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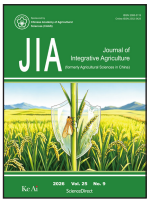

LETTER

ACC1 mutations in wheat for quizalofop-p-ethyl resistance: An expansion and their incorporation into Chinese breeding lines

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Highlights

- In wheat, the G2084S substitution renders ACC1 insensitive to the herbicide quizalofop-p-ethyl (QPE).
- In hexaploid wheat, stacking the G2084S allele across subgenomes confers enhanced resistance to QPE.
- A low-dose (2.50‰) of QPE can effectively control weeds in wheat fields without causing a yield penalty.

Wheat (*Triticum aestivum*) supplies approximately 20% of the protein and calories of the human diet (Apples *et al.* 2018). Wheat yield and quality are influenced by numerous factors, including weeds. Competition between wheat and weeds occurs throughout the wheat life cycle, affecting wheat growth and productivity. Managing weedy grasses has long been a challenge in wheat fields, particularly in seed production fields.

Quizalofop-p-ethyl, an aryloxyphenoxypropionate (FOPs) herbicide, controls weedy grasses by inhibiting Acetyl-CoA Carboxylases (ACCase or ACC1) (de Andrade *et al.* 2018). In plants, ACC1 is a biotin-dependent enzyme that carboxylates acetyl-CoA into malonyl-CoA, which plays critical roles in fatty acid biosynthesis (Sasaki and Nagano 2004; Bough and Dayan 2022). Quizalofop, which was developed in the 1980s, has been widely used in agriculture to manage weedy grasses (Chen *et al.* 2022; Liu *et al.* 2023). Quizalofop binds the carboxyltransferase (CT) domain in ACC1, inhibits malonyl-CoA synthesis, and consequently results in plant death (Sasaki and Nagano 2004; Bough and Dayan 2022).

The literature indicates that the C to T transition within the CT domain of *ACC1* results in an A2004V (alanine to valine at position 2004) amino acid substitution in *Alopecurus myosuroides* (Délye *et al.* 2002) and in wheat (Ostlie *et al.* 2015); however, in the wheat ACC1 protein, this A to V substitution actually occurs in position 1992 (Lin *et al.* 2020). A1992V mutations were used in the CoAXium® wheat (Bough *et al.* 2021), which are resistant to quizalofop and allow effective control of weedy grasses (Ribeiro *et al.* 2023). Among the subgenome mutations (*ACC-A1*, *ACC-B1*, and *ACC-D1*), the B-allele A1992V only confers weak quizalofop resistance (Bough *et al.* 2021); however, the B-allele A1992V, by base editing, was effective against 10 g ai ha^{-1} of quizalofop (Zhang *et al.* 2019). So far, only the A- and D-allele A1992V mutations were incorporated as the two components for herbicide resistance in the AXigen® trait in wheat (Bough *et al.* 2021).

In this study, we aimed to develop the *ACC1*-based quizalofop resistance in Chinese wheat. We first validated the dose effect of quizalofop-p-ethyl (QPE, 10% emulsion; PD20081191, KESAI AGROCHEM, China) on wild-type (WT) wheat and one noxious weedy grass (*Aegilops tauschii* Coss.). In the greenhouse, plants of the Chinese wheat cultivars 'Luyan 128' (or LY128), 'Jimai 38' (or JM38), 'Jimai 44' (or JM44) and 'Shannong 30' (or SN30) at 10 wk after planting were sprayed with 0, 0.3125, 0.625, 1.25, and 2.50‰ of QPE (10% emulsion/water, v/v). As shown in Fig. 1-A and B, the QPE dose was positively correlated with tiller mortality in wheat and *Ae. tauschii*. All plants died at 2.50‰ QPE, which was thus accepted as an optimal lethal dose for wheat and *Ae. tauschii*.

Previously, we created a Mega-scale EMS population using LY128, JM38, JM44, and SN30 (Wang *et al.* 2024). To identify mutants for QPE resistance, approximately 1,000 kg of M_2 seeds were sown in the field of 2.67 ha (Fig. 1-C). At the 3-leaf stage, seedlings were first sprayed with 10‰ QPE for a more stringent selection and re-sprayed seven days later (Fig. 1-C). Ten days after the second spraying, all

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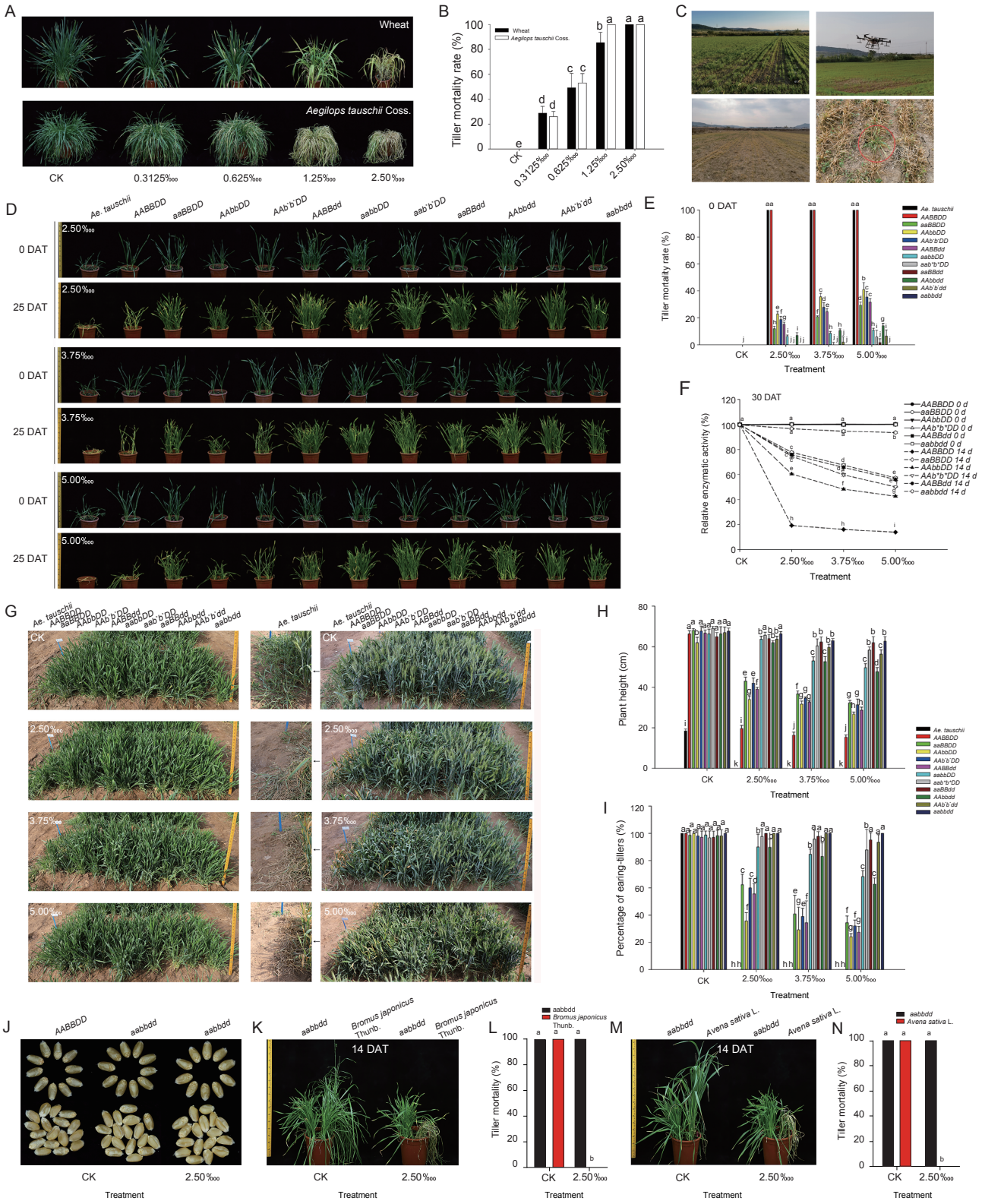

Fig. 1 Wheat *ACC1* mutants and their responses to the herbicide quizalofop-p-ethyl. A, selection of the lethal dose of quizalofop-p-ethyl (QPE, 10% emulsion) in 4-wk-old wheat (Jimai 38) and the weed *Aegilops tauschii*. B, the tiller mortality rate in wheat and *Ae. tauschii* treated with 0–2.50‰ QPE. C, screening for the QPE-resistant mutants, from left to right, the wheat field, applying QPE by an unmanned aerial vehicle, the resultant dying phenotype in most of the plants, and an example of one resistant mutant in the field selection. D, QPE resistance in 4-wk-old mutants in the greenhouse. * indicates the G6250A (or G2084S) allele; DAT, days after QPE treatment. E and F, greenhouse trials. E, tiller mortality rate (n=6); F, the relative ACC1 enzymatic activity (n=5) of mutants treated with QPE compared to untreated controls. G, the QPE resistance in the field. The middle column pictures highlight the *Ae. tauschii* phenotype at 30 DAT. H, plant height (n≥20). I, percentage of fertile tillers (n≥20) of the QPE-treated mutants in the field. J, grains of the *aabbdd* genotype from plants that were or were not treated with 2.50‰ QPE. K–N, the effect of QPE (2.50‰) on the growth (in K and M) and the tiller mortality rate (in L and N) of 4-wk-old *Bromus japonicus* (in K and I, n≥10) and *Avena sativa* (in M and N, n≥10). Means were separated with Duncan's multiple range test with α=0.01; error bars represent one standard error of the mean (in B, E, F, H, I, L, and N). Untreated control (=0 QPE).

seedlings were visually evaluated, resulting in the discovery of only 10 plants that grew well with tillers and green leaves (Fig. 1-C). Approximately one QPE-resistant mutant was recovered from every two million M_2 seeds. To genotype the *ACC1* gene in QPE-resistant mutants, the subgenome-specific primers were designed for PCR and for amplicon sequencing (Appendices A and B). Surprisingly, nine mutants shared the C5975T transition (at the cDNA level of TraesCS2A02G069400, TraesCS2B02G082500, and TraesCS2D02G068100; IWGSC CS RefSeq v2.1) among subgenomes, which corresponds to the A1992V substitution in ACC1 (Ostlie *et al.* 2015). In addition, a novel G to A transition was detected in the *ACC-B1* allele at position 6250 (at the cDNA level of TraesCS2B02G082500), resulting in a glycine to serine substitution at position 2084 (=G2084S) (Appendix C).

Both ACC1 variants, A1992V and G2084S, were evaluated for herbicide resistance to 2.50‰ QPE. In WT hexaploid wheat (hereinafter, we use the notation AABBDD to represent the WT *ACC1* alleles across the A, B, and D subgenomes; corresponding lowercase letters denote mutant alleles) and WT diploid *Ae. tauschii* (DD), tiller mortality rates were 100%. The *aaBBDD*, *AAbbDD*, and *AABBdd* genotypes (with A1992V) had significantly lower tiller mortality rates of approximately 12, 23, and 15%, respectively (Fig. 1-D and E). The *AAb*b*DD* genotype (*=G2084S) had a lower mortality, approximately 5% less than that in the *AAbbDD* genotype (23%; Fig. 1-E). Thus, the novel ACC1 variant (G2084S) apparently improved QPE resistance. Because the WT LY128, JM38, JM44, and SN30 were fully sensitive to QPE, their cross progeny should have the same response. Consequently, we used the intercultival crosses to further stack the subgenome variations. Wheat plants with two or more subgenome mutations in *ACC1* showed obvious herbicide resistance (Fig. 1-D and E). For example, the *AAbbdd*, *aabbDD*, *aab*b*DD*, *aaBBdd*, *AAb*b*dd*, and *aabbdd* genotypes had low tiller mortality rates, at approximately 7, 6, 0, 0, 0, and 0%, respectively when treated with 2.50‰ QPE. We additionally tested all mutant genotypes with 3.75 and 5.00‰ of QPE; only the *aabbdd* genotype grew vigorously and showed no tiller mortality (Fig. 1-E). However, neither A1992V nor G2084S conferred resistance to clethodim or haloxyfop-p, two other ACC1 inhibitors.

We further examined the relative enzymatic activity of ACC1 (using the ACCase/ACC Activity Assay Kit, Cat.# BC6020, Solarbio, Beijing, China) after treatment with 2.50‰ QPE (Fig. 1-F). In the WT wheat (*AABBDD*), plants treated with QPE had significantly lower ACC1 activity than untreated controls; however, in the *aaBBDD*, *AAbbDD*, *AAb*b*DD*, and *AABBdd* genotypes, plants treated with QPE had no or limited inhibition of ACC1 activity. Similar to A1992V (Bough and Dayan 2022), G2084S likely prevented QPE from binding ACC1. In particular, in the *aabbdd* genotype, the ACC1 activity in those treated with QPE was only 3% less than that of the untreated control. Even with 5.00‰ QPE, the relative AAC1 activity in the *aabbdd* genotype remained at 94% of the untreated control. Therefore, 2.50–5.00‰ of QPE is practical for weed control in the *aabbdd* wheat with either the A1992V and/or G2084S variants of ACC1.

We then evaluated the herbicide resistance in selected *ACC1* genotypes in fields (Fig. 1-G–I). With 2.50–5.00‰ QPE, 100% of the *Ae. tauschii* control died. In contrast, the single subgenome mutants, including *aaBBDD*, *AAbbDD*, *AAb*b*DD*, and *AABBdd*, were significantly suppressed in plant height and in the percentage of tillers that were fertile. The double subgenome mutants, including *aabbDD*, *aab*b*DD*, *aaBBdd*, *AAbbdd*, and *AAb*b*dd*, were somewhat dwarfed and lower in fertile tillers. However, the *aabbdd* genotype was normal in plant height and fertile tillers with 2.50‰ QPE. Although the 3.75–5.00‰ QPE resulted in a slight reduction in plant height in the *aabbdd* genotype, it had no effect on the fertile tillers. The *aab*b*dd* genotype would therefore likely confer vigorous growth under QPE, similar to that of the *aabbdd* genotype. With 2.50‰ QPE, the *aabbdd* genotype produced grains that were comparable in grain length, width, and 1,000-kernel weight to those from the untreated *AABBDD* and *aabbdd* genotypes (Fig. 1-J; Appendix D). Therefore, 2.50‰ QPE is a practical application to control *Ae. tauschii* in wheat fields with the *aabbdd* genotype of *ACC1*.

We tested whether 2.50‰ of QPE can kill other weedy grasses (i.e., *Bromus japonicus* Thunb. and *Avena sativa* L.). The *aabbdd* mutants and the tested weeds were planted in the same pot (as the untreated seedling controls in Fig. 1-K–N). After the QPE treatment, both *B. japonicus* and *A. sativa* were eliminated, whereas the *aabbdd* mutants grew vigorously. Thus, 2.50‰ QPE effectively controlled noxious grass weeds in this test.

In summary, we identified 10 QPE-resistant mutants, one of which included a novel G2084S substitution in ACC1. The pyramiding of subgenome mutations significantly increased QPE resistance in Chinese wheat. Furthermore, a low dose (2.50‰) of QPE effectively killed the tested weedy grasses with limited effects on the *aabbdd* genotype of *ACC1* in wheat. This study demonstrates an effective weed management method in wheat fields and supports the development of a sustainable crop production system for non-GMO wheat.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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