

RESEARCH ARTICLE

# Determination of Ploidy Level and Isolation of Genes Encoding Acetyl-CoA Carboxylase in Japanese Foxtail (*Alopecurus japonicus*)

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**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. All sequence files are available from the GenBank database (accession numbers KJ781292, KJ781293, KJ781294 and KJ781295).

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## Abstract

Ploidy level is important in biodiversity studies and in developing strategies for isolating important plant genes. Many herbicide-resistant weed species are polyploids, but our understanding of these polyploid weeds is limited. Japanese foxtail, a noxious agricultural grass weed, has evolved herbicide resistance. However, most studies on this weed have ignored the fact that there are multiple copies of target genes. This may complicate the study of resistance mechanisms. Japanese foxtail was found to be a tetraploid by flow cytometer and chromosome counting, two commonly used methods in the determination of ploidy levels. We found that there are two copies of the gene encoding plastidic acetyl-CoA carboxylase (ACCCase) in Japanese foxtail and all the homologous genes are expressed. Additionally, no difference in ploidy levels or ACCCase gene copy numbers was observed between an ACCCase-inhibiting herbicide-resistant and a herbicide-sensitive population in this study.

## Introduction

Ploidy level, defined as the number of sets of chromosomes in the nucleus, is an important genomic characteristics in biodiversity studies and developing strategies for isolating important plant genes [1, 2, 3]. Although knowledge on ploidy levels continues to improve in angiosperms, little is known regarding those

of weeds. Nevertheless, it has been shown that polyploidy is more frequent in weeds than in other species, especially in natural area invaders and agricultural weeds [4, 5]. The advantages of being polyploidy may predispose such weeds to be biologically invasive species [5, 6]. The intensive and global use of herbicides to control agricultural weeds has resulted in the evolution of resistance, and herbicide-resistant weeds pose a great threat to global agriculture. To date, resistance is documented in 431 resistant biotypes in 235 species [7]. Furthermore, compared with the knowledge of diploid weeds, our understanding of the evolution of herbicide resistance in polyploid weeds is limited [8].

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) is a key enzyme in most organisms, catalyzing the carboxylation of acetyl-CoA to form malonyl-CoA [9, 10]. Plants have two ACCase isoforms [11, 12]. Cytosolic ACCase, encoded by a single nuclear gene, is a multidomain and homodimeric enzyme in all plants. Plastidic ACCase is a multisubunit and heteromeric enzyme consisting of three nuclear-encoded subunits and one plastidic-encoded subunit in most plants [11, 12, 13]. The exception is the plastidic ACCase in the Poaceae family, which is a multidomain and homodimeric enzyme, encoded by a nuclear gene [11, 12, 13, 14]. The difference among plastidic ACCase between broadleaf and grass weeds is the basis for the selectivity of ACCase-inhibiting herbicides [9]. The cytosolic ACCase and the plastidic heteromeric ACCase are relatively insensitive, but the plastidic homomeric ACCase is sensitive to ACCase-inhibiting herbicides [9, 14, 15]. The continuous and extensive use of these herbicides has resulted in resistance evolution in at least 46 weeds [7]. Two mechanisms known to confer ACCase-inhibiting herbicide resistance to plants are target site resistance (TSR) and non-target site resistance [14, 15, 16, 17]. To date, substitutions at seven codon positions: (1781, 1999, 2027, 2041, 2078, 2088 and 2096) in the carboxyl transferase domain of plastidic ACCase genes have been identified to endow resistance to ACCase-inhibiting herbicides [18, 19, 20, 21, 22]. However, the ploidy level of many weeds is unknown. This may influence the isolation of target genes of interest since the ploidy level of the weed will determine the number of target genes. Thus, determining the ploidy of weeds is meaningful in studying resistance.

Japanese foxtail (*Alopecurus japonicus* Steud.), an annual weed of the Poaceae family, is one of the most noxious weeds infesting cereal and oilseed rape fields in China and Eastern Asia [23]. Resistance to ACCase and acetolactate synthase (ALS)-inhibiting herbicides has been documented in Japanese foxtail [24, 25, 26, 27, 28, 29]. Although Japanese foxtail is one of the most studied herbicide-resistant grass weeds in China, most of the previous studies ignored the multiple copies of target genes. The traditional method for determining ploidy level is chromosome counting, which has proven reliable in many different species [30, 31]. Flow cytometry, a convenient, fast and reliable method, has also been used recently to determine the DNA content and ploidy levels in many plant species [32]. The objectives of this study are to determine the ploidy level of Japanese foxtail and to assess any difference in this respect between a sensitive and resistant population.

## Materials and Methods

### Plant materials

Seeds from a resistant (R) Japanese foxtail population JLGY-4 were collected in 2011 from a wheat field in Ganyu, Lianyungang, Jiangsu province in China (34°78'N, 119°12'E). The R population evolved resistance to the ACCase-inhibiting herbicides fenoxaprop-P-ethyl and pinoxaden [29]. A sensitive (S) population (JLGY-1) was collected near the R population on a river bank that had never been treated with herbicide (34°78'N, 119°06'E). This population has been documented to be sensitive to ACCase-inhibiting herbicides [29]. The distance between the two sites is approximately 5 km. Seeds from both R and S populations were sown in 12-L pots filled with a 2:1 (wt/wt) mixture of sand and soil, and seedlings were grown in the greenhouse. The growth conditions were 25/20°C day/night temperatures ( $\pm 3^\circ\text{C}$ ) and 12/12 h cycles of light/dark with a light intensity of 450  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . All plants were actively growing and healthy at the time of the sampling. Both R and S plants were used in the following study.

“No specific permissions were required for the location where the Japanese foxtail seeds were collected. This study did not involve any endangered or protected species”.

### Flow cytometry

Eight samples from each of R and S populations were analyzed by flow cytometry. Flow cytometry was performed essentially as described by Doležal et al. [32]. Briefly, approximately 100 mg of fresh Japanese foxtail leaf tissue was harvested and transferred to a glass Petri-dish (on ice). Approximately 80 mg of fresh perennial ryegrass (*Lolium perenne* L.,  $2n = 4X = 28$ ) leaf tissue served as an external reference standard. Tissues were finely chopped with a razor blade in ice-cold LB01 lysis buffer (15 mM Tris, 2 mM  $\text{Na}_2\text{EDTA}$ , 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, and 0.1% (v/v) Triton X-100) [33]. After chopping, the suspension was filtered through a 50- $\mu\text{m}$  nylon mesh, and RNase A and propidium iodide (both to 50 mg/ml final concentrations) were added. A suspension of isolated nuclei was then incubated on ice in darkness for 30 min prior to analysis.

Flow cytometry was performed with an Accuri C6 flow cytometer (BD Accuri, USA). For each sample, 5000 to 10,000 nuclei were collected and analyzed. The results were displayed as one-parameter DNA histograms ( $G_0/G_1$  peak). Flow cytometer records the fluorescence intensity of cells in  $G_0/G_1$  and  $G_2$  periods. Cells in the  $G_0/G_1$  period precede DNA synthesis. Cells in the  $G_2$  period have finished DNA replication but have not divided. Thus, the ploidy level of the sample can be determined by the equation [32]: Sample ploidy = reference ploidy  $\times$  (mean position of sample  $G_0/G_1$  peak)/(mean position of standard  $G_0/G_1$  peak)

### Chromosome counts preparation and cytological measurement

Japanese foxtail root tips were harvested from germinated seeds, pretreated with 2 mM 8-hydroxyquinoline for 4–6 h at 20°C to accumulate metaphase cells, and fixed in methanol–acetic acid (3:1) fixative. Root tips were macerated in 1 M HCl at 20°C for 1h and squashed in 45% acetic acid. All slides were stored at 70°C. After removing the coverslips, slides were dehydrated using 100% ethanol prior to the chromosome counting. Slides were stained in 4,6-diamidino-2-phenylindole (Roche Diagnostics, Switzerland) for 5 min at room temperature, and finally, anti-fade (Vector, USA) was applied under the coverslip. Slides were examined under an Olympus BX51 fluorescence microscope. Chromosome images were captured using an Evolution VF CCD camera (Media Cybernetics, USA) and merged using Image-Pro Express software.

### DNA isolation and Southern blot analysis

Southern blotting was carried out to confirm the existence of multiple copies of genes encoding plastidic ACCase in Japanese foxtail. The genomic DNA of Japanese foxtail was isolated from the young leaf tissues of R and S individuals according to Xu et al. [24]. Southern blot analysis was performed according to the manufacturer's instructions using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics). In brief, 30 µg of genomic DNA was digested with *Hae*III, *Eco*RI and double digested with both enzymes, and then electrophoresed through a 1.0% agarose gel. DNA was transferred onto Pure Nitrocellulose Blotting Membranes (Solarbio Science&Technology Co., China) by capillary transfer and crosslinked under UV light. The hybridization probe labeled with digoxigenin was a 553 bp PCR fragment amplified by the primer pair ACCp1F/ACCp1R (Table 1). After hybridization, the membrane was washed and analyzed following the manufacturer's instructions.

### Total RNA extraction, cDNA synthesis and PCR amplification

Total RNA was extracted from 100 mg young shoot tissue of individuals using RNAiso Plus (TaKaRa Biotech, China) according to the manufacturer's instructions. Genomic DNA contamination was removed using the Recombinant DNase I (TaKaRa). cDNA was synthesized following the manufacturer's instructions using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa). The primer pairs ACCp1F/ACCp1R and ACCp2F/ACCp2R were used to amplify a 1230 bp region containing the resistance-endowing ACCase gene mutation sites (Table 1) [29]. The PCR procedure was conducted as described in Xu et al. [24]. Purification of the PCR products, cloning and sequencing protocols were as described previously [29]. At least eight clones for each biological replicate were sequenced. BioEdit Sequence Alignment Editor software was used to align and compare the sequence data.

**Table 1.** List of primers.

Primer	Sequence (5'-3')	Usage	Product size (bp)	Annealing temperature (°C)	References
ACCp1F	GCAAACCTCIGGTGCTCGGATTGGCA	Southern probe and sequencing	553	60	<a href="#">[29]</a>
ACCp1R	GAACATAICTGAGCCACCTCAATATATT				
ACCp2F	TGCATACAGCGTATTGACCAG	Sequencing	873	60	<a href="#">[29]</a>
ACCp2R	CTCTGACCTGAACTTGATCTC				

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## Results

### Flow cytometry

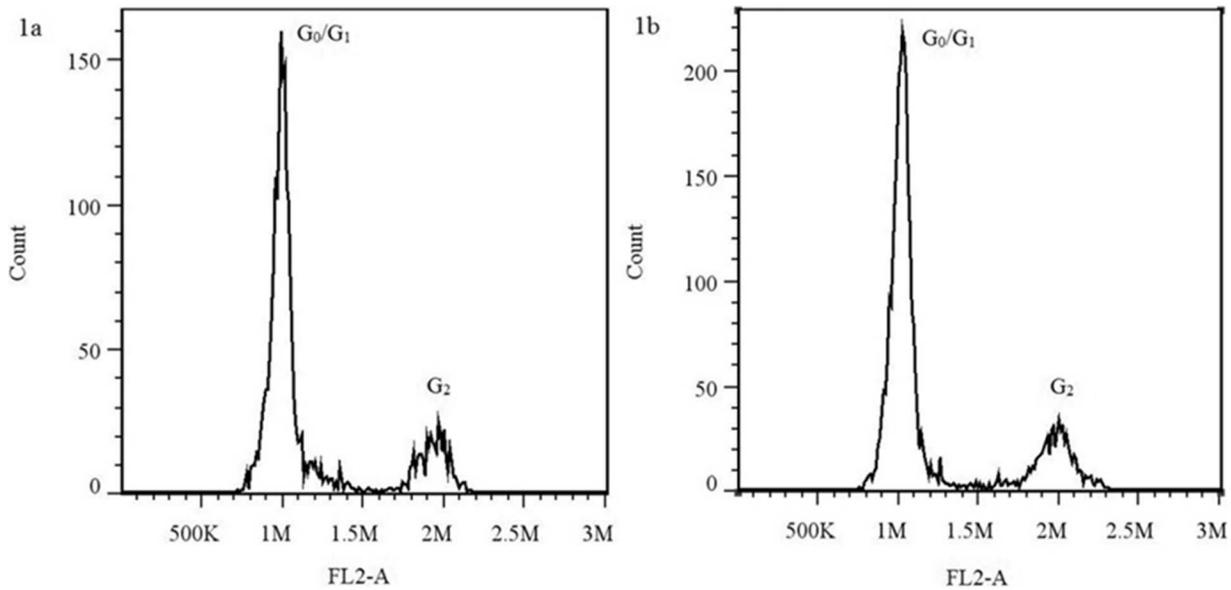
The mean fluorescence value of the  $G_0/G_1$  peak positions for the reference standard was  $9.84 \times 10^5$ . The mean fluorescence values of the  $G_0/G_1$  peak positions for the R and S samples were  $1.01 \times 10^6$  and  $1.00 \times 10^6$ , respectively. The coefficient of variation of the cytometry values, as a parameter of the reliability of the measurement, varied between 2.3% and 4.7%. These values were considered acceptable [\[32\]](#). The flow cytometry results revealed that all these samples were tetraploid. Thus, Japanese foxtail is a tetraploid grass weed species ([Fig. 1](#)).

### Chromosome count

Mitotic metaphase chromosomes are advantageous for counting chromosomes. As previously reported, chromosome numbers were used to confirm the ploidy levels of different species. To determine the ploidy level of Japanese foxtail, chromosome counting was used to corroborate the flow cytometry results. More than 20 excellent mitotic cells with dispersed metaphase chromosomes were obtained and counted. The result showed 28 chromosomes in each cell ([Fig. 2](#)). Interestingly, because of the basic chromosome number in *Alopecurus*, which is  $X = 7$ , the result is in accordance with  $2n = 4X = 28$  [\[34\]](#). Thus, Japanese foxtail was confirmed to be tetraploid.

### Southern blot analysis

A genomic hybridization analysis was performed to estimate the copy number of the gene encoding plastidic ACCase in Japanese foxtail. The hybridization pattern after digestion with *EcoRI*, *HaeIII* or both enzymes displayed two, three and three fragments, respectively ([Fig. 3](#)). The same hybridization pattern was observed in the R and S populations of Japanese foxtail (data not shown). Since there is no restriction site for either enzyme within the probe, these data suggest that at least two copies of the ACCase gene should be present in the Japanese foxtail genome. The presence of multiple copies of the ACCase gene would be in accordance with the tetraploid nature of Japanese foxtail. This result confirmed the previous finding by our group [\[29\]](#).

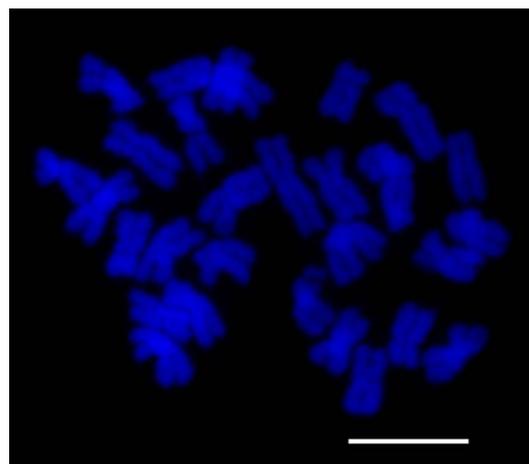


**Fig. 1. Flow cytometric analysis of homogenates prepared from perennial ryegrass (a) and Japanese foxtail (b).**

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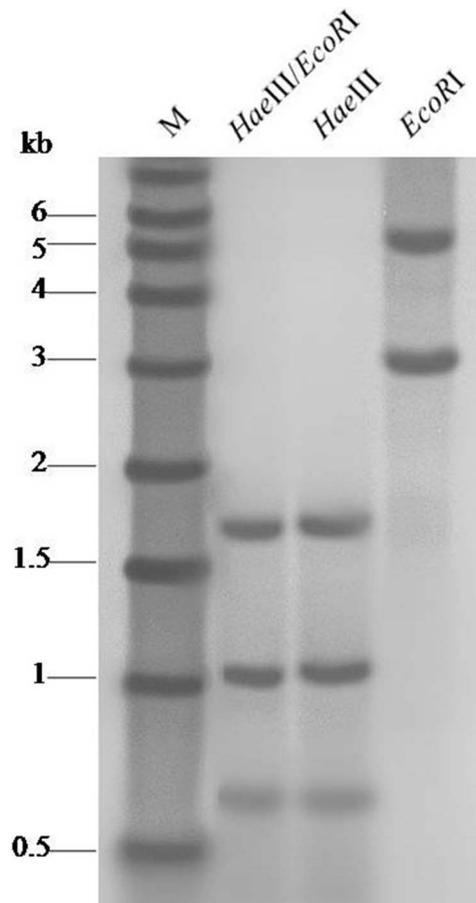
### ACCase gene isolation and sequencing

Since two copies of the ACCase genes have been isolated previously, this study was conducted to confirm that both of the homologous genes isolated are expressed. Reverse transcriptase-PCR analysis revealed that two homologous genes can be transcribed. The isolated cDNA lengths of *Acc1;1* and *Acc1;2* were all 1230 bp (GenBank accession numbers KJ781292 and KJ781293 for the S population, and KJ781294 and KJ781295 for the R population). No intron was found for either gene when comparing the genomic sequences obtained previously. The deduced amino acid sequences of *Acc1;1* and *Acc1;2* showed 99% identity. When



**Fig. 2. Mitotic metaphase chromosomes of Japanese foxtail.** Scale bar is 10  $\mu$ m.

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**Fig. 3. Genomic southern blot hybridization analysis of Japanese foxtail digested with *EcoRI*, *HaeIII* and both enzymes.** M, DIG labeled DNA molecular weight.

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comparing the sequences derived from six plants of both the R and S populations, it was discovered that the R population plants contained a TGG to TGC mutation, causing a tryptophan to cysteine mutation at codon position 1999 on locus *Acc1;1*. This finding is in agreement with our previous report that the mutation Trp-1999-Cys was responsible for the resistance to ACCase-inhibiting herbicides [29].

## Discussion

In the present study, Japanese foxtail was determined to be a tetraploid weed by two commonly used methods, flow cytometer and chromosome counting. Flow cytometry is a convenient, fast and reliable method that has been used to determine the ploidy level of many species [32]. Since the reliability of flow cytometry compares with that of chromosome counting, this method has become

widespread in biomedical research [32]. Here, flow cytometry offered a rapid method to determine the ploidy level of Japanese foxtail.

No difference of ploidy levels was observed between R and S populations, suggesting that resistance was irrelevant to ploidy level in this study. Puri et al. [35] found that there were ploidy variations among different biotypes of *Hydrilla verticillata* (Linn. f.) Royle, a serious problematic aquatic weed in the United States, with varying levels of resistance to fluridone. The differential resistance levels in different biotypes may also be due to ploidy variations [35]. Polyploidy is an important feature of evolution for many plant species [6, 36]. Polyploidy may confer new attributes, allowing plants to enter new environments or adapt to changing environments [6]. This may be significant to the weed's ability to evolve resistance under herbicide stress. Whether ploidy level can affect resistance in agricultural weeds is still unknown.

Multiple copies of the plastidic ACCase gene would be predicted based on the tetraploid nature of Japanese foxtail, and two copies of this gene were recently isolated by our group [29]. However, in the present study, the hybridization pattern after genomic DNA was digested with *EcoRI*, *HaeIII* or both enzymes displayed two, three and three fragments, respectively. These results indicate the presence of at least two ACCase genes in Japanese foxtail. Similarly, when confirming the presence of multiple ALS genes in *Schoenoplectus mucronatus* (L.) Palla (three ALS genes have been isolated) by Southern blot, four fragments were observed [37]. The presence of at least three ALS genes in the *S. mucronatus* genome was concluded by Scarabel et al. [37]. In hexaploid *Avena sterilis* L. and *Avena fatua* Linn., three plastidic ACCase gene loci were isolated [8, 38]. In the tetraploid *Echinochloa phyllopogon* (Stapf.) Koss., four plastidic ACCase gene loci were isolated [39]. In addition, multiple copies of target genes have been observed in polyploid weed species when isolating the genes of ALS, which is another important herbicide-targeted enzyme [37, 39, 40, 41, 42]. Two partial cDNA sequences were isolated in this study. By comparing the sequences isolated from genomic DNA previously [29], we found that all of the homologous ACCase genes isolated were transcribed. Similarly, the three homologous ACCase genes in *A. fatua* were documented to be transcribed [8]. The mutation(s) in either of the ACCase gene loci may confer resistance since each gene was able to maintain its own mutation in Japanese foxtail and *A. fatua*. However, Iwakami et al. [39] reported that one of the four ACCase gene loci was not transcribed in *E. phyllopogon*. This may be due to the pseudogenization of duplicated genes or gene silencing [43].

Although the ploidy levels of many plants have been documented, limited knowledge is available regarding that of agricultural weeds. For instance, the ploidy levels of the problematic weed species *Descurainia sophia* (Linn.) Webb. ex Prantl, *Myosoton aquaticum* (Linn.) Moench, *Galium aparine* Linn. and *P. fugax* are still unknown. In recent research, *D. sophia*, *M. aquaticum* and *G. aparine* were documented to confer resistance to tribenuron [44, 45, 46, 47, 48], and a population of *P. fugax* evolved resistance to ACCase-inhibiting herbicides [49]. Because of the unknown ploidy levels of these weeds, multiple copies of target

genes may be ignored when they are studied. Even when the ploidy level was known for some polyploid weed species, multiple copies of target genes have been ignored [50, 51, 52]. Derived cleaved amplified polymorphic sequence (dCAPS), a simple technique to detect herbicide resistance-conferring gene mutations, has been widely used [8, 29, 53, 54, 55, 56]. This technique was considered able to detect heterozygous and homozygous loci with herbicide-resistant mutations. However, the presence of more than one target gene confounded the ability of this technique to accurately identify “true” heterozygotes [8, 29, 41]. Recently, we found that plants of the R population of Japanese foxtail carrying ACCase-inhibiting herbicide resistance-conferring mutations were always determined to be heterozygous using dCAPS [29], which suggested that multiple plastidic ACCase genes existed. The heterozygotes detected were probably homologous heterozygotes, not “true” allelic heterozygosity. These findings were in agreement with those of Yu et al. and Warwick et al. [8, 41]. In addition, the dilution effect could make the resistance more complex in polyploidy weed species [8, 39]. For multiple copies of target genes, the protein products of each gene can be diluted by the others. Under the same herbicide selection pressure, polyploid weed species may evolve resistance more slowly than diploids because of the dilution effect [39]. Relatively low-level resistance was observed in the polyploid *A. fatua* in contrast to high-level resistance conferred by the same mutations in unrelated diploid weed species [8]. This may in part be due to polyploidy and the dilution of resistance genes by susceptible genes. Thus, before studying the mechanism of resistance, the ploidy level and the target gene’s copy number should be determined.

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## Author Contributions

Conceived and designed the experiments: HLX JL LYD. Performed the experiments: HLX WPZ TZ XW. Analyzed the data: HLX WPZ. Contributed reagents/materials/analysis tools: LYD. Wrote the paper: HLX WPZ.

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