Theor Appl Genet (2015) 128:343–351
DOI 10.1007/s00122-014-2434-4

ORIGINAL PAPER

Development and characterization of mutant winter wheat (*Triticum aestivum* L.) accessions resistant to the herbicide quizalofop

Michael Ostlie · Scott D. Haley · Victoria Anderson · Dale Shaner · Harish Manmathan · Craig Beil · Phillip Westra

Received: 7 July 2014 / Accepted: 18 November 2014 / Published online: 29 November 2014

Abstract
Key message **New herbicide resistance traits in wheat were produced through the use of induced mutagenesis.**
Abstract While herbicide-resistant crops have become common in many agricultural systems, wheat has seen few introductions of herbicide resistance traits. A population of Hatcher winter wheat treated with ethyl methanesulfonate was screened with quizalofop to identify herbicide-resistant plants. Initial testing identified plants that survived multiple quizalofop applications. A series of experiments were designed to characterize this trait. In greenhouse studies the mutants exhibited high levels of quizalofop resistance compared to non-mutant wheat. Sequencing *ACC1* revealed a novel missense mutation causing an alanine to valine change at position 2004 (*Alopecurus myosuroides* reference sequence). Plants carrying single mutations in wheat's three genomes (A, B, D) were identified. Acetyl co-enzyme A carboxylase in resistant plants was 4- to 10-fold more tolerant to quizalofop. Populations of segregating backcross progenies were developed by crossing each of the three individual mutants with wild-type wheat. Experiments conducted with these populations confirmed largely normal segregation, with each mutant allele conferring an additive level of resistance. Further tests showed that the A genome mutation conferred the greatest resistance and the B genome mutation conferred the least resistance to quizalofop. The non-transgenic herbicide resistance trait identified will enhance weed control strategies in wheat.

Communicated by Aimin Zhang.

M. Ostlie (✉)
Carrington Research Extension Center, North Dakota State University, Carrington, ND 58421, USA
e-mail: mike.ostlie@ndsu.edu

S. D. Haley · V. Anderson · H. Manmathan · C. Beil
Department of Soil and Crop Sciences, Colorado State University, Fort Collins, CO 80523, USA

D. Shaner
USDA/ARS, Fort Collins, CO 80523, USA

P. Westra
Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523, USA

Introduction

ACC1 (plastidic acetyl co-enzyme A carboxylase; ACCase) is a well characterized single copy gene in each wheat genome (Gornicki et al. 1997). *ACC1* has been extensively studied and characterized in wheat (Gornicki and Haselkorn 1993; Gornicki et al. 1994; Konishi and Sasaki 1994; Zuther et al. 2004) and utilized in phylogenetic analysis due to its high degree of conservation across the Poaceae family (Chalupska et al. 2008; Huang et al. 2002). Other than being an herbicide target, *ACC1* modification has also been associated with altered seed oil content, plant cuticle formation, secondary metabolites, and general plant health (Chalupska et al. 2008; Sasaki and Nagano 2004).

ACCase (EC 6.4.1.2) 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 membranes for new cells. It is well known that single point mutations in the *ACC1* gene can cause resistance to ACCase-inhibiting herbicides (Delye et al. 2005; Kukorelli et al. 2013). Some ACCase inhibitors, such as fenoxaprop-P-ethyl and clodinafop, can be applied to wheat safely due to the inclusion of safening compounds in

the herbicide formulation that aid in herbicide detoxification (Hacker et al. 2000). ACCase herbicides that are registered in broadleaf crops, but not cereal crops, such as quizalofop-P-ethyl, do not contain "safeners" and have activity on a wider range of grass species.

Eleven *ACC1* single nucleotide polymorphisms (SNPs) that confer herbicide resistance have been reported (Delye et al. 2005; Yu et al. 2007; Kukorelli et al. 2013), with more resistant species and likely more new SNPs discovered each year, such as in Tang et al. (2014). Each SNP has the potential to confer resistance to a different subset of herbicides within the ACCase inhibitor family and the response can vary by species (Martins et al. 2014). Each SNP occurs in the carboxyltransferase domain of *ACC1*, and all but one SNP is contained within a 300 nucleotide region of the gene. Recent crystallography studies and simulations of susceptible and resistant ACCase have indicated that altering protein structure of ACCase causes herbicide resistance by preventing or reducing normal herbicide binding to the enzyme and allowing the enzyme to remain largely functional in the presence of the inhibitor (Xiang et al. 2009; Zhang et al. 2004; Zhu et al. 2009).

In wild oat (*Avena fatua* L.), another hexaploid, a single SNP on one genome can confer whole-plant resistance to the ACCase inhibitor (Christoffers et al. 2002). It is unknown if a single mutant copy in wheat would confer commercially acceptable resistance to ACCase inhibitors. Evidence suggests that the effect of resistant alleles would be diluted by an abundance of susceptible alleles in polyploid species (Iwakami et al. 2012; Yu et al. 2013). Fortunately, ACCase in vitro assays have been developed to infer herbicide binding affinity to ACCase (Bradley et al. 2001; Seefeldt et al. 1996; Yu et al. 2003). This assay can 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 plants resistant to quizalofop herbicide were identified by phenotypic screening of M_2 plants following ethyl methanesulfonate (EMS) mutagenesis of Hatcher winter wheat (PI 638512; Haley et al. 2005) seeds. The following experiments were designed to: identify the wheat accessions containing the highest level of resistance; genotype the resistant plants; confirm that the resistance is caused by a change in the target gene; and determine the gene action and segregation pattern among the progeny of resistant plants.

Materials and methods

Mutant population development

A mutagenesis dose response study was conducted to identify the optimum level of ethyl methanesulfonate (EMS) to balance the degree of potential genetic alteration with plant mortality (data not shown). The optimum method included submersing 2.27 kg of wheat seed in a solution of 60 mM EMS (in 5 L of water) with an exposure time of 2.5 h. The seeds were then removed from the solution and allowed to dry in a fume hood. The seed viability was 80 % when screened in a germination test and the newly germinated plants exhibited a varying degree of seedling vigor (data not shown). This population was planted in the field. In field conditions, there was only a 55 % survival rate resulting in approximately 40,000 M_1 plants that reached maturity. The M_1 plants were bulk harvested generating an M_2 seed lot.

There were 800,000 M_2 seeds sown in the fall of 2010 and the plants were challenged with a highly discriminating dose of 21 g ai ha^{-1} of quizalofop herbicide (Assure II, E.I. du Pont de Nemours and Company, Wilmington, DE, 19898) at the three-leaf stage in the spring. This herbicide dose was chosen after testing rates of quizalofop on commercial winter wheat varieties (data not shown). Plants were treated a second time with the same dose of quizalofop 3 weeks later, in order to reduce the chance for identifying false positives. Heads from 46 plants were selected (based on survival and recovery from herbicide damage) for further investigation. Each of the 46 accessions was named with an AF prefix to denote that the plants were screened with quizalofop (Assure II) in field conditions. Initial greenhouse screening was conducted with 20 individuals from each $M_{2:3}$ (M_2-derived M_3) accession. The plants were challenged twice with the herbicide, as described above, and scored individually for survival and vigor. Twenty accessions were identified that appeared resistant to quizalofop. The remaining accessions had less than a 5 % survival rate after being challenged twice. Leaf samples were collected from the surviving plants for genetic analysis.

Dose response study

The $M_{2:3}$ quizalofop-resistant accessions were evaluated in a greenhouse study to determine their relative resistance to the herbicide. The experiment was conducted in a greenhouse at 22 °C with a 14-h day length and natural lighting supplemented with 400 J s^{-1} sodium halide lamps. The study was conducted twice as a randomized complete block design with a split-plot arrangement and three replicates. Each replicate consisted of two seeds (due to limited seed quantities for some accessions) planted in 7.6 × 7.6 × 8.9 cm inserts with potting soil (Fafard #2 SV, Conrad Fafard, Inc, Agawam, MA 01001). Herbicide dose was the main plot effect and wheat accession was the subplot effect. The study included five quizalofop doses (11, 23, 46, 92, and 184 g ai ha^{-1}) and a non-treated check.

Table 1 Primer sequences for PCR of genome-specific DNA fragments and for subsequent sequencing reactions of the *ACC1* gene in wheat

Primer specificity	Primer sequence (5′ → 3′)
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[a]	
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

[a] Modified for wheat specificity from Delye and Michel (2005)

Herbicide application was made at the three- to four-leaf growth stage 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} set to a pressure of 206 kPa. At 7 days after treatment (DAT) the tops of seedlings were clipped above the newest above-ground growing point to evaluate regrowth of the treated plants objectively. Categorical evaluation (alive/dead) of plant survival was performed 28 DAT. Data were analyzed using the logistic function in the probit procedure of SAS (9.2, SAS Institute, Cary, NC) and an LD_{50} was calculated for each accession based on the generated model. An LD_{50} represents the herbicide dose resulting in 50 % plant mortality.

DNA extraction and sequencing

Genetic analysis was done using $M_{2:3}$ wheat plants that survived two applications of quizalofop herbicide. Five centimeters of leaf tissue was collected from 20 wheat accessions and frozen in liquid nitrogen. DNA was extracted using a CTAB extraction method (Doyle and Doyle 1987). After extraction, DNA was resuspended and stored in 100 μL of 10 mM TE buffer and stored at −20 °C.

To develop genome-specific primers, generic ACC1 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) using *E. coli* competent DH5α cells and sequenced using those same primers. Three unique sequence sets were identified and matched to each wheat genome by comparison to EU660900, EU6600901, and EU660902 (Chalupska et al. 2008). The cloned fragments 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 *ACC1*s reported in Chalupska et al. (2008) were used to design genome-specific primers in the intron regions surrounding the carboxyltransferase domain to exclude the highly similar *ACC2* sequence. These primers were able to amplify a single sequence without inclusion of homoeologs from other genomes (Table 1). This was tested by PCR analysis with the diploid species *Triticum urartu* (A genome), *Aegilops speltoides* and *Triticum monococum* (B genome), and *Aegilops tauschii* (D genome).

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 2 min followed by 30 cycles of 98 °C for 30 s, annealing temperatures for 30 s, extension at 72 °C for 1.5 min, and final extension for 10 min 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 PCR protocol was slightly different from the A and B genome protocols including: 14 cycles beginning at 63 °C and decreasing 0.5 °C each cycle to end at 56 °C before 35 cycles of the protocol listed above.

DNA sequencing was performed with purified PCR product using the sequencing primers in Table 1. These primers were designed for wheat with the intention of amplifying the DNA that codes for the two herbicide/protein interaction sites with known resistance mutations that exist in weed populations (Delye et al. 2005; Yu et al. 2007). Each area had a sequencing overlap of 400–550 bp between forward and reverse primers, with additional extension beyond due to the original amplification length of over 2,000 bp from the PCR reaction. All sequences were compiled and aligned using Geneious Pro 5.5.8 (Biomatters Ltd., Auckland, New Zealand, 1010).

Table 2 Mutant *ACC1* accessions identified for the A, B, and D genomes of common wheat

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

ACCase enzyme activity

An in vitro enzyme assay (adapted from Seefeldt et al. 1996; Yu et al. 2003) was conducted to confirm that the mutations conferred enzymatic herbicide resistance by measuring 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, together with a radio-labeled reagent for the reaction, to measure the ATP-dependent incorporation of sodium bicarbonate (tracing compound) into malonyl CoA. If the herbicide interacts with ACCase, it reduces or prevents the incorporation of sodium bicarbonate into the final product.

The most actively growing shoot tissue of three-leaf $M_{2:4}$ plants (screened for resistance in both the M_2 and M_3 generations) for each of three highly quizalofop-resistant accessions (AF10, AF26, AF28) was pooled to provide 3 g of ACCase-rich fresh plant material. 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 after grinding, for a total of 10 mL. The solution was centrifuged at 25,000*g* for 15 min and the pellet was discarded. Saturated ammonium sulfate was added, dropwise, to a final concentration of 66 % V/V of the original supernatant volume. The solution was stirred for 1 h to allow the protein to precipitate. Centrifugation was performed at 25,000*g* for 30 min. 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. Glycerol was added to the samples at a concentration of 25 % of the final volume for storage in −80 °C until the assay was conducted.

Four quizalofop concentrations (0.1, 1, 10, 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 $NaHCO_3$ containing 2 kBq of radio-labeled $NaH^{14}CO_3$, and 50 μL of enzyme extract (the enzyme concentration varied for each accession and experiment). This solution was held at 30 °C for 3 min. Acetyl CoA was excluded from the negative control for each accession but included at 3 mM in the remaining solutions. The reaction was carried out at 30 °C for 10 min and stopped by the addition of 20 μL glacial acetic acid. Since a byproduct of the reaction is $^{14}CO_2$, the addition of acid was conducted in a sealed 46 × 46 × 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 1 h and were then transferred to glass fiber paper inside liquid scintillation vials. Samples remained in the vials one additional hour before 10 mL scintillation cocktail (Ultima Gold LLT, Perkin Elmer Life and Analytical Sciences, Inc. Waltham, MA 02451) was added. Samples were then measured for radioactivity via liquid scintillation spectroscopy.

Residual ACCase activity was determined on a percentage basis by comparison to the no-herbicide controls. The experiment included two replicates each of four treatments, which included non-mutagenized Hatcher and the three quizalofop-resistant accessions (AF10, AF26, AF28), each representing a different newly discovered mutation (Table 2). The experiment was conducted twice. Regression analysis was performed in SigmaPlot 11 (Systat Software, Inc., Chicago, IL 60606) using a three-parameter logistic curve

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

where a is the maximum value, x is the dose, x_0 is the LD_{50} and b is the slope near the LD_{50}. Data were also analyzed with analysis of variance and subjected to pair-wise analysis across accessions in SAS.

Genetic segregation, expression of resistance, and gene action

Greenhouse studies were done to assess the genotypic segregation of mutant and wild-type alleles for each of three herbicide-resistant individuals, characterize expression of resistance conferred by the mutant alleles, and determine gene action for resistance conferred by the mutant alleles. Seed was harvested from plants derived from crosses with individual A (AF28), B (AF26), and D (AF10) genome mutant lines with resistance to quizalofop. For the A and B genomes, F_2 progeny from heterozygous F_1 plants were used while BC_1F_2 progeny from heterozygous BC_1F_1 plants were used for the D genome. Seeds were planted into individual cells of 96-cell horticultural flats containing a greenhouse potting soil medium. At the two-leaf stage, tissue samples were collected from each plant for DNA sequence

analysis. At 13 days after planting, segregating populations were treated with 10.5 and 21 g ai ha^{-1} (LD_{90}) of quizalofop herbicide (96 plants per population for both rates) in a controlled spray-chamber. At 15 DAT, plants were scored for percent injury with a 0 indicating no damage and 100 being plant death. At 30 DAT, individual plants were scored categorically for survival (alive or dead). Plants were genotyped using PCR products resulting from gene-specific PCR amplification as described previously. Based on DNA sequence analysis, each plant was categorized as being homozygous for the respective mutant allele conferring resistance, homozygous for the respective wild-type allele conferring susceptibility, or heterozygous for mutant and wild-type alleles. Statistical analyses (conducted using SAS-JMP Pro, SAS Institute, Cary, NC) included a Chi-square goodness-of-fit test for genotypic segregation ratios of mutant and wild-type alleles; a Chi-square goodness-of-fit test for phenotypic segregation ratios of alive and dead plants 30 DAT; and analysis of variance for percent damage scores 15 DAT. An arcsine transformation of the individual-plant percent damage ratings was done to enable statistical comparisons though least-square means from analyses with untransformed data will be presented. Logistic regression was done to enable statistical comparisons among genotypic categories based on categorical assessments (alive and dead) made 30 DAT. Pair-wise comparisons for the categorical data were performed without Bonferroni adjustments due to the a priori nature of the comparisons of interest.

Results

Quizalofop dose response

Differences were observed in the whole-plant sensitivity to increasing application rates of quizalofop in select M3 accessions. Accession-wide LD_{50}s ranged from 10 g ai ha^{-1} (Hatcher) to 76 g ai ha^{-1}. The field use-rate for quizalofop in the United States ranges from 31 to 92 g ai ha^{-1} in most legumes (soybeans, field peas, lentils, etc.). The values observed in this experiment were largely within these labeled rates and many are greater than non-mutant wheat. Accessions AF10, AF26, and AF28 were identified as good donors for the herbicide resistance trait based on results from this study, observations of plant vigor after clipping, and the gene sequencing conducted below. Each of these accessions had a higher resistance level than non-mutant Hatcher (Table 3). Large upper bounds above the LD_{50} value of AF10 and AF28 were observed in response to quizalofop due to a few individuals surviving the highest doses applied, indicating potential for trait improvement within the accessions once plants are genotyped and susceptible biotypes discarded.

Table 3 The response of Hatcher and select mutant wheat accessions to application of five rates of quizalofop herbicide

Accession	95 % confidence limit			R:S[b]
	LD_{50} (g ai ha^{-1})[a]	Lower (g ai ha^{-1})	Upper (g ai ha^{-1})	
Hatcher	10.2	9.6	11.3	–
AF28[c] (A)	65.3	43.5	103.2	6.4
AF26 (B)	23.7	16.8	32.8	2.3
AF10 (D)	38.2	23.2	156.2	3.8

[a] The LD_{50} represents the dose with which half the plants were killed by the herbicide

[b] R:S is the ratio between the LD_{50} of an accession and that of Hatcher

[c] AF indicates accessions that were originally selected following quizalofop application to the M_2 population in field conditions

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 relative quizalofop resistance of the mutant wheat accessions compared to non-mutant Hatcher wheat at the LD_{50} value. The R to S ratios for accessions AF10, AF26, and AF28 demonstrate that the resistance to quizalofop in the mutant accessions is more than twice that of Hatcher (Table 3). This trait did not confer resistance to other ACCase-inhibiting herbicides from the cyclohexanedione family (data not shown).

Sequencing

Three missense mutations were discovered, via Sanger sequencing, in the *ACC1* carboxyltransferase domain among the 20 quizalofop resistant mutants, all within amino acid position 2004 using the *Alopecurus myosuroides* reference sequence. The mutation was a C to T substitution resulting in an alteration from alanine to valine (Table 4). The substitution on the A genome was found in five accessions, in the B genome in six accessions, and in the D genome in nine accessions (Table 2). No accession contained more than one of these mutations. The consensus for each mutation was submitted to Genbank (KM056766, KM056767, KM056768).

The A2004V (alanine to valine at position 2004) amino acid substitution is located between two other known herbicide resistance mutations within positions 1999 and 2026, providing evidence that alteration at this position influences herbicide binding affinity. Additionally, each accession identified with increased resistance contained a mutation, increasing confidence that the mutation is causing a functional change in the enzyme.

Accessions AF12, AF26, and AF33 contained a second missense mutation of G to C in the A genome (E1946Q).

Table 4 Comparison of wild type and mutant ACCase amino acid and DNA sequences in wheat A, B, D genomes, including a newly discovered non-synonymous mutation in each mutant sequence

Amino acid position	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Amino acid sequence	**F**	**P**	**D**	**S**	**.**	**T**	**K**	**T**	**A**	**Q**	**A**
Hatcher A genome	TTTCCAGATTCAG**C**TACTAAGACAGC**T**CA**A**GCA
Mutant A genome	TTTCCAGATTCAG**T**TACTAAGACAGC**T**CA**A**GCA
Hatcher B genome	TTTCCAGATTCAG**C**TACTAAGACAGCGCAGGCA
Mutant B genome	TTTCCAGATTCAG**T**TACTAAGACAGCGCAGGCA
Hatcher D genome	TTTCCAGATTCAG**C**TACTAAGACAGCGCAGGCA
Mutant D genome	TTTCCAGATTCAG**T**TACTAAGACAGCGCAGGCA

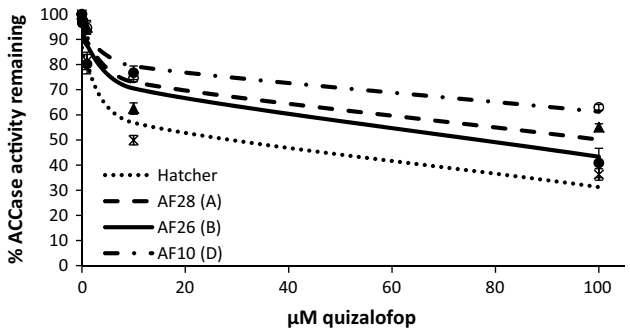

Fig. 1 Means and predicted response of ACCase enzyme activity in increasing quizalofop concentrations. Model information is listed in Table 5

Table 5 Variables used to describe the trend of ACCase decline in the presence of quizalofop

Accession	Variable[a]			
	a	*b*	x_0	R^2
Hatcher	1.03	0.45	15.72	0.87
AF28[b] (A)	1.04	0.40	83.12	0.80
AF26 (B)	0.98	0.51	62.19	0.67
AF10 (D)	1.01	0.37	310.08	0.84

The listed values were used to generate Fig. 1

[a] Listed variables fit into $y = \frac{a}{1+(\frac{x}{x_0})^b}$

A third mutation of C to T in the B genome (S1895F) was identified in AF8, AF41, and AF39. These mutations were in addition to A2004V. Neither E1946Q nor S1895F increased herbicide resistance more than plants that only contained A2004V, nor is either site known to interact with herbicides.

ACCase enzyme activity

ACCase activity in non-mutagenized Hatcher winter wheat had the greatest decline in response to quizalofop when compared to mutant accessions AF10 (D genome), AF26 (B genome), and AF28 (A genome) (Fig. 1; Table 5). When using pair-wise comparisons at the highest dose applied, accessions AF28 ($P = 0.031$) and AF10 ($P = 0.003$) had greater quizalofop resistance than non-mutant Hatcher. AF26 was similar to non-mutant Hatcher ($P = 0.360$) at 100 µM quizalofop, but also similar to AF28 ($P = 0.633$) and AF10 ($P = 0.178$) at that dose.

The herbicide dose that results in 25 % enzyme inhibition (I_{25}) was calculated. At this level of inhibition, the R to S ratio for the A genome was 4.6, the B genome was 3.6, and the D genome was 10.9. These values are a result of a reduction in the binding affinity between ACCase and quizalofop, likely due to a conformational change in the enzyme of the mutants compared to Hatcher.

Genotypic segregation

F_2 (AF28-A genome and AF26-B genome) and BC_1F_2 (AF10-D genome) progeny screening revealed genotypic segregation ratios of mutant and wild-type alleles for each of the three genomes, as shown in Table 6. Normal 1:2:1 genotypic segregation was observed for the A genome ($P = 0.11$) and for the B genome ($P = 0.12$) traits, whereas non-normal segregation was observed for the D genome trait ($P = 0.04$). For the D genome, compared to the genetic expectations, greater numbers of homozygous mutant and wild-type classes and lower numbers of the heterozygote class were observed. While none of the mutant alleles appeared to segregate in a perfect 1:2:1 ratio, no particular pattern was evident and it is unlikely that this would compromise the ability to recover progenies carrying the mutant alleles.

Gene action

In F_2 and BC_1F_2 progenies, phenotypic expression of resistance and susceptibility to quizalofop was assessed 15 days after herbicide application with quantitative damage scores (Table 7). While the differences were not significant in each case, expression of resistance conferred by each trait appeared to be additive (i.e., incomplete dominance) with respect to mutant allele number as plants with only one mutant copy (i.e., heterozygotes) showed damage

Table 6 Genotypic segregation ratios of mutant and wild-type alleles at loci conferring resistance to quizalofop herbicide on the A, B, and D genomes of winter wheat

Category[a]	AF28 (A genome)		AF26 (B genome)		AF10 (D genome)	
	Observed	Expected	Observed	Expected	Observed	Expected
Homo mutant	45	44	34	44.25	51	41.5
Hetero	76	88	101	88.5	67	83
Homo wild type	55	44	42	44.25	48	41.5
X2 *P* value (1:2:1)	0.110 NS[b]		0.119 NS		0.043*	

[a] For each category and genome, data represent the number of plants observed and the numbers of plants expected based on a 1:2:1 genotypic segregation ratio. A and B genome observations were done with F_2 progeny, while D genome observations were made on BC_1F_2 progeny

[b] "NS" indicates lack of statistical difference between the observed and expected ratio, whereas "*" indicates that the observed ratio did not conform to the expected 1:2:1 ratio

Table 7 Mean visual damage score of homozygous mutant, heterozygous, and homozygous wild-type genotypes of three genomes following application of quizalofop herbicide at 10.5 and 21 g ha^{-1} (LD_{90})

Category	AF28 (A genome)[a]		AF 26 (B genome)		AF10 (D genome)	
	10.5 g ha^{-1}	21 g ha^{-1}	10.5 g ha^{-1}	21 g ha^{-1}	10.5 g ha^{-1}	21 g ha^{-1}
Homo mutant	7.7 a	22.9 a	12.9 a	34.3 a	14.1 a	20.2 a
Hetero	30.3 b	30.1 a	24.3 b	50.0 a	24.6 a	31.9 b
Homo wild type	76.2 c	91.7 b	78.1 c	86.2 b	82.1 b	90.5 c

[a] Assessments were made 15 days after herbicide treatment. A and B genome observations were done with F_2 progeny, while D genome observations were made on BC_1F_2 progeny. Means within a column followed by different letters are significantly different at the 0.05 probability level (Tukey's honestly significant difference test)

Table 8 Phenotypic segregation ratios of resistant (alive) and susceptible (dead) phenotypes among F_2 (A and B genomes) and BC_1F_2 (D genome) progenies at loci conferring resistance to quizalofop from the A, B, and D genomes of winter wheat

Category	AF28 (A genome)[a]				AF26 (B genome)				AF10 (D genome)			
	10.5 g ai ha^{-1}		21 g ai ha^{-1}		10.5 g ai ha^{-1}		21 g ai ha^{-1}		10.5 g ai ha^{-1}		21 g ai ha^{-1}	
	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp
Alive	51	22	28	23.25	22	21.75	3	22.5	33	21.75	23	19.75
Dead	37	66	65	69.75	65	65.25	87	67.5	54	65.25	56	59.25
X2 *P* value (1 alive:3 dead)	0.000*		0.255 NS[b]		0.951 NS		0.000*		0.005*		0.398 NS	

[a] Quizalofop was applied at 10.5 and 21 g ai ha^{-1}. Data were collected 30 days after herbicide treatment. Data represent the numbers of plants observed for each class and genome and the numbers of plants expected based on completely normal segregation

[b] "NS" indicates lack of statistical difference between the observed and expected ratio whereas "*" indicates that the observed ratio did not conform to the expected 1:3 ratio

intermediate to that of either homozygote. While the statistical comparison could not be made, the data also suggested that the B genome trait conferred the lowest level of resistance as plants carrying the B genome trait showed greater damage than plants carrying either the A or D genome traits, particularly at the 21 g ai ha^{-1} rate.

Phenotypic segregation ratios for qualitative resistance (alive) and susceptibility (dead) assessed 30 DAT are shown in Table 8. The 1 alive:3 dead segregation ratios tested would pertain to a scenario whereby both mutant alleles are required for resistance (resulting in plant survival) and heterozygotes and homozygous wild-types are both susceptible (resulting in plant death). Focusing on the 21 g ai ha^{-1} treatment, both the A and D genome traits conformed to the 1:3 segregation ratio, whereas the B genome trait did not. This observation confirms observations made with the 15 DAT quantitative damage score that suggested the B genome trait conferred a lower degree of resistance than either the A or D traits. The B genome trait did conform to the 1:3 segregation ratio at the 10.5 g ai ha^{-1} dose,

whereas the A and D genome traits did not, again demonstrating the higher degree of resistance conferred by the A and D genome traits compared to the B genome trait.

Discussion

A single mutation resulting in an Ala to Val substitution at amino acid position 2004 (A2004V) was present in each of the 20 accessions with increased resistance to quizalofop. This mutation has not previously been documented in ACCase-resistant weed populations. C to T substitutions are preferentially induced by EMS application (Greene et al. 2003). None of the previously documented ACCase resistance traits could develop from a C to T substitution (Delye et al. 2005; Yu et al. 2007), explaining why a novel herbicide resistance mutation was discovered with this method and not a previously documented resistance mutation. This transition was found in each of the wheat genomes. Based on previous calculations, which include an estimated mutation density of 1 per 96 kb, roughly 2,200 non-synonymous mutations could exist in the herbicide sensitivity region of the gene from the original M_2 population of 800,000 individuals screened. Since the M_1 population was bulk harvested, it is unknown if more than three separate mutation events occurred to generate the three mutations found among the 20 resistant accessions as multiple events could have generated one or more of the discovered mutations. Newhouse et al. (1992) screened 120,000 M_2 plants and identified four resistant phenotypes in large-scale screening of a mutagenized wheat population with imazethapyr. These plants were later determined to originate from a single mutation event.

An herbicide-resistant individual carries many non-target site mutations of unknown function due to the random nature of EMS mutation induction. As a result, challenging the M3 population with quizalofop generated a varied response across accessions that share a common mutation. Nevertheless, the observed pattern of response in the mutant accessions is enough to confirm the presence of herbicide resistance, as described by Beckie et al. (2000). Since the ACCase 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. The degree of enzymatic resistance to the herbicide in the mutant plants indicates that the genetic change observed in wheat *ACC1* is the mechanism of herbicide resistance. None of the tested accessions had more than one homoeologous *ACC1* mutation in the plant, meaning there were both wild type and mutant isozymes in the pool of ACCase screened in the assay. In these plants, approximately 66 % of the ACCase would be the susceptible isozyme, as each copy of the gene contributes to the ACCase pool equally (Gornicki et al. 1997). With a second copy of the resistant gene in a single plant (i.e., A + D genome mutants), it is likely that whole-plant resistance would increase since the quantity of active ACCase after herbicide application would at least double compared to a single resistant copy, and the majority of the plant ACCase would be the resistant isozyme.

Comparisons can be made with other polyploid species. In the cases of both the whole-plant and enzymatic dose responses in wheat, the R:S values aligned closely with values recorded in hexaploid but single-gene ACCase-resistant *Avena fatua* (Cruz-Hipolito et al. 2011; Uludag et al. 2008). In a study looking at one, two, and three gene resistant *Avena. f*, Yu et al. (2013) found that multiple resistant genes additively affected the resistance to ACCase-inhibiting herbicides. Just as it was observed in wheat, Iwakami et al. (2012) found that each allele influences ACCase resistance in polyploid *Echinochloa phyllopogon*. Since three resistance genes were discovered in wheat, one originating from each genome, it is likely that up to six additive levels of herbicide resistance can be achieved by increasing the resistant gene and allele dose in a plant.

It is unclear at this time why differences exist in the strength of the resistance trait based on genomic origin (i.e., the B genome mutation appears to confer a lower resistance level). There are a few possible explanations for this. Although the three copies of wheat *ACC1* are highly similar, small differences in the nucleotide sequence can result in conformational variances that influence herbicide binding affinity. It is unknown how, individually, the native or mutant ACCase from each wheat genome interacts with quizalofop. A second scenario is that ACCase activity is reduced as a result of one or more of the mutations, as has been documented in weed populations containing ACCase resistance mutations (Yu et al. 2013). This could result in a difference of performance when comparing the same mutation across genomes after an application of quizalofop.

Three quizalofop-resistant wheat accessions were identified with a common mechanism of resistance. Mutations in the ACCase gene sequence were discovered, and enzyme activity and whole-plant assay data demonstrate that the mutation increases wheat ACCase resistance to quizalofop. F_2 and BC_1F_2 progeny screening demonstrated normal or near-normal segregation patterns, while phenotypic and genotypic screening of segregating progenies determined that the traits respond additively within each locus. Furthermore, differences in the degree of resistance among the three mutant accessions were determined. A patent application was filed for the utility of the trait. Future experiments will characterize the resistance level conferred when multiple homoeologous mutant *ACC1* copies are combined into a single plant.

Author contribution statement MO, DS, and PW conducted the initial selection experiments; VA, HM, and MO conducted genotyping experiments; VA, CB, and SH conducted the segregation and gene action experiments. MO drafted the manuscript.

Acknowledgments This research was supported by the Colorado Wheat Administrative Committee and the Colorado Wheat Research Foundation. Thanks to Galen Brunk who provided guidance in the initiation of mutagenesis and the ACCase assay.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards These experiments were conducted in accordance with the laws of the United States of America.

References

Beckie HJ, Heap IM, Smeda RJ, Hall LM (2000) Screening for herbicide resistance in weeds. Weed Technol 14:428–445

Bradley KW, Wu J, Hatzios KK, Hagood ES Jr (2001) The mechanism of resistance to aryloxyphenoxypropionate and cyclohexanedione herbicides in a johnsongrass biotype. Weed Sci 49:477–484

Chalupska D, Lee HY, Faris JD, Avrard A, Chalhoub B, Haselkorn R, Gornicki P (2008) Acc homoeoloci and the evolution of wheat genomes. Proc Natl Acad Sci 105:9691–9696

Christoffers MJ, Berg ML, Messersmith CG (2002) An isoleucine to leucine mutation in acetyl-CoA carboxylase confers herbicide resistance in wild oat. Genome 45:1049–1056

Cruz-Hipolito H, Osuna MD, Dominguez-Valenzuela JA, Espinoza N, De Prado R (2011) Mechanism of resistance to ACCase-inhibiting herbicides in wild oat (*Avena fatua*) from Latin America. J Agric Food Chem 59:7261–7267

Delye C, Michel S (2005) Universal primers for PCR-sequencing of grass chloroplastic acetyl-CoA carboxylase domains involved in resistance to herbicides. Weed Res 45:323–330

Delye C, Zhang XQ, Michel S, Matejicek A, Powles SB (2005) Molecular bases for sensitivity to acetyl-coenzyme A carboxylase inhibitors in black-grass. Plant Physiol 137:794–806

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Gornicki P, Haselkorn R (1993) Wheat acetyl-CoA carboxylase. Plant Mol Biol 22:547–552

Gornicki P, Podkowinski J, Scappino LA, DiMaio J, Ward E, Haselkorn R (1994) Wheat acetyl-coenzyme A carboxylase: cDNA and protein structure. Proc Natl Acad Sci 91:6860–6864

Gornicki P, Faris J, King I, Podkowinski J, Gill B, Haselkorn R (1997) Plastid-localized acetyl-CoA carboxylase of bread wheat is encoded by a single gene on each of the three ancestral chromosome sets. Proc Natl Acad Sci 94:14179–14184

Greene EA, Codomo CA, Taylor NE, Henikoff JG, Till BJ, Reynolds SH, Enns LC, Burtner C, Johnson JE, Odden AR, Comai L, Henikoff S (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. Genetics 164:731–740

Hacker E, Bieringer H, Willms L, Rosch W, Kocher H, Wolf R (2000) Mefenpyr-diethyl: a safener for fenoxaprop-P-ethyl and iodosulfuron in cereals. J Plant Dis Prot 17:493–500

Haley SD, Quick JS, Johnson JJ, Peairs FB, Stromberger JA, Clayshulte SR, Clifford BL, Rudolph JB, Seabourn BW, Chung OK, Jin Y, Kolmer J (2005) Registration of 'Hatcher' wheat. Crop Sci 45:2654–2655

Huang S, Sirikhachornkit A, Su X, Faris J, Gill B, Haselkorn R (2002) Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the *Triticum/aegilops* complex and the evolutionary history of polyploid wheat. Proc Natl Acad Sci 99:8133–8138

Iwakami S, Uchino A, Watanabe H, Yamasue Y, Inamura T (2012) Isolation and expression of genes for acetolactate synthase and acetyl-CoA carboxylase in *Echinochloa phyllopogon*, a polyploid weed species. Pest Manag Sci 68:1098–1106

Konishi T, Sasaki Y (1994) Compartmentalization of two forms of acetyl-CoA carboxylase in plants and the origin of their tolerance toward herbicides. Proc Natl Acad Sci 91:3598–3601

Kukorelli G, Reisinger P, Pinke G (2013) ACCase inhibitor herbicides—selectivity, weed resistance and fitness cost: a review. Intern J Pest Manag 59:165–173

Martins BAB, Sanchez-Olguin E, Perez-Jones A, Hulting AG, Mallory-Smith C (2014) Alleles contributing to ACCase-resistance in an Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) population from Oregon. Weed Sci 62:468–473

Newhouse KE, Smith WA, Starrett MA, Schaefer TJ, Singh BK (1992) Tolerance to imidazolinone herbicides in wheat. Plant Physiol 100:882–886

Sasaki Y, Nagano Y (2004) Plant acetyl-CoA carboxylase: structure, biosynthesis, regulation and gene manipulation for plant breeding. Biosci Biotechnol Biochem 68:1175–1184

Seefeldt SS, Fuerst EP, Gealy DR, Shukla A, Irzyk GP, Devine MD (1996) Mechanisms of resistance to diclofop of two wild oat (*Avena fatua*) biotypes from the Willamette Valley of Oregon. Weed Sci 44:776–781

Tang W, Zhou F, Chen J, Zhou X (2014) Resistance to ACCase-inhibiting herbicides in an Asia minor bluegrass (*Polypogon fugax*) population in China. Pest Biochem Physiol 108:16–20

Uludag A, Park KW, Cannon J, Mallory-Smith CA (2008) Cross resistance of acetyl-CoA carboxylase (ACCase) inhibitor-resistant wild oat (*Avena fatua*) biotypes in the Pacific Northwest. Weed Technol 22:142–145

Xiang S, Callaghan MM, Watson KG, Tong L (2009) A different mechanism for the inhibition of the carboxyltransferase domain of acetyl-coenzyme A carboxylase by tepraloxydim. Proc Natl Acad Sci 106:20723–20727

Yu Q, Friesen LJS, Zhang XQ, Powles SB (2003) Tolerance to acetolactate synthase and acetyl-coenzyme A carboxylase inhibiting herbicides in *Vulpia bromoides* is conferred by two co-existing resistance mechanisms. Pest Biochem Physiol 78:21–30

Yu Q, Collavo A, Zheng M, Owen M, Sattin M, Powles SB (2007) Diversity of acetyl-coenzyme A carboxylase mutations in resistant *Lolium* populations: evaluations using clethodim. Plant Physiol 145:547–558

Yu Q, Ahmad-Hamdani MS, Han H, Christoffers MJ, Powles SB (2013) Herbicide resistance-endowing ACCase gene mutations in hexaploid wild oat (*Avena fatua*): insights into resistance evolution in a hexaploid species. Heredity 110:220–231

Zhang H, Tweel B, Tong L (2004) Molecular basis for the inhibition of the carboxyltransferase domain of acetyl-coenzyme-A carboxylase by haloxyfop and diclofop. Proc Natl Acad Sci 101:5910–5915

Zhu XL, Ge-Fei H, Zhan CG, Yang GF (2009) Computational simulations of the interactions between acetyl-coenzyme-A carboxylase and clodinafop: resistance mechanism due to active and nonactive site mutations. J Chem Inf Model 49:1936–1943

Zuther E, Huang S, Jelenska J, Eilenberg H, Arnold EM, Su X, Sirikhachornkit A, Podkowinski J, Zilberstein A, Haselkorn R, Gornicki P (2004) Complex nested promoters control tissue-specific expression of acetyl-CoA carboxylase genes in wheat. Proc Natl Acad Sci 101:1403–1408