Running title: Quinclorac resistance

Quinclorac resistance induced by the suppression of the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase genes in *Echinochloa crus-galli* var. *zelayensis*

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Abbreviations[1]

[1] (Boc-aminooxy)acetic acid, AOA; 1-aminocyclopropane-1-carboxylate synthase, ACS; 1-aminocyclopropane-1-carboxylate oxidase, ACO; 1-aminocyclopropane-1-carboxylic acid, ACC; reactive oxygen species, ROS; superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; S-adenosylmethionine, SAM; real-time polymerase chain reaction, real-time PCR

Abstract

We previously reported that the mechanism of quinclorac resistance in *Echinochloa crus-galli* var. *zelayensis* may be closely related to ethylene biosynthesis and the detoxification of cyanide. Differences in EcCAS gene sequences and expression levels may result in higher capacity to detoxify cyanide in resistant biotypes, which may avoid cyanide accumulation and avoid more ethylene and cyanide production and then avoid damage. In the present study, we focused on the mechanism of resistance related to ethylene biosynthesis in *E. crus-galli* var. *zelayensis*. The fresh weight of susceptible and moderately resistant biotypes were significantly reduced after treatment with quinclorac. However, AOA, an ethylene biosynthesis inhibitor, reduced the impact of quinclorac. On pretreatment with AOA, ethylene production was significantly reduced in the three biotypes. The highly resistant biotype produced less ethylene compared to the other two biotypes. Three ACS and seven ACO genes, which are the key genes in ethylene biosynthesis, were obtained. The expression levels of *EcACS-like*, *EcACS7*, and *EcACO1* varied in the three biotypes upon treatment with quinclorac, which could be manipulated by AOA. In summary, it is inferred that the expression of *EcACS-like*, *EcACS7*, and *EcACO1* can be stimulated to varying extent after quinclorac treatment in three *E. crus-galli* var. *zelayensis* biotypes, which consequently results in varying levels of ethylene production. Lower expression of these three genes results in more resistance to quinclorac, which may also be related to quinclorac resistance in *E. crus-galli* var. *zelayensis*.

Keywords: Quinclorac resistance; *Echinochloa crus-galli* var. *zelayensis*; Ethylene; ACS gene; ACO gene

1. Introduction

Barnyardgrass is one of the most troublesome weeds of rice fields in China [1, 2]. In the past twenty years, barnyardgrass has been effectively controlled by a highly selective herbicide, quinclorac, which is a synthetic auxin [3, 4]. However, various *Echinochloa* spp. have evolved resistance to quinclorac in many countries and regions

of China [5-11]. *E. crus-galli* var. *zelayensis* is also becoming increasingly resistant to quinclorac [1].

Similar to other synthetic auxins, quinclorac can cause symptoms of growth inhibition, foliar chlorosis, epinasty, and tissue decay at high concentrations [12]. The production of two substances induced by quinclorac are closely related to the mechanism of action of quinclorac. Similar to other auxin herbicides, quinclorac can lead to production of ROS resulting in lipid peroxidation in sensitive plants [13-16]. Research has shown that quinclorac causes oxidation and results in chlorosis and lipid peroxidation in crabgrass (*Digitaria adscendens*) [15]. It can also induce the generation of ROS in maize (*Zea mays* L.) root tissue [17] and rice (*Oryza sativa*) plants [16]. Another is that quinclorac acts by inducing ethylene biosynthesis in plants [1, 11, 18], including its co-product, cyanide [19, 20]. When subjected to stress induced by quinclorac, ACC is produced in the roots of plants, which is then transported to the shoot, and results in the production of ACC and ethylene in large amounts [18, 19]. Quinclorac can rapidly stimulate ethylene formation in sensitive *E. phyllopogon* plants and pretreatment with malathion, which can be used as a P450 inhibitor, can reduce the ED_{50} value, but not by reducing the production of ethylene [11]. Quinclorac can also induce the biosynthesis of ethylene in sensitive *E. crus-galli* var. *zelayensis*[1] and *maize* [17], ethylene and cyanide in sensitive crabgrass [20]. In addition, quinclorac can also induce electrolyte leakage as a secondary response in crabgrass and other susceptible grasses, such as barnyardgrass, maize, and foxtail, green (*Setaria viridis*) [21]. Furthermore, quinclorac cannot inhibit the synthesis of cellulose [22].

Although resistance to quinclorac in barnyardgrass has been known for nearly two decades [6], the exact mechanism of resistance remains unclear. Quinclorac resistance in barnyardgrass was not found to be caused by differential absorption, translocation or metabolism [7, 11, 23, 24]. Studies have shown that after treatment with quinclorac, the expression levels of glutamate receptor-like gene and *GH3* gene were different in susceptible and resistant barnyardgrass biotypes; these genes may contribute to the mechanism of resistance [25, 26]. The difference in activity of

antioxidant enzymes may also be involved in the mechanism of quinclorac resistance and antioxidant enzyme assays shows that the constitutive activities of SOD, CAT, APX, and GR in the shoots of quinclorac-sensitive *E. oryzicola* are lower than those in rice, which is tolerant to quinclorac [14]. At present, the most studied is the relationship between quinclorac resistance and ethylene biosynthesis [1, 11, 12, 18], including the detoxification of cyanide, the co-product of ethylene biosynthesis [11, 19, 20]. Different studies in this context have shown that ethylene biosynthesis is limited in the resistant plants [1, 11, 18] and resistant plants have stronger capacity for cyanide detoxification [1, 11, 18, 27]. Our previous study also found that differences in *EcCAS* gene sequences and expression levels may lead to higher ability to detoxify cyanide in resistant biotypes, which maybe one of the mechanisms of quinclorac resistance in *E. crus-galli* var. *zelayensis* [28].

SAM is converted to ACC by the activity of ACS, which is then oxidized to ethylene, carbon dioxide, and cyanide by ACO [29]. AOA is an inhibitor of ethylene biosynthesis that acts on the process of SAM conversion to ACC [30, 31]. Studies have also shown that AOA inhibits ethylene synthesis associated with it as an ACC synthase for pyridoxal supplementation [32]. The ethylene biosynthetic enzymes, ACS and ACO, are encoded by multigene families in various plant species [33-38]. At present, there are more studies on ACS and ACO genes in rice, *Arabidopsis thaliana*, tomato, and melon (*Cucumis melo* L) [33, 39-43]. However, little research has been done on *ACS* and *ACO* in *E. crus-galli*.

To understand the mechanism of resistance related to ethylene biosynthesis of *E. crus-galli* var. *zelayensis* in East China, we studied and quantified the response of three biotypes with different levels of resistance to quinclorac. On the basis of differences in the fresh weight of *E. crus-galli* var. *zelayensis*, the amount of ethylene produced, *ACS* and *ACO* sequence alignment, and *ACS* and *ACO* expression patterns, among the three biotypes were compared. Moreover, the ethylene biosynthesis inhibitor, AOA, was used to further test our hypothesis that the suppression of *ACS* and *ACO* genes expression may be one of the important causes of quinclorac resistance. Thus, mechanism of quinclorac resistance associated with ethylene

biosynthesis and its molecular mechanism of *E. crus-galli* var. *zelayensis* in East China were elucidated.

2. Materials and methods

2.1 Plant material.

Seeds of sensitive and moderately resistant *E. crus-galli* var. *zelayensis* biotypes were collected from Xuanwu District, Nanjing, and Wujin District, Changzhou, Jiangsu Province, China (32.04°N,118.88°E; 31.71°N, 119.92°E) and seeds of highly resistant *E. crus-galli* var. *zelayensis* biotype were collected from Songjiang District, Shanghai, China (30.94°N, 121.07°E). In May 2015, the seeds of all three biotypes were planted in pots (white and plastic pot with small holes at the bottom, size: 47.5 mm * 35.5 mm * 30.5 mm) containing a 1:1 (wt/wt) mixture of sand and clay (pH 5.6 and 1.4% organic matter) and 50% organic fertilization (one fifth of the total weight). The pots were placed in a growth chamber at 30/25°C with 12-h light (300 μmol m^{-2} s^{-1}) [44] and 12-h darkness each day, and watering every three days. When the plants reached the 2.5–3.5-leaf stage, quinclorac at 300 g ai ha^{-1} was applied to all the seedlings of the two resistant biotypes and half of the seedlings of the susceptible biotype. After three weeks, all the seedlings of resistant biotypes and half of the sensitive seedlings, which were not sprayed with quinclorac, survived, but half of the sensitive seedlings, which were sprayed with quinclorac, died. The surviving seedlings were transplanted into larger pots (size: 1m * 1m) containing the same growth media, in the same growth environment. In the autumn of 2015, seeds were harvested from all the surviving biotypes. The sensitive, moderately resistant, and highly resistant biotypes were 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) [1], respectively. The seeds were dried in the sun and stored at 4 °C.

2.2 Chemicals and herbicide treatment.

Quinclorac (50% WP) used in this study was provided by Jiangsu Xinyizhongkai (Xinyi, China). Ethylene biosynthesis inhibitor, AOA (Adamas, 98 %+), was

purchased from Shanghai Titan, China. In this experiment, the germinated seeds of the three biotypes were transplanted into the Kasugai nutrient solution [45] for hydroponic cultivation in a plant incubator at 30/25°C with 12-h light (300 μmol m^{-2} s^{-1}) [44] and 12-h darkness each day. When seedlings (grown in disposable paper cups that contained 300 mL of Kasugai nutrient solution) reached the 2.5–3.5-leaf stage, quinclorac was added to the nutrient solution to a final concentration of 50 μ mol/ L (μM) [17] for 6, 9, 12, 18, or 24 h. For the ethylene biosynthesis inhibitor treatment group, AOA was dissolved with Kasugai nutrient solution to prepare a 2.5 m mol/ L (mM) solution, and then the solution was added to the Kasugai nutrient solution to a final concentration of 50 μM [31] for 24 h, prior to the addition of quinclorac. The AOA was removed when quinclorac was added.

2.3 Effects of quinclorac and AOA on fresh weight of shoots.

To examine the effect of ethylene biosynthetic pathway on fresh weight of the three biotypes, plants of each biotype were divided into four groups. Each group contained four biological replicates, each containing 12 plants at the same growth stage. "W" represents a control group treated with water. "A" represents the group treated only with AOA. The group "A+Q" was pretreated with AOA for 24 h and then treated with quinclorac for 48 h. The fourth group, "Q", was treated only with quinclorac for 48 h. The growth conditions were similar for all the four groups except for the difference in the chemicals and herbicide treatment at the 2.5–3.5-leaf stage. At 48 h after treatment, the fresh weight of shoots of all the four groups was determined.

The experiment was conducted twice. All studies were carried out with percentages of fresh weight which is based on fresh weight of group W. The groups were randomly arranged. Data of percentages of fresh weight were subjected to ANOVA. For comparison of the differences in the percentages of fresh weight among the four groups, the Duncan's Multiple Range Test ($P < 0.05$) was used. ANOVA was conducted using SPSS version 20 (SPSS, Chicago, IL, USA).

2.4 Effects of quinclorac and AOA on ethylene production.

To understand whether ethylene biosynthesis is associated with resistance to quinclorac and whether AOA has any effect on ethylene production, ethylene production of the three biotypes was determined using gas phase chromatography following the method of Abdallah and Xu [1, 20] with slight modifications. The three biotypes were divided into two groups respectively, one of which was pretreated with 50 μM of AOA for 24 h and then with 50 μM quinclorac, the other group was treated only with quinclorac. A set of intact seedlings was sampled at 0 (untreated with quinclorac), 6, 9, 12, 18, and 24 HAT and was sealed in screw-neck vials (20 mL). The samples were weighed before sealing. After incubation at 25 °C under light conditions (300 μmol m^{-2} s^{-1}) for 5 h, 1 mL of gas was extracted using a syringe to measure the ethylene content. Gas was injected into a chromatograph (GC 5890C, Nanjing, China) equipped with a 30 m × 0.32 mm × 0.25 mm Al_2O_3 column, with a column temperature of 45 °C and a gasification chamber temperature of 150 °C. Detection was performed using a hydrogen flame ionization detector at 50 °C. The flow rate of carrier gas (N_2) was 90 mL min^{-1} and the flow rate of combustion gas (air) was 75 mL min^{-1}. The amount of ethylene produced was expressed as pmol g^{-1} FW h^{-1}.

Each experimental treatment was performed in triplicate, and the experiment was conducted twice. Significant differences in the production of ethylene were analysed as described in section 2.3 using SPSS version 20 (SPSS, Chicago, IL, USA).

2.5 ACS and ACO gene sequences and sequence analysis.

We performed transcriptome sequencing of JNNX-S and SSXB-R2 biotypes of *E. crus-galli* var. *zelayensis* (SRP130228) and searched for *ACS* and *ACO* genes in the transcriptome sequences according to genes function annotation. Then we performed sequence alignment of these genes on the NCBI website (http://www.ncbi.nlm.nih.gov/.) for confirmation and screened for correct *ACS* and *ACO* gene fragments. We finally identified three *ACS* genes and seven *ACO* genes that could be used for primer design for Q-PCR. The ten genes in the three biotypes were amplified using the primers for Q-PCR (Table 2), sequenced, and analysed by BioEdit Sequence Alignment Editor Software to further confirm whether the

fragments obtained by amplification were corresponding *ACS* and *ACO* gene fragments. The names, accession numbers in gene bank, lengths, and similarities with the corresponding genes from other species of the ten genes are shown in Table1. The sequence of ten genes is shown in Supporting table S1.

2.6 Expression analysis of ACS and ACO based on real-time PCR.

The expression levels of 10 *ACS* and *ACO* genes in three *E. crus-galli* var. *zelayensis* biotypes were analysed by real-time PCR. The plant samples of the three biotypes were collected at 0 (untreated with quinclorac), 6, 12, and 24 HAT with 50 μM quinclorac and stored at -70 °C. Total RNA was extracted using RNAsimple Total RNA Kit (TIANGEN, China). Subsequently, total RNA was reverse transcribed to cDNA using PrimeScript^{TM} RT Reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan). All the cDNA samples were adjusted to the same concentration for the real-time PCR. The *EcActin* gene (GenBank accession HQ395760) was used as the reference sequence (Table 2) [25]. All the primers (Table 2) for the target genes were designed using the online tool available at http://bioinfo.ut.ee/primer3-0.4.0/, based on the obtained sequences of *ACS* and *ACO* genes in section 2.5. The reactions were performed in a volume of 20 μL, using SYBR ® Premix Ex Taq^{TM} II (Tli RNAaseH Plus; TaKaRa, Otsu, Japan), with each reaction mixture containing 10 μL SYBR® Premix Ex Taq™, 0.4 μL 10 μM primers (Table 2), 0.4 μL 50× Rox, 2 μL diluted cDNA, and 6.8 μL ddH_2O. The PCR products were detected by SYBR Green fluorescent dye. To ensure the accuracy of the operation, at least two duplicate amplifications were conducted. The following protocol was used for real time PCR on the ABI-7500 Fast Real-Time PCR System (ABI, USA): 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 34 s.

The relative mRNA levels were calculated by the *ΔΔC*t method with at least six technical and biological replicates. Based on the Ct values of a series of diluted cDNA templates, the amplification efficiency of each primer was determined from the standard curve, and approximately equal amplification efficiencies of the target and internal control, which is a requirement for the *ΔΔC*t method [46] were confirmed. The

t-test ($P < 0.05$) and the fold change (three-fold) were used to determine the up- or down-regulation of gene expression caused by quinclorac treatment [44].

2.7 Effect of AOA on EcACS-like, EcACS7, and EcACO1 gene expression.

The expression levels of *EcACS-like*, *ACS7*, and *ACO1* in the sensitive and moderately resistant *E. crus-galli* var. *zelayensis* biotypes that were pretreated with AOA for 24 h before the exposure to quinclorac were analysed using real-time PCR. The plant samples of the JNNX-S and JCWJ-R1 biotypes were collected at 0 (untreated with quinclorac), 6, and 12 HAT with quinclorac. The primers used, the experimental procedure for the real-time PCR of the three genes, and the analysis of the experimental data was as described in section 2.6.

Table 1 Information on the ACS and ACO genes obtained in *E. crus-galli* var. *zelayensis*.

Name of the genes	GenBank accession number	Length (bp)	Genes with a high similarity in other plants (GenBank accession number) (Ident)
EcACS-like	KY963549	1491	*ACS* of *E. crus-galli* (KT245168.1) (97%) *ACS-like* of *S. italica* (XM_004976514.3) (91%)
EcACS3	KY963551	853	*ACS3* of *Zea mays* (XM_008660261.2) (91%)
EcACS7	KY963550	415	*ACS7* of the *S. italica* (XM_004968450.1) (93%) *ACS2* of *Saccharum* hybrid cultivar (XM_ JF274986.1) (92%)
EcACO-like	KY963552	694	*ACO* of *E. crus-galli* (KP795111.1) (99%)
EcACO homolog 1-like	KY963553	446	*ACO homolog 1-like* of *S. italica* (XM_004975083.2) (83%)
EcACO1	KY963554	368	*ACO1* of *S. italica* (XM_004965982.2) (96%)
EcACO homolog 4-like	KY963555	278	*ACO homolog 4-like* of *S. italica* (XM_004965098.3) (92%)
EcACO homolog 4	KY963556	606	*ACO homolog 4* of *O. sativa* (XM_015786977.1) (79%)
EcACO5-like	KY963548	1448	*ACO5-like* of *S. italica* (XM_012847269.1) (90%)
EcACO homolog 6-like	KY963557	316	*ACO homolog 6-like* of *S. italica* (XM_004968833.2) (90%)

Table 2 Primers used to amplify the *EcACS* and *EcACO* genes in real-time PCR.

Primer	Sequence (5′ to 3′)	Annealing temperature (°C)	Length of product
EcActin-Real time-F	GTGCTGTTCCAGCCATCGTTCAT	60	171
EcActin-Real time-R	CTCCTTGCTCATACGGTCAGCAATA		
EcACS-like-Real time-F	GATCTCCATGGTCTGGTCGT	60	186
EcACS-like-Real time-R	CTCTTCTCGTGGATGGACCT		
EcACS3-Real time-F	TCTCTTGCTGGGTTTCGAGT	60	233
EcACS3-Real time-R	AAGCATTCAATCCCCACTTG		
EcACS7-Real time-F	GAGGTGAAGCTCAACATCTCG	60	173
EcACS7-Real time-R	TGTTCTTGCTGCGTTGACAT		
EcACO-like-Real time-F	GACATGGACTGGGAGGACAT	60	232
EcACO-like-Real time-R	GGTAGTGGCTGACCTTGGTG		
EcACO homolog 1-like-Real time-F	CTCGGGCTTTCTGACCATAG	60	209
EcACO homolog 1-like-Real time-R	AGCACGCAATCGAAATTCTT		
EcACO1-Real time-F	TCCCAGGTTTGGAGTTTCTG	60	169
EcACO1-Real time-R	ATAGATAGGCGGCTCCCATT		
EcACO homolog 4-like-Real time-F	TAATCAATGTCGGGGACCTC	60	233
EcACO homolog 4-like-Real time-R	CCCAGAAACTCAGGCATTGT		
EcACO homolog 4-Real time-F	TGCAGATGATGTCCAACGAT	60	160
EcACO homolog 4-Real time-R	TTCTCCTCCGATACCAGCTC		
EcACO5-like-Real time-F	CGTACAGGCCCTTCACCTAC	60	211
EcACO5-like-Real time-R	GGGGAATCCGCCAGTATTAT		
EcACO homolog 6-like-Real time-F	CTTCTTCAATCCTGCCAAGC	60	157
EcACO homolog 6-like-Real time-R	TCGTTCAATGGATGATCTGG		

3. Results

3.1 Differences in fresh weight of shoots under the influence of quinclorac and AOA in the three biotypes.

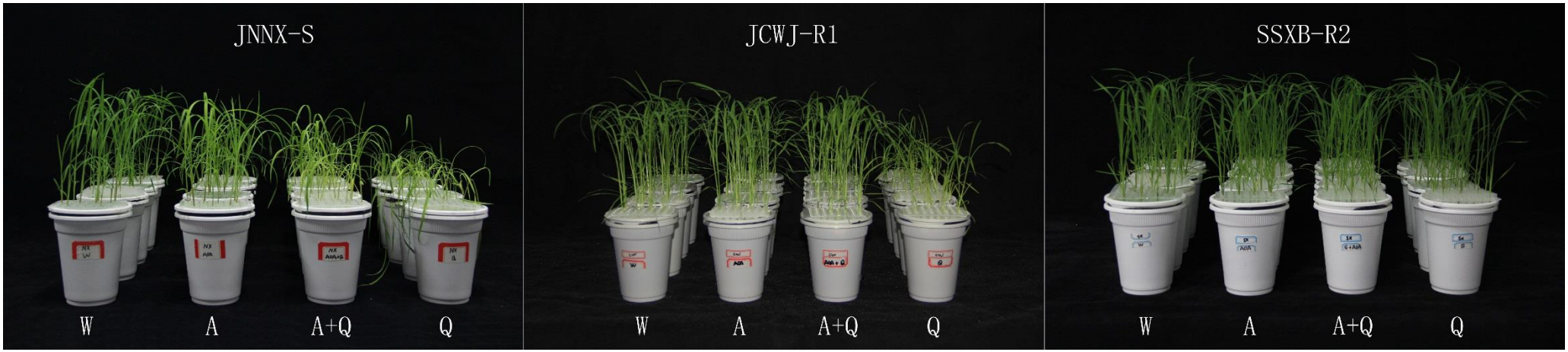
The percentages of fresh weight in the four treatment groups, namely W, A, A+Q, and Q, were determined in the three biotypes (Fig.1a, Fig.1b). After 48 h of treatment with quinclorac, the percentage of fresh weight of group Q of the JNNX-S decreased significantly compared to group W ($P < 10^{-12}$). However, the percentage of fresh weight in group A + Q of JNNX-S was significantly higher than that of group Q, although a significant decrease was also observed compared to group W ($P < 10^{-12}$). There was also a significant decline in the percentage of fresh weight in group Q of JCWJ-R1 ($P < 10^{-6}$). However, the percentage of fresh weight of group A + Q of JCWJ-R1 showed no obvious decrease compared to group W. There was no significant difference in the percentages of fresh weight for the four groups of the SSXB-R2 biotype ($P < 0.5$). The results also showed that the treatment with AOA alone did not affect the fresh weight in all three biotypes.

3.2 Difference in the release of ethylene and the effect of AOA on ethylene production.

The production of ethylene and the effect of AOA on ethylene production were studied in the three biotypes (Table 3). Ethylene production in the JNNX-S biotype increased continuously within 12 h after treatment with quinclorac, and the amount of ethylene produced at 12 h was 5-fold the initial amount, which then gradually decreased after 12 h. Ethylene production in the JCWJ-R1 biotype also increased after treatment with quinclorac, which increased at 18 h by about 3-fold compared to the initial amount. However, ethylene production in the JCWJ-R1 biotype was always lower than that in the JNNX-S biotype. The SSXB-R2 biotype with the highest resistance level had no significant change in ethylene production before and after the treatment with quinclorac for 24 h.

However, if pretreated with AOA for 24 h, ethylene production was significantly reduced in the three biotypes both before and after treatment with quinclorac. Moreover, only ethylene production in the JNNX-S biotype increased. The differences in ethylene production were consistent with the results of the differences in fresh weight in three biotypes (Fig. 1b).

A


JNNX-S
JCWJ-R1
SSXB-R2
W
A
A+Q
Q
W
A
A+Q
Q
W
A
A+Q
Q

B

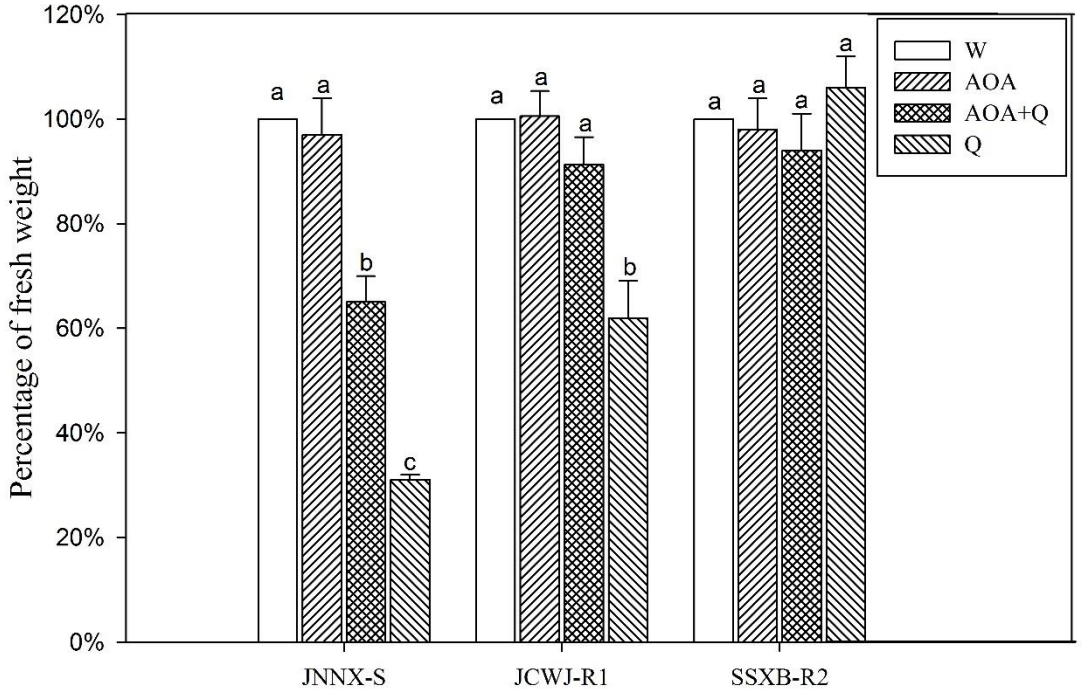

120%
100%
80%
60%
40%
20%
0%
Percentage of fresh weight
a
a
b
c
a
a
a
b
a
a
a
a
W
AOA
AOA+Q
Q
JNNX-S
JCWJ-R1
SSXB-R2

Fig. 1. Susceptibility and resistance to quinclorac in three *E. crus-galli* var. *zelayensis* biotypes. a. Differences in morphology of shoots after treatment with water (W), AOA (A), AOA and quinclorac (A+Q), or quinclorac (Q) for all three biotypes. The treatment time of quinclorac was 48 h and the pretreatment time of AOA was 24 h.

b. The fresh weights of plants from each biotype at the end of equivalent treatment periods plotted as a percentage of the water treatment control (W). "A" means treated only with AOA, "A+Q" means pretreated with AOA for 24 h and then treated with quinclorac for 48 h, and "Q" means treated only with quinclorac for 48h. The AOA and quinclorac were added to the Kasugai nutrient solution to a final concentration of 50 μM. Data are the mean values of at least eight biological replicates. The standard errors of the means are described by vertical bars. ANOVA significance groupings are shown as a, b, and c

Table 3 Release of ethylene in three biotypes at 0, 6, 9, 12, 18, and 24 h after quinclorac treatment, and the amount of ethylene released from the individual biotypes after AOA pretreatment for 24 h. The unit of ethylene production is pmol g^{-1} FW h^{-1}.

Biotype and treatment	0h	6h	9h	12h	18h	24h	df	*F*-ratio	*P*>*F*
JNNX-S Q	185.9 ± 10.4^{eA}	510.9 ± 18.8^{cA}	620.9 ±11.2^{bA}	993.8 ± 55.5^{aA}	535.7 ± 40.2^{bcA}	338.9 ±13.5^{dA}	5,30	83.18	<10^{-6}
JNNX-S A+Q	79.8 ± 13.2^{dB}	143.8 ± 21.9^{dC}	249.3 ± 28.2^{bcB}	309.7 ± 24.9 ^{abB}	373.6 ± 29.5^{aC}	233.5 ± 15.0^{cB}	5,29	19.96	<10^{-6}
JCWJ-R1 Q	168.3 ± 16.5^{cA}	265.6 ± 26.6^{bB}	224.4 ± 6.1^{bcB}	277.2 ± 25.1^{bB}	451.8 ± 28.4^{aB}	102.3 ± 8.8^{dD}	5,30	33.54	<10^{-6}
JCWJ-R1 A+Q	76.2 ± 8.3^{aB}	78.8 ± 5.2^{aD}	70.9 ± 13.5^{aD}	92.5 ± 16.2^{aCD}	90.5 ± 14.9^{aDE}	63.6 ± 6.0^{aE}	5,29	0.97	<1
SSXB-R2 Q	161.9 ± 10.7^{aA}	166.8 ± 19.4^{aC}	157.9 ± 18.3^{aC}	163.5 ± 8.7^{aC}	153.4 ± 17.1^{aD}	150.1 ± 11.5^{aC}	5,30	0.18	<1
SSXB-R2 A+Q	55.9 ± 6.4^{aB}	63.9 ± 5.8^{aD}	53.0 ± 7.8^{aD}	53.2 ± 11.3^{aD}	58.5 ± 7.6^{aE}	62.7 ± 9.8^{aE}	5,30	0.31	<1
df	5,29	5,30	5,30	5,29	5,30	5,30	-	-	-
F-ratio	25.40	83.87	168.22	146.57	63.43	95.30	-	-	-
P>*F*	<10^{-9}	<10^{-9}	<10^{-9}	<10^{-9}	<10^{-9}	<10^{-9}	-	-	-

“Q” means treated with quinclorac only.

“A+Q” means pretreated with AOA for 24 h and then treated with quinclorac.

a–e Significance level at different treatment time of each *E. crus-galli* var. *zelayensis* biotype.

A-E Significance level of different *E. crus-galli* var. *zelayensis* biotype at the same treatment time.

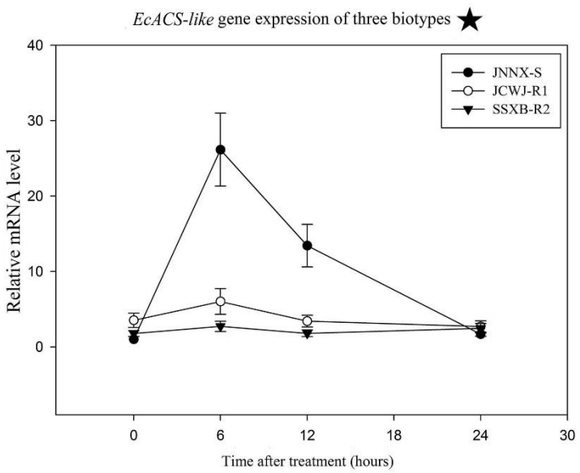
3.3 cDNA sequences of ACS and ACO genes of three E. crus-galli var. zelayensis biotypes.

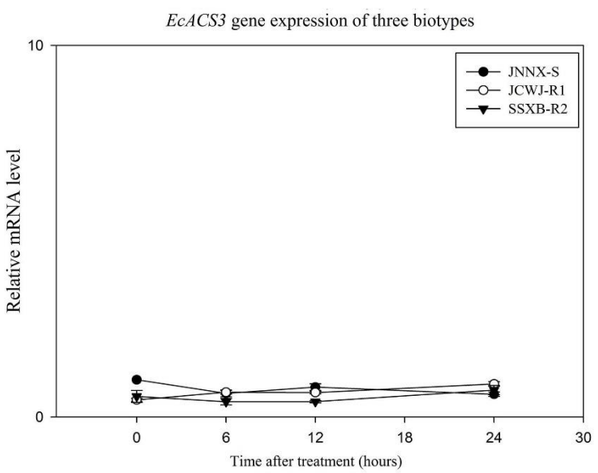
Three *ACS* and seven *ACO* genes were identified as described in Section 2.5. After amplification using the primers in Table 2, sequencing, and alignment analysis, it was revealed that all of the ten genes were present in all three biotypes and there was no difference in the amino acid sequences of each amplified cDNA sequence encoded among the three biotypes.

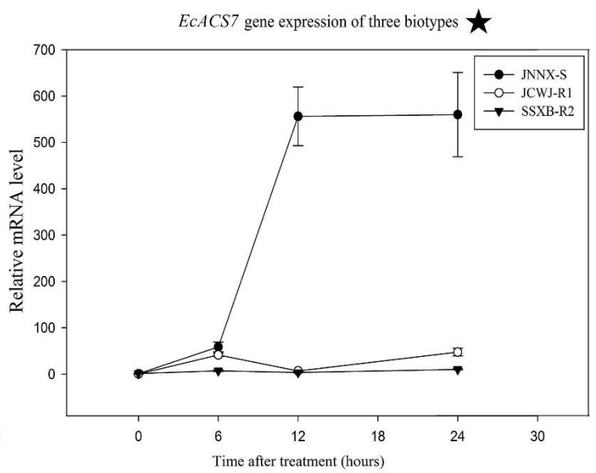
3.4 Expression patterns of ACS and ACO genes under quinclorac stress in the three E. crus-galli var. zelayensis biotypes.

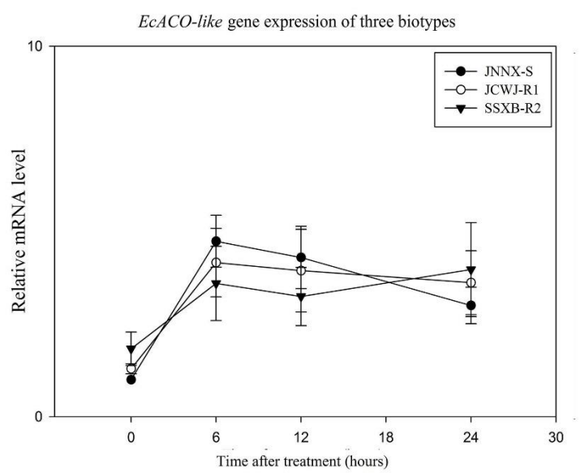
The relative expression levels of *ACS* and *ACO* mRNA in the shoots of the three *E. crus-galli* var. *zelayensis* biotypes at the seedling stage was assessed by real-time PCR (Fig. 2). The mRNA levels of all the 10 genes did not change significantly within 24 h of the treatment with quinclorac in the SSXB-R2 biotype. However, the expression levels of *EcACS-like*, *EcACS7*, and *EcACO1* in the JNNX-S biotype were dramatically upregulated compared to those at 0 HAT ($P < 0.001$). In the JNNX-S biotype, the expression of *EcACS-like* increased by 26-fold at 6 HAT with quinclorac and the expression of *EcACS7* gene increased by about 560-fold at 12 HAT, and the expression of *EcACO1* increased by 38- and 47-fold at 6 and 12 HAT, respectively, compared to its expression at 0 HAT. Moreover, the expression levels of *EcACS7* and *EcACO1* were also increased in the moderately resistant biotype ($P < 10^{-6}$), JCWJ-R1, although its expression level was lower than that in the sensitive biotype ($P < 10^{-4}$); the expression levels of *EcACS7* and *EcACO1* increased by 48- and 34-fold, respectively.

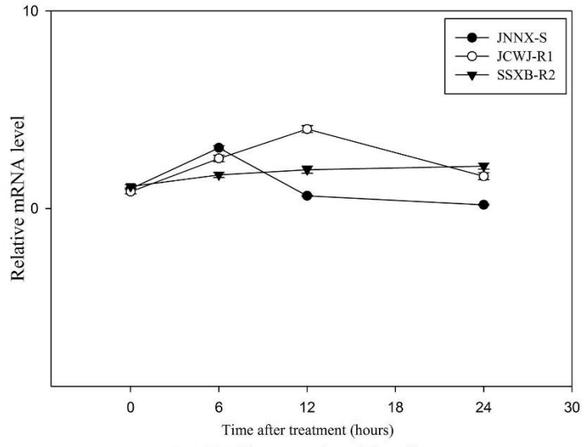
In addition, the expression levels of three other genes, *EcACO-like*, *EcACO5-like*, and *EcACO homolog 6-like*, were slightly induced by quinclorac in JNNX-S and JCWJ-R1 biotypes. The expression levels of *EcACO homolog 1-like* and *EcACO homolog 4* stimulated by quinclorac in JNNX-S peaked at 6 HAT, whereas in JCWJ-R1, the peak levels were present at 12 HAT, i.e., the maximum level of expression was delayed compared to that in JNNX-S and it positively correlated with their resistance level to quinclorac, although the rate of change in gene expression is relatively small.

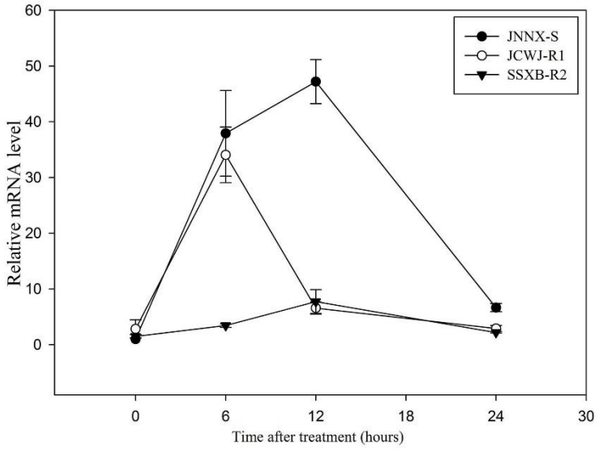


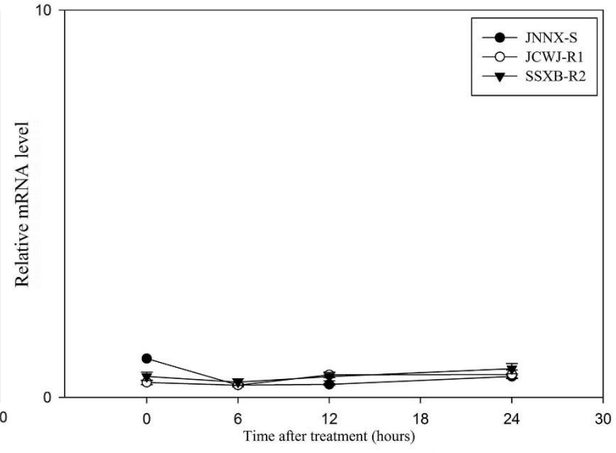


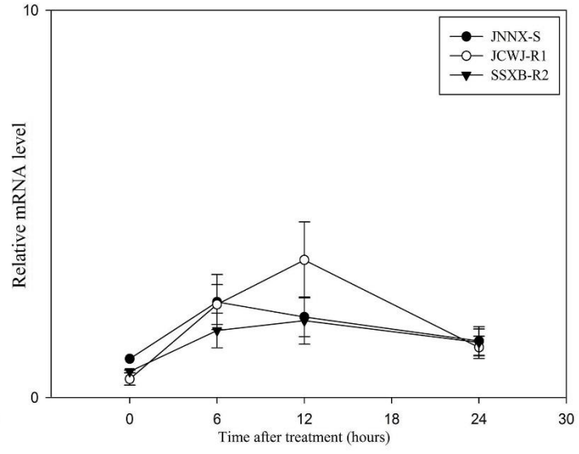


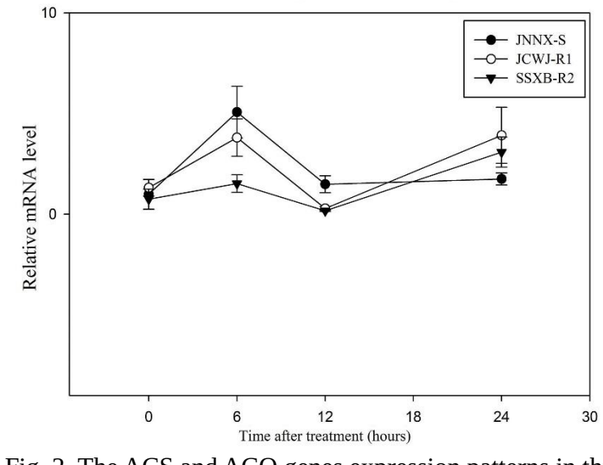












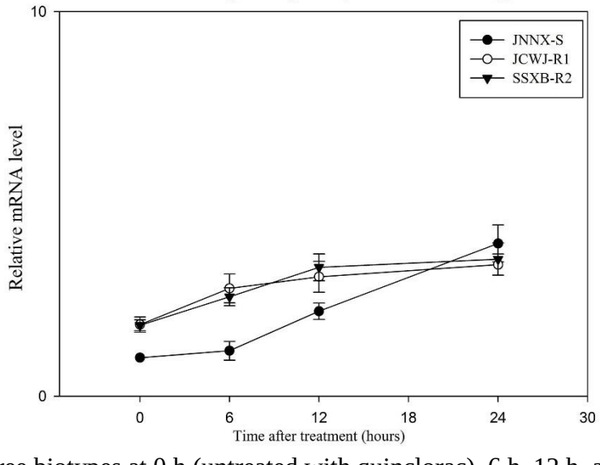


Fig. 2. The ACS and ACO genes expression patterns in three biotypes at 0 h (untreated with quinclorac), 6 h, 12 h, and 24 h after treated with quinclorac. Quinclorac was added to the Kasugai nutrient solution to a final concentration of 50 μM. The X-axis represents the time after quinclorac treatment. The Y-axis represents transcript levels relative to 0 h untreated with quinclorac in the susceptible line. Data are the mean values of at least six technical and biological replicates. The standard errors of the means are described by vertical bars. "★" represents genes that were upregulated more than tenfold by quinclorac treatment.

3.5 Effects of AOA on EcACS-like, EcACS7, and EcACO1 expression.

To test the hypothesis mentioned above, the correlation among the expression patterns of *EcACS-like*, *EcACS7*, and *EcACO1*, ethylene biosynthesis and quinclorac resistance, the expression levels of these three genes in the JNNX-S and JCWJ-R1 biotypes were evaluated after AOA pretreatment (Fig. 3). The expression of *EcACS-like* and *EcACS7* after the AOA pretreatment showed a significant decline ($P < 10^{-5}$). The expression of *EcACS-like* gene in the JNNX-S biotype decreased by about 7-fold at 12 HAT, and by 5-fold at 0 HAT in the JCWJ-R1 biotype after pretreatment with AOA ($P < 10^{-5}$). The expression of *EcACS7* decreased by 5-fold at 6 HAT and by 14-fold at 12 HAT in the JNNX-S biotype, and by 6-fold at 0 HAT in the JCWJ-R1 biotype ($P < 10^{-5}$). At almost all other time points, the expression levels of these two genes decreased slightly (< 3-fold) after AOA pre-treatment in the two biotypes.

However, the expression of *EcACO1* increased after AOA pretreatment, especially in the JCWJ-R1 biotype ($P < 0.01$). It increased by about 7-fold at 6 HAT and by 3-fold at 12 HAT in the JCWJ-R1 biotype after pretreatment with AOA. At almost all other time points, the expression level of *EcACO1* increased slightly (< 3-fold) after AOA pre-treatment in the two biotypes.

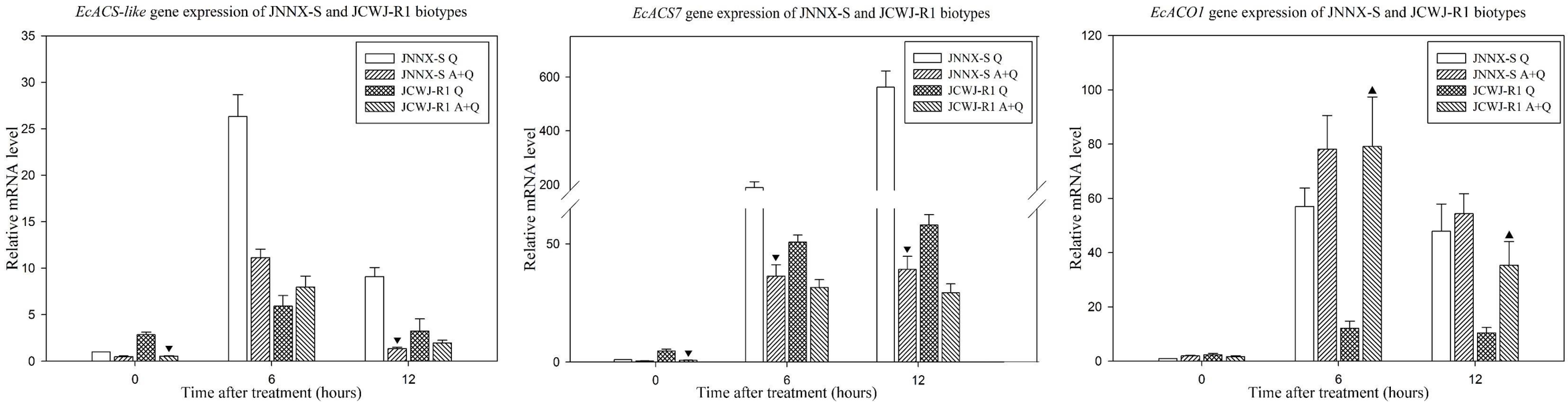


Fig. 3. The EcACS-like, EcACS7, and EcACO1 genes expression patterns in JNNX-S biotype and JCWJ-R1 biotype at 0 h (untreated with quinclorac), 6 h, and 12 h after treated with quinclorac or treated with AOA and quinclorac. "Q" means treated with quinclorac only, and "A+Q" means pretreated with AOA for 24 h and then treated with quinclorac. The AOA and quinclorac were added to the Kasugai nutrient solution to a final concentration of 50 μM, respectively. The X-axis represents the time after quinclorac treatment. The Y-axis represents transcript levels relative to 0 h (untreated with AOA and quinclorac) in the susceptible line. Data are the mean values of at least six technical and biological replicates. The folded part of the Y-axis is from 65-180 in the middle picture (*EcACS7* gene expression of JNNX-S and JCWJ-R1 biotypes). The standard errors of the means are described by vertical bars. "▼" means the gene at this timing point was downregulated more than threefold compared to that untreated with AOA. "▲" means the gene at this timing point was upregulated more than threefold compared to that untreated with AOA.

4. Discussion

The mechanism of resistance of barnyardgrass to quinclorac is closely related to ethylene biosynthesis [1, 18]. It is generally believed that ethylene and its co-product, cyanide, both of which are induced by quinclorac, affect the normal growth of sensitive plants [11, 12, 19]. Ethylene is a plant hormone that promotes senescence and death of plants [47-51], whereas cyanide damages the electron transport chain in respiration [52]. In addition, ethylene is also a gaseous signalling molecule that causes other physiological and biochemical reactions [53, 54]. In a previous study, we observed that the enzymatic activity of ACS and ACO in the highly-resistant barnyardgrass biotype was inhibited after treatment with quinclorac, and increase in the ACC content was also inhibited, which eventually resulted in no increase in ethylene content [1]. These results demonstrated that quinclorac resistance may be closely related to the biosynthesis of ethylene in *E. crus-galli* var. *zelayensis*. AOA is a known ethylene biosynthesis inhibitor, which can inhibit ethylene production in plants. In the present study, AOA pre-treatment before the quinclorac treatment could reduce the injury caused by quinclorac to JNNX-S and JCWJ-R1. In other words, JNNX-S and JCWJ-R1 become more resistant to quinclorac after AOA treatment, which suggests that quinclorac resistance in *E. crus-galli* var. *zelayensis* is related to the inhibition of ethylene biosynthesis.

In several sensitive plants, ethylene production could be induced after treatment with quinclorac [4, 17, 18, 20]. In the JNNX-S biotype, a rapid increase in ethylene production induced by quinclorac was also observed. The amount of ethylene production in the JNNX-S biotype was significantly higher than in the two resistant biotypes. AOA pretreatment before application of quinclorac could significantly decrease ethylene production in all three biotypes, which was consistent with their resistance levels and the differences in fresh weight after quinclorac treatment or pretreatment with AOA. This may further indicate the importance of inhibition of ethylene biosynthesis in the mechanism of quinclorac resistance in *E. crus-galli* var. *zelayensis*.

Ethylene biosynthesis is controlled by two gene families, namely the ACS gene family and ACO gene family [33-40]. Twelve ACS genes are found in *Arabidopsis*, and most of them can be induced by auxin [40]. ACO gene expression was found in tomato, melon, and deep-water rice [33, 41, 42]. However, studies on ACS and ACO genes in barnyardgrass are rare. In this study, three ACS genes and seven ACO genes

were obtained. No mutations were found in these 10 genes. This is consistent with our hypothesis that the change of ethylene production is closely related to the expression of *ACS* and *ACO* genes.

The expression levels of three genes, *EcACS-like*, *EcACS7*, and *EcACO1*, were very different among the three biotypes and increase in expression of these genes was limited in the resistant biotypes. Changes in the tertiary structure of the herbicide target at the herbicide binding site or changes in the expression levels can lead to resistance [44, 55]. In fact, the catalytic capacity of ACS and ACO in the resistant biotypes were much lower than those in the susceptible biotype, according to our previous studies [1]. The present study shows that, in JNNX-S, the expression levels of *EcACS-like*, *EcACS7*, and *EcACO1* increased to more than 20-fold compared to the initial level, whereas in JCWJ-R1 and SSXB-R2, a lower induction of expression of these three genes was observed. We hypothesize that less inducible expression of these three genes in JCWJ-R1 and SSXB-R2 led to lower ACS and ACO activities, resulting in lower ethylene production, conferring resistance to quinclorac.

AOA pretreatment could decrease the expression of *EcACS-like* and *EcACS7* in both the sensitive and moderately resistant biotypes induced by quinclorac, which was consistent with the results of ethylene inhibition caused by AOA (Table 3). This may indicate that the expression levels of *EcACS-like* and *EcACS7* are closely related to ethylene biosynthesis induced by quinclorac. However, the expression of *EcACO1* was upregulated after AOA treatment, especially in the moderately resistant biotype, which was not consistent with the results of our study on ethylene production. First, AOA does not act in the conversion of ACC to ethylene [30]. Second, there may exist an unclear regulatory mechanism, such as negative feedback regulation. After pretreatment with AOA, the content of ACC decreased [30], leading to negative feedback regulation, thereby causing upregulation of *EcACO1* gene expression. Perhaps the increased expression of *EcACO1* was a natural response of plants to decreased ACS gene expression after AOA treatment. However, this may also indicate that the *EcACO1* gene plays a role in the biosynthesis of ethylene.

As an auxin-type herbicide [3, 4], quinclorac may directly induce the expression of these downstream genes, *ACS* [40] and *ACO*, resulting in a large amount of ethylene production in sensitive biotypes (Table 3). The large amount of ethylene produced will inevitably lead to the accumulation of cyanide [19, 20]. Moreover, the capacity for cyanide detoxification in sensitive biotype is relatively low [28], which

increases the damage from quinclorac in sensitive biotypes. Cyanide can induce ethylene biosynthesis [18-20]. Thus, there is a another possibility that quinclorac maybe induce the production of ethylene and cyanide first, leading to the accumulation of cyanide due to the low capacity of cyanide detoxification in sensitive populations [28]. Then the accumulated cyanide may induce the expression of *EcACS-like*, *ACS7* and *ACO1* (Fig. 2), resulting in more ethylene production (Table 3), and more cyanide. However, the resistant biotypes with a higher cyanide detoxification capacity [28] and suppression of the expression of *ACS* and *ACO* (Fig. 2), avoid large increases in ethylene production (Table 3), and have resistance to quinclorac.

5. Conclusion

In *E. crus-galli* var. *zelayensis*, the expression of *EcACS-like*, *EcACS7*, and *EcACO1* were induced dramatically in susceptible and moderately resistant biotypes, especially in the JNNX-S biotype after treated with quinclorac (Fig. 2), resulting in increased ethylene biosynthesis (Table 3). In the highly resistant biotype, these three genes responsible for ethylene biosynthesis were not upregulated after quinclorac treatment (Fig. 2); thus, the ethylene biosynthesis pathway was not activated (Table 3) and injury was avoided (Fig.1 a, b), resulting in a highly resistant phenotype. In the moderately resistant biotype, these three genes were induced after quinclorac treatment, but to a lower level than JNNX-S (Fig. 2). Correspondingly, the production of ethylene also increased moderately (Table 3), and moderate resistance to quinclorac results (Fig.1 a, b). However, whether quinclorac acts as a signalling substance directly inducing the upregulation of expression of *EcACS-like*, *EcACS7*, and *EcACO1*, or by inducing a small amount of ethylene and cyanide first, or by other unknown pathways, requires further study. Further research is also required to understand the reasons for the differences in the response time of gene expression to quinclorac between the JNNX-S and JCWJ-R1 biotypes. In addition, how ethylene causes damage to the fresh weight of the JNNX-S and JCWJ-R1 biotypes, direct action or by induction of other physiological and biochemical pathways, also requires further studies.

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