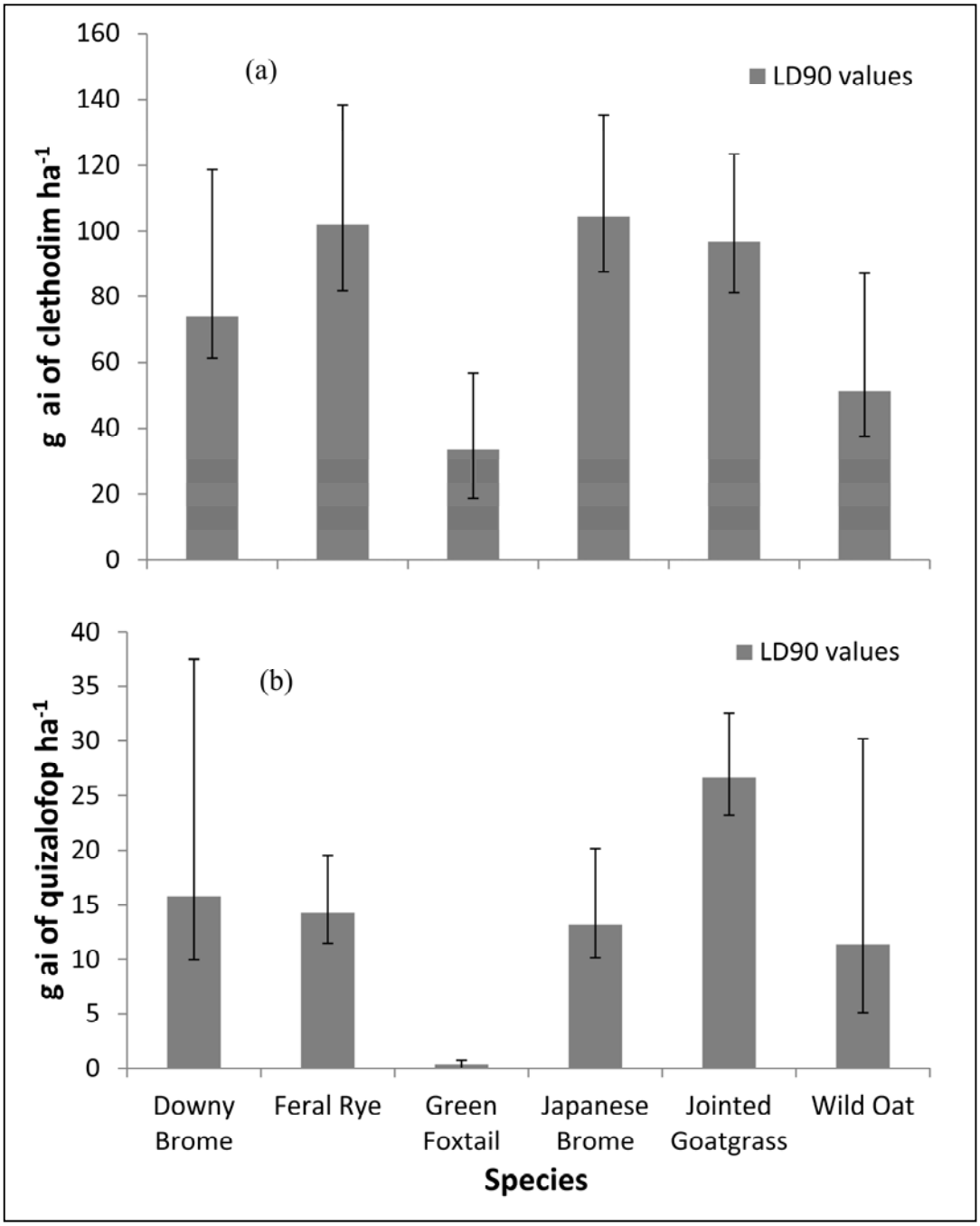

Figure 1. Control of common grass species with clethodim (a) and quizalofop (b) herbicides. LD90 values represent the herbicide dose in which 90% plant mortality was achieved.

Wheat dose response

Rates of fluazifop and sethoxydim were not high enough to accurately detect the LD_{90} values for the herbicides. Wheat appears to be inherently more tolerant to these herbicides (Table 1) than some of the weed species (Figure 1). Quizalofop had the greatest level of inhibition in respect to its use-rate, resulting in an LD_{90} at roughly 66% of the minimum labeled application rate. The clethodim LD_{90} was achieved within labeled application rates, but roughly double the minimum rate. Fluazifop and sethoxydim lethal doses were well above typical use-rates for the herbicides.

Table 1. Hatcher winter wheat susceptibility to ACCase herbicides as measured by 90% plant mortality (LD_{90}).

		-----95% confidence limit------		
Herbicide	LD_{90}	Lower limit	Upper limit	Labeled use-rates[a]
	g ai ha^{-1}	g ai ha^{-1}	g ai ha^{-1}	g ai ha^{-1}
Clethodim	129.4	107.5	164	70-280
Fluazifop	658.3[b]	448.7	1085.4	87-210
Quizalofop	21.1	15.6	29.3	31-92
Sethoxydim	528.3[b]	420.4	633.8	105-315

[a]These correspond to application rates used in legume crops for grass weed control in the U.S.
[b]These calculated rates are higher than the actual tested rates

Discussion

Dose response studies. Identifying a discriminating dose for large-scale herbicide resistance screening is paramount (Beckie et al. 2000). This allows for rapid identification of resistant and susceptible individuals in a large population. A discriminating dose for each herbicide was identified by measuring plant survival (Table 2). Clethodim and quizalofop are good herbicides to use in the development of an

herbicide resistant cropping system due to the relatively low use-rates required for control of weed species and wheat.

Fluazifop and sethoxydim would not perform as well as clethodim and quizalofop in large-scale screening trials. Wheat has a greater inherent tolerance to fluazifop and sethoxydim compared to clethodim and quizalofop, resulting in a high dose requirement relative to their typical use-rates. Using more effective herbicides will likely generate more discreet plant responses. When resistance to ACCase herbicides occurs, plants can become many fold resistant. Using a single rate or multiple rates at or above the calculated LD_{90} values should be an effective method of uncovering this phenotype.

ACCase wheat development. The development and release of ACCase resistant wheat would require a complex coordination of many research disciplines. Since genetically engineered wheat is currently not sold, mutation induction is likely the most effective method for developing a new commercial herbicide resistance trait. This could generate random mutations across the ACCase gene and by chance, an ACCase resistant mutant could be generated.

Identifying a herbicide resistant plant from a large mutant population will be difficult due to the hexaploid nature of wheat. Compared to a diploid, the phenotype will not be as distinct since there are three unique copies of ACCase in wheat (Gornicki et al 1997). To isolate the three individual ACCase genes in wheat, a primer set would be needed for each ACCase copy, increasing the time and resources required for genotyping. If the mutation conferring resistance occurs at a different locus, many additional experiments evaluating translocation, metabolism, copy number and others will be

required to characterize the mechanism of resistance. A likely non-ACCase target would be in the cytochrome P450 family, a group known to control metabolism of herbicides.

Due to the high diversity in naturally occurring resistance mutations (Delye et al. 2005), it would seem the odds of uncovering a novel mutation, even within the ACCase gene, are high compared to some other modes of action with more limited known resistance mutations. Characterizing the effect of mutations at the enzyme level would be necessary to demonstrate function through in-vitro studies and protein/herbicide interaction modeling. This would also limit the interference random mutations could cause in any identified herbicide resistant plants. Enzyme sensitivity studies were an important component in the early characterization of imidazolinone resistant wheat (Newhouse et al. 1992)

Stewardship. The key to success with a new herbicide resistance trait is to ensure the technology will remain useful. This should focus primarily on avoiding further ACCase resistance development in weed species.

Current information suggests that high selection pressure, such as application of a highly effective herbicide, enables rapid development of target-site mutations. Conversely, low herbicide use-rates, or applying herbicides when plants are too large, selects for minor allele/quantitative resistance development (Busi and Powles 2009). Both of these situations can occur readily with ACCase inhibitors (Delye et al. 2005; Holtum et al. 1991). In either scenario the resistant allele is likely to quickly spread in a population due to the out-crossing nature of many weedy grasses.

Known target-site mutations in ACCase do not cause resistance to all ACCase inhibitors. Each herbicide interacts with the ACCase molecule in a unique manner, the

result being that each mutation causes whole-plant resistance to a unique spectrum of ACCase inhibitors (Delye et al 2005). This is both an opportunity and a challenge in weed management. This is an opportunity in an ACCase -resistant cropping system to avoid certain ACCase herbicides, while still utilizing others if a particular mutation is known to be present. The challenging aspect will be identifying that mutation and educating the local growers and crop consultants on which herbicides are no longer as useful while other similar herbicides still are effective.

The implications of these situations are that increased use of ACCase herbicides is likely to generate more resistant weed populations. As such, if a herbicide-resistant cropping system is developed around the ACCase inhibitor chemistry, a rigorous stewardship program will have to be conducted simultaneously to delay herbicide resistance development in weed species. ACCase herbicides are often heavily relied upon in broadleaf crops. If ACCase herbicides are used on a wider range of crops, the outcome could parallel glyphosate resistance development following glyphosate resistant crop release world-wide. Herbicide resistance development is largely linked to the frequency of herbicide applications and equipment sanitation (Legere et al 2000, Davis et al 2009). It took 20 years for the first incidence of glyphosate resistance to develop, compared to five years for ACCase resistance, indicating a much more rigorous stewardship approach will be needed in conjuction with ACCase resistant crops.

To complicate the potential herbicide resistance development problem in wheat is the potential for gene flow between wheat and jointed goatgrass (Gandhi et al 2006). Wheat and jointed goatgrass share the ancestral D genome of *Aegilops tauschii*. This leads to very rare crossing between the species, but gene flow has been implicated in

studies to spread imazamox tolerance to jointed goatgrass from imazamox-tolerant wheat (Perez-Jones et al. 2010).

For much of the wheat growing areas in the United States, winter annual grass species account for the majority of grass weeds. For this reason, it should be recommended that a cover crop or tillage be used following an ACCase resistant wheat crop to compete with or eliminate weed seedlings in the fall. If tillage is not an option, the optimum non-chemical method of weed control seems to be a cover crop to provide a repression of resistance development (Beckie et al 2004). Incorporating tank-mixes with other modes of action, or herbicides that contain a residual component also seem to reduce herbicide resistance development (Neve et al 2007, Davis et al 2009). Ultimately, any practice that can minimize or eliminate seed production of weed species will reduce the risk of annual weed resistance development.

The key to the success of a stewardship program is grower education. In many cases, growers are not aware of a resistance problem or how to effectively manage it (Legere et al 2000, Davis et al 2009). With recent advances and accessibility to molecular biology, rapid screening of point mutations can be conducted (Delye and Michel 2005, Delye et al 2011). If growers are aware of ACCase resistance in the area, and have a rapid way to test for it, better management practices can be recommended. This approach would not account for many non-target-site based mutations due to the lack of information regarding the genetic control of these traits.

In summary, ACCase herbicide resistant crops would be a useful addition to weed management options in wheat. The highly effective nature of the chemistry allows for grass species control in cereal crops, an area with limited options in the past. However,

much caution and education is needed to prevent the spread of current ACCase resistant species and to reduce the introduction of new resistance alleles into a population. It would be ill-advised to release an ACCase resistant cereal crop without a long term stewardship program in place.

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Chapter 2: Ethyl Methanesulfonate Application to Wheat and Initial Screening for Acetyl Co-enzyme A Carboxylase Resistant Mutants

Summary

Wheat is one of the world's leading food crops, in acreage and consumption. The lack of market acceptance of wheat genetic engineering makes wheat a good candidate for mutation induction for new trait development. Acetyl co-enzyme A carboxylase (ACCase) point-mutations are known to be selected in weedy species, creating the potentialfor ACCase inhibitor resistance through mutation induction. A mutagenesis dose response study was conducted with EMS and sodium azide to obtain a high mutation density in wheat based on germination and seedling vigor reduction. An EMS concentration of 0.75% at an exposure length of 2.5 hours was found to provide the most adequate results. This population was grown in the field and the seed bulked to develop the M2 generation which was used for both forward and reverse genetics experiments. Over 3000 M2 individuals were incorporated into a DNA library. Herbicide selection was applied to M2 field- and greenhouse-grown populations to identify herbicide resistant mutants. In the M3 generation these mutants still often exhibited lower emergence rates than the non-mutagenized check but many accessions also displayed higher resistance to the herbicides used than non-mutant wheat. Overall, roughly 2 million M2 plants were screened with herbicides and by the M3 generation, 37 herbicide resistant phenotypes were identified.

Introduction

Acetyl co-enzyme A carboxylase (ACCase) inhibitors are potent graminicides. This herbicide family targets the plastidic isozyme of grass species' ACCase exclusively. ACCase inhibitors work by binding to the carboxyltransferase (CT) domain of the ACCase enzyme, preventing de-novo fatty acid biosynthesis (Zhang et al 2004, Catanzaro et al 1993, Rendina et al 1988, Burton et al 1987).

Resistance to ACCase inhibitors in weedy populations was discovered shortly after the commercialization of the first ACCase herbicide diclofop (Heap and Knight 1982). It was later determined that single nucleotide polymorphisms (SNPs) were a major cause for resistance to ACCase herbicides, with several mutations occurring in the CT domain (Delye et al 2005, Yu et al 2007). This demonstrates the potential for the use of mutation induction in wheat to find a mutation conferring resistance to ACCase inhibiting herbicides.

Mutagenesis has long been a tool for plant breeders (Muller 1927). With the increasing accessibility to molecular genetics, mutagenesis is once again becoming a powerful tool in trait discovery and plant improvement (McCallum et al 2000). Efficient, high throughput systems have been developed for screening populations of mutant wheat. Targeting Induced Local Legions IN Genomes (TILLING) is one such platform, relying on genotyping specific loci and correlating discovered mutations to a phenotype (Slade et al 2005). Interest in mutagenesis and TILLING has been growing in wheat with some early successes in new trait variations (Uauy et al. 2009; Dong et al. 2009; Till et al. 2007; Slade and Knauf 2005; Slade et al. 2005)

Genotyping random mutations can be problematic when a specific phenotype is desired. Synonymous or nonfunctional mutations may be generated via mutagenesis,

which may lead to false positives at the nucleotide level. In the case of screening for herbicide resistance, plant survival after herbicide application is a highly efficient method for detecting the desired phenotype. Such a large scale technique was used in the discovery of imidazolinone-tolerant wheat (Newhouse et al 1992). Thousands of plants were screened in greenhouse conditions in this case. ACCase resistant *Zea mays* was also developed via large screening populations with tissue culture selection (Parker et al 1990). Even with redundancy in the genome due to its hexaploid nature, phenotypic screening of wheat mutants via herbicides should be possible for detecting an ACCase resistance trait, especially if the population is large enough.

The objective of this research was to establish a population of plants suitable for both forward and reverse genetics approaches, with investigation into new trait discovery.

Materials and methods

Mutagenesis dose response

Two germination experiments were conducted to determine an appropriate level of mutagenic compound to optimize mutation frequency and survival in Hatcher winter wheat (Haley et al. 2005). These tests used two common mutagens, ethyl methanesulfonate (EMS) and sodium azide (SA). Before application of the mutagen, 100 g samples of wheat seed were soaked in tap water overnight (approximately 16 hours). The mutagen concentrations and exposure times ranged from 20 to 120 mM from 1 to 4 hours for EMS and 1 to 5 mM from 1 to 4 hours for SA. Solutions containing SA were pre-treated with 3% v/v of a 1M stock solution of KH_2PO_4 (adjusted to pH 3 with phosphoric acid). The wheat seed was treated in a fume hood with proper protective gear.

Samples were stirred every half hour while exposed to mutagen. Following application of mutagen, seeds were rinsed in flowing tap water for 1 hour and left to dry for 24 hours.

A germination test was conducted with 50 seeds of each of sample. Two replications of twenty five seeds from each mutagen treatment were placed in germination paper (Anchor Paper Co, Saint Paul, Mn 55101) and incubated in a growth chamber at 15 °C for seven days in darkness. Percent germination was determined after seven days. Also, a damage rating was taken on a scale of 1 to 5, based on the unmutagenized samples. This rating system is described in Table 2.

Table 2. Rating system used for wheat injury due to mutagen application.

Damage rating	Description
1	Little to no damage
2	Visibly shorter shoot than untreated
3	Shoot/root roughly half the length of untreated
4	Very little elongation of shoot/root
5	Germination occurred but no elongation of shoot/root

M1 population development

A larger sample of wheat seed was mutagenized for developing a mutant trait screening population. The procedure was largely the same as above, with the exception that 2.27 kg of wheat seed was used in ~19 liter containers. Seeds were soaked in 5 liters water containing an EMS concentration and exposure of 0.75% for 2.5 hours; for SA it was 5 mM for 1.5 hours in five liters of water pre-treated with KH_2PO_4 as above. Before seeds were rinsed in tap water, the seeds were washed in three additional 19 liter containers containing 2.5 L water. The samples were planted within 48 hours after removal from mutagen.

An estimated 106,000 EMS- and SA-treated wheat seeds were planted in an irrigated field site at the Agricultural Research Development and Education Center (ARDEC) of Colorado State University, Fort Collins, CO in February, 2009. Plants were harvested in August 2009. Roughly 3000 individual spikes were selected at random from each population for use in DNA library construction. The remaining plants were bulk harvested with a plot combine and the bulk seed was stored for future use.

M2 population development

Compared with SA, seeds treated with EMS had lower vigor and a higher percentage of unique phenotypes (such as leaf bleaching and albino plants) indicating a higher mutation rate and thus were used for the remaining experiments. The EMS M2 generation was divided into three lots. The first lot was established from the 3000 individually collected heads for use in TILLING. One lot consisting of approximately 250,000 seeds was grown in the greenhouse, and a third lot consisting of approximately 2.5 million seeds was grown on an irrigated field site.

TILLING population. One seed from each head of wheat from the TILLING population was soaked for 1 minute in 0.1% of a formulation containing 200 g L^{-1} carboxin and 200 g L^{-1} thiram (Vitavax, Chematura Agrosolutions, Adelaide, SA Australia). One seed per well of a 24-well plate was placed on 2 mL agar (Phytagar, Gibco-BRL Life Technologies, Rockville, MD, 20852). The seeds were then vernalized for six weeks at 4 °C with an eight hour day cycle. Plants were watered weekly to start, with 1/4x Hoagland's solution (Hoagland's no. 2 basal salt mixture, Sigma-Aldrich Corp, St. Louis, MO 63103), then watered more frequently as the seedlings grew larger. After vernalization, seedlings were transplanted, with agar, into 6.5 by 20 cm cones with

potting soil (Fafard #2 SV, Conrad Fafard, Inc, Agawam, MA 01001) and grown at 18/22 °C with a 14 daylength. Plants were top-watered daily and bottom watered weekly with 20-20-20 fertilizer (Miracle Gro, Scotts Miracle-Gro Products, Inc., Marysville, OH 43041).

At the 6-8 leaf stage approximately 15 cm of leaf tissue from each plant was collected and frozen. DNA was extracted with an SDS extraction method. DNA concentration was measured with a spectrophotometer and each sample was standardized to a 50 ng ul^{-1} concentration.

M2 greenhouse population. Over 250,000 plants were screened in the greenhouse for detecting resistance to ACCase inhibiting herbicides. Seeds were planted, 500 at a time, into 54.5 by 28 by 6.3 cm flats with potting soil. Greenhouse conditions were 22 °C with a 14 hour daylength and natural lighting supplemented with 400 joule sec^{-1} sodium halide lamps.

At the three-to-four leaf stage, plants were treated with either clethodim (Select 2 EC, Valent U.S.A. Corporation, Walnut Creek, CA, 94596), a member of the cyclohexanedione herbicide family at a rate of 129.4 g ai ha^{-1}, or quizalofop (Assure II, E.I. du Pont de Nemours and Company, Wilmington, DE, 19898), a member of the aryloxyphenoxypropionate acid family, at a rate of 21.1 g ai ha^{-1}. Application rates were calculated from a previous dose response study and included 1% methylated seed oil (Destiny, Winfield Solutions, St. Paul, MN 55164). Treatments were applied with an overhead track sprayer at a spray volume of 187 L ha^{-1} at 206 kPa. Seven days after treatment (DAT) seedlings were clipped above the base of the newest emerging leaf. Surviving plants were allowed to recover and at 28 DAT seedlings were treated then

clipped again as above. Survivors were transplanted individually into round 15 by 15 cm pots with potting soil and M2:M3 seed was collected at maturity. Seeds collected from an individual M2 plant will be referred to as an accession.

M2 field population. Approximately two ha were planted under irrigation with EMS mutagenized wheat in Oct 2009. In April 2010, two-thirds of the field was treated with clethodim, while the remaining portion was treated with quizalofop at the above rate and sprayer conditions. A second dose of quizalofop was applied in late May 2010 due to a high wheat survival rate. The clethodim portion of the field was not treated a second time due to the maturity of the wheat and lower incidence of wheat survival compared to quizalofop treatment. M3 seed from individual survivors was collected on July 20 2010. Seeds collected from an individual M2 plant will be referred to as an accession.

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M3 population screening

Seed of field and greenhouse M2 selections were grown in greenhouse conditions similar to above. Five seeds per accession were evenly planted into 12.7 by 12.7 by 5 cm inserts with potting soil.. At the three leaf stage, quizalofop-selected seedlings were treated with 21.05 g ai ha^{-1} quizalofop, and clethodim-selected seedlings were treated with 129.44 g ai ha^{-1} clethodim at sprayer settings listed above. Seedlings were clipped above the base of the newest emerging leaf 7 DAT and treated with their respective herbicides 28 days after initial treatment. Plants were clipped once again 7 days after this treatment. Data were recorded categorically as plant survival or death. DNA was collected from survivors and the plants were transplanted as above.

This study was organized in a randomized complete block design with two replicates and repeated once. Data were analyzed with a general linear model in SAS 9.2 (SAS Institute, Inc., Cary, NC, 27513) and means separation was performed using Fisher's protected LSD at the 0.05 probability level.

Results

Mutagenesis dose response

EMS concentration and exposure times provided an adequate range of germination and damage ratings for optimizing future mutagenesis experiments (Table 3). Both concentration and exposure times were important factors in determining damage to seed. At an average damage rating of 5, seeds would almost always terminate growth before or shortly after emergence, even though germination was initiated (data not shown). This created problems determining an exact dose to be used in seeds planted at the field level since a higher vigor would likely be needed to survive outdoor conditions than growth chamber conditions, where these seeds were tested. A target rate of 50-60% germination is typically the benchmark for many mutagenesis studies in wheat to optimize mutation density, while retaining fertile seed production capacity (Dong et al 2009, Uauy et al 2009). Since a large portion of the M2 seed would be used for large-scale phenotypic screening, a slightly more conservative rate was adopted to limit deleterious mutations while still maintaining a high chance for herbicide resistance trait discovery. As such, a rate of 0.75% for 2.5 hours was chosen for further EMS mutagenesis.

The SA treatments yielded a similar range of data for germination reduction and damage (Table 4). Using the same logic as above, a rate of 5 mM for 1.5 hours was

chosen for the field level trial. Relatively speaking, damage due to SA application was more mild than EMS application, even when germination rates were similar. In fact, SA treated plants that emerged in the field displayed little or no phenotypic injury. Plants treated with EMS had a higher, yet still small number of unusual phenotypes (<1%) such as albinism and variegated chlorotic patterns.

Table 3. Ethyl methanesulfonate dose response conducted on winter wheat.

EMS concentration	Exposure	Germination	Damage rating
mM	hour(s)	%	
0		100	1
20	1	100	1
20	2	100	1
20	4	98	2
60	1	100	3
60	2	90	3
60	4	68	4
120	1	58	4
120	2	2	5
120	4	4	5

Table 4. Sodium azide dose response conducted on winter wheat.

SA concentration	Exposure	Germination	Damage rating
mM	hour(s)	%	
0		100	1
1	4	100	1
2	2	86	3
2	4	94	3
5	1	82	3
5	2	38	4
5	3	14	4
5	4	14	5

Based on stand counts taken at the three leaf stage from the field, the EMS population had an emergence rate of approximately 55%, and the SA population had an

emergence rate of 52%. For EMS, this is lower than would be indicated by Table 4. The SA population seems similar with predicted results. The EMS population was used for all future experiments due to the indication of higher mutation frequency based on mutant phenotype occurrence in a subset of the population.

M1 seed was not screened for any mutations due to the nature of mutagenesis. The first generation of mutants are heterozygous at mutant loci (Slade et al 2005). By the M2 generation, homozygous plants can be produced via segregation. This is likely very important for phenotypic screening of a hexaploid plant known for redundancy in its genetic code.

M2 population development

TILLING population. Cel1 digestion was utilized in mutation detection from PCR product, as per Till et al. (2006). A 1.5% agarose gel was substituted for the polyacrylamide gel used in this procedure. Screening two stress-related genes with genome specific primers a density of 1 mutation per 96 kb was calculated in the TILLING population based on the number of mutations found in each gene (Harish Manmathan, personal communication). This density was lower than other examples (Slade et al 2005, Dong et al 2009, and Uauy et al 2009).. Approximately 15% of plants failed to produce seed either through lack of growth or infertility. This was assumed to be related to deleterious mutations. Any DNA collected from non-reproducing plants was excluded from further studies. M3 seed was harvested from M2 plants and stored for use in mutation detection assays on the M2 generation.

M2 greenhouse population. Herbicides can be used very effectively to identify herbicide resistance and make large-scale, whole-plant screening just as effective.

Herbicide screening allows identification of the target phenotype, regardless of which gene the herbicide resistance mutation occurs. Of the 157,000 mutants screened with clethodim, 42 herbicide resistant mutants were identified. Over 99,000 mutants were treated with quizalofop and 26 mutants were identified. This equates to one in 3738 mutants resistant to clethodim and one in 3826 mutants resistant to quizalofop, although there is potential for multiple plants sharing a single mutation event since the M1 was bulk harvested. Subsequent screening of greenhouse-selected mutants in the M3 resulted in little evidence of increased resistance (data not shown).

M2 field population. Roughly 800,000 plants were treated with quizalofop and 1.7 million plants were treated with clethodim. This led to the collection of 167 potential clethodim resistant mutants and 46 quizalofop mutants. Based on the mutation frequency, and known herbicide/ACCase interaction zones (Delye and Michel 2005) an estimated 2200 and 4400 potential nonsynonymous mutations exist among the quizalofop and clethodim selected plants, respectively.

M3 population screening

A total of 213 field-selected accessions were screened for resistance in the M3 generation. A wide range of responses were observed. Seed germination was still reduced in many accessions, even though parents were still able to reproduce. Roughly 20% of total accessions tested had an emergence level lower than non-mutagenized wheat (Table 5). Less than half of the M2 accessions selected showed any resistance to the herbicides in the M3. This is likely due to selecting herbicide escapes following M2 screening. After a second herbicide application less than 10% of the clethodim selected accessions survived.

Table 5. Response of M2 derived M3 accessions (M2:M3) during herbicide screening. The data represent a mean response based on emergence reduction or plant survival across an accession.

Accession type	Total accessions	Reduced Emergence rate	Survived one herbicide dose	Survived two herbicide doses
	No.	No.	No.	No.
Clethodim	167	33	72	9
Quizalofop	46	9	18	18

The majority of accessions screened showed some emergence reduction, although many did not differ from the non-mutagenized check. There were also still a small number of accessions with similar emergence to the M1 generation.

Even with a high level of variability in the data, differences were noted in plant survival rate to a single application of clethodim (Figure 2a). In fact, the data were surprisingly well distributed across the range of outcomes and nearly half of clethodim-selected mutant accessions exhibited increased survival over non-mutant Hatcher. Even though only nine accessions had greater plant survival than the background after two applications, it is apparent that high survival rate of one application was not a predictor of success for two applications (Figure 2b). This is demonstrated with multiple accessions showing survival in the 80-90% range after one application yet being completely desiccated after the second application.

The clethodim response is in contrast to accessions screened with quizalofop (Figure 4), where plant survival was nearly identical between the first and second dose. Not only that, but the data were distributed over a much narrower range. The narrower range tends to suggest definitive presence or absence of a resistance trait, compared to the

data in Figure 2. This was even more apparent in personal observation of the surviving plants' increased vigor after quizalofop treatment, compared to clethodim.

The most accessions resistant to both clethodim and quizalofop were used for genotyping and phenotyping experiments to further characterize the mechanism of resistance.

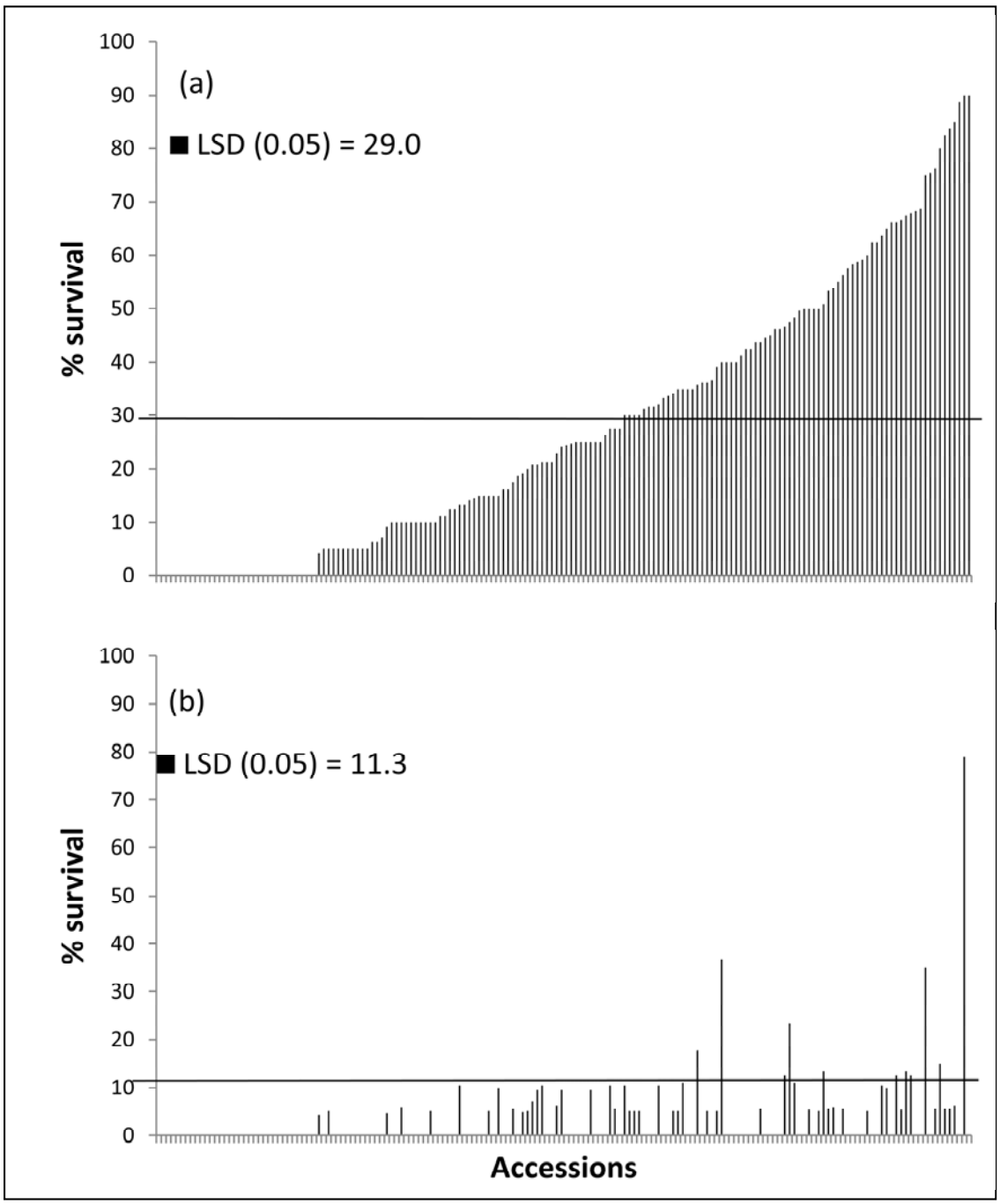

Figure 2. Survival rate of SF accessions after one (a) or two (b) applications of clethodim. Accessions are listed in the same order for a and b. Survival rates above the horizontal line are higher than non-mutagenized wheat.

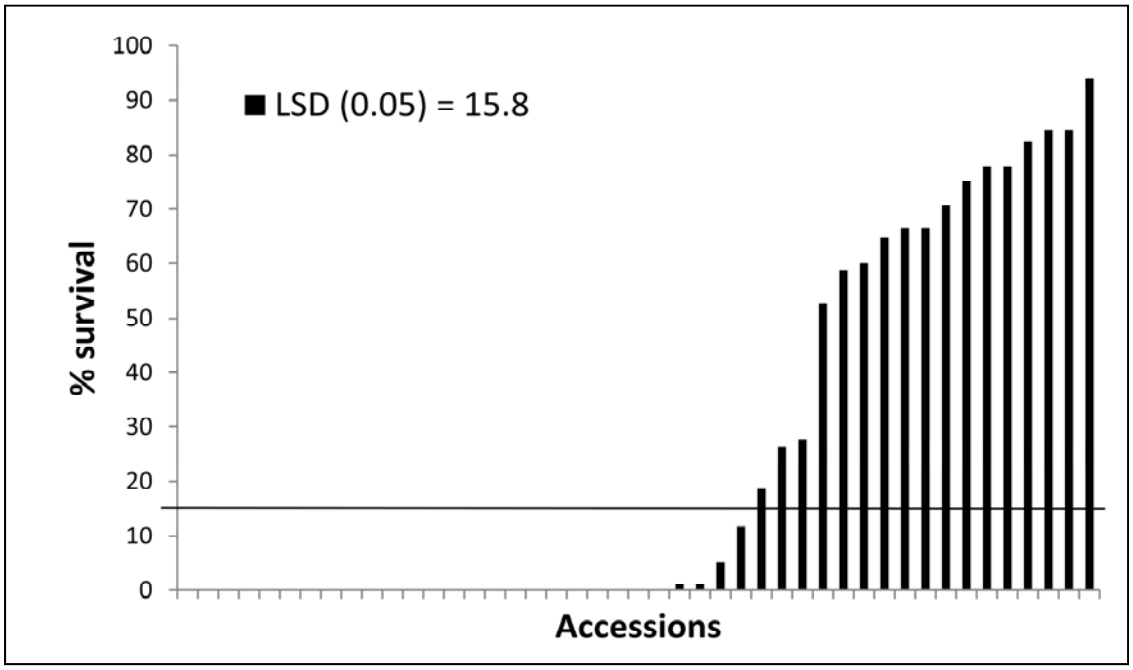

Figure 3. Survival rate of quizalofop-resistant accessions after two applications of quizalofop. There were no differences between one and two applications of this herbicide. Survival rates above the horizontal line are higher than non-mutagenized wheat.

Discussion

The data in Figures 2 and 3 allow for some conjecture. Since the quizalofop resistant mutants tended to survive multiple herbicide applications equally well, a target-site mutation is a likely cause of the resistance. If ACCase changes conformation due to mutation and no longer interacts with the herbicide there would be no enzyme inhibition essentially making the enzyme immune to the presence of the herbicide at any concentration.

The mechanism of action in the identified clethodim mutants is difficult to hypothesize based on their phenotype. The low survival number after a second application indicates that the plant was eventually overwhelmed by the herbicide, which is more characteristic of a non-target site mutation. Based on observation of plant vigor (data not shown), some of the most resistant accessions were not necessarily accessions

with the highest survival after two herbicide applications. Some of the clethodim resistant accessions also appeared to have a segregating response based on survival rates, something not noted with the quizalofop resistant population.

The CT domain of wheat is just under 1650 bps. With the size of the population screened (~3 million individuals) and mutation rate documented (1 per 96 kb), just over 100,000 nonsynonymous mutations would be generated in the population. Even with this knowledge, it would be difficult to preemptively estimate the number of herbicide resistant mutants generated since the herbicide binding site is a smaller portion of the CT domain and each mutation causes unique herbicide-enzyme interaction differences. It also remains to be seen how many resistance mutations would be required in a single wheat plant for commercial-level resistance to ACCase inhibitors. The experiments were designed for mutant detection via phenotypic screening and did not take fitness into consideration, something that will need to be evaluated before the trait can be incorporated into commercial germplasm.

This study demonstrates the successful use of a chemical mutagen for uncovering trait variation in response to selection, even with a genome as complex as wheat. The interval between initial mutation induction and detection of mutant phenotypes was just under 2 years, making this an efficient method of trait discovery.

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Chapter 3. Characterization of Mutant Winter Wheat Accessions Resistant to Quizalofop

Summary

While herbicide resistant crops have become common in many agricultural systems, wheat has had few introductions of this technology. Quizalofop resistant wheat accessions were identified in herbicide screening studies of mutagenized plants. A series of experiments were designed to phenotypically and genotypically characterize this resistance mechanism. These 18 accessions were found to have a 1.5 to 7.5 fold increase in tolerance over non-mutant wheat. Some accessions also contained a low level of resistance to clethodim and sethoxydim. DNA sequencing revealed a novel C to T substitution resulting in a change from alanine to valine in acetyl co-enzyme A carboxylase (ACCase) at position 2004 based on *Alopecurus myosuroides* notation. This mutation was discovered multiple times on each of the three homologous wheat chromosomes among the accessions studied. Enzyme activity of representatives for the three mutations revealed a 4 to 10 fold increase in tolerance to quizalofop. The data from these experiments suggest that a single new point mutation in the ACCase gene confers whole plant resistance to quizalofop.

Introduction

Acetyl co-enzyme A carboxylase catalyzes the first committed step to de-novo fatty acid biosynthesis in the chloroplast, converting acetyl co-enzyme A to malonyl co-enzyme A, the plant's only known source for malonyl CoA (Sasaki and Nagano 2004).

This makes ACCase a potent herbicidal target, where inhibition effectively stops synthesis of essential building material for new cells. It is well known that single point mutations in this gene can cause resistance to ACCase inhibiting herbicides (Delye et al. 2005).

Eight ACCase single nucleotide polymorphisms (SNPs) that confer herbicide resistance have been reported (Delye et al. 2005, Yu et al. 2007). Each SNP confers resistance to a different subset of herbicides within the ACCase inhibitors. Each SNP occurs in the carboxyltransferase domain of ACCase within 1200 nucleotides of each other, all but one of which are contained within a 300 nucleotide region. Recent crystallography studies and simulations of wild type and resistant ACCase have indicated altered protein structure of ACCase prevents or reduces normal herbicide binding to the enzyme and allow the enzyme to remain largely functional in the presence of the inhibitor (Xiang et al. 2009, Zhang et al. 2004, Zhu et al. 2009).

ACC1 (plastidic ACCase) is a well characterized single copy gene in each wheat genome (Gornicki et al. 1997). ACCase has been extensively studied in wheat for phylogenetic analysis due to its high degree of conservation across species (Chalupska et al. 2008, Huang et al 2002) as well as various other experiments (Gornicki and Haselkorn 1993, Gornicki et al. 1994, Konishi and Sasaki 1994, Zuther et al. 2004). Other than being an herbicide target, ACCase modification has also been associated with modifications in seed oil content, plant cuticle formation, secondary metabolites and improving general plant health (Chalupska et al. 2008, Sasaki and Nagano 2004).

In wild oat (*Avena fatua* L.), another hexaploid, a single SNP on one genome can confer whole plant resistance (Christoffers et al. 2002). It is unknown if a single mutant

copy in wheat would confer commercially acceptable resistance to ACCase inhibitors. Fortunately, ACCase in-vitro assays have been developed to infer herbicide binding affinity to ACCase (Bradley et al. 2001, Seefeldt et al. 1996, and Yu et al. 2003). This assay will help determine the effect of individual mutations on a phenotype, and potentially provide clues toward the efficiency of combining multiple mutations in a single plant.

Winter wheat accessions resistant to quizalofop herbicide have recently been developed by phenotypic screening of M2 plants from ethyl methanesulfonate (EMS) treated Hatcher winter wheat seeds. The following experiments were designed to genotypically and phenotypically characterize potential mutations conferring this resistance.

Materials and methods

Greenhouse evaluations

M2:3 (M2-derived M3) plants selected for quizalofop (Assure II, E.I. du Pont de Nemours and Company, Wilmington, DE, 19898) resistance were evaluated in greenhouse studies. Plant material was selected from previous studies (Ostlie 2011) and represented the accessions most tolerant to quizalofop. Each accession was given an AF prefix to designate that the selection pressure was Assure II applied to a field population

Three studies were conducted in a greenhouse at 22 °C with a 14 hour daylength and natural lighting supplemented with 400 joule sec^{-1} sodium halide lamps. Each replicate consisted of two seeds planted in 7.6 by 7.6 by 8.9 cm inserts with potting soil (Fafard #2 SV, Conrad Fafard, Inc, Agawam, MA 01001). All herbicide applications were made at the three to four leaf stage of wheat and included 1% methylated seed oil

(Destiny, Winfield Solutions, St. Paul, MN 55164). Treatments were applied with an overhead track sprayer at a spray volume of 187 L ha^{-1} at 206 kPa. All studies were conducted as a randomized complete block design and repeated once.

An herbicide injury study was conducted with 15 replicates. Treated accessions included a non-mutagenized Hatcher wheat check plus 18 quizalofop- resistant M2:3 accessions. Seedlings were treated with 21g ai ha^{-1} quizalofop, a discriminating dose based on previous studies (data not shown). Each accession was represented with a non-treated check. Seedlings were evaluated 28 days after treatment (DAT) for visible injury to quizalofop on a scale of 0 to 100%, with 0 being no injury and 100 being complete desiccation. The data were analyzed using a general linear model with SAS 9.2 (SAS Institue, Inc., Cary, NC, 27513) and means separation was conducted using Fisher's protected LSD (α=0.05).

A dose response study was conducted as a randomized complete block design with a split-plot arrangement. Herbicide dose was the main plot effect and wheat accession was the sub-plot effect. The study included five herbicide doses, a non-treated check, and three replicates for each treatment combination. Quizalofop doses were 11, 23, 46, 92, and 184 g ai ha^{-1}. Seven DAT the tops of seedlings were excised above the newest above-ground growing point. Categorical evaluation of plant survival was performed 28 DAT. Data were analyzed using the logistic function in the probit procedure of SAS and an LD_{50} was calculated for each accession. An LD_{50} represents the herbicide dose resulting in 50% plant mortality based on logistic analysis.

A cross resistance study was conducted within the ACCase herbicide mode of action using herbicides normally lethal to wheat. Clethodim (Select 2 EC, Valent U.S.A.

Corporation, Walnut Creek, CA, 94596) was applied at 65 g ai ha^{-1} and sethoxydim (Poast, BASF Corporation, Research Triangle Park, NC, 27709) was applied at 264 g ai ha^{-1}, representing members of the cyclohexanedione family; while fluazifop (Fusilade DX, Syngenta Crop Protection, Inc. Greensboro, NC, 27419), another member of the aryloxyphenoxypropionate acid family, was applied at a rate of 361 g ai ha^{-1}. An additional treatment of clethodim at the above rate plus quizalofop at a rate of 10.5 g ai ha^{-1} was included. These rates are roughly half an LD_{90} calculated from a previous dose response study on non-mutant wheat (data not shown). Seven DAT plants were clipped as above and categorical evaluation was performed 28 DAT. The experiment was designed as a randomized complete block with a split-plot arrangement with three replicates. The main plot effect was herbicide treatment and the sub-plot effect was wheat accession. Data were analyzed with a general linear model in SAS. Means separation was calculated using a weighted T statistic to account for differences in variance between the two main effects of accession and herbicide treatment and allowed for comparison of the interaction.

DNA Extraction and Sequencing

Quizalofop resistant plants were identified in a previous greenhouse screening study (Ostlie 2011). Two inches of leaf tissue was collected from one- to- two quizalofop resistant plants from 22 accessions and frozen in liquid nitrogen. DNA was extracted using a CTAB extraction method. Extraction buffer consisted of 5% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCL, 1% w/v PVP 20, and 100mM 2-Mercapto-ethanol. Leaf material was ground to fine powder in 1.5 mL tubes. 800 µL of extraction buffer was added and incubated for 30 minutes at 65 °C with tube inversion occurring

every 10 minutes. Samples were centrifuged at 12,000 g for 5 minutes. The supernatant was transferred to a new 1.5 mL tube and 1 volume of 24:1 chloroform:iso-amyl alcohol was added. Samples were kept on ice for 30 minutes with inversion occurring every 10 minutes. Samples were then centrifuged at 20,000 g for 15 minutes. Supernatant was transferred to a new 1.5 mL tube and 1 volume of chilled (-20 °C) isopropanol was added. The sample was centrifuged at 20,000 g for 30 minutes. Supernatant was discarded. The samples were washed with 500 µL of 70% ethanol and centrifuged at 20,000 g for 5 more minutes. The ethanol was discarded and samples dried. DNA was resuspended and stored in 100 µL of 10 mM TE buffer.

To develop genome specific primers, generic ACCase primers from Delye and Michel (2005) were used with standard polymerase (NEB Taq, New England Biolabs, Inc., Ipswich, MA 01938). Samples were then cloned with an *E. coli* vector (pGEM-T Easy, Promega Corporation, Madison, WI 53711) and sequenced using those same primers. Three unique sequence sets were identified and correlated to a specific genome by comparison to EU660900, EU6600901, and EU660902 (Chalupska et al 2008). The cloned sequences were identical to the Chalupska et al. (2008) sequences and submitted to GenBank (JQ073901, JQ073902, JQ073903; http://www.ncbi.nlm.nih.gov/genbank/).

Due to the similarity, the full length ACC-1's reported in Chalupska et al (2008) were used to design genome specific primers in the intron regions surrounding the carboxyltransferase domain to avoid any chance of ACC-2 sequence contamination and maximize the chance of discovering herbicide resistance mutations. These primers were able to amplify a single sequence without inclusion of paralogs from other genomes (Table 6). This was tested by PCR analysis with *Triticum urartu*, *Aegilops speltoides*, and

Aegilops tauschii as the closest diploid A, B, and D genome progenitors, respectively (Chalupska et al 2008).

PCR with genome specific primers was carried out with a high fidelity polymerase (Velocity DNA Polymerase, Bioline USA, Inc, Tauton, MA 02780). The thermocycler protocol used for the primers consisted of an initial denaturation temperature of 98 °C for two minutes followed by 30 cycles of 98 °C for 30 seconds, annealing temperatures for 30 seconds, extension at 72 °C for 1.5 minutes, and final extension for 10 minutes at 72 °C. Annealing temperatures were 62 °C for A genome primers, 70 °C for B genome primers and 56 °C for D genome primers. The D genome primer set had some differences from the A and B genome primer sets including: 14 cycles of touchdown beginning at 63 °C and decreasing 0.5 °C each cycle to end at 56 °C before 35 cycles of the protocol listed above.

Table 6. Primer sequences for PCR of genome specific DNA fragments and for subsequent sequencing reactions. All sequences are 5' – 3'.

Primer specificity	**Primer Sequence**
PCR primers	
A genome forward	CTC CCT CTC TAT CTC TAT ACA TGT ATG
A genome reverse	GGA TCC ATC TGA ACA AGT GAC
B genome forward	ATT TTC CGT TGG TGA GTA TCT CCC TCT CTG TCT CTA TAC
B genome reverse	AGG GAT CCA TCT GAA CAA GCG AG
D genome forward	TTC CGT TGG TGA GTA TCT CGC TCT CTT
D genome reverse	CCT CGA ATA ACA GTT GCC TCC AAT AAC AG
Sequencing primers	
ACCp1*	CAA ACT CTG GTG CTC GGA TCG
ACCp1R*	GAA CAT AGC TGA GCC ACC TCA
ACCp4*	CAG CTT GAT TCC CAT GAG CGG
ACCp2R*	CCA TGC ACT CTT GGA GTT CCT

*modified for wheat specificity from Delye and Michel (2005).

Sanger sequencing was conducted with purified PCR product using the sequencing primers in Table 6. These primers were designed for wheat with the intention

of amplification around the two herbicide/protein interaction sites with known resistance mutations existing in weedy populations. Each area had a sequencing overlap of 400-550 bps between forward and reverse primers, with additional extension beyond due to the original amplification length of over 2000 bps from the PCR reaction. If All sequences were compiled and aligned using Geneious (Biomatters Ltd., Auckland, New Zealand, 1010).

ACCase enzyme activity

To confirm that identified mutations conferred whole plant resistance, an in-vitro enzyme assay was conducted to measure ACCase activity in conjunction with quizalofop-P-ethyl (98.2%, Sigma-Aldrich Corp, St. Louis, MO 63103) directly. This assay uses a partially purified plant protein extraction, in concert with a radio-labeled reagent for the reaction, to measure the ATP-dependent incorporation of tracing compound (sodium bicarbonate) into malonyl CoA. If the herbicide interacts with ACCase, it reduces or prevents the incorporation of the tracing compound into the final product.

The procedure was adapted from Seefeldt et al. (1996) and Yu et al. (2003). The most actively growing shoot tissue of three-leaf M2 derived M4 plants was pooled together to gain three grams of ACCase-rich material per accession tested. The protein extraction was conducted in a cold chamber at 3 °C. The tissue was ground in liquid nitrogen and sand in a mortar and pestle with 5 mL extraction buffer (100 mM Tris (pH 8), 1 mM EDTA, 10% glycerol, 2 mM L-ascorbic acid, 1 mM PMSF, 20 mM DTT, 0.5% PVP40, 0.5% PVPP). An additional 5 mL of extraction buffer was used to clean the mortar and pestle. The solution was centrifuged at 25,000 g for 15 mins and the The

pellet was discarded. Saturated ammonium sulfate was added, dropwise, to a final concentration of 66% of the original supernatant volume. The solution was stirred for one hour to allow the protein to precipitate. Centrifugation was performed at 25,000 g for 30 mins. The supernatant was discarded and the pellet was dissolved in 2.7 mL elution buffer (50 mM Tricine, 50 mM KCl, and 2.5 mM $MgCl_2$). The solution was then placed on an equilibrated Sephadex G-25 column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ 08855) and eluted with 2.8 mL elution buffer. Samples were stored with 25% glycerol in -80 C until the assay was conducted.

Four quizalofop concentrations of 0.1, 1, 10, and 100 μM were included in the assay along with a non-treated control. The assay solution included 20 mM tricine (pH 8.3), 10 mM KCl, 2 mM $MgCl_2$, 2.5 mM DTT, 0.1% BSA, 10 mM ATP, 40 mM $NaHCO_3$ which included 2 kBq of $NaH^{14}CO_3$, and 50 uL of enzyme extract. This solution was prepped at 30 °C for 3 mins. Acetyl CoA was left out of a set of blanks for each accession but included at 3 mM in the remaining solutions. The reaction was carried out at 30 °C for 10 minutes and stopped by the addition of 20 uL glacial acetic acid. Since a byproduct of the reaction is $^{14}CO_2$, the addition of acid was conducted in a sealed 46 by 46 by 61 cm plexiglas container with air being pulled through a CO_2 absorbing compound (Ascarite, Sigma-Aldrich Corp, St. Louis, MO 63103). The samples remained in the container for one hour, at which point they were transferred to glass fiber paper inside liquid scintillation vials. Samples remained an additional hour in the vials before 10 mL scintillation cocktail (Ultima Gold LLT, PerkinElmer Life and Analytical Sciences, Inc. Waltham, MA 02451) was added. Samples were then measured for radioactivity via liquid scintillation spectroscopy.

ACCase activity was determined on a percentage basis by comparison to the no-herbicide controls. The experiment included two reps each of four accessions, which included non-mutagenized Hatcher wheat and three quizalofop resistant accessions, each containing a single ACCase mutation. The experiment was conducted twice. Regression analysis was performed in SigmaPlot 11 (Systat Software, Inc., Chicago, IL 60606) using a three parameter sigmoidal curve (Figure 4). Mean separation was performed with a general linear model in SAS using Fisher's protected LSD (α=0.05).

$$y = \frac{a}{1 + (\frac{x}{x_0})\text{^}b}$$

Figure 4. Model used in regression analysis of ACCase assay.

Results

Injury rating

Nearly all accessions evaluated in this study appeared more resistant to quizalofop than non-mutant Hatcher wheat (Figure 5). Damage to quizalofop resistant mutants ranged from 40 to 70% following quizalofop treatment, with the exception of AF21. The most common symptom of herbicide damage was necrosis of the newest leaf while new growth initiated from the crown. Another common symptom was initial necrosis of existing leaf material at a newly formed growing point, with subsequent growth from the growing point forming healthy green tissue.

Most seedlings screened were highly uniform in herbicide response within accessions. Each quizalofop resistant accession had very few completely dead plants, the exceptions being AF17, 19, and 21.

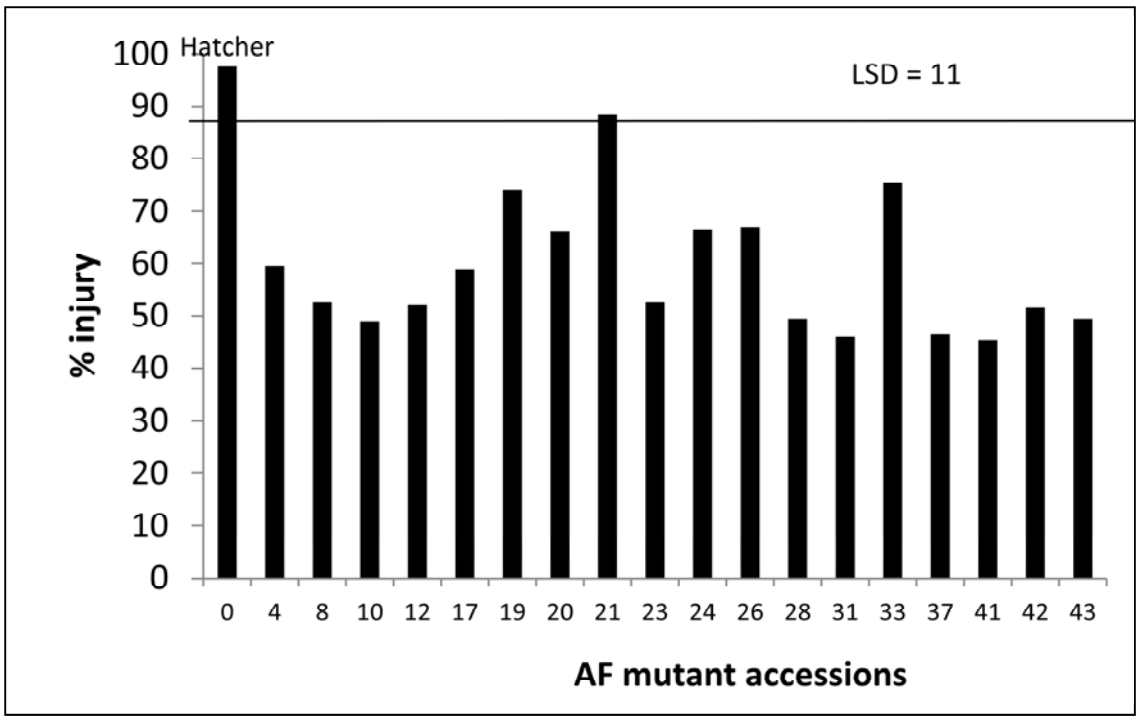

Figure 5. Visual injury of M2-selected M3 mutants screened with quizalofop. Values below the horizontal line are different than non-mutagenized Hatcher wheat, represented by accession 0.

This was the only test conducted which allowed plants to remain intact for the full length of the experiment and is the only study that does not base results on life/death of the plant alone. As such, it appears the level of herbicide resistance in any of the accessions would not fulfill commercialization goals of plant fitness after quizalofop application.

Dose response

Differences were uncovered in the whole plant sensitivity to increasing application rates of quizalofop. LD_{50}'s ranged from 10 g ai ha^{-1}, with the non-mutagenized wheat, to 76 g ai ha^{-1} with AF23 (Figure 6). The field use-rate for quizalofop in the United States ranges from 31 to 92 g ai ha^{-1} in most legumes. The

values observed in this experiment were largely within these labeled rates and many are higher than non-mutant wheat.

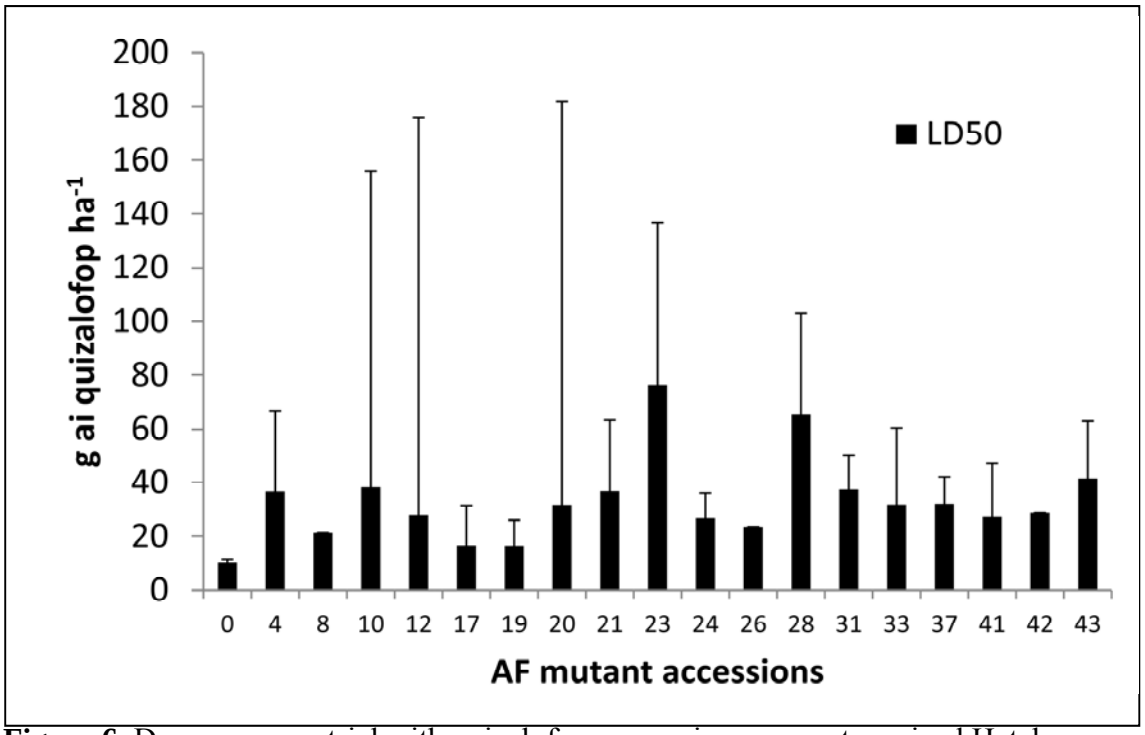

Figure 6. Dose response trial with quizalofop comparing non-mutagenized Hatcher wheat, represented by accession 0, with M2-derived M3 accessions.

Resistant to Susceptible (R to S) ratios for this experiment ranged from 1.6 to 7.5 based on survival/death of the plants. This ratio indicates the increase in survival of the mutant wheat accessions compared to non-mutant Hatcher in response to quizalofop treatment. The resulting survivors recovered in a similar manner to above, with the exception being that recovery started sooner. Since most plant material was manually excised seven DAT, the length of time for the sloughing process was reduced. The rate and degree of recovery was observed anecdotally and used to aid in the determination of the most fit accessions for future experiments.

Cross Resistance

When investigating cross resistance to other ACCase herbicides, few herbicide responses were noted among the quizalofop resistant mutants (Table 7). Plants that survived were not vigorous, but eventually produced seed. No survivors were observed after fluazifop application, a herbicide in the same family as quizalofop. It should be noted that the fluazifop rate used for this experiment was based on a theoretical LD_{90} predicted beyond the range tested in the study and was likely artificially high.

The quizalofop mutants' resistance to clethodim and sethoxydim was low. Accessions AF21, 23, and 28 had the most cross resistance with AF28 showing slight but non-significant resistant to sethoxydim in addition to clethodim. The presence of any cross resistance in the quizalofop resistant mutants is encouraging. In all likelihood, a single mutation per plant is responsible for the herbicide resistance observed. If more than one mutation event is present among the quizalofop resistant population, the resistant alleles could be compiled into a single plant, increasing herbicide resistance further.

Table 7. Quizalofop resistant mutant survival after application of other ACCase herbicides. Accession 0 is non-mutant Hatcher wheat.

Accession	----------Herbicide treatment----------			
	Clethodim	Sethoxydim	Fluazifop	Cleth. + quiz.
No.	%	%	%	%
0	0	0	0	0
4	10	0	0	0
8	0	0	0	0
10	0	0	0	0
12	0	0	0	0
17	0	0	0	0
19	0	0	0	0
20	0	0	0	0
21	25	0	0	0
23	17	0	0	0
24	0	0	0	0
26	0	8	0	0
28	17	8	0	0
31	0	0	0	0
33	0	0	0	0
37	0	0	0	0
41	0	0	0	0
42	0	0	0	0
43	0	0	0	0
LSD = 16				

Sequencing

When comparing sequences from non-mutant Hatcher to 22 quizalofop resistant mutants, three non-synonymous mutations were revealed in the ACCase carboxyltransferase domain, all at position 2004 in the *Alopecurus myosuroides* amino acid reference sequence. This mutation on the A genome was found in seven accessions, on the B genome in six accessions, and on the D genome in nine accessions (Table 8). No accession had more than one of these mutations. The mutation was a C to T substitution resulting in an alanine to valine change (Figure 7).

Table 8. Wheat accessions containing a newly discovered ACCase variant and the corresponding genome in which they were found.

Mutation location	Accession
A genome	AF12, AF20 AF25, AF28, AF31, AF33, AF43
B genome	AF17, AF21, AF23, AF24, AF26, AF33
D genome	AF4, AF8, AF10, AF12, AF37, AF39, AF40, AF41, AF42

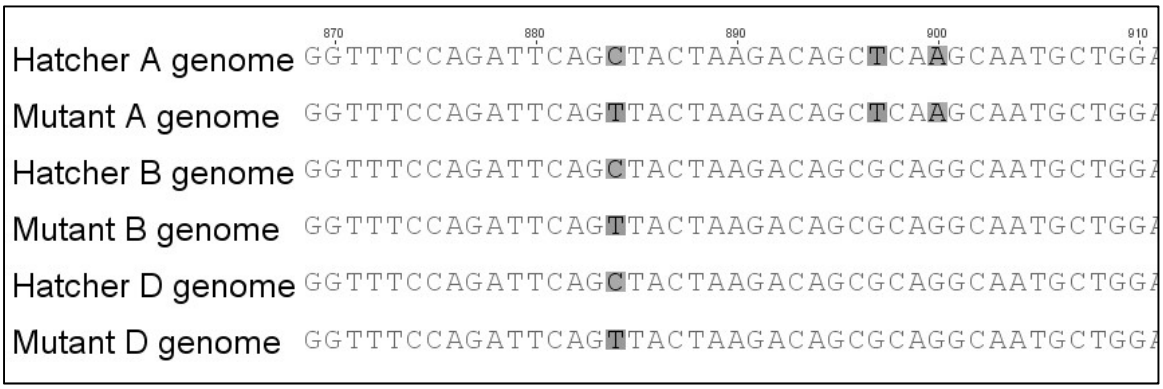

Figure 7. Comparison of wild type and mutant ACCase sequences in wheat A, B, D genomes, including a newly discovered non-synonymous mutation in each mutant sequence.

The 2004 mutation is located between two other known resistance-causing mutations at positions 1999 and 2026. Additionally, each accession with higher survival than the non-mutant Hatcher contained one of the mutations, adding confidence to a functional change caused by the mutation. Based on the chromatograph patterns, the majority these mutations are also believed to be homozygous in the plant. AF26 contained a second heterozygous G to C transition (Glu to Gln) at position 1946 on the A genome, an area not known to interact with herbicides, and is thought to be unrelated to herbicide resistance.

The genome location of the new variant does not appear to affect the whole plant response to quizalofop uniformly. For instance, AF23 and 28 were the accessions that performed best in the dose response study, but AF23 contains the mutation on the B genome while AF28 has the mutation on the A genome (Figure 6). Since the plants in the studies have only been selfed, some of the variation between accessions is undoubtedly due to random mutations still present in the plants. Also, in wheat each genome contributes equally toward the expression of ACCase (Gornicki et al. 1997). Once the mutation is transferred to non-mutant wheat, the level of whole-plant resistance is expected to increase and accessions with the new mutation would likely behave similarly.

ACCase enzyme activity

Non-mutagenized Hatcher winter wheat had greater sensitivity to quizalofop than the mutant accessions (Figure 8). Accessions with the B and D genome mutations resulted in higher levels of quizalofop resistance at the 10 μM, level than non-mutant Hatcher (LSD 14.5). The A and D genome mutations had higher quizalofop resistance than non-mutant Hatcher at the 100 μM concentration (LSD 21.6)

An herbicide dose resulting in 50% enzyme inhibition (I_{50}) could not be calculated due to lack of inhibition at the highest concentration of quizalofop for the A and D genome mutants. The representative B genome mutant line had poor enzyme activity in the first run of the experiment, resulting in overly low activity in the presence of herbicide, particularly at 100 μM.

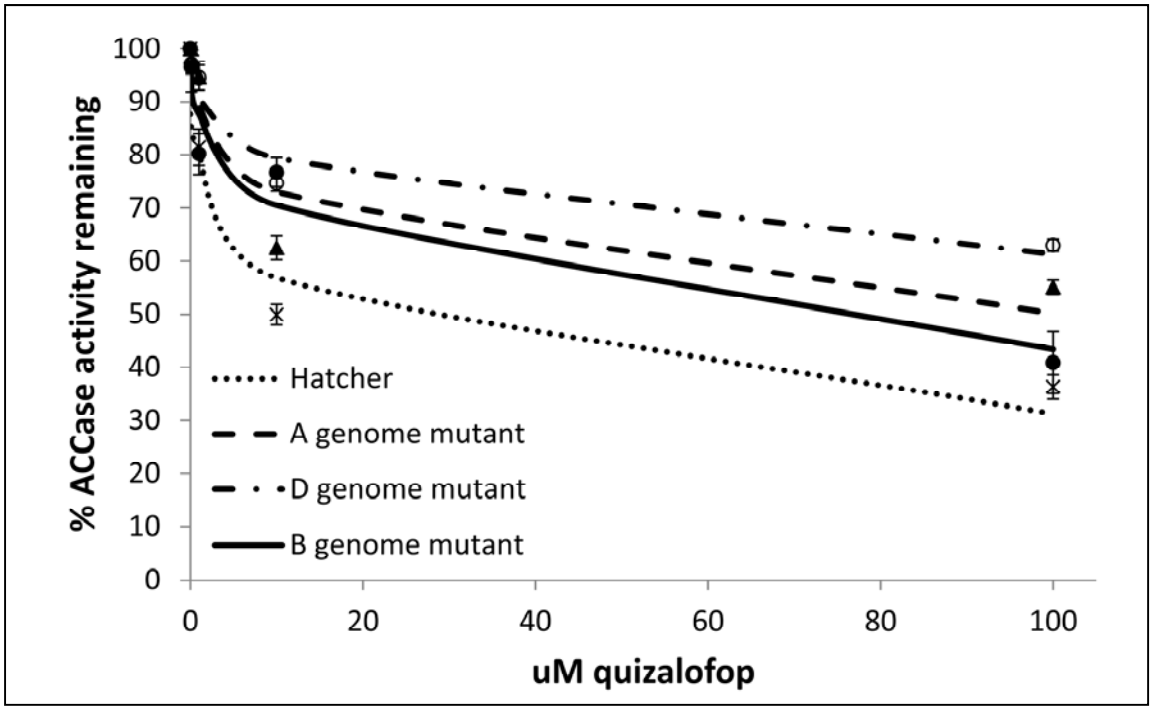

Figure 8. Means and predicted response of ACCase enzyme activity in increasing quizalofop concentrations. Model information is listed in Table 9.

Table 9. Variables and variance information for equations relating to ACCase activity reduction in Figure 8. Variables listed fit into the equation from Figure 4.

Accession	Variable			
	a	b	x_0	R^2
Non-mutant Hatcher	1.03	0.45	15.72	0.87
A genome mutants	1.04	0.4	83.12	0.80
B genome mutants	0.98	0.51	62.19	0.67
D genome mutants	1.01	0.37	310.08	0.84

None of the 22 tested accessions had multiple mutant ACCase copies in the plant, meaning there were both R and S isozymes in the pool of ACCase screened in the assay. Regardless, calculated at the I_{25} level, the R to S ratio for the A genome was 4.57, the B genome was 3.57 and the D genome was 10.86. When calculated at a theoretical I_{50} value, the R to S ratios become even larger. Similar ratios were observed for ACCase inhibitor resistant hexaploid *Avena fatua* to fenoxaprop (Cruz-Hipolito et al 2011) and on-par with

values from the above dose-response. A resistant phenotype includes many non-target site mutations of unknown function due to the random nature of EMS. Since this assay is measuring ACCase efficiency in the presence of quizalofop, it is a good method of removing random non-target site mutations from resistance evaluations.

Discussion

A single mutation resulting in an Ala -> Val substitution at amino acid position 2004 was present in all wheat accessions with increased resistance to quizalofop. This mutation has not previously been documented in ACCase resistant weed populations, likely due to the lack of mutations resulting from C to T changes. C to T substitutions are preferentially selected by EMS application (Greene et al 2003) largely explaining why a novel mutation was discovered in this experiment. This transition was found in each of the genomes, a fairly unlikely event. However, based on previous calculations which include an estimated mutation density of 1 per 96 kb, roughly 2200 non-synonymous mutations could exist in the herbicide sensitivity region of the original M2 population with 800,000 individuals screened (Ostlie 2011). The number of independent mutation events resulting in the three detected herbicide resistance mutations is unknown. Newhouse et al. (1992) screened 120,000 M2 plants and identified four resistant phenotypes in large scale screening with imazethapyr. These plants were later determined to originate from a single mutation event.

Quizalofop-resistant wheat was successfully generated and one mechanism of resistance was identified. Mutations in the ACCase gene sequence were discovered, and enzyme activity and whole-plant assay data suggest that the presence of the mutation increases wheat ACCase resistance to quizalofop. Phenotypic data also indicate the

increased level of resistance. Future experiments will be able to characterize the resistance level when multiple mutant ACCase copies are compiled into a single accession, compared to a single copy. Incorporating more than one resistant gene copy into a plant is likely to increase the whole plant tolerance several fold and potentially lead to a higher level of cross resistance across ACCase chemistries.

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