Biochemical and Structural Characterization of Quizalofop-Resistant Wheat Acetyl-CoA Carboxylase

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Research Article

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Abstract

A novel nucleotide mutation in *ACC1* resulting in an alanine to valine amino acid substitution in acetyl-CoA carboxylase (ACCase) at position 2004 of the *Alopecurus myosuroides* reference sequence (A2004V) imparts quizalofop resistance in wheat. Genotypes endowed with one or two homozygous mutant *ACC1* homoeologs are 7- and 68-fold more resistant to quizalofop than a wildtype variety in greenhouse experiments, respectively. *In vitro* assays of ACCase activities in protein extracts from these varieties reveal a 3.8- and 39.4-fold increase in resistance to quizalofop in the single and double-mutants relative to the wildtype. The A2004V mutation does not alter the specific activity of wheat ACCase, suggesting that ACCase mutants retain their normal catalytic functions. Modeling of wildtype and quizalofop-resistant wheat ACCase demonstrates that the A2004V amino acid substitution causes a reduction in the volume of the binding pocket that hinders quizalofop's interaction with ACCase. Docking studies confirm that the mutation reduces the binding affinity of quizalofop. Interestingly, the models suggest that the A2004V mutation does not affect haloxyfop binding. Follow up *in vivo* and *in vitro* experiments reveal that the mutation, in fact, imparts negative cross-resistance to haloxyfop, with quizalofop-resistant varieties exhibiting more sensitivity to haloxyfop than the wildtype variety.

1. Introduction

Acetyl-CoA carboxylase (ACCase or EC 6.4.1.2) catalyzes the first committed and rate-limiting step of fatty acid biosynthesis, converting acetyl-CoA to malonyl-CoA¹. The three primary functional components of ACCase are biotin carboxylase, biotin carboxyl carrier protein, and carboxyl transferase (see Fig. 1 in Takano et al., 2021)². Mechanistically, biotin carboxylase first reduces a molecule of ATP to transfer a carboxyl group from bicarbonate to the prosthetic biotin arm of the biotin carboxyl carrier protein domain. The biotin arm then moves the newly acquired carboxyl group to the carboxyl transferase domain, which carboxylates acetyl-CoA to form malonyl-CoA. Most plants have two forms of ACCase, a eukaryotic multifunctional homomeric form located in the cytosol and a prokaryotic multi-subunit form localized to plastids³. Grasses differ from most plants by having only the eukaryotic form of the enzyme in both the cytosol and plastids⁴. Group 1 herbicides specifically target the eukaryotic form of ACCase by binding to the carboxyl transferase domain and inhibiting the second catalytic step⁵, whereas these herbicides have little activity on the prokaryotic form. Consequently, group 1 herbicides are excellent graminicides⁶.

The recently commercialized CoAXium® winter wheat production system consists of a specialized quizalofop formulation under the tradename Aggressor® for use on wheat varieties with the AXigen® quizalofop resistance trait coupled with a stewardship program aiming to delay resistance in weeds. Quizalofop, applied as the proherbicide form quizalofop-p-ethyl, controls grasses postemergence. Following cuticle penetration, the proherbicide is bioactivated to the active form quizalofop acid *in planta*. Plants transport the active quizalofop to meristematic tissue, which contains a large concentration of plastid-bound ACCase. Plastid-bound wheat ACCase encoded by the gene *ACC1* occurs as a homodimer with three functional domains per monomer⁷. Though biochemical characterization of quizalofop's

binding to ACCase has not been studied, work on other aryloxyphenoxy-propionate herbicides demonstrates that these are reversible, noncompetitive inhibitors of grass ACCase^{8,9}.

The AXigen® trait is a mutant quizalofop-resistant wheat ACCase. Researchers discovered the trait via ethyl methanesulfonate (EMS) mutagenesis and subsequent screening for resistance to quizalofop¹⁰. The nucleotide substitution in *ACC1* imparting quizalofop resistance in CoAXium® wheat corresponds to position 2004 in the ACCase amino acid sequence from the *Alopecurus myosuroides* reference sequence¹⁰. This mutation causes an amino acid substitution of an alanine for a slightly larger valine residue in ACCase (A2004V). Initially, the researchers identified three accessions with heterozygous resistance-conferring mutations for each of three *ACC1* gene homoeologs in hexaploid wheat (6x = 42) on chromosome 2 and later developed a line with a homozygous mutation for each homoeolog. Not all homoeologs contribute equally to quizalofop resistance, with the A to V mutation on the D homoeolog providing the greatest level of resistance, followed by the same mutation in the A and B homoeologs. The Wheat Breeding and Genetics Program at Colorado State University introgressed the mutations from the A and D *ACC1* homoeologs into an elite genetic background and developed the initial CoAXium® varieties featuring the mutation in both homoeologs.

Our objectives were to determine if the amino acid substitution affects the specific activity of wheat ACCase and to describe how the substitution imparts resistance at biochemical and structural levels. We further quantified fold-differences in whole-plant and enzymatic resistance between wheat with wildtype ACCase and wheat with one or two mutant ACCase homoeologs.

We first determined whole-plant quizalofop resistance in wheat with one or two mutant ACCase homoeologs by comparison to wildtype, susceptible wheat and correlated these experiments to the effect of quizalofop in *in vitro* assays of wheat ACCase. We then modeled the changes in the ACCase protein structure and its interaction with the herbicide using homology modelling, molecular dynamic simulations, and docking.

2. Results

2.1. Herbicide Dose Effect on Plant Growth

Three wheat genotypes were grown in the greenhouse to assess the contribution of a single or double homozygous *ACC1* mutation leading to whole-plant quizalofop resistance and to examine cross-resistance to haloxyfop. In general, whole-plant resistance to either quizalofop or haloxyfop varies by the number of mutant *ACC1* homoeologs present in a wheat genotype. The residual standard error of the 2-parameter log-logistic regression model with Box-Cox transformation of quizalofop doses is 11.5 with 97 degrees of freedom. For the haloxyfop doses, the residual standard error of the 4-parameter Brain-Cousens model with Box-Cox transformation is 2.14 with 92 degrees of freedom.

The estimated quizalofop doses that reduce fresh biomass growth normalized as a percent of untreated controls by 50% (GR₅₀) range from 7.13 to 485 g ae·ha⁻¹, while haloxyfop GR₅₀ values range from 26.5 to 125 g ae·ha⁻¹ (Table 1 and Fig. 1). Based on GR₅₀ values, single- and double-mutant genotypes are 7.0 and 68.0 times more resistant to quizalofop than the wildtype, susceptible genotype, respectively. Both quizalofop-resistant genotypes are more sensitive to haloxyfop than the wildtype. The single-mutant is about three-fold and the double-mutant is about five-fold more sensitive, respectively, than the susceptible genotype (Table 1). According to Student's t-tests, single- and double-mutant genotype resistance is significantly different from the susceptible genotype for both herbicides.

Table 1
Relative herbicide dose effects on whole-plant growth by genotype¹ for 50% growth reduction (GR₅₀)²

Herbicide ae	Mutant <i>ACC1</i> Homoeologs	GR ₅₀	R:S ³	R:S p-value ⁴
Quizalofop	0	7.13 (1.0)	-	-
	1	50.1(5.8)	7.0 (1.31)	< 0.0001
	2	485 (43.6)	68.0 (11.6)	< 0.0001
haloxyfop	0	125 (28.7)	-	-
	1	40.4 (13.4)	0.32 (0.130)	< 0.0001
	2	26.5 (6.1)	0.21 (0.07)	< 0.0001

¹Genotype refers to the number of homozygous mutations in *ACC1* that result in a A2004V amino acid substitution, with up to three possible on group 2 chromosomes in hexaploid wheat (2n = 6x).

²Leaf fresh weight values (g) normalized to percent untreated controls and transformed with Box-Cox transformation per genotype for each herbicide. Quizalofop dose effect modeled with a 2-parameter log-logistic function and haloxyfop dose effect modeled with a 4-parameter Brain-Cousens function to estimate GR₅₀ values.

³Resistance factor, calculated by dividing the GR₅₀ of a quizalofop-resistant genotype by the susceptible genotype GR₅₀.

⁴Student's t-test p-value (α = 0.05) of resistance factor ratios.

2.2. Enzyme Specific Activity Assay

Crude enzyme preparations containing ACCase activity, the target enzyme of quizalofop and other group 1 herbicides, were extracted from wheat to compare specific activity and to determine enzyme-level resistance of the same three genotypes used in the whole-plant resistance studies. Enzyme extract

specific activity range from 2.66 to 3.91 nmol·mg protein⁻¹ (Fig. 2). Values are not significantly different between genotypes ($F_{2,6} = 0.278$).

2.3. Herbicide Dose Effect on ACCase Activity

Enzyme-level resistance to either quizalofop or haloxyfop varies by the number of mutant *ACC1* homoelogs present in a wheat genotype. The residual standard error of the 2-parameter log-logistic regression model with Box-Cox transformation of quizalofop doses is 0.435 with 48 degrees of freedom. A similar model of haloxyfop doses has a residual error of 0.940 with 55 degrees of freedom.

The estimated quizalofop doses that inhibit ACCase activity normalized as a percent of untreated controls to 50% (I_{50}) range 0.486 to 19.3 μ M, while haloxyfop I_{50} values range from 0.968 to 7.63 μ M (Table 2 and Fig. 3). ACCase extracts from single- and double-mutant genotypes are 3.8 and 39.4 times more resistant to quizalofop than the wildtype, susceptible genotype, respectively, based upon estimated I_{50} values. Conversely, ACCase extracts from the same quizalofop-resistant genotypes are less resistant to haloxyfop than the susceptible genotype. The single- and double-mutants are between eight- and seven-fold more sensitive to haloxyfop than the wildtype (Table 2 and Fig. 3).

Table 2

Relative herbicide dose effects on ACCase activity by genotype¹ for 50% inhibition (I_{50})²

Herbicide ae	Mutant <i>ACC1</i> Homoelogs	I_{50}	R:S ³	R:S p-value ⁴
Quizalofop	0	0.49 (0.13)	-	-
	1	1.84 (0.52)	3.8 (1.5)	0.0634
	2	19.30 (4.01)	39.4 (13.3)	0.00571
Haloxyfop	0	7.63 (1.08)	-	-
	1	0.97 (0.21)	0.13 (0.003)	< 0.0001
	2	1.12 (0.25)	0.15 (0.004)	< 0.0001

¹Genotype refers to the number of homozygous mutations in *ACC1* that result in a A2004V amino acid substitution, with up to three possible on group 2 chromosomes in hexaploid wheat (2n = 6x). These mutations result in quizalofop-resistant wheat ACCase.

²Activity values (DPM) normalized to percent untreated controls and transformed with Box-Cox transformation per genotype for each herbicide. The dose effect of each herbicide is modeled with a 2-parameter log-logistic function to estimate I_{50} values.

³Resistance factor, calculated by dividing the I_{50} of a quizalofop-resistant genotype by the susceptible genotype I_{50} .

⁴Student's t-test p-value ($\alpha = 0.05$) of resistance factor ratios.

While the resistance factor for the double-mutant genotype is statistically significant for quizalofop in Student's t-test, the resistance factors for the single-mutant genotype is not statistically significant though the p-value is close to the significance threshold. Resistance factors for the single- and double-mutant genotypes are significantly different from the susceptible genotype for haloxyfop.

Though the resistance factors are not significantly different for the single- and double-mutants compared to the susceptible in the enzyme-level dose-response trial with quizalofop, the values are nearly equal to the resistance factors calculated from the whole-plant dose-response trial. This disparity may be due to the smaller sample size used in the enzyme dose-responses, which causes larger confidence intervals for I_{50} estimates across genotypes. Nonetheless, our results suggest that quizalofop resistance conferred by the AXigen® trait is additive at both the whole-plant and enzyme-level. Estimated GR_{50} values in the whole-plant dose response trials for the susceptible and single-mutant genotype were within confidence intervals provided by Ostlie et al. in 2015¹⁰, however, our resistance factor estimate for the single-mutant genotype based on these values is nearly double.

The single- and double-mutants are more sensitive to haloxyfop relative to the susceptible genotype in both whole-plant and enzyme-level dose-response trials. These results suggest that while the ACCase conformational change induced by the A2004V amino acid substitution increases quizalofop-resistance, the change causes negative cross-resistance to haloxyfop (e.g., increases susceptibility to haloxyfop). The negative cross-resistance to haloxyfop is not concerning for the CoAXium® Wheat Production System because the system is only available in the United States, where haloxyfop is not labeled for use in wheat.

2.4. Homology Modeling of Wheat Carboxyl Transferase Domain and Docking of Quizalofop and Haloxyfop

Both quizalofop and haloxyfop are group 1 herbicides that target eukaryotic forms of ACCase. However, our biological studies in the greenhouse and biochemical characterization of ACCase reveal that the alanine to valine mutation imparts tolerance to quizalofop both *in planta* and *in vivo*, while causing negative cross-resistance to haloxyfop.

Though quizalofop and haloxyfop are structurally related (Fig. 4A and B), quizalofop is a slightly larger molecule due to its heterobicyclic 6-chloro-quinoxalin-2-yl rings relative to the monocyclic 3-chloro-5-(trifluoromethyl)pyridin-2-yl ring of haloxyfop, with CPK volumes of 303.97 and 317.09 Å³, respectively (Table 3). Quizalofop has a smaller molecular weight than haloxyfop despite a larger volume. Both herbicides are lipophilic, though quizalofop is more so according to a greater partition coefficient ($\log P$). Quizalofop has a slightly larger dipole moment than haloxyfop, indicating it is more polar. The herbicide molecules have a similar electrostatic potential (Fig. 4C and D).

Table 3
Physicochemical properties of quizalofop and haloxyfop

Parameters	Quizalofop	Haloxyfop
Mw	344.75	361.70
log <i>P</i>	1.89	0.65
CPK volume A ³	317.09	303.97
Energy (1000 kJ·mol ⁻¹)	-4,006.2	-4,445.5
Dipole	3.51	3.08

The docking energy of quizalofop increases from - 6.07 to -5.70 kcal·mol⁻¹, whereas the docking score of haloxyfop decreases slightly from - 5.074 to -5.211 kcal·mol⁻¹ (Table 4). The number of correct docking poses and root-mean-square deviation of atomic poses for all docking combinations are within acceptable limits. Clearly, both quizalofop and haloxyfop bind to the catalytic domain of the wildtype carboxyl transferase (Figs. 5A and C). The A2004V amino acid substitution is located at the bottom left of the herbicide binding domain, where the methyl side chain of alanine occupies a smaller volume than that of the isopropyl side chain of valine. The asterisk in Fig. 5B highlights the strong steric hindrance between the volume occupied by the valine side chain and the 6-chloro-quinoxalin-2-yl group of quizalofop. The same A2004V amino acid substitution does not prevent binding of haloxyfop (Fig. 5D).

Table 4
Docking parameters of quizalofop and haloxyfop on wheat ACCase

	Quizalofop	Haloxyfop
wildtype wheat ACCase		
Correct docking	92/110	82/110
Docking energy	-6.070 ± 0.095	-5.074 ± 0.206
RMSD	0.273 ± 0.021	0.245 ± 0.033
quizalofop-resistant wheat ACCase		
Correct docking	95/110	104/110
Docking energy	-5.700 ± 0.230	-5.211 ± 0.221
RMSD	0.434 ± 0.034	0.242 ± 0.035

3. Discussion

Whole-plant quizalofop dose greenhouse experiments confirm resistance in quizalofop-resistant wheat genotypes (Table 1). Dose-response curves shift right with an increasing number of *ACC1* resistance alleles (Fig. 1A), therefore the double- is more resistant than the single-mutant genotype, and the AXigen® quizalofop resistance trait appears to be additive. The level of resistance to quizalofop at the enzyme-level parallels that observed in the greenhouse study (Table 2 and Fig. 3A), establishing a strong relationship between the number of mutant resistant homoeologs with phenotypic and enzymatic responses.

Though the resistance factors are not significantly different for the single- and double-mutants compared to the susceptible in the enzyme-level dose-response trial with quizalofop, the values are nearly equal to the resistance factors calculated from the whole-plant dose-response trial. This disparity may be due to the smaller sample size used in the enzyme dose-responses, which causes larger confidence intervals for I_{50} estimates across genotypes. Nonetheless, our results suggest that quizalofop resistance conferred by the AXigen® trait is additive at both the whole-plant and enzyme-level. Estimated GR_{50} values in the whole-plant dose response trials for the susceptible and single-mutant genotype were within confidence intervals provided by Ostlie et al. in 2015¹⁰, however, our resistance factor for the single-mutant genotype based on these values is nearly double.

The location of the A2004V mutation is within the binding domain of ACCase inhibitors. While the conformation change imparted by this mutation reduces the volume of the binding pocket sufficiently to impact the binding of quizalofop because of its large heterobicyclic group (Figs. 5A and B), it does not affect the binding of haloxyfop with its smaller monocyclic 3-chloro-5-(trifluoromethyl)pyridin-2-yl group (Figs. 5C and D). This was further supported computational docking energy calculation. The mutation increased the docking energy of quizalofop, relative to the wildtype, whereas this same mutation decreased the docking of haloxyfop. Followup experiments confirmed these computational experiments, where quizalofop-resistant genotypes are more sensitive to haloxyfop than the wildtype genotype in both whole-plant (Table 1) and enzyme-level (Table 2) dose-response trials. The leftward shift of whole-plant dose-response curves with an increasing number of resistance mutations indicates that the quizalofop resistance mutation imparts negative cross-resistance to haloxyfop (Fig. 1B). Additionally, single- and double-mutant genotypes are more sensitive to haloxyfop than the susceptible genotype at the enzyme-level (Fig. 3B), confirming the negative cross-resistance pattern suggested by our computational calculation and observed in the greenhouse. These results suggest that while the ACCase conformational change induced by the A2004V amino acid substitution increases quizalofop-resistance, the change causes negative cross-resistance to haloxyfop (e.g., increases susceptibility to haloxyfop). The negative cross-resistance to haloxyfop is not concerning for the CoAXium® Wheat Production System because the system is only available in the United States, where haloxyfop is not labeled for use in wheat.

A lack of statistically significant differences in specific activity between genotypes with susceptible *ACC1* and one or two resistant *ACC1* homoeologs suggests that the conformational change induced by the

A2004V amino acid substitution does not affect ACCase activity. This finding supports field trials that found comparable CoAXium® wheat yields following quizalofop treatment to non-treated, quizalofop-susceptible wheat yields (data not shown).

Both quizalofop and haloxyfop are group 1 herbicides that target eukaryotic forms of ACCase, though quizalofop occupies more volume (Table 3 and Fig. 4). Our biological studies in the greenhouse and biochemical characterization of ACCase reveal that the *ACC1* mutation resulting in the A2004V amino acid substitution imparts tolerance to quizalofop both *in planta* and *in vivo*, while causing negative cross-resistance to haloxyfop. Interestingly, the homology modeling docking scores agree with the biological and biochemical observations made on these herbicides (Table 4). The docking energy of quizalofop increases from the wildtype to quizalofop-resistant wheat ACCase model, indicating that the A2004V substitution reduces quizalofop binding affinity. Conversely, the docking energy of haloxyfop decreases. This decrease implies that haloxyfop binds more tightly to the mutated enzyme relative to the wildtype enzyme, therefore supporting negative cross-resistance. A similar observation was reported in mutations imparting resistance to the phytoene desaturase inhibitor fluridone resulting negative cross-resistance to some of the other phytoene desaturase-inhibiting herbicides¹¹.

4. Methods

4.1. Plant Material and Growing Conditions

Four wheat genotypes were used to compare whole-plant resistance of lines with wildtype and mutant ACCase as well as specific activity and resistance of extracted ACCase. The AF10 and Hatcher lines were provided by Dr. Scott Haley from the Department of Crop and Soil Science, and the varieties Byrd, and Incline AX were obtained from the Colorado Wheat Research Foundation. All experiments carried out on these wheat varieties were done with their permission and complied with local and national regulations. Byrd is a quizalofop-susceptible variety that was the recurrent parent for Incline AX, a CoAXium® variety, whereas Hatcher is the original mutagenized variety. AF10 is one of the initial quizalofop-resistant lines, which contains a homozygous mutant *ACC1* homoeolog in sub-genome D¹⁰. Incline AX exhibits two mutant homoeologs in sub-genomes A and D. Byrd was the wildtype, null mutant in all physical experiments except for the quizalofop plant growth dose-response experiment, where Hatcher was substituted. AF10 and Incline AX were consistently used as single and double mutants, respectively, in the same experiments.

Plants for the whole-plant dose responses and ACCase extractions were grown from seed in soilless media. Plants were maintained in a greenhouse environment with temperatures from 20 to 25°C, relative humidity between 50 to 70%, and a 14-hour daylength with natural lighting supplemented by sodium halide lamps.

4.2. Herbicide Dose Effect on Plant Growth

Prior to reaching the jointing stage, wheat seedlings were treated with herbicide. Untreated seedlings of each line were also retained to normalize data. Eleven rates of herbicide were used in each dose-response experiment. Quizalofop herbicide doses for the susceptible line ranged from 3.85 and 108 g ae ha⁻¹, whereas doses for resistant lines ranged from 15.4 and 493 g ae ha⁻¹. Haloxifop doses ranged from 15.4 and 616 g ae ha⁻¹ for all wheat lines. Non-ionic surfactant was added to each dose (0.25% v/v). All treatments were applied in a single pass at 40 cm above the plant canopy using a spray chamber. Each treatment was triplicated, with four plants comprising a replicate. Fresh leaf biomass was harvested three weeks after treatment. Biomass was adjusted in some instances to account for germination of less than four plants per replicate.

4.3. Enzyme Extraction

Approximately 5.0 g of crown tissue was harvested from each genotype at the two-leaf stage, flash frozen in liquid nitrogen, and stored at -80°C. Enzyme extraction and activity assay were modified from previously published methods¹²⁻¹⁴. Frozen wheat tissue was powdered in liquid nitrogen. All subsequent extraction steps were carried out on ice or in a cold room to maintain an extract temperature of 4°C. Ground tissue was transferred to 14.85 mL extraction buffer (100 mM Trizma, 20 mM DTT, 2 mM l-ascorbic acid, 1 mM EDTA, 0.5% w/v PVP-40, 0.5% w/v insoluble PVP-20, and 10% v/v glycerol; pH 8.0) and 150 µL of 100 mM PMSF (dissolved in 100% ethanol). The tissue was homogenized at max speed for 30 s with a homogenizer probe. Homogenate was filtered by hand with Miracloth, followed by centrifugation for 30 min at a speed of 25,000 x g. The resulting supernatant was slowly brought to 66% saturation with solid ammonium sulfate and stirred for an hour.

Ammonium sulfate solutions were centrifuged using the same parameters as the previous centrifugation step to precipitate ACCase and similar proteins. After discarding the supernatant, the protein pellets were resuspended with 2.5 mL elution buffer (50 mM tricine, 50 mM KCl, 2.5 mM MgCl₂, and 1 mM DTT; pH = 8.0). The protein extracts were desalted using a gravity protocol with conditioned PD-10 columns. Desalted protein extracts were eluted with 3.5 mL of elution buffer and mixed with 25% v/v glycerol.

4.4. Enzyme Specific Activity Assay

To determine ACCase specific activity, four 40 µL aliquots of fresh protein extract per genotype were preincubated for 3 min in assay solution without acetyl-CoA. Acetyl-CoA was subsequently added to three aliquots to initiate enzyme activity, whereas water was added in lieu of acetyl-CoA for a background readings. Preincubation and reaction steps were maintained at 32°C with moderate shaking. Final concentrations of assay solution components for each 200 µL reaction (including enzyme aliquot) were as follows: 20 mM tricine (pH = 8.3), 10 mM KCl, 10 mM MgCl₂, 5 mM ATP, 3.24 mM NaHCO₃, 2.5 mM DTT, 0.1% w/v BSA, 0.25 mM acetyl-CoA, and 0.25 mM NaH¹⁴CO₃ (provides 18.5 kBq per reaction). After 10 min, enzyme reactions were quenched with 20 µL of 12 M hydrochloric acid. Solutions were transferred to 20 mL scintillation vials and left uncapped overnight to enable volatilization of unincorporated carbonate to CO₂. Reactions were carried out in a fume hood equipped with a ¹⁴C filter.

The following day, 10 mL of scintillation cocktail was added to each vial. Vials were capped and vortexed for 20 s prior to analysis. Radioactivity was measured with a liquid scintillation analyzer in disintegrations per min (DPM).

4.5. Herbicide Dose Effect on ACCase Activity

Specific activity dose-responses were conducted using the same protocol as the specific activity assay but with the addition of herbicide immediately preceding the preincubation step. Dilution series were prepared with quizalofop acid and haloxyfop acid analytical standards dissolved in 100% acetonitrile. Triplicated dose treatments per genotype included background readings (water added in lieu of acetyl-CoA), null herbicide doses, and doses that provided 0.0100, 0.100, 1.00, 10.0, 100, and 1000 μ M herbicide in the final assay solution.

4.6. Homology Modeling of Wheat Carboxyl Transferase Domain

The segments of wheat ACCase sequence where the A2004V substitution is located were aligned to the sequence of the CT domain of yeast ACCase using EMBOSS Needle¹⁵. This information was used to build a homology model of wheat ACCase based on the crystal coordinates of the carboxyl transferase (CT) domain of yeast ACCase (1UYS)¹⁶ available from the RCSB protein data bank. The homology modeling pipeline was similar to that reported previously to model the binding of glufosinate on resistant glutamine synthetase from *Lolium perenne*¹⁷. A preliminary model was obtained using Modeller 10.0^{18,19}. The model was assembled in its functional dimer configuration and refined using GROMACS (version 2018.3)^{20,21} on a workstation with two processors (96 threads) and video cards (10 GB GPU memory each). Steric clashes or inappropriate geometries were corrected through molecular dynamics simulation and evaluated using MolProbity^{22,23} as described before.

Proteins and ligand interactions were visualized using PyMOL v.2.3.3²⁴. Additionally, the docking of quizalofop and haloxyfop to native and mutant carboxyl transferase dimers of wheat was measured using established protocols developed for Autodock^{25,26}. Briefly, the receptor was defined to encompass residues lining the herbicide binding domain. Amino acid charges were calculated using Gasteiger charges including all polar hydrogens²⁷. The docking imposed a covalent map to involve the nitrogen hydrogen of ILE 222 in the docking of the herbicides within the gridbox. A total of 100 poses were generated.

4.7. Statistical Analysis

All statistical analysis was conducted with packages in RStudio v.1.2.5033 with R "Orange Blossom"²⁸. To model the effect of herbicide on plant growth, fresh weight data was first normalized to percent of control means. Data was transformed with a Box-Cox transformation. For quizalofop data, a two-parameter log-logistic regression was fit using the LL.4 [drc] function:

$$y = c - \frac{d - c}{1 + \exp(m[\log(x) - \tilde{e}])}$$

1

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where lower limit c is fixed at 0, upper limit d is fixed at 100, m is the slope, and e is the 50% growth reducing dose (GR_{50}). A four-parameter Brain-Cousens model was fit using the BC.4 [drc] function for haloxyfop data:

$$y = c + \frac{d - c + f}{1 + \exp(m[\log(x) - \tilde{e}])}$$

2

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where lower limit c is fixed at 0, d is the upper limit, f is the hormesis effect, with m and e as additional parameters without direct interpretation. The GR_{50} of the haloxyfop model was estimated through parameterization with the ED [drc] function. A resistance factor ($R:S_{50}$) was calculated by dividing GR_{50} 's of quizalofop-resistant wheat lines by the GR_{50} of a wildtype, susceptible line with the EDcomp [drc] function. Student's t-tests ($\alpha = 0.05$) of resistance factor ratios were used to identify significant fold differences in resistance between estimated GR_{50} doses.

A linear calibration curve of absorbance versus BSA concentration was fit using the stats [lm] function to estimate wheat ACCase extract protein concentrations:²⁹

$$y = mx + b$$

3

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where m and b constants and standard deviations were $2.76 \cdot 10^{-3} \pm 3.21 \cdot 10^{-4}$ and $1.40 \cdot 10^{-1} \pm 1.32 \cdot 10^{-3}$, respectively. The residual standard error of the calibration was $1.06 \cdot 10^{-3}$ with a multiple R-squared value of 0.987. Genotype protein concentrations were calculated from the calibration and adjusted for dilution.

Per genotype, the DPM measurement of a reaction to which water was added instead of acetyl-CoA was subtracted from replicate specific activity measurements as background activity. Mean DPM enzyme specific activity measurements were converted to ^{14}C pmol units using manufacturer provided activity of ^{14}C -labeled sodium bicarbonate. Specific activity was then weighted by protein concentration to yield units of ^{14}C pmol·mg⁻¹ protein. Standard errors reflect error propagation of DPM measurement replicates and protein concentration. Specific activity was compared between genotypes using ordinary one-way

ANOVA F-protected Student's t-tests ($\alpha = 0.05$, $n = 3$) with anova [stats] and emmeans [emmeans] functions.

For specific activity dose-response analysis, background activity was subtracted from DPM readings. Background corrected DPM readings were normalized to percent residual activity using control means for each genotype. Data was modelled using the same methods as for the effect of quizalofop on whole-plant growth, with the exception that e represents the 50% enzyme inhibition dose (I_{50}). Similar resistance factors (R:S) were calculated using I_{50} estimates, followed by Student's t-tests ($\alpha = 0.05$) of ratios to identify significant fold differences in resistance between estimated doses.

Declarations

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Models1 of quizalofop (A) and haloxyfop (B) herbicide dose effects on whole-plant growth by genotype2
 1Leaf fresh weight values (g) normalized to percent untreated controls and transformed with Box-Cox
 transformation per genotype for each herbicide. Quizalofop dose effect modeled with a 2-parameter log-
 logistic function (A) and haloxyfop dose effect modeled with a 4-parameter Brain-Cousens function (B).
 2Genotype refers to the number of homozygous mutations in ACC1 that result in a A2004V amino acid

substitution, with up to three possible on group 2 chromosomes in hexaploid wheat ($2n = 6x$). These mutations result in quizalofop-resistant wheat ACCase.

Figure 2

ACCase specific activity by genotype^{1,2} 1Genotype refers to the number of homozygous mutations in *ACC1* that result in a A2004V amino acid substitution, with up to three possible on group 2 chromosomes in hexaploid wheat ($2n = 6x$). These mutations result in quizalofop-resistant wheat ACCase. 2Genotype specific activities labeled with the same capital letter are not significantly different according to Student's t-tests ($\alpha=0.05$).

Figure 3

Models1 of quizalofop (A) and haloxyfop (B) herbicide dose effects on ACCase activity by genotype2
 1Genotype refers to the number of homozygous mutations in ACC1 that result in a A2004V amino acid substitution, with up to three possible on group 2 chromosomes in hexaploid wheat (2n = 6x). These mutations result in quizalofop-resistant wheat ACCase. 2Activity values (DPM) normalized to percent

untreated controls and transformed with Box-Cox transformation per genotype for each herbicide. The dose effect of each herbicide is modeled with a 2-parameter log-logistic function.

Figure 4

Structures of A) quizalofop and B) haloxyfop showing the carbons in gray, hydrogen in white, oxygen in red, nitrogen in blue, chlorine in green and fluorine in cyan on the left side. Electrostatic potential maps of C) quizalofop and D) haloxyfop. Colors on the volume represent range from electronegative (red) to electropositive (blue) partial charges.

Figure 5

Comparison of the docking of quizalofop on A) wildtype carboxyl transferase dimer of wheat with alanine (in red) and B) mutated carboxyl transferase dimer of wheat with valine mutation (in red) to the docking of haloxyfop on C) wildtype carboxyl transferase dimer of wheat with alanine (in red) and D) mutated carboxyl transferase dimer of wheat with valine mutation (in red). Asterisk in panel B denotes the breach of the surface of the protein by quizalofop due to the increase volume of the valine side chain in the mutated ACCase.