Research Article

Received: 25 July 2023 Revised: 7 September 2023 Accepted article published: 25 September 2023 Published online in Wiley Online Library: 17 October 2023

(wileyonlinelibrary.com) DOI 10.1002/ps.7789

Syntenic analysis of *ACCase* loci and target-site-resistance mutations in cyhalofop-butyl resistant *Echinochloa crus-galli* var. *crus-galli* in Japan

Satoshi Iwakami,[a*] Hinata Ishizawa,[a] Kai Sugiura,[a] Keisuke Kashiwagi,[b] Toshiya Oga,[b] Shota Niwayama[c] and Akira Uchino[d*]

Abstract

BACKGROUND: Recently, suspected cyhalofop-butyl-resistant populations of allohexaploid weed *Echinochloa crus-galli* var. *crus-galli* were discovered in rice fields in Aichi Prefecture, Japan. Analyzing the target-site *ACCase* genes of cyhalofop-butyl helps understand the resistance mechanism. However, in *E. crus-galli*, the presence of multiple *ACCase* genes and the lack of detailed gene investigations have complicated the analysis of target-site genes. Therefore, in this study, we characterized the herbicide response of *E. crus-galli* lines and thoroughly characterized the *ACCase* genes, including the evaluation of gene mutations in the *ACCase* genes of each line.

RESULT: Four suspected resistant lines collected from Aichi Prefecture showed varying degrees of resistance to cyhalofop-butyl and other FOP-class ACCase inhibitors but were sensitive to herbicides with other modes of action. Through genomic analysis, six *ACCase* loci were identified in the *E. crus-galli* genome. We renamed each gene based on its syntenic relationship with other *ACCase* genes in the Poaceae species. RNA-sequencing analysis revealed that all *ACCase* genes, except the pseudogenized copy *ACCase2A*, were transcribed at a similar level in the shoots of *E. crus-galli*. Mutations known to confer resistance to FOP-class herbicides, that is W1999C, W2027C/S and I2041N, were found in all resistant lines in either *ACCase1A*, *ACCase1B* or *ACCase2C*.

CONCLUSION: In this study, we found that the *E. crus-galli* lines were resistant exclusively to ACCase-inhibiting herbicides, with a target-site resistance mutation in the *ACCase* gene. Characterization of *ACCase* loci in *E. crus-galli* provides a basis for further research on ACCase herbicide resistance in *Echinochloa* spp.

Supporting information may be found in the online version of this article.

Keywords: barnyardgrass; TSR; polyploid; allohexaploid

1 INTRODUCTION

Acetyl-CoA carboxylase (ACCase)-inhibiting herbicides play an important role in the control of grass weeds. Since the discovery of this group of herbicides in the 1970s, they have been intensively used globally in various crops.[1] These herbicides target the grass-specific plastidic ACCase.[2] Non-synonymous mutations in the target-site *ACCase* genes, known as target-site resistance (TSR) have been identified as the main mechanism of resistance to these herbicides, although other mechanisms, known as non-target-site resistance (NTSR), were also reported.[1,3]

ACCase is encoded by a single nuclear gene often present as a single copy in the genome of diploid grasses, but polyploid species or even some diploid species have multiple copies of *ACCase* genes in their genome or subgenome.[4–7] When there is only one gene in the genome that produces the target-site protein of an herbicide, a heterozygous state of TSR mutation results in 50% of resistant-type proteins in the plant. However, when multiple genes for the target protein of an herbicide exist, the effect of the TSR mutation can be further diluted by the presence of sensitive alleles, which can result in a lower resistance level.[4,8–10] Thus, elucidating the copy number

* *Correspondence to: S Iwakami, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan, E-mail: iwakami.satoshi.2v@kyoto-u.ac.jp; or A Uchino, Central Region Agricultural Research Center, National Agriculture and Food Research Organization, Tsu 514-2392, Japan, E-mail: uchino@affrc.go.jp*

a *Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake-cho, Kyoto, Japan*

b *Aichi Agricultural Research Center, Nagakute, Japan*

c *Corteva Agriscience Japan Limited, Tokyo, Japan*

d *Central Region Agricultural Research Center, National Agriculture and Food Research Organization, Tsu, Japan*

and expression of each copy is important for understanding the modes of herbicide resistance evolution.

Echinochloa crus-galli is a common and noxious weed in crop production worldwide.[11] The species is classified into multiple varieties, for example, *E. crus-galli* var. *crus-galli* and var. *formosensis* based on the morphological, ecological, and genetic characteristics.[12–14] It occurs in various cropping systems, such as rice, maize, and cotton, where herbicides in each cropping system act as selective pressures for herbicide resistance. Herbicide resistance within these two varieties has been recorded for herbicides from eight modes of action,[15] causing problems in crop production.

Echinochloa crus-galli, an allohexaploid species, has six copies of plastidic *ACCase* genes, with each of the subgenomes estimated to carry two copies of the gene.[5] The presence of six copies of *ACCase* genes is the largest number in agricultural weeds reported so far, but accessing the exact copy number and gene expression has been difficult because of the lack of a reference genome. A recent report of a high-quality reference genome in *E. crus-galli*[12] opened the door for a more detailed study of TSR.

Cyhalofop-butyl, an ACCase-inhibiting herbicide, has been widely used to control *Echinochloa* weeds since its introduction in Japan in 1996. It is an effective herbicide for both direct-seeded and transplanted rice. However, the repeated use of cyhalofop-butyl resulted in the evolution of non-target-site-based cyhalofop-butyl resistance in *E. crus-galli* var. *formosensis* in 2010.[5] Two lines derived from distinct fields in Okayama Prefecture exhibited distinct resistance profiles: the Ecf108 line was resistant to only cyhalofop-butyl, whereas the Ecf27 line was resistant to acetolactate synthase (ALS)-inhibiting herbicides in addition to cyhalofop-butyl. Both lines did not carry known TSR mutations in *ACCase* and *ALS* genes. Thus, both lines were estimated to be exhibiting non-target-site-based resistance. In recent years, there has been a growing incidence of ineffective *E. crus-galli* var. *crus-galli* control in multiple rice fields located in Aichi Prefecture. These fields have been rotated with rice, wheat, and soybeans for more than 10 years, with cyhalofop-butyl being the major selection pressure for *Echinochloa* spp. in rice fields. In the current study, we reveal that the four cyhalofop-butyl-resistant lines from Aichi Prefecture all carry TSR mutations in well-transcribed *ACCase* genes. Furthermore, we conducted a detailed analysis of the number and loci of *ACCase* genes in *E. crus-galli* and proposed a new nomenclature for each gene. These analyses provide the foundation for further research on ACCase inhibitor resistance in *Echinochloa* spp.

2 MATERIALS AND METHODS

2.1 Plant materials

In 2019, the lines of *E. crus-galli* var. *crus-galli* with suspected resistance (Ec1902, Ec1909, Ec1914, and Ec1916) were collected from four severely infested dry-seeded rice fields in Aichi Prefecture. The susceptible *E. crus-galli* var. *crus-galli* (Ec1906) was collected from a dry-seeded rice field in Aichi Prefecture. The origins of the cyhalofop-butyl-resistant lines (Ecf108 and Ecf27) and susceptible line (Ecf99, previously named Ec/99-J-197) of *E. crus-galli* var. *formosensis* were described by Iwakami *et al.*[5] All the lines were derived from a single seed and had undergone self-propagation for at least one generation before being utilized in the experiments.

2.2 Sensitivity to cyhalofop-butyl and fluazifop-*p*-butyl

Susceptibility to cyhalofop-butyl and fluazifop-*p*-butyl was assessed in 2021 and 2022 in Kyoto, Japan (35°01′55.4″ N 135° 46′59.7″ E), respectively. Seeds were treated with sodium hypochlorite solution (~2.5% active chlorine) for 10 min, washed with tap water three times and kept at 4 °C for 4 days under water. Seeds were incubated at 30 °C under a photoperiod of 13 h for germination. Three days later, the germinated seeds were planted in a 50% mixture of soil (Bonsol-nigou; Sumitomo Chemicals, Tokyo, Japan) and non-nutrient soil (Inaho-baido; Inaho-Kako, Toyama, Japan) in 113 cm^2 polypropylene pots. The plants were grown in a glasshouse under drained wet conditions. Two days before the cyhalofop-butyl and fluazifop-*p*-butyl treatments, each pot was thinned to four and two plants per pot, respectively. The commercial formulation of cyhalofop-butyl (Clincher 30% EW; Corteva Japan Ltd, Tokyo, Japan) or fluazifop-*p*-butyl (Onecide P 17.5% EC; ISK BIOSCIENCES, Tokyo, Japan) was sprayed using a hand sprayer (DIA spray swing 500; Furupla, Tokyo, Japan) on four-leaf stage plants with four replicates at each dose. The pots were placed randomly within a 1 m^2 area, and a prescribed amount of herbicide was uniformly applied across the entire area. Cyhalofop-butyl was applied at ×1/100, ×1/30, ×1/10, ×1/3, ×1, ×3, and ×10 doses of the recommended rate [300 g active ingredient (a.i.)/ha) and supplemented with 0.1% detergent (Surfactant 30; Maruwa Biochemical, Tokyo, Japan). Fluazifop-*p*-butyl was administered at ×1/3 and ×1 doses of the recommended rate (131 g a.i./ha). Two weeks later, the shoots were collected and dried for 3 days at 80 °C, followed by dry weight measurement. Dose–response curves were plotted using the drc package[16] (version 3.0-1) in R (version 4.2.2)[17] with a two-parameter log-logistic function. The experiments yielded consistent results in herbicide responses through multiple iterations.

2.3 Sensitivity to other herbicides for rice cultivation

Experiments were conducted in three replicates during the summer of 2022 in Mie, Japan (34°46′25.6″ N 136°25′36.1″ E). Seeds were immersed in water at 5 °C for 1 to 16 months to break seed dormancy and germinated at 30 °C under constant light for 5 days. Six germinated seeds were transplanted to loam soil in 200 cm^2 polyethylene pots. Plants were grown in a glasshouse under flooded conditions with a 3–6 cm water depth and thinned to three plants per pot just before herbicide treatment. The herbicides were sprayed at the five-leaf stage of the plants using the hand sprayer after the water in the pot was drained. The pots were flooded again 1 day after treatment. Commercial formulations of metamifop (Todome MF 4.9% EC; Kaken Pharmaceutical Co., Tokyo, Japan), bispyribac-sodium (Nominee 2% L; Kumiai Chemical Industry Co., Tokyo, Japan), penoxsulam (Wide Attack 3.6% SC; Corteva Japan Ltd), and florpyrauxifen-benzyl (Loyant 2.7% EC; Corteva Japan Ltd) were treated at the recommended rates of 99, 40, 37.5, 50 g a.i./ha, respectively. Shoot dry weights were measured 3 weeks after treatment as described earlier. The experiments were conducted twice with consistent results in herbicide responses.

2.4 Genome survey for *ACCase* loci in *E. crus-galli*

The *ACCase* genes of each species were identified using blastp in diamond (version 2.0.15.153)[18] with rice protein sequences as a query. Genome sequence and gene structural annotation of each species were obtained from the respective database as follows: National Genomics Data Center for *E. crus-galli* (GWHBDNR00000000),[12] and *Leptochloa chinensis* (GWHBJVB00000000)[19]; Phytozome for rice (Phytozome genome ID: 323), *Setaria italica* (Phytozome genome ID: 4555), *Zea mays* (Phytozome genome ID: 4577), *Brachypodium distachyon* (Phytozome genome ID: 15368), *Hordeum vulgare*

(Phytozome genome ID: 112509), *Pharus latifolius* (Phytozome genome ID: 38686); National Center for Biotechnology Information (NCBI) for *Lolium rigidum* (GCA_022539505.1 APGP_CSIRO_Lrig_0.1)[20]; and CoGe for *Poa annua* (Genome ID: 63982).[21] A pseudogenized *ACCase* gene and one of the *ACCase* genes, BH09.424, which has an unusually long sequence at the 5′ region, in *E. crus-galli* were annotated using miniprot (version 0.7)[22] with CH05.2958 as a query. The output gff3 format was converted to gtf format using gffread (version 0.12.7),[23] which was used for RNA-sequencing (RNA-Seq) analysis. The gene structure was visualized using the ggtrasncript (version 0.99.9) package.[24] The syntenic relationships of *ACCase* loci among Poaceae species were analyzed using the GENESPACE (version 0.9.4) package.[25]

2.5 Phylogenetic analysis

Since *ACCase2A* carries multiple indels leading to frameshifts and stop codons, these indels were modified based on other *ACCase* genes in *E. crus-galli* so that they could be used for protein alignment. Full-length protein sequences of the plastidic *ACCase* genes of each species, together with those of *Alopecurus myosuroides* (NCBI accession number, AJ310767.1), were subjected to MAFFT version 7.508.[26] The aligned sequences were used to construct a phylogenetic tree using the maximum likelihood method in RAxML-NG (version 1.1.0)[27] with 1000 bootstraps, following best model selection with ModelTest-NG (version 0.1.7).[28]

2.6 RNA-Seq analysis

Echinochloa crus-gali lines (Ec1902, Ec1906, Ec1914, and Ec1916) were germinated as described earlier for the cyhalofop-butyl sensitivity assay. The plants were cultivated for a 14-h thermal period at 25 ± 5 °C in a growth chamber with a high-pressure sodium lamp. RNA was extracted from the shoots of two-leaf-stage plants using a Plant Total RNA Mini Kit (Favorgen, Pingtung, Taiwan), followed by DNase treatment using a TURBO DNA-free Kit (Thermo Fisher Scientific, Tokyo, Japan). The messenger RNA (mRNA)-Seq libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB, Tokyo, Japan). Sequencing of the libraries was performed on a NovaSeq 6000 platform (150-bp paired-end reads). Raw read data were filtered using fastp (version 0.19.5)[29] with the following parameters: -q 30 -l 120–detect_adapter_for_pe–trim_poly_x. The clean reads were mapped to the reference genome using STAR (version 2.7.9)[30] with the outFilterMultimapNmax 1 option, followed by read quantitation using HTSeq (version 2.0.2).[31] The count data were transformed into transcripts per million (TPM) with R. Principal component analysis (PCA) was conducted on the $\log_2$(TPM + 1) transformed data using the prcomp() function in R. RNA-Seq analysis was conducted with three biological replicates.

2.7 Sequencing *ACCase* genes in *E. crus-galli* populations

Ec1902, Ec1906, Ec1909, Ec1914, and Ec1916 lines were cultivated as described in the cyhalofop-butyl sensitivity assay. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) from the leaves of *E. crus-galli* lines. The carboxy transferase (CT) domains of six *ACCase* genes were amplified using KOD-FX Neo (Toyobo, Japan) with previously reported primers (Supporting Information Table S1).[5] The polymerase chain reaction (PCR) product was treated with ExoSAP-IT (Thermo Fisher Scientific) and directly sequenced. The amino acid positions are described based on the reference *Alopecurus myosuroides* ACCase (NCBI accession number, AJ310767.1).

3 RESULTS

3.1 Dose response to cyhalofop-butyl

The rice fields in Aichi Prefecture, where suspected cyhalofop-butyl resistance was found, are ~350 km away from the fields in Okayama Prefecture, where the first and only cases of herbicide resistance in *E. crus-galli*[5] were reported in Japan (Fig. 1(a)). The seed morphology, especially the awn length, is distinct: the Aichi and Okayama lines carry long and short awns, respectively (Fig. 1(a)). In this study, we first evaluated the cyhalofop-butyl sensitivity of four suspected resistant lines from Aichi Prefecture, together with two resistant lines found in Okayama Prefecture.

All suspected resistant lines exhibited a marked decrease in cyhalofop-butyl sensitivity compared with the two sensitive lines (Fig. 1(b),(c); Table 1). Resistance levels differed significantly among the resistant lines, with Ec1902 and Ec1914 showing lower resistance levels, similar to the previously characterized[5] Ecf27 and Ecf108 lines. The Ec1909 and Ec1916 strains exhibited the highest levels of resistance. These results suggest differences in resistance mechanisms.

3.2 Sensitivity to other herbicides

Next, we examined the sensitivity of the resistant lines to other foliar herbicides registered for rice cultivation in Japan. Four resistant lines from Aichi Prefecture (Ec1902, Ec1909, Ec1914, and Ec1916) exhibited resistance to the FOP ACCase inhibitor metamifop (Fig. 2). In contrast, the four lines were severely suppressed by the ALS inhibitors bispyribac-sodium and penoxsulam, and the auxin herbicide florpyrauxifen-benzyl, similar to the susceptible lines. The resistance profiles of the two resistant lines from Okayama Prefecture differed from those of the Aichi lines. Ecf27, a multiple-herbicide-resistant line, exhibited marked resistance to metamifop and penoxsulam (Supporting Information Fig. S1). Ecf108, a cyhalofop-butyl-resistant line, did not show resistance to any of the other herbicides, including the ACCase inhibitor metamifop (Fig. S1).

In the area where Aichi lines were collected, most farmers rotated paddy rice and soybean as summer crops. As *E. crus-galli* plants grows in both flooded and dry conditions, the cyhalofop-butyl-resistant *E. crus-galli* may potentially cause a problem in soybean cropping where herbicides with the same mode of action are used. Therefore, we tested the sensitivity of the resistant *E. crus-galli* lines to one of the major FOP ACCase herbicides, fluazifop-*p*-butyl. Although the sensitive line was controlled by the recommended field rate, Ec1902, Ec1909, and Ec1916 exhibited marked resistance (Fig. 3). In contrast to the cyhalofop-butyl response, no clear differences in fluazifop-*p*-butyl sensitivity were observed among the three lines. Decreased sensitivity to fluazifop-*p*-butyl was also observed in Ec1914, although it was well-suppressed by the field rate.

3.3 ACCase genes in *E. crus-galli*

Because the herbicides to which Aichi lines exhibited resistance were limited to ACCase herbicides, we analyzed their target-site genes. In a previous study, partial sequences of six different *ACCase* genes were cloned in *E. crus-galli* by PCR-based approach.[5] Now that the whole genome sequence of *E. crus-galli* is available, we looked into the copy number and their loci in the genome. Five genes, previously named *ACC1*, *ACC2*, *ACC3*, *ACC5*, *ACC6* were identified on the 05 and 09 chromosomes (Fig. 4(a)). The missing gene, *ACC4*, is a frameshift-based pseudogene with a single nucleotide deletion in its CT domain,[5] which

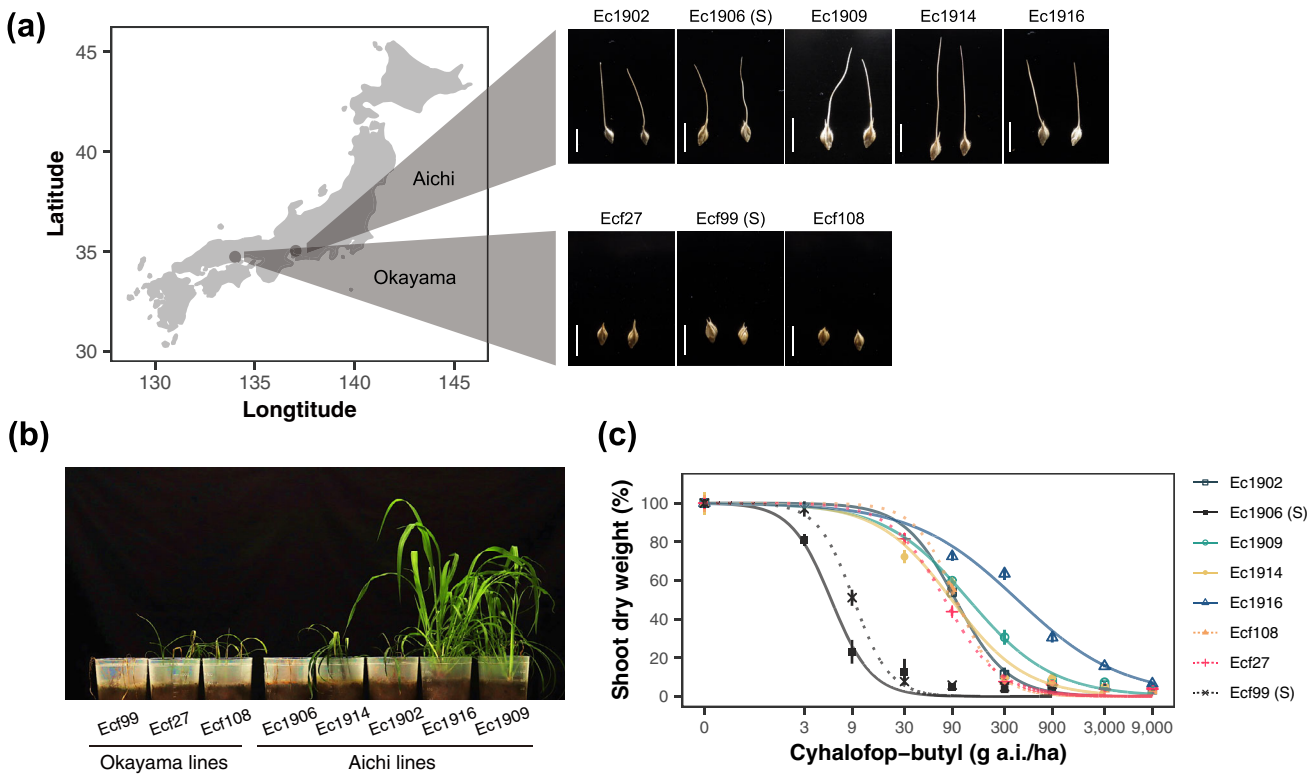

Figure 1. Cyhalofop-butyl responses of the Japanese *Echinochloa crus-galli* var. *crus-galli*. Ec1906 and Ecf99 are the sensitive (S) lines. (a) The collection sites and seed shapes of *E. crus-galli* var. *crus-galli* and *E. crus-galli* var. *formosensis*. Bar in the photograph represents 0.5 cm. (b) Responses to the field dose cyhalofop-butyl (300 g a.i./ha). (c) Dose responses to cyhalofop-butyl. Bars, standard error ($n = 4$).

Table 1. *Echinochloa crus-galli* lines and their cyhalofop-butyl sensitivity

Species	Line	Location	Year	GR_{50}[a]	Response[b]	R/S[c]
Echinochloa crus-galli var. *crus-galli*	Ec1902	Aichi Prefecture	2019	97.4	R	10.4
Echinochloa crus-galli var. *crus-galli*	Ec1906	Aichi Prefecture	2019	5.6	S	0.6
Echinochloa crus-galli var. *crus-galli*	Ec1909	Aichi Prefecture	2019	131.5	R	14.1
Echinochloa crus-galli var. *crus-galli*	Ec1914	Aichi Prefecture	2019	88.9	R	9.5
Echinochloa crus-galli var. *crus-galli*	Ec1916	Aichi Prefecture	2019	405.0	R	43.3
Echinochloa crus-galli var. *formosensis*	Ecf27	Okayama Prefecture	2010	76.0	R	8.1
Echinochloa crus-galli var. *formosensis*	Ecf108	Okayama Prefecture	2010	99.4	R	10.6
Echinochloa crus-galli var. *formosensis*	Ecf99	Okayama Prefecture	1999	9.3	S	—

[a] The dose of 50% growth reduction (g a.i./ha).
[b] Lines were classified as cyhalofop-butyl resistant (R) or sensitive (S).
[c] The GR_{50} value of Ecf99 was used for calculation.

should have prevented its structural annotation in the genome sequencing study.[12] Thus, we searched the sequence in the genome using miniprot[22] and found *ACC4* gene on the AH05 chromosome (Fig. 4(a); Table S2). This gene carries multiple indels, causing frameshifts and substitutions at intron-exon junctions. The corresponding nucleotide sequence of *ACC4* gene to the coding sequence of CH05.2958 determined using miniprot shows a 96% similarity to CH05.2958. The nucleotide sequence is listed in Supporting Information Dataset S1 together with the coding sequences of the other five *ACCase* genes.

Phylogenetic clustering revealed that the six copies were grouped into two groups (Fig. 4(b)). In both groups, ACCase in subgenomes B and C showed higher similarity than that in subgenome A, which is in accordance with a previous analysis conducted using the partial sequence,[5] reflecting the whole subgenome relationship.[32] As previously reported,[6,7] diploid *Z. mays* and *Lolium rigidum* carried two copies of *ACCase* genes (Fig. 4(b)). Tetraploid *Leptochloa chinensis* and *Poa annua* carry two *ACCase* genes.

Syntenic analysis showed that *ACCase* loci that correspond to the AH09, BH09, and CH09 chromosomes in *E. crus-galli* are well conserved among Poaceae species, except for rice (*Oryza sativa*) (Fig. 4(c)). These *ACCase* genes were renamed as *ACCase1A*, *ACCase1B*, and *ACCase1C* (Table 2). In contrast, no syntenic relationships were observed in Poaceae for *ACCase* loci on the AH05, BH05, and CH05 chromosomes of *E. crus-galli*. These

enzymes were renamed *ACCase2A*, *ACCase2B*, and *ACCase2C*. Similarly, non-syntenic *ACCase* genes were found in one copy of *Z. mays* and *Lolium rigidum*. These additional copies were not related among the species (Fig. 4(b)), suggesting that the duplicated genes were acquired in a species/lineage-specific manner. This is in accordance with the findings that the divergence of two *ACCase* copies dates back to 16 Mya,[33] > 10 Mya later than the divergence of Andropogoneae (e.g., *Z. mays*) and Paniceae (e.g., *Echinochloa* spp.) occurred.[34] Another interesting finding was that *ACCase* locus in rice (LOC_0s05g22940) was located only on chromosome 5, and there was no syntenic relationship with the *ACCase* loci in other species. The observation suggests the possibility that rice experienced *ACCase* gene duplication followed by loss of the copy that are conserved in other Poaceae species. Similary, the loss of one *ACCase* gene has also been pointed out in *Aegilops*.[33]

Next, we investigated the transcript levels of *ACCase* genes in the shoots of two-leaf-stage plants using RNA-Seq analysis except Ec1909. The libraries from each line were clustered together (Fig. S2), indicating that the sequencing, including library preparation and subsequent analyses, was conducted properly. Next, we examined the transcription of *ACCase* genes. In the five lines from Aichi Prefecture, no prominent differences in transcript levels were observed for any of the *ACCase* genes (Fig. 5(a)). Therefore, we averaged the five lines for each *ACCase* gene and calculated the percentage transcript level of each gene. We excluded *ACCase2A* as it was pseudogenized and did not comprise the ACCase pool in *E. crus-galli*. The transcript levels of the five genes were within a two-fold difference, although some differences in transcription were observed, with genes of *ACCase1* loci being more highly expressed than those of *ACCase2* (Fig. 5(b)).

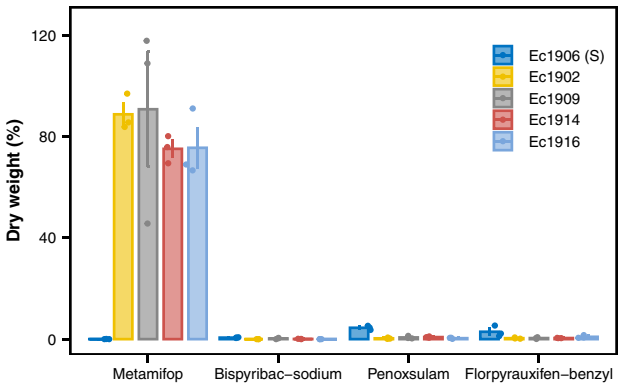

Figure 2. Responses of cyhalofop-butyl resistant *Echinochloa crus-galli* var. *crus-galli* to foliar-applied herbicides used for rice cropping in Japan. Ec1906 is the sensitive (S) line. Bars, standard error ($n = 3$).

3.4 TSR mutations in *ACCase* genes

Finally, the CT domains of each *ACCase* gene were sequenced. Non-synonymous mutations known to confer resistance to FOP ACCase herbicides, including cyhalofop-butyl[3,35–39] were identified as homozygous state in all four resistant lines in either *ACCase1A*, *ACCase1B*, and *ACCase2C* (Table 3). These results strongly suggest that these mutations play an important role in the resistance to FOP-ACCase herbicides, and all the subgenomes can contribute to resistance evolution to ACCase inhibitors in *E. crus-galli*.

4 DISCUSSION

In this study, cyhalofop-butyl resistance was confirmed in four *E. crus-galli* populations from Aichi Prefecture, marking the second cases of herbicide resistance in *E. crus-galli* in Japan, subsequent to the cases observed in Okayama Prefecture.[5] The analysis of *ACCase* loci within the recently assembled chromosome-level genome of *E. crus-galli* unveiled the presence of six *ACCase* loci, consistent with previous findings.[5] Through full-length gene structure and RNA-Seq analyses, we identified five loci as potential contributors to resistance. Sequencing on each copy from the four resistant lines unveiled a single distinctive non-synonymous TSR mutation across three of the five *ACCase* loci. Notably, these results contrast with previously reported resistant *E. crus-galli* lines from Okayama Prefecture, where no TSR mutations were detected.[5] The distinctive TSR mutation patterns suggest independent evolution of cyhalofop-butyl resistance occurring in multiple regions in Japan.

In Japan, the first resistance cases in *E. crus-galli* were found in Okayama Prefecture in 2010, where dry direct-seeded rice was most widely practiced for a long time. The second cases of resistance in Aichi Prefecture were also from areas where dry direct-seeded rice has been widely practiced since the 1990s. In dry-seeded rice cultivation in Japan, various one-shot herbicides

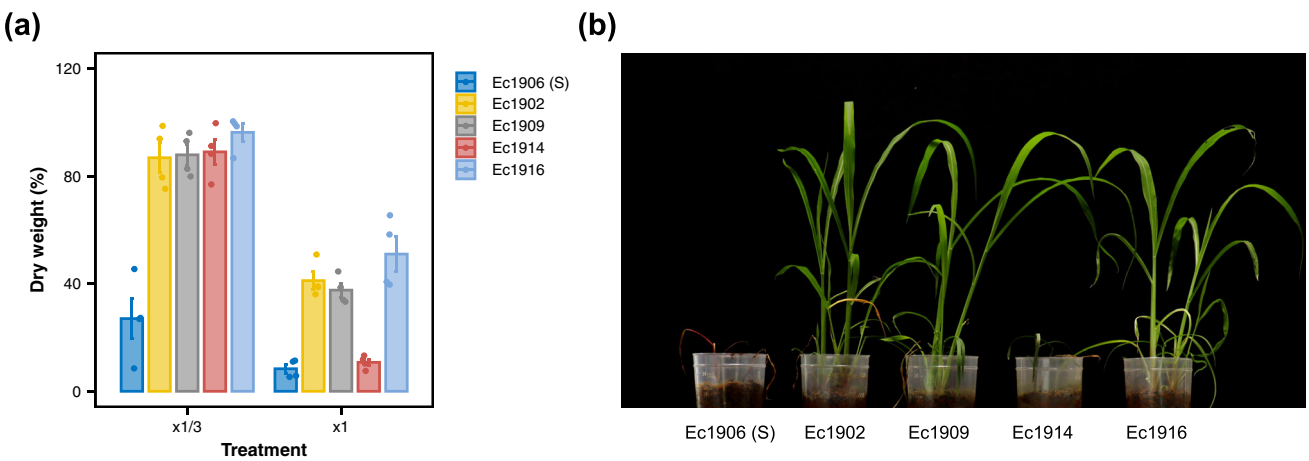

Figure 3. Responses of cyhalofop-butyl resistant *Echinochloa crus-galli* var. *crus-galli* to fluazifop-*p*-butyl. Ec1906 is the sensitive (S) line. (a) Fluazifop-*p*-butyl was treated with 1/3 (×1/3) and the recommended dose (×1). Bars, standard error ($n = 4$). (b) Plant responses to the recommended dose.

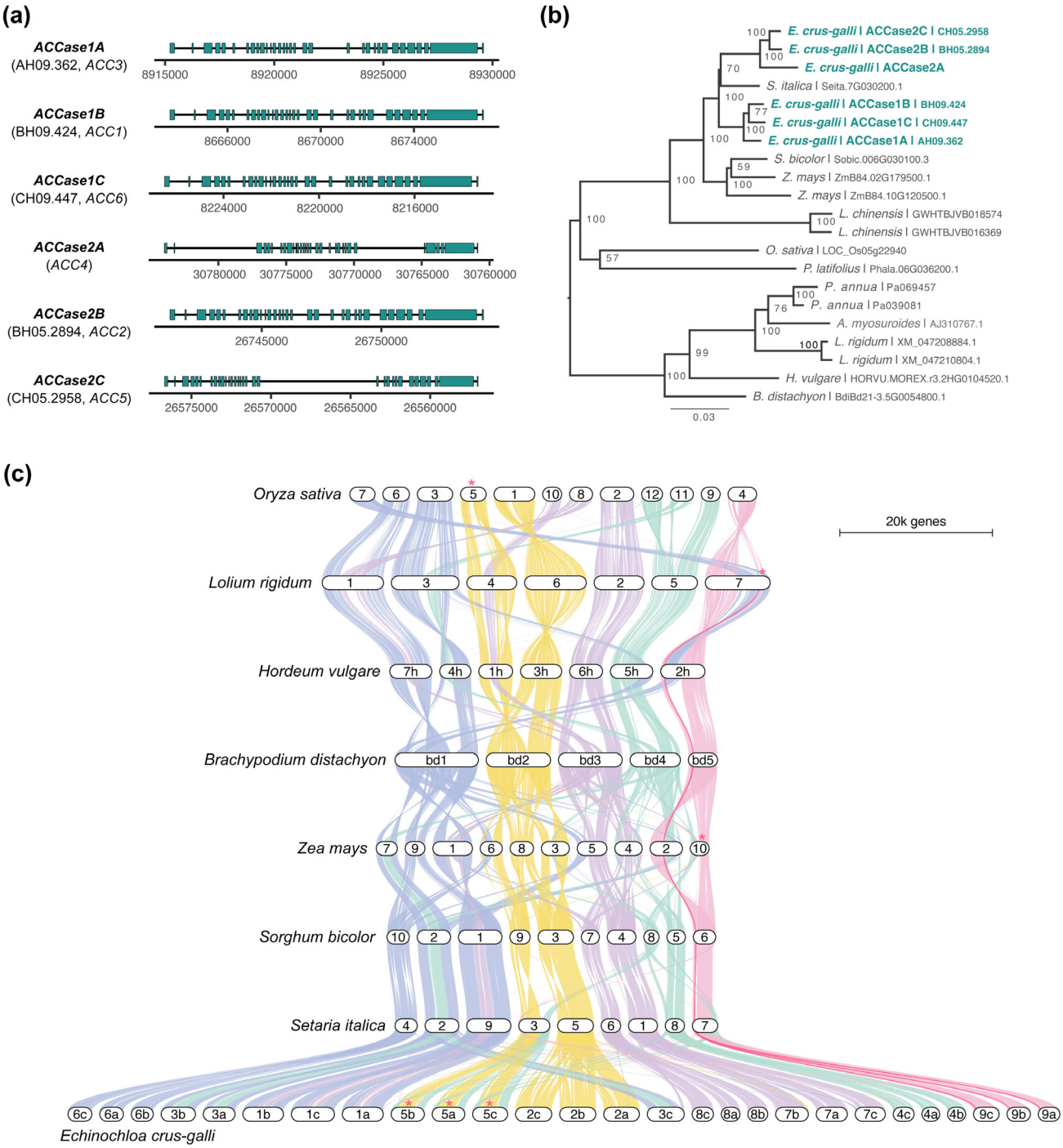

Figure 4. The *ACCase* genes of *Echinochloa crus-galli*. (a) Schematic representation of the coding sequences (green boxes) of *ACCase* genes in *E. crus-galli*. The former gene names are shown alongside the current names. The gene models from *E. crus-galli* genome version 3[12] were used except *ACCase1B* and *ACCase2A*, whose gene structure are annotated in this study (Supporting Information Table S1). The *ACCase2A* representation does not reflect coding sequence, but corresponds to coding sequence of *ACCase2C*. The numbers indicate the nucleotide positions of the version 3 genome. (b) Phylogenetic tree of ACCase of Poaceae species, that is, *E. crus-galli*, *Setaria italica*, *Sorghum bicolor*, *Zea mays*, *Leptochloa chinensis*, *Oryza sativa*, *Pharus latifolius*, *Poa annua*, *Alopecurus myosuroides*, *Lolium rigidum*, *Hordeum vulgare*, and *Brachypodium distachyon*. The numbers represent bootstrap percentage values. (c) Synteny of *ACCase* loci among Poaceae species. Red line indicates where conserved *ACCase* locus was found in each species. *Represents the *ACCase* locus other than the conserved locus.

available for transplanted rice cannot be applied until the paddy fields are flooded after the rice seedlings are fully established. There are very few options for effective herbicides against *Echinochloa* spp. that can be used before flooding, although the control at that time is critical to the success of *Echinochloa* management. One notable option is cyhalofop-butyl, which was introduced as early as 1996 and has been extensively employed in *Echinochloa* management. Thus, the evolution of cyhalofop-butyl resistance in *E. crus-galli* in Japan has been distinctly influenced by the dry-seeded rice system.

Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Table 2. *ACCase* loci in *Echinochloa crus-galli*

Gene name	Gene ID[a]	Chromosome	Previous gene name[d]
ACCase1A	AH09.362	AH09	*ACC3*
ACCase1B	BH09.424[b]	BH09	*ACC1*
ACCase1C	CH09.447	CH09	*ACC6*
ACCase2A	—[c]	AH05	*ACC4*
ACCase2B	BH05.2894	BH05	*ACC2*
ACCase2C	CH05.2958	CH05	*ACC5*

[a] Gene identifier was obtained from version 3 genome of *E. crus-galli*.[12]
[b] Annotation was corrected in this study.
[c] The locus was annotated in this study.
[d] Each gene was named based on the order of identification in Iwakami *et al.*[5]

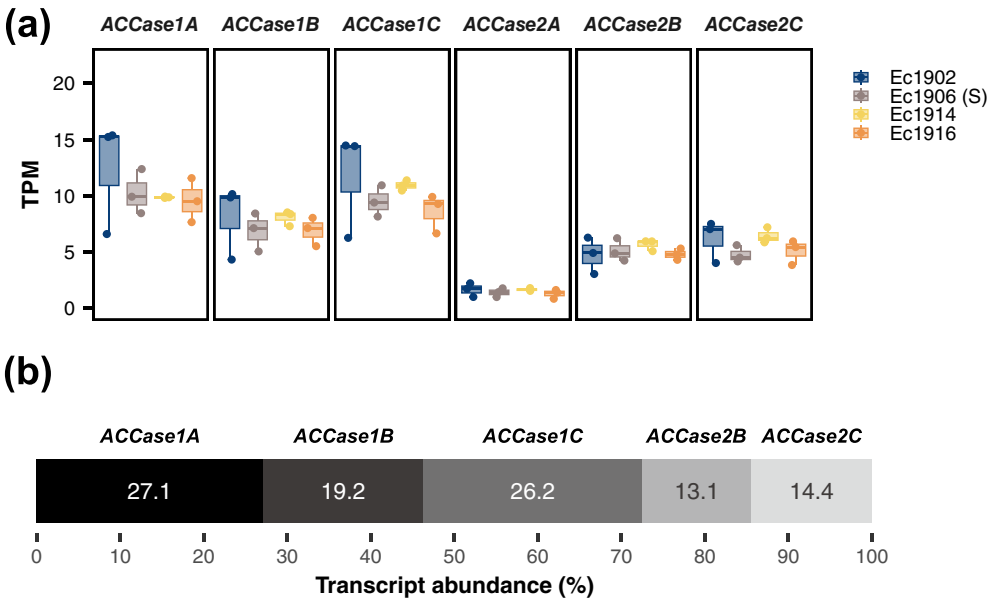

Figure 5. Transcription of each *ACCase* gene in *Echinochloa crus-galli* var. *crus-galli*. (a) Transcription of *ACCase* genes evaluated by RNA-Seq analysis. Ec1906 is the sensitive (S) line. TPM, transcripts per million. (b) Transcript abundance of each ACCase gene. *ACCase2A* was excluded since it is a frameshift-based pseudogene.

Table 3. Target-site resistance mutations identified in *Echinochloa crus-galli* lines

Line	*ACCase1A*	*ACCase1B*	*ACCase1C*	*ACCase2A*	*ACCase2B*	*ACCase2C*
Ec1902	W2027C	—	—	—	—	—
Ec1906 (S)	—	—	—	—	—	—
Ec1909	W2027S	—	—	—	—	—
Ec1914	—	W1999C	—	—	—	—
Ec1916	—	—	—	—	—	I2041N

Note: —, no known mutation that confers resistance to ACCase inhibitors. The amino acid positions correspond to ACCase in *Alopecurus myosuroides* (NCBI accession number, AJ310767.1). Ec1906 is the sensitive (S) line.

Currently, several alternative herbicides in dry-seeded rice are available: ALS inhibitor penoxsulam, ACCase inhibitor metamifop, and auxin mimic florpyrauxifen-benzyl, which were introduced to Japan in 2007, 2017, and 2020, respectively. ALS inhibitor bispyribac-sodium, which was introduced earlier than these (in 1997), can also be used for *Echinochloa* control in dry-seeded rice, although it is less popular in Japan due to its slight phytotoxic effect on *Japonica* rice varieties.[40] The *E. crus-galli* lines found in Aichi Prefecture were sensitive to these alternative herbicides except for metamifop (Fig. 2). Therefore, it is important to control these lines using herbicides with other modes of action. In contrast, the other two cyhalofop-butyl-resistant lines from Okayama

Prefecture (Ecf108 and Ecf27) showed different cross-resistance patterns (Fig. S1) compared to the lines collected in Aichi Prefecture. Ecf108 exhibited high susceptibility to metamifop, whereas Ecf27 demonstrated broad resistance to both penoxsulam and metamifop. Neither line has known resistance mutations in *ACCase* genes,[5] implying that they carry different mechanisms from the lines derived from Aichi Prefecture. These resistant lines require further detailed analysis for their proper control and management.

In weed species with multiple copies of herbicide-target-site genes, identifying the gene copies that can confer resistance is important for understanding the modes of resistance evolution. Studies have revealed that some species make use of all copies of their target-site genes in the resistance evolution, and some species that exploit only few copies.[10,41–45] To accumulate evidence on mutated copies, it is important to use a uniform nomenclature across studies. However, with regard to *ACCase* gene in *E. crus-galli*, consistent nomenclature has not always been employed[5,46–49] likely because the presence of many copies with non-organized nomenclature and partial isolation of the gene sequences complicate sequence classification. Therefore, we utilized the recently deciphered whole-genome sequence of *E. crus-galli* to rename and classify each gene based on syntenic relationships (Table 2). Although we did not analyze the genome of the allotetraploid *Echinochloa oryzicola* (syn. *Echinochloa phyllopogon*), which is a progenitor of *E. crus-galli* (A and B genome donors), the same nomenclature should be applied.

Transcription of target-site genes is strongly linked to herbicide resistance. In *Monochoria vaginalis*, resistance mutations have only been found in two highly transcribed *ALS* genes among the four *ALS* genes all of which encode functional ALS protein.[10] The findings suggest that TSR mutations in genes with low expression may not confer sufficient herbicide resistance to plants. We found that the transcript levels of all genes in *E. crus-galli* except pseudogenized *ACCase2A* did not differ substantially, suggesting that resistance may be expressed regardless of which *ACCase* gene undergoes a TSR mutation. Notably, a TSR mutation was detected even in *ACCase2B*, one of the independently duplicated genes in *ACCase2* clade of *Echinochloa* spp. This indicates that *ACCase2*, along with the syntenically conserved *ACCase1*, plays a vital role in *E. crus-galli*. It is important to examine whether resistance can be conferred on genes other than *ACCase1A*, *ACCase1B*, and *ACCase2C*, by analyzing the resistant *E. crus-galli* from different populations.

In the current study, all lines with a TSR mutation, that is W1999C, W2027C/S, and I2041N, exhibited resistance to both metamifop and fluazifop-*p*-butyl although the resistance level to fluazifop-*p*-butyl in W1999C mutant (Ec1914) was only marginal (Figs 2 and 3). Weed biotypes with these TSR mutations exhibited resistance to metamifop.[35,36,38,39] Additionally, at least the W2027C and I2041N mutations have been reported to be associated with resistance to fluazifop-*p*-butyl.[3,35,39] Hence, the resistance to these herbicides in *E. crus-galli* lines likely resulted from cross-resistance due to the mutations. In the case of ACCase TSR biotypes, some ACCase herbicides can still effectively control them.[1] While the options of ACCase inhibitors are limited to cyhalofop-butyl and metamifop in rice, the options for the farmers in Aichi areas extend to fluazifop-*p*-butyl, quizalofop-*p*-ethyl, clethodim, and sethoxydim as they also engage in soybean for crop rotation. Among these, sethoxydim is considered effective against such mutations.[3,35,39,50] Hence, investigating the efficacy of this compound is warranted.

Meanwhile, it is imperative to ascertain whether the resistance observed in this study can be entirely attributed to TSR mutations. In *Avena fatua*, which has three copies of *ACCase* genes, the decrease in ACCase sensitivity to ACCase inhibitors was marginal when mutations occur in only one copy.[8] Similar cases have been observed in glyphosate-resistance in *Echinochloa colona*, which carries three copies of glyphosate target-site genes.[9] This phenomenon is known as the dilution effect where the effect of a mutated target-site gene is diluted by the presence of non-mutated target-site genes.[4,8] Given that only a marginal resistance level was observed in *Avena fatua* with a TSR mutation that confers high-level resistance in diploid plants,[8] it is questionable that the identified TSR mutations in this study solely underlie the FOP herbicide resistance in the *E. crus-galli* lines, especially for Ec1916 that exhibited high-level resistance to cyhalofop-butyl. There is a possibility that TSR mutations have accumulated in individuals with weaker NTSR mutations, akin to instances observed in Ecf27 or Ecf108 (Fig. 1), or conversely, the cumulative occurrence of NTSR mutations within individuals bearing TSR mutations.

The coexistence of TSR and NTSR is often observed in grass weeds, especially in obligate outcrossing species.[51,52] Although the accumulation of multiple mechanisms does not easily occur in predominantly self-pollinating species such as *E. crus-galli*, the lines in this study, especially those with high resistance levels, may have accumulated more than one mechanism in the history of more than 10 years of cyhalofop-butyl application. Although the NTSR mechanisms of cyhalofop-butyl are not well understood, the involvement of cytochrome P450 monooxygenases and glutathione *S*-transferases, both of which play a central role in herbicide detoxification,[53,54] is often discussed in the study of cyhalofop-butyl-resistant weeds.[39,55,56] In the last 5 years, several molecular players conferring weed resistance to ACCase inhibitors have been identified, although most of them do not seem to be active against cyhalofop-butyl. In *E. phyllopogon*, a close relative of *E. crus-galli*, cytochrome P450 genes (CYP81A12, *CYP81A15*, *CYP81A21*, and *CYP81A24*) that detoxify ACCase inhibitors have been identified,[57] although our analysis failed to confirm any cyhalofop-butyl-metabolizing activity of these enzymes (data not shown). CYP709C69, a newly identified P450 in *E. phyllopogon*, was only active against diclofop-methyl and not cyhalfop-butyl.[58] However, *CYP81A68*, a likely the subgenome C homeolog of *CYP81A24* in E. *phyllopogon*, has been reported to metabolize cyhalofop-butyl in *E. crus-galli*.[59] Thus, some of the homeologs of these CYP families may be involved in high-level resistance to cyhalofop-butyl in Ec1916, although the key NTSR players in cyhalofop-butyl resistance are not clearly understood. Further in-depth analysis of these lines is necessary to fully understand the underlying mechanisms of resistance.

AUTHOR CONTRIBUTIONS

Experiment design, SI, AU, and SN; material collection, KK and TO; herbicide sensitivity assay, AU, SI, and KS; RNA extraction, SI and HI; RNA-Seq library preparation, KS; bioinformatics analysis, SI and HI; writing, SI and AU.

ACKNOWLEDGEMENTS

The authors thank Tomomi Kubo and Yuuri Nakamura for technical assistance. Computations were partially performed on the NIG supercomputer at ROIS National Institute of Genetics. The

research was partially supported by JSPS KAKENHI (19K06066, 22H02347) and by Japan Association for Advancement of Phyto-Regulators (JAPR) grant no. JA-22050.

DATA AVAILABILITY STATEMENT

The mRNA-Seq data have been deposited in the DDBJ Sequence Read Archive (DRA) database (DRR493742-DRR493753).

CONFLICT OF INTEREST STATEMENT

SN is an employee of Corteva Agriscience Japan, which markets herbicide products containing cyhalofop-butyl, penoxsulam, or florpyrauxifen-benzyl for use in rice.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Kaundun SS, Resistance to acetyl-CoA carboxylase-inhibiting herbicides. *Pest Manag Sci* **70**:1405–1417 (2014).
2 Délye C, Weed resistance to acetyl coenzyme a carboxylase inhibitors: an update. *Weed Sci* **53**:728–746 (2005).
3 Takano HK, Ovejero RFL, Belchior GG, Maymone GPL and Dayan FE, ACCase-inhibiting herbicides: mechanism of action, resistance evolution and stewardship. *Sci Agric* **78**:e20190102 (2021).
4 Iwakami S, Uchino A, Watanabe H, Yamasue Y and Inamura T, 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 (2012).
5 Iwakami S, Hashimoto M, Matsushima K-I, Watanabe H, Hamamura K and Uchino A, Multiple-herbicide resistance in *Echinochloa crus-galli* var. *formosensis*, an allohexaploid weed species, in dry-seeded rice. *Pestic Biochem Physiol* **119**:1–8 (2015).
6 Ashton AR, Jenkins CL and Whitfeld PR, Molecular cloning of two different cDNAs for maize acetyl CoA carboxylase. *Plant Mol Biol* **24**:35–49 (1994).
7 Huang S, Sirikhachornkit A, Faris JD, Su X, Gill BS, Haselkorn R *et al*., Phylogenetic analysis of the acetyl-CoA carboxylase and 3-phosphoglycerate kinase loci in wheat and other grasses. *Plant Mol Biol* **48**:805–820 (2002).
8 Yu Q, Ahmad-Hamdani MS, Han H, Christoffers MJ and Powles SB, Herbicide resistance-endowing ACCase gene mutations in hexaploid wild oat (*Avena fatua*): insights into resistance evolution in a hexaploid species. *Heredity* **110**:220–231 (2013).
9 Han H, Yu Q, Widderick MJ and Powles SB, Target-site EPSPS Pro-106 mutations: sufficient to endow glyphosate resistance in polyploid *Echinochloa colona*? *Pest Manag Sci* **72**:264–271 (2016).
10 Tanigaki S, Uchino A, Okawa S, Miura C, Hamamura K, Matsuo M *et al*., Gene expression shapes the patterns of parallel evolution of herbicide resistance in the agricultural weed *Monochoria vaginalis*. *New Phytol* **232**:928–940 (2021).
11 Damalas CA and Koutroubas SD, Herbicide-resistant barnyardgrass (*Echinochloa crus-galli*) in global rice production. *Weed Biol Manag* **23**:23–33 (2023).
12 Wu D, Shen E, Jiang B, Feng Y, Tang W, Lao S *et al*., Genomic insights into the evolution of *Echinochloa* species as weed and orphan crop. *Nat Commun* **13**:689 (2022).
13 Yabuno T, Cytotaxonomic studies on the two cultivated species and the wild relatives in the genus *Echinochloa*. *Cytologia* **27**:296–305 (1962).
14 Aoki D and Yamaguchi H, Genetic relationship between *Echinochloa crus-galli* and *Echinochloa oryzicola* accessions inferred from internal transcribed spacer and chloroplast DNA sequence. *Weed Biol Manag* **8**:233–242 (2008).
15 Heap I, The international survey of herbicide resistant weeds https://www.weedscience.com (2023).
16 Ritz C, Baty F, Streibig JC and Gerhard D, Dose-response analysis using R. *PloS One* **10**:e0146021 (2015).
17 R Core Team, *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria (2022).
18 Buchfink B, Reuter K and Drost H-G, Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nat Methods* **18**:366–368 (2021).
19 Wang L, Sun X, Peng Y, Chen K, Wu S, Guo Y *et al*., Genomic insights into the origin, adaptive evolution, and herbicide resistance of *Leptochloa chinensis*, a devastating tetraploid weedy grass in rice fields. *Mol Plant* **15**:1045–1058 (2022).
20 Paril J, Pandey G, Barnett EM, Rane RV, Court L, Walsh T *et al*., Rounding up the annual ryegrass genome: High-quality reference genome of *Lolium rigidum*. *Front Genet* **13**:1012694 (2022).
21 Robbins MD, Bushman BS, Huff DR, Benson CW, Warnke SE, Maughan CA *et al*., Chromosome-scale genome assembly and annotation of allotetraploid annual bluegrass (*Poa annua* L.). *Genome Biol Evol* **15**:evac180 (2023).
22 Li H, Protein-to-genome alignment with miniprot. *Bioinformatics* **39**: btad014 (2023).
23 Pertea G and Pertea M, GFF utilities: GffRead and GffCompare. *F1000Res* **9**:304 (2020).
24 Gustavsson EK, Zhang D, Reynolds RH, Garcia-Ruiz S and Ryten M, Ggtranscript: an R package for the visualization and interpretation of transcript isoforms using ggplot2. *Bioinformatics* **38**:3844–3846 (2022).
25 Lovell JT, Sreedasyam A, Schranz ME, Wilson M, Carlson JW, Harkess A *et al*., GENESPACE tracks regions of interest and gene copy number variation across multiple genomes. *Elife* **11**:e78526 (2022).
26 Katoh K and Standley DM, MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**:772–780 (2013).
27 Kozlov AM, Darriba D, Flouri T, Morel B and Stamatakis A, RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* **35**:4453–4455 (2019).
28 Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B and Flouri T, ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Mol Biol Evol* **37**:291–294 (2020).
29 Chen S, Zhou Y, Chen Y and Gu J, fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**:i884–i890 (2018).
30 Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S *et al*., STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**:15–21 (2013).
31 Putri GH, Anders S, Pyl PT, Pimanda JE and Zanini F, Analysing high-throughput sequencing data in python with HTSeq 2.0. *Bioinformatics* **38**:2943–2945 (2022).
32 Ye CY, Wu D, Mao L, Jia L, Qiu J, Lao S *et al*., The genomes of the allohexaploid *Echinochloa crus-galli* and its progenitors provide insights into polyploidization-driven adaptation. *Mol Plant* **13**:1298–1310 (2020).
33 Chalupska D, Lee HY, Faris JD, Evrard A, Chalhoub B, Haselkorn R *et al*., *Acc* homoeoloci and the evolution of wheat genomes. *Proc Natl Acad Sci U S A* **105**:9691–9696 (2008).
34 Guo L, Qiu J, Ye C, Jin G, Mao L, Zhang H *et al*., *Echinochloa crus-galli* genome analysis provides insight into its adaptation and invasiveness as a weed. *Nat Commun* **8**:1031 (2017).
35 Yu J, Gao H, Pan L, Yao Z and Dong L, Mechanism of resistance to cyhalofop-butyl in Chinese sprangletop (*Leptochloa chinensis* (L.) Nees). *Pestic Biochem Physiol* **143**:306–311 (2017).
36 Deng W, Cai J, Zhang J, Chen Y, Chen Y, Di Y *et al*., Molecular basis of resistance to ACCase-inhibiting herbicide cyhalofop-butyl in Chinese sprangletop (*Leptochloa chinensis* (L.) Nees) from China. *Pestic Biochem Physiol* **158**:143–148 (2019).
37 Peng Y, Pan L, Liu D, Cheng X, Ma G, Li S *et al*., Confirmation and characterization of cyhalofop-butyl–resistant Chinese sprangletop (*Leptochloa chinensis*) populations from China. *Weed Sci* **68**:253–259 (2020).
38 Jiang M, Wang Y, Li W, Li Q, Zhang J, Liao M *et al*., Investigating resistance levels to cyhalofop-butyl and mechanisms involved in Chinese sprangletop (*Leptochloa chinensis* L.) from Anhui Province, China. *Pestic Biochem Physiol* **186**:105165 (2022).
39 Zhang Y, Chen L, Song W, Cang T, Xu M and Wu C, Diverse mechanisms associated with cyhalofop-butyl resistance in Chinese sprangletop (*Leptochloa chinensis* (L.) Nees): characterization of target-site mutations and metabolic resistance-related genes in two resistant populations. *Front Plant Sci* **13**:990085 (2022).

40 Saika H, Horita J, Taguchi-Shiobara F, Nonaka S, Nishizawa-Yokoi A, Iwakami S *et al.*, A novel rice cytochrome P450 gene, *CYP72A31*, confers tolerance to acetolactate synthase-inhibiting herbicides in rice and Arabidopsis. *Plant Physiol* **166**:1232–1240 (2014).
41 Sada Y and Uchino A, Biology and mechanisms of sulfonylurea resistance in *Schoenoplectiella juncoides*, a noxious sedge in the rice paddy fields of Japan. *Weed Biol Manag* **17**:125–135 (2017).
42 Délye C, Causse R and Michel S, Genetic basis, evolutionary origin and spread of resistance to herbicides inhibiting acetolactate synthase in common groundsel (*Senecio vulgaris*). *Pest Manag Sci* **72**:89–102 (2016).
43 Du Y, Wang M, Chen Y, Deng Y, Zhang L, Bai T *et al.*, Occurrence and mechanism of target-site resistance to bensulfuron-methyl in *Monochoria korsakowii* from China. *Pestic Biochem Physiol* **191**:105379 (2023).
44 Iwakami S, Tanigaki S, Uchino A, Ozawa Y, Tominaga T and Wang GX, Characterization of the acetolactate synthase gene family in sensitive and resistant biotypes of two tetraploid *Monochoria* weeds, *M. vaginalis* and *M. korsakowii*. *Pestic Biochem Physiol* **165**:104506 (2020).
45 Löbmann A, Schulte M, Runge F, Christen O and Petersen J, Occurrence, resistance factors and cross-resistance patterns to herbicides inhibiting acetolactate synthase (ALS) of *Echinochloa crus-galli* (L.) pal. Beauv in Central Europe. *J Plant Dis Prot* **128**:843–852 (2021).
46 Fang J, He Z, Liu T, Li J and Dong L, A novel mutation Asp-2078-Glu in ACCase confers resistance to ACCase herbicides in barnyardgrass (*Echinochloa crus-galli*). *Pestic Biochem Physiol* **168**:104634 (2020).
47 Yang Q, Yang X, Zhang Z, Wang J, Fu W and Li Y, Investigating the resistance levels and mechanisms to penoxsulam and cyhalofop-butyl in barnyardgrass (*Echinochloa crus-galli*) from Ningxia Province, China. *Weed Sci* **69**:422–429 (2021).
48 González-Torralva F and Norsworthy JK, Overexpression of *acetyl CoA carboxylase 1* and *3* (*ACCase1* and *ACCase3*), and *CYP81A21* were related to cyhalofop resistance in a barnyardgrass accession from Arkansas. *Plant Signal Behav* **18**:2172517 (2023).
49 Li Q, Zhao N, Jiang M, Wang M, Zhang J, Cao H *et al.*, Metamifop resistance in *Echinochloa glabrescens* via glutathione S-transferases-involved enhanced metabolism. *Pest Manag Sci* **79**:2725–2736 (2023).
50 Jang S, Marjanovic J and Gornicki P, Resistance to herbicides caused by single amino acid mutations in acetyl-CoA carboxylase in resistant populations of grassy weeds. *New Phytol* **197**:1110–1116 (2013).
51 Han H, Yu Q, Owen MJ, Cawthray GR and Powles SB, Widespread occurrence of both metabolic and target-site herbicide resistance mechanisms in *Lolium rigidum* populations. *Pest Manag Sci* **72**:255–263 (2016).
52 Kersten S, Chang J, Huber CD, Voichek Y, Lanz C, Hagmaier T *et al.*, Standing genetic variation fuels rapid evolution of herbicide resistance in blackgrass. *Proc Natl Acad Sci U S A* **120**:e2206808120 (2023).
53 Dimaano NG and Iwakami S, Cytochrome P450-mediated herbicide metabolism in plants: current understanding and prospects. *Pest Manag Sci* **77**:22–32 (2021).
54 Edwards R and Dixon DP, Plant glutathione transferases, *Methods Enzymol* **401**:169–186 (2005).
55 Chen K, Peng Y, Zhang L, Wang L, Mao D, Zhao Z *et al.*, Whole transcriptome analysis resulted in the identification of Chinese sprangletop (*Leptochloa chinensis*) genes involved in cyhalofop-butyl tolerance. *BMC Genomics* **22**:521 (2021).
56 Zhao N, Jiang M, Li Q, Gao Q, Zhang J, Liao M *et al.*, Cyhalofop-butyl resistance conferred by a novel Trp-2027-leu mutation of acetyl-CoA carboxylase and enhanced metabolism in *Leptochloa chinensis*. *Pest Manag Sci* **78**:1176–1186 (2022).
57 Iwakami S, Kamidate Y, Yamaguchi T, Ishizaka M, Endo M, Suda H *et al.*, CYP81A P450s are involved in concomitant cross-resistance to acetolactate synthase and acetyl-CoA carboxylase herbicides in *Echinochloa phyllopogon*. *New Phytol* **221**:2112–2122 (2019).
58 Suda H, Kubo T, Yoshimoto Y, Tanaka K, Tanaka S, Uchino A *et al.*, Transcriptionally linked simultaneous overexpression of P450 genes for broad-spectrum herbicide resistance. *Plant Physiol* **192**:3017–3029 (2023).
59 Pan L, Guo Q, Wang J, Shi L, Yang X, Zhou Y *et al.*, CYP81A68 confers metabolic resistance to ALS and ACCase-inhibiting herbicides and its epigenetic regulation in *Echinochloa crus-galli*. *J Hazard Mater* **428**:128225 (2022).

