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Resistance to quinclorac caused by the enhanced ability to detoxify cyanide and its molecular mechanism in *Echinochloa crus-galli* var. *zelayensis*

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ABSTRACT

Quinclorac, an auxin-type herbicide, is widely used to control barnyardgrass and some dicotyledon weeds. *Echinochloa crus-galli* var. *zelayensis*, a variety of *E. crus-galli* (L.) Beauv., is widespread in China and some populations have resistance to quinclorac. *E. crus-galli* var. *zelayensis* seeds with varying sensitivity to quinclorac were used in the present study. The expression of the ADP/ATP carrier protein (*ANT*) gene, which plays an important role in the maintenance of cellular energy balance, dramatically rose in the S biotype after exposure to quinclorac, while no change was found in two R biotypes. The activity of *β*-cyanoalanine synthase (*β*-CAS), which is the key enzyme for cyanide degradation, was higher in two R biotypes than in the S biotype before and after treatment with quinclorac. One single-nucleotide difference was detected in the *EcCAS* gene of two R biotypes compared with the S biotype. The nucleotide change, which caused one amino acid substitution, replacing Methionine (Met)-295 with Lysine (Lys)-295 in the two R biotypes, which are same as the rice *β-CAS* gene at this position. In addition, *EcCAS* gene expression was higher in the two R biotypes than in the S biotype. In conclusion, *β*-CAS may play a crucial role in the resistance of *E. crus-galli* var. *zelayensis* to quinclorac. *EcCAS* gene mutation and higher gene expression may enhance the activity of *β*-CAS to avoid the accumulation of toxic cyanide in resistant populations, thus contributing to the resistance mechanism of *E. crus-galli* var. *zelayensis*. to quinclorac.

1. Introduction

Echinochloa crus-galli (barnyardgrass) is the sixth most troublesome herbicide-resistant weed around the world [1] and is also one of the most harmful weeds in China [2,3]. *E. crus-galli* var. *zelayensis* is one of the most serious weeds in rice fields in China due to its strong adaptability and causes a serious decline in rice yield [4].

Quinclorac (3,7-dichloro-8-quinolinecarboxylic acid), which belongs to the class of quinolinecarboxylic acid, is one of the auxin herbicides [5,6]. It is a highly efficient and selective herbicide that can effectively control *Echinochloa* spp. as well as some other common monocot weeds, including *Digitaria*, *Setaria*, and *Brachiaria* spp. It also controls certain dicot weeds, such as *Sesbania* spp., *Cassia* spp., *Oenanthe javanica*, and *Monochoria* spp. with excellent crop safety [5,6]. Because of its economy, efficiency, and low toxicity, quinclorac had been the first choice to control barnyardgrass in paddy fields in the past three decades.

Like other natural or synthetic auxins, quinclorac causes symptoms of growth inhibition, epinasty, foliar chlorosis, and tissue decay in high concentrations [7]. However, its mechanism of action remains unclear. There are two main views regarding the mechanism of quinclorac. One theory is that reactive oxygen species (ROS) induced by quinclorac are the most important factor responsible for the herbicide's effectiveness. Research has shown that quinclorac induced ROS production and lipid peroxidation in corn roots, causing cell death [8]. Another argument is that quinclorac induces ethylene biosynthesis via the 1-aminocyclopropane-1-carboxylic acid (ACC) pathway. This process produces a toxic coproduct, cyanide, which is the main quinclorac action [9,10]. When ACC production is induced by quinclorac, it is initially produced in the roots; then the accumulated ACC is transported from the root to the shoot, where it promotes the generation of ethylene and cyanide. This is why shoot growth was more susceptible to damage caused by quinclorac than root growth [10,11]. Exogenously applied KCN led to an increase in ACC synthase activity and induced the generation of

Abbreviations: ANT, ADP/ATP carrier protein; *β*-CAS, *β*-cyanoalanine synthase; Met, methionine; Lys, lysine; Val, valine; CCO, cytochrome *c* oxidase; ATP, adenosine triphosphate; Ala, alanine; Asp, aspartic acid; Gly, glycine; Arg, arginine; Ile, isoleucine; Thr, threonine; PCR, polymerase chain reaction; RACE, rapid-amplification of cDNA ends

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endogenous cyanide in the shoots, finally causing inhibition of plant growth similar to quinclorac [10–12]. Cyanide can inactivate a mitochondrial enzyme, cytochrome *c* oxidase (CCO), which is a key enzyme of the respiratory chain. This damage to the respiratory electron-transport chain can cause a reduction in the generation of adenosine triphosphate (ATP) [13]. ADP/ATP carrier protein (ANT) is a carrier protein that is also closely related to mitochondrial energy metabolism. ANT is positioned in the mitochondria and functions as the transport carrier of ADP in the cytoplasm and ATP in mitochondria; it is essential for the exchange of ATP and ADP [14]. In addition, ANT plays a key role in cell apoptosis [15]. *ANT* gene mutation or dysregulation is related to a variety of diseases in humans and animals [16–18]. Little research has been done on *ANT* genes in plant cells. One study reported that under low temperature and salt stress, the expression of *ANT* genes in rice was increased [19]. The homologous sequence for the *ANT* gene in *E. crus-galli* has not been reported previously. The two alternatives are not necessarily conflicting; whether ROS or cyanide is primarily responsible for quinclorac-induced phytotoxicity in susceptible plants might be determined by the plants' individual capacity for cyanide detoxification [8].

In recent years, there have been many reports of resistance of barnyardgrass to quinclorac. This resistance was first reported in 1997 in Brazil, Spain, and the United States. Various *Echinochloa* spp. have evolved resistance to quinclorac in different countries and regions, including *E. crus-pavonis* [1], *E. crus-galli* [20–24], *E. colona* [22], *E. phyllopogon* [25], *E. hispidula* [26], and *E. oryzicola* [26], The resistance problem is worsening, but the mechanism of that resistance remains unclear. It is noteworthy that the resistance is not based on differences in absorption, translocation, and metabolism of quinclorac, because there are no significant differences in these factors between resistant and susceptible plants [21,27,28]. Secondly, unlike other auxin herbicides, the mechanism of resistance is irrelevant to cellulose biosynthesis [29]. It is generally believed that resistance to quinclorac is mainly conferred by inducing a smaller change to the ACC pathway and producing only a small amount of cyanide. Tolerance to quinclorac could be favored by higher *β*-CAS activity [11,25,30,31]. In rice, *β*-CAS is mainly located in the mitochondria and one of its known functions is to degrade toxic cyanide during the biosynthesis of ethylene [32]. These studies suggest that the detoxification of cyanide by *β*-CAS may be one of the important mechanisms of resistance, even as its real role and the molecular mechanism by which it degrades cyanide remain unknown.

To clarify quinclorac resistance mechanism associated with cyanide detoxification and its molecular mechanism in *E. crus-galli* var. *zelayensis* biotypes in east China, we studied quantified responses to quinclorac of three biotypes with different resistance levels. The mechanisms of quinclorac resistance were investigated on the basis of observed differences in the *ANT* gene expression patterns, the activity of *β*-CAS, the *β-CAS* gene sequence alignment, and the *β-CAS* gene expression patterns among the three biotypes.

2. Materials and methods

2.1. Plant material

E. crus-galli var. *zelayensis* seeds for the F_0 generation were collected in the autumn of 2010 from different paddy fields in Jiangsu and Shanghai in China. In May 2014, seeds of three biotypes were planted in a growth chamber containing a 1:1 (wt/wt) mixture of sand and soil (pH 5.6 and 1.4% organic matter), mimicking natural conditions. All of the resistant seedlings and half of the sensitive seedlings at the 2.5- to 3.5-leaves stage were treated with quinclorac at the recommended dosage (300 g AI ha^{-1}). Three weeks after treatment, all the resistant populations had survived, but the sensitive individuals had not. Seeds were harvested from the surviving plants and marked as JNNX-S (ED_{50} value 36.75 g·ha^{-1}, ED_{50} ratio 1.00), JCWJ-R1 (ED_{50} value 355.85 g·ha^{-1}, ED_{50} ratio 9.68) and SSXB-R2 (ED_{50} value 2457.79 g·ha^{-1}, ED_{50} ratio 66.88), respectively. Seeds were dried in open trays in the sun and then stored at 4 °C.

Germinated seeds of the three biotypes were grown hydroponically in Kasugai nutrient solution [33] in a growth chamber at 30/25 °C with 12 h of light (300 μmol m^{-2} s^{-1}) [34] and 12 h of darkness per day. Seedlings at the 2.5- to 3.5-leaf stage were used for all experiments.

2.2. Chemicals and herbicide treatment

Quinclorac (50% WP) was provided by Jiangsu Xinyizhongkai. (Xinyi, China). Plants were grown in disposable paper cups that contained 300 mL of nutrient solution and 50 μM quinclorac [8] at the 2.5- to 3.5-leaf stage.

2.3. EcANT gene expression analysis based on real-time PCR

The expression of the *EcANT* gene in the three *E. crus-galli* var. *zelayensis* biotypes was analyzed with real-time PCR. Shoots of susceptible and resistant plants were sampled at the same time under light conditions at 0 h (untreated with quinclorac) and 6 h, 12 h, 18 h, and 24 h after treatment with 50 μM of quinclorac and stored at − 80 °C. In addition, the expression of the sensitive population's *EcANT* gene within 24 h after treatment with 10 μM and 25 μM of quinclorac was also investigated. The method of sampling and storage is as described above. Total RNA was isolated with the RNAsimple Total RNA Kit (TIANGEN). Total RNA of all samples was reverse transcribed to cDNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan). Reactions in 20 μL were performed using SYBR ® Premix Ex Taq™II(Tli RNAaseH Plus) (TaKaRa, Otsu, Japan), each containing 10 μL SYBR® Premix Ex Taq™ (TaKaRa), 2 μL diluted cDNA, 0.4 μL 10 μM *EcANT*-Real time-F (CGCATACATTTGCTGATTGC)/-R (GATGACTGCTG GTGCTTTGA) or *EcActin*-Real time –F (GTGCTGTTCCAGCCA TCGTTCAT)/-R (CTCCTTGCTCATACGGTCAGCAATA) primers, 0.4 μL Rox, and 6.8 μL ddH_2O. The product lengths were 171 bp for the reference sequence and 183 bp for the *EcANT*. Each sample was amplified using an equal amount of cDNA template at least three times. PCR products were detected by SYBR Green fluorescence dye. The threshold values (Ct) were determined automatically by the onboard software. Real-time PCR was performed on the ABI-7500 Fast Real Time PCR System (ABI, USA) using the following protocol: 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Relative transcript levels were calculated by the ΔΔCt method with at least six technical and biological replicates.

The *EcActin* gene (GenBank accession HQ395760) was used as the reference sequence [35]. According to the conserved region of the *Camelina sativa ANT* gene (GenBank accession XM_010493807.1), *Arabidopsis thaliana ANT* gene (GenBank accession AY050857.1), *Vitis vinifera ANT* gene (GenBank accession XM_010658690.1), and *Cleome spinosa ANT* gene (GenBank accession XM_010559490.1), a pair of primers *ANT*-F (CTTATTGTCTGGCTCGGTA)/-R (TGTCATCATCATCC-TCCTC) were designed to obtain a partial *EcANT* sequence and the product was about 1068 bp. Then the sequence was used to design Q-PCR primers, *EcANT*-Real time -F/-R primers, using the website http://bioinfo.ut.ee/primer3-0.4.0/. PCR amplification efficiencies for each primer set were determined from a standard curve based on Ct values against serial dilutions of cDNA template, and approximately equal amplification efficiencies of the target and internal control, a requirement for the ΔΔCt method [36], were confirmed. The primer sets were tested by dissociation curve analysis and verified for the absence of non-specific amplification.

Significant differences in expression levels were analyzed by SPSS version 20 statistical software (SPSS, Chicago, IL, USA). The degree of change was used to determine up- or downregulation by quinclorac treatment.