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Confirmation and characterization of cyhalofop-butyl–resistant Chinese sprangletop (*Leptochloa chinensis*) populations from China

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Research Article

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Abstract

Chinese sprangletop [*Leptochloa chinensis* (L.) Nees] is one grass weed severely affecting rice (*Oryza sativa* L.) growth in paddies in China. Cyhalofop-butyl is the main herbicide used to control grass weeds in Chinese paddy fields, especially for controlling *L. chinensis*; however, *L. chinensis* has evolved resistance to cyhalofop-butyl due to continuous and extensive application. To investigate cyhalofop-butyl resistance levels and mechanisms in *L. chinensis* in some of the Chinese rice areas, 66 field populations were collected and treated with cyhalofop-butyl. Of these tested populations, 10 showed a high level of resistance to cyhalofop-butyl; the 50% effective dose ranged within 108.4 to 1,443.5 g ai ha^{-1} with resistance index values of 9.1 to 121.8 when compared with the susceptible population. Acetyl-coenzyme A carboxylase genes (*ACCase*) of susceptible and all 10 resistant populations were amplified and sequenced. Among them, Ile-1781-Leu, Trp-2027-Cys, Trp-2027-Ser, and Ile-2041-Asn mutations were found in five resistant populations. No known resistance-related mutations were found in the other five resistant populations, indicating that resistance to cyhalofop-butyl in these populations was likely to be endowed by non–target site resistance mechanisms. Notably, the Ile-1781-Leu and Trp-2027-Cys substitutions have previously been reported, but this is the first report of Trp-2027-Ser and Ile-2041-Asn mutations in *L. chinensis*. Furthermore, three derived cleaved amplified polymorphic sequence methods were developed to rapidly detect these mutations in *L. chinensis*.

Introduction

Rice (*Oryza sativa* L.) feeds more than half of the world's population and is an important food crop in Asia (Kuenzer and Knauer 2013). In addition, rice has the highest per-unit yield and accounts for 32.2% of China's total crop yield (NBSC 2019). Weeds compete with rice for soil nutrients, space, light, and water, and are the most problematic pests (Oerke and Dehne 2004). Chinese sprangletop [*Leptochloa chinensis* (L.) Nees] is a grass weed severely affecting rice growth in paddies in China. Continuous use of acetolactate synthase enzyme inhibitors (such as penoxsulam and bispyribac-sodium) and auxin herbicides (such as quinclorac), which are the most popular rice herbicides among farmers in China for controlling barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.], has resulted in the dominance of *L. chinensis* (Cheng et al. 2011).

Acetyl-coenzyme A carboxylase (ACCase) inhibitors kill grass weeds by inactivating ACCase and blocking fatty-acid biosynthesis (Devine 1997). The ACCase inhibitors include three dissimilar classes of herbicides—aryloxyphenoxypropionates (APPs), cyclohexanediones, and phenylpyrazoline—and target the plastid ACCase carboxyltransferase (CT) domain in grass weeds (Yu et al. 2010). However, widespread use of these three herbicides for controlling weeds has increased herbicide resistance. To date, more than 48 weed species resistant to ACCase inhibitors have been reported (Heap 2019).

Resistance to ACCase inhibitors is caused by two mechanisms: target-site resistance (TSR) and non–target site resistance (NTSR) (Délye 2005, 2013). The TSR to ACCase inhibitors involves conformational changes of herbicide target-site proteins resulting from amino acid substitution in the CT domain of ACCase (Powles and Yu 2010) or by increased abundance of the target protein due to gene overexpression or amplification. In contrast, NTSR embraces

all resistance mechanisms not related to target enzymes (Yuan et al. 2007). Up to now, 13 amino acid substitutions in the ACCase CT domain have been recognized to confer ACCase-inhibitor resistance in grass weeds: Ile-1781-Val/Leu/Thr (Délye et al. 2002, 2005; Deng et al. 2019; Kaundun et al. 2013b), Trp-1999-Cys/Leu/Ser (Deng et al. 2019; Kaundun et al. 2013a; Liu et al. 2007; Xu et al. 2014), Trp-2027-Cys (Deng et al. 2019; Xu et al. 2013; Yu et al. 2017), Ile-2041-Asn/Val (Délye et al. 2003), Asp-2078-Gly (Délye et al. 2005), Cys-2088-Arg (Yu et al. 2007), and Gly-2096-Ala/Ser (Cruz-Hipolito et al. 2012; Délye et al. 2005).

Cyhalofop-butyl is the main ACCase inhibitor used for selective control of *L. chinensis* in Chinese paddy fields, and it is the only herbicide that is highly effective against *L. chinensis* and very safe for rice. Because of great reliance on cyhalofop-butyl, the susceptibility of *L. chinensis* to cyhalofop-butyl has decreased and resistant populations have gradually evolved. It was reported that *L. chinensis* resistant to cyhalofop-butyl contained the Ile-1781-Leu, Trp-1999-Cys (Deng et al. 2019), and Trp-2027-Cys mutations (Deng et al. 2019; Yu et al. 2017). In recent years, a phenomenon of increasing numbers of *L. chinensis* populations that cannot be controlled by cyhalofop-butyl has become apparent to farmers in several provinces in China. To sustain herbicide efficacy for weed control, it is necessary to identify the resistance level and the underlying mechanisms.

In this study, we aimed to (1) determine the level of resistance in *L. chinensis* to cyhalofop-butyl collected from rice fields in China, (2) investigate the resistance mechanism to cyhalofop-butyl in *L. chinensis*, and (3) develop derived cleaved amplified polymorphic sequence (dCAPS) markers to identify the TSR related to resistance in *L. chinensis*.

Materials and Methods

Plant Materials

Sixty-six field populations were collected from paddy fields in Jiangsu, Hunan, Hainan, Hubei, Shanghai, and Zhejiang provinces between 2016 and 2018. Farmers found that the recommended field dose of cyhalofop-butyl failed to control *L. chinensis* in rice fields after several years of successful control. The known susceptible population, LC17041, was collected from Qinglongzui, Dingcheng District in Hunan Province. Seeds from at least 20 mature plants were collected and bulked. Information of the collection locations is shown in Supplementary Table S1.

Cyhalofop-Butyl Single-Rate Test

Seeds were germinated and planted in 9.5-cm-diameter plastic cups (35 seeds per cup). Next, the seedlings were transferred to a greenhouse (temperature maintained at approximately 25 to 35 C night/day temperature with natural sunlight) and watered as needed. The plants were thinned to 20 evenly sized plants per cup at the 2-leaf stage. The plants were sprayed with 90 g ai ha^{-1} (1×) cyhalofop-butyl (Clincher®, 100 g L^{-1} EC; Dow AgroSciences, Indianapolis, IN, USA). Herbicide treatment methods and spray facilities were as described by Yu et al. (2017). The plants treated with water were selected as a control. A single cup was considered an experimental unit, and the treatments were replicated four times. Plant survival was assessed visually at 21 d after herbicide was applied. Plants that died or were severely injured were regarded as susceptible, and plants surviving that produced expanded new green leaves were regarded as resistant.

Table 1. Herbicide dose used to test the resistance index in *Leptochloa chinensis* populations.

Populations	Dose
	—g ai ha^{-1}—
LC17041, LC17007, LC17008	0, 4.5, 13.5, 40.5, 121.5
LC17005, LC17026, LC17036	0, 3, 9, 27, 81, 243
LC16001, LC17050	0, 75, 150, 300, 600, 1,200
LC18014	0, 600, 900, 1,350, 3,037.5, 4,556.25
LC18011, LC18015, LC17009, LC18002, LC18006	0, 600, 1,200, 2,400, 4,800
LC18012, LC18013	0, 600, 900, 1,350, 2,025, 3,037.5, 6,834.375

Cyhalofop-Butyl Dose–Response Experiment

Ten populations of *L. chinensis* that survived the labeled dose (1×) application of cyhalofop-butyl and the susceptible population were considered further for the dose–response bioassay to evaluate their level of resistance. The plant and spray procedures were performed as described earlier. Initial dose–response experiments were conducted to determine the dose–response rates for different populations. The herbicide application doses are described in Table 1. Aboveground fresh weights of the surviving plants were assessed at 21 d after treatment. Each treatment was replicated four times, and experiments were performed twice.

Statistical Analysis

The herbicide rate causing 50% growth reduction (GR_{50}) was determined by fitting the data to a four-parameter log-logistic curve using Sigmaplot v. 12.5 (Systat Software, San Jose, CA, USA). The fitted model is shown below:

$$y = C + \frac{D - C}{1 + (x/\mathrm{GR}_{50})^b} \quad [1]$$

where C is the lower limit, D is the upper limit, b is the slope of the curve in GR_{50} (50% effective dose), x is the herbicide dose (g ai ha^{-1}), and y is the percentage of the control. The resistance index (RI), which is used to determine the level of resistance, was calculated as the ratio between the GR_{50} of each resistant population and the GR_{50} of the susceptible population.

Identification of Resistant Amino Acid Substitution

The seeds were germinated and planted as described earlier, except each cup only included 1 seedling. Cups were watered as needed, and seedlings were managed as described earlier. Approximately 100 mg of shoot tissue from each individual plant was collected for further analyses. Survivors from dose–response treatments were also selected for identification. Total DNA was extracted using the Trelief™ Plant Genomic DNA Kit (Beijing TsingKe Biotech, Beijing, China) according to the manufacturer's instructions.

The software Primer Explorer V4 v. 4 (http://primerexplorer.jp/elamp4.0.0/index.html) was used to design two pairs of primers (Table 2) to amplify the entire CT domain of *L. chinensis ACCase*. The primers were designed based on the description of Yu et al. (2017). The PCR mixture contained 45 µl of Golden Star T6 Super PCR Mix, 2 µl of each primer, and 1 µl of genomic DNA in a total volume of 50 µl. The PCR program was as follows: 98 C for 2 min, 30 cycles of 98 C for 10 s, 53 C for 10 s, and 72 C for 15 s, followed by a final extension step of 5 min at 72 C. The resulting PCR product fragments were sequenced by a commercial sequencing company (BioSune Biote [Shanghai], Shanghai,

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Table 2. Polymerase chain reaction primers used to amplify the acetyl-CoA carboxylase gene of *Leptochloa chinensis* and those used for the derived cleaved amplified polymorphic sequence (dCAPS) technique for screening resistance-related mutations in *L. chinensis*.

Primers	Sequence (5′→3′)	Usage
Lc-ACC-F4	TCTCGTATTGGCTCATCTGT	Sequencing
Lc-ACC-R4	CATCCTCAGAGATCCTCCTC	
Lc-ACC-F5	ATTCTCGTATTGGCTCATCT	Sequencing
Lc-ACC-R5	CGACTCTTCCCAATCAACAA	
1781-*Hinf*I-F	CTGTTGTGGGCAAGGAGGAT	dCAPS
1781-*Hinf*I-R	ACCCAACTGCATATGGGAGC	
2027-*Xho*I-F	AGTTGGTGTCATAGCTGTGGAG	dCAPS
2027-*Xho*I-R	AACACTCAATGCGGTCTGGA	
2041-*Eco*RI-F	GCTGTGGAGACACACAGACCAT	dCAPS
2041-*Eco*RI-R	TGGAGTTCCTCTGACCTGAAC	

China). Ten individual plants from each population were sequenced. The sequence data for the resistant and susceptible populations were compared. The BioEdit Sequence Alignment Editor Software was used to align and compare sequence data for each population.

dCAPS Method for Three Resistant Mutations

Three new dCAPS markers were developed for detecting three different mutations (Table 2). A forward dCAPS primer (1781-*Hinf*I-F) was designed to identify the Ile-1781-Leu mutation caused by an ATA–CTA codon mutation, which was introduced by a forced mismatch to create a restriction site for *Hinf*I. Along with primer 1781-*Hinf*I-R, the mutant amplicons were digested by *Hinf*I. A forward dCAPS primer (2027-*Xho*I-F) was designed to identify the Trp-2027-Ser mutation caused by a TGG–TCG codon mutation, which was introduced by a forced mismatch to create a restriction site for *Xho*I. Along with primer 2027-*Xho*1-R, the mutant amplicons were digested by *Xho*I. A forward dCAPS primer (2041-*Eco*RI-F) was designed to identify the Ile-2041-Asn mutation caused by an ATT–AAT codon mutation, which was introduced by a forced mismatch to create a restriction site for *Eco*RI. Along with primer 2041-*Eco*RI-R, the wild-type amplicons were digested by *Eco*RI. The PCR procedure for generating the gene fragments and PCR conditions was as described by Pan et al. (2015). After digestion, the products were visualized on 3% agarose gels.

Results and Discussion

Cyhalofop-Butyl Single-Rate Test

In this research, 66 *L. chinensis* field populations were screened for their resistance to cyhalofop-butyl. For the single-rate test, 56 populations were severely injured, and no new leaves expanded after treatment with cyhalofop-butyl at the recommended field dose (90 g ai ha^{-1}) compared with the control. Growth of two populations (LC16001 and LC17050) was obviously inhibited, while the remaining eight populations (LC17009, LC18002, LC18006, and LC18011–LC18015) were entirely unaffected.

Cyhalofop-Butyl Dose–Response Experiments

The whole-plant dose–response bioassay results showed no significant differences; therefore, data were combined for the subsequent analyses. Ten field populations tested showed high levels of resistance to cyhalofop-butyl. The GR_{50} value for LC17050 was 108.4 g ai ha^{-1}, which was the lowest among all resistant populations, but still was higher than the recommended field rate of 90 g ai ha^{-1}. The GR_{50} values ranged within 108.4 and 1,443.5 g ai ha^{-1}, with RI values of 9.1 to 121.8, and these were compared with the susceptible LC17041 population (Table 3; Figure 1).

Table 3. The level of resistance to cyhalofop-butyl in 11 *Leptochloa chinensis* populations.[a]

Populations	GR_{50} (SE)[a]	*b*[b]	R^2	RI (>)
	—g ai ha^{-1}—			
LC17041	11.9 (0.4)	1.6	0.99	—
LC16001	128.3 (0.2)	1.1	0.97	10.8
LC17009	1,443.5 (1.4)	2.0	1.00	121.8
LC17050	108.4 (0.4)	0.5	0.97	9.1
LC18002	1,167.7 (0.4)	2.2	0.98	98.5
LC18006	1,052.3 (0.2)	1.3	1.00	88.8
LC18011	1,138.0 (0.9)	2.0	1.00	96.0
LC18012	1,188.2 (0.3)	0.9	1.00	100.2
LC18013	1,210.9 (0.3)	2.1	0.98	102.1
LC18014	1,151.1 (0.4)	1.0	0.99	97.1
LC18015	1,183.8 (0.4)	1.2	0.99	99.9

[a]GR_{50}, herbicide dose required to decrease plant fresh weight by 50%; each value represents the mean ± SE. *b*, slope of the curve in GR_{50}. R, correlation coefficient of the dose–response curve.
RI, resistance index calculated by dividing the GR_{50} value of the resistant population by the susceptible population; because all 10 resistant populations never achieved 100% mortality, the RIs in the table are the relative values, expressed as "> value."

Previous studies reported that natural selection of resistance in gramineous weeds required several years of continuous use of ACCase inhibitors (Hochberg et al. 2009). Thus, it is speculated that the resistance of *L. chinensis* populations to cyhalofop-butyl resulted from strong selection pressure, imposed by the past few years of consecutive cyhalofop-butyl applications. An *L. chinensis* field population resistant to cyhalofop-butyl was first identified in 2014 in Yuhang, Zhejiang Province (Yu et al. 2017). Then, Deng et al. (2019) found five *L. chinensis* populations also resistant to cyhalofop-butyl. In this research, resistant populations were collected in Jiangsu, Hunan, Shanghai, and Zhejiang provinces.

Identification of Resistant Amino Acid Substitution

The amplified fragment of the ACCase amino acid sequence in *L. chinensis* showed 81% similarity to *Alopecurus myosuroides* plastidic ACCase sequences (AJ310767). A portion of the CT fragment was amplified and sequenced to determine the resistance mechanism. Sequence comparison showed several key amino acid changes in the CT region of the resistant populations (Table 4). Four substitutions at three positions (Table 4) were identified in this research, which included the less-reported Trp-2027-Ser. The changes Ile-1781-Leu, Trp-2027-Cys, Trp-2027-Cys, Trp-2027-Ser, and Ile-2041-Asn were identified in the LC18015, LC16001, LC17050, LC18011, and LC17009 populations, respectively. These results indicated that these key substitutions were the TSR mechanism in the corresponding cyhalofop-butyl–resistant populations. Some researchers reported Trp-2027-Cys (Deng et al. 2019; Yu et al. 2017), Ile-1781-Leu, and Trp-1999-Cys (Deng et al. 2019) substitutions as the main mechanism of resistance in *L. chinensis*, and our study showed that Trp-2027-Ser and Ile-2041-Asn substitutions also endowed cyhalofop-butyl resistance in *L. chinensis*. These results indicate that *L. chinensis* exhibits rich diversity in TSR mechanisms for cyhalofop-butyl

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Table 4. Key amino acids in the carboxyltransferase domain of *Alopecurus myosuroides* and cyhalofop-butyl–resistant and cyhalofop-butyl–susceptible *Leptochloa chinensis*.

	Amino acid position and relative nucleotide and amino acid sequence[a]						
Populations	1781	1999	2027	2041	2078	2088	2096
LC17041	Ile	Trp	Trp	Ile	Asp	Cys	Gly
LC18011	Ile	Trp	**Ser**	Ile	Asp	Cys	Gly
LC18012	Ile	Trp	Trp	Ile	Asp	Cys	Gly
LC18013	Ile	Trp	Trp	Ile	Asp	Cys	Gly
LC18014	Ile	Trp	Trp	Ile	Asp	Cys	Gly
LC18015	**Leu**	Trp	Trp	Ile	Asp	Cys	Gly
LC16001	Ile	Trp	**Cys**	Ile	Asp	Cys	Gly
LC17009	Ile	Trp	Trp	**Asn**	Asp	Cys	Gly
LC17050	Ile	Trp	**Cys**	Ile	Asp	Cys	Gly
LC18002	Ile	Trp	Trp	Ile	Asp	Cys	Gly
LC18006	Ile	Trp	Trp	Ile	Asp	Cys	Gly
Alopecurus myosuroides	Ile	Trp	Trp	Ile	Asp	Cys	Gly

[a]The known resistance-conferring amino acid substitution is in bold for the resistant populations.

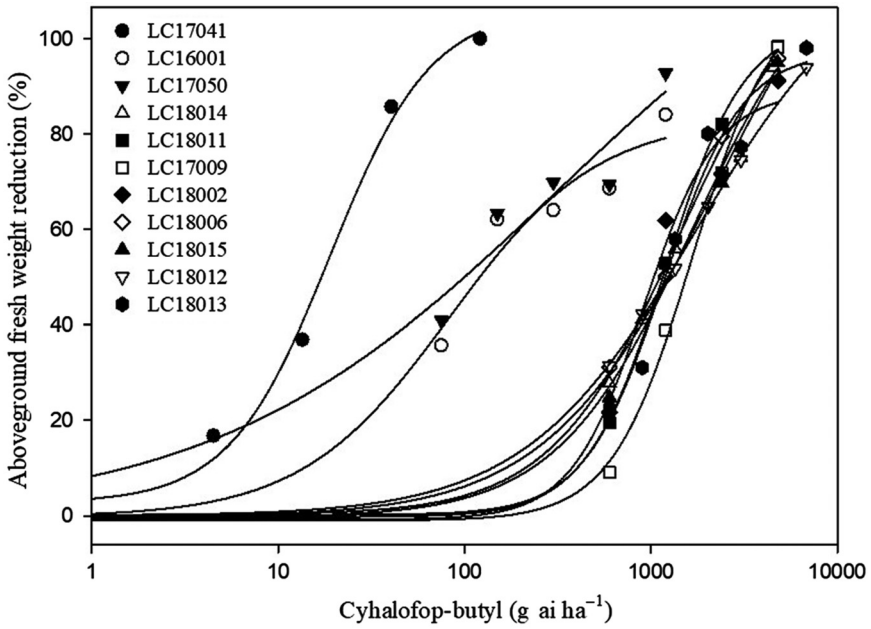

Figure 1. Dose–response curves of 11 *Leptochloa chinensis* population to cyhalofop-butyl. LC17041 was the susceptible populaiton, the others were resistant populations.

resistance. Due to the different cross-resistance patterns associated with different ACCase substitutions, this diversity will make it more difficult to control cyhalofop-butyl–resistant *L. chinensis*.

In the whole-plant experiment, the LC18011 and LC17009 populations showed high levels of cyhalofop-butyl resistance. The Ile-1781-Leu, Trp-2027-Cys, Trp-2027-Ser, and Ile-2041-Asn substitutions have been reported to endow APP resistance (Beckie and Tardif 2012; Jang et al. 2013; Liu et al. 2007), with resistance more than 100-fold those of another two ACCase inhibitors, fenoxaprop and clodinafop (Jang et al. 2013). Thus, it is not surprising that the LC18011 and LC17009 populations are highly resistant to cyhalofop-butyl. However, in the LC16001 and LC17050 populations, which were weakly resistant to cyhalofop-butyl, the mutation frequencies were under 30%. These results indicated that the resistance level is relevant to the mutation frequency.

Moreover, no known substitutions conferring resistance to ACCase inhibitor were identified in the LC18002, LC18006, LC18012, LC18013, and LC18014 populations. Resistance to cyhalofop-butyl in these populations is suspected to be conferred by the NTSR mechanism. Furthermore, little is known about other resistance mechanisms, except for the insensitive target enzyme in *L. chinensis*, especially the NTSR mechanism. Compared with the single gene–encoded TSR mechanism, the NTSR mechanism, which is likely encoded by multiple genes, has unpredictable cross-resistance patterns and more complex genetic patterns (Délye 2013). As far as we know, this study is the second report on NTSR in cyhalofop-resistant *L. chinensis* following that of Deng et al. (2019). Thus, further research is needed to confirm the existence of NTSR in these cyhalofop-resistant *L. chinensis* populations.

dCAPS Method for Three New Resistant Mutations

The restriction enzyme used to digest the Ile-1781-Leu mutation was *Hinf*I. The wild-type alleles showed a single undigested 260-bp band (Figure 2), mutant alleles showed a 220-bp band

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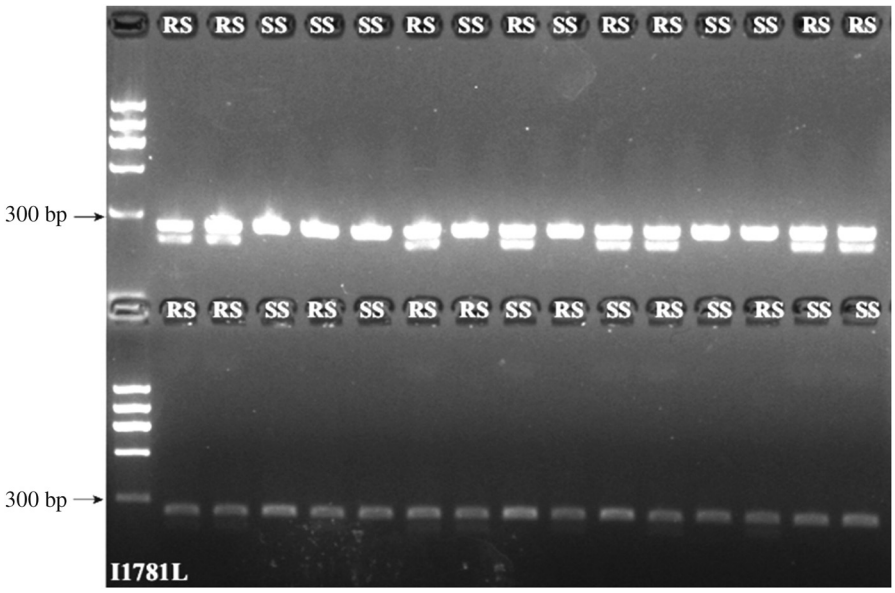

Figure 2. The derived cleaved amplified polymorphic sequence (dCAPS) method was developed to detect the Ile-1781-Leu mutations in *Leptochloa chinensis*. Homozygous sensitive (SS) and heterozygous resistant (RS) genotypes are shown.

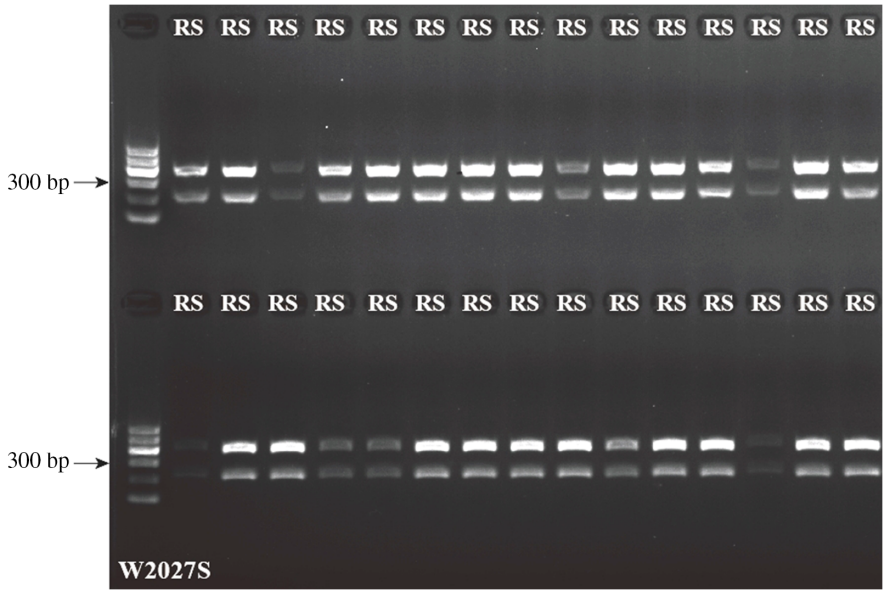

Figure 3. The derived cleaved amplified polymorphic sequence (dCAPS) method was developed to detect the Trp-2027-Ser mutations in *Leptochloa chinensis*. Heterozygous resistant (RS) genotypes are shown.

and an undetectable 40-bp fragment, and the heterozygous type showed both bands (Figure 2). The restriction enzyme used to digest the Trp-2027-Ser mutation was *Xho*I. The wild-type alleles showed a single undigested 377-bp band, mutant alleles showed 190- and 187-bp bands, and the heterozygous type showed both types of bands (Figure 3). The restriction enzyme used to digest the Ile-2041-Asn mutation was *Eco*RI. Mutant alleles showed a single undigested 443-bp band, wild-type alleles showed a 223-bp band and a 220-bp fragment (Figure 4), and the heterozygous type showed both bands (Figure 4).

Eighty plants of the resistant LC18015 population were analyzed using the established dCAPS method. Forty-six heterozygous resistant, thirty-four homozygous sensitive, and no homozygous resistant plants were identified. The results of analyzing the DNA samples using the dCAPS method were consistent with the previous sequencing results. Therefore, this method was confirmed to be accurate and effective in detecting the three ACCase mutations in *L. chinensis*.

In this study, we established the first dCAPS method to rapidly detect the Ile-1781-Leu, Trp-2027-Ser, and Ile-2041-Asn mutations in *L. chinensis*. The dCAPS method is useful to detect sequence polymorphisms. However, the results of dCAPS suggested that no homozygous *ACCase* loci were present. Two copies of the plastidic *ACCase* gene were identified in *L. chinensis* (Deng et al. 2019), indicating that the heterozygotes detected were not the "true" allelic heterozygotes and might be the homologous

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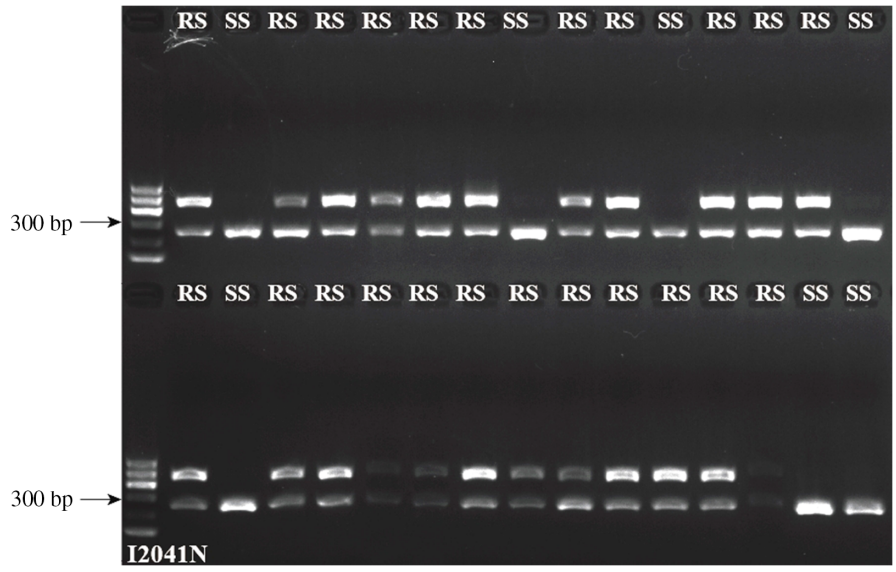

Figure 4. The derived cleaved amplified polymorphic sequence (dCAPS) method was developed to detect the Ile-2041-Asn mutations in *Leptochloa chinensis*. Homozygous sensitive (SS) and heterozygous resistant (RS) genotypes are shown.

heterozygotes (Warwick et al. 2010; Yu et al. 2013). Thus, heterozygosity was reported in *L. chinensis* at the whole-genome level.

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Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2020.15

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