Different regulation of auxin homeostasis would be a possible mechanism conferring quinclorac resistance in *Echinochloa crusgalli* var. *zelayensis*

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Abstract

Differences in ethylene biosynthesis, and cyanide detoxification have been reported to be mechanisms of quinclorac resistance in *Echinochloa crusgalli* var. *zelayensis*. Resistant phenotypes could be a consequence of the altered endogenous IAA homeostasis induced by the herbicide. In this study we determined the IAA content and expression levels of auxin homeostasis-related genes in susceptible and resistant biotypes of *E. crusgalli* var. *zelayensis* after quinclorac treatment. The results showed that the IAA content of JNNX-S (susceptible biotype) was significantly higher than that of SSXB-R (resistant biotype) after treatment with 50 μM quinclorac. To better understand this rise in IAA, the expression profiles of seven genes (one for auxin synthesis, five for IAA conjugation, and one for IAA oxidation) and the biochemical activities of two oxidases involved in IAA homeostasis were measured. The expression of *EcYUCCA10* was significantly higher in JNNX-S than in SSXB-R. The expression levels of the *EcGH3*s were significantly lower in JNNX-S than in SSXB-R. These expression profiles were consistent with the elevation of IAA levels in the susceptible biotype. In contrast, *EcUGT* and *EcDAO* were induced in each biotype, but a smaller increase was observed in SSXB-R than in JNNX-S. The enzymatic activities of IAA oxidases and peroxidases were higher in SSXB-R than in JNNX-S 24 h after treatment. It was inferred that altered expression of specific genes involved in IAA synthesis, conjugation, and oxidation resulted in less IAA being induced in the resistant biotype, resulting in a lower ethylene burst and the associated quinclorac resistance. These results suggest novel layers of complexity in the mechanism of quinclorac resistance.

KEYWORDS

Auxin homeostasis; Quinclorac resistance mechanism; IAA content

1 INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops in China (Fang Fuping, 2018). However, *Echinochloa spp.* are the predominant gramineous weeds in rice cropping systems, with up to 14 million hectares of infested area (Gibson et al., 2002). The application of herbicides is the primary measure of weed control in rice fields. In China, quinclorac, an auxin-type herbicide, has been widely used for more than 20 years to control *Echinochloa spp*. in rice fields owing to its high effectiveness and low cost. However, resistance to quinclorac has evolved because of its continuous and widespread use worldwide (Malik et al., 2010).

Synthetic auxin herbicides (SAHs) have a unique mode of action that mimics the function of indole acetic acid (IAA). They exhibit systemic mobility and selectivity, and are widely used to control weeds in cereal crops. One of the early responses of plants to exogenous auxin addition is the up-regulation of ethylene. The enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase is responsible for the first step in ethylene synthesis in plants, and quinclorac stimulates ethylene production by promoting expression of ACC synthase (Grossmann & Kwiatkowski, 1995, Yasuor et al., 2012). In susceptible dicots and monocots, a burst in ethylene biosynthesis leads to the production of abscisic acid (ABA), which plays an important role in growth inhibition (Grossmann, 1998, Gaines, 2020, Wang et al., 2022). Studies have also shown that cyanide, a co-product of ethylene biosynthesis, may be induced by quinclorac and may contribute to toxicity in susceptible plants (Grossmann & Kwiatkowski, 1995, Gao et al., 2017).

To date, the full details of the resistance mechanism of *Echinochloa spp.* to quinclorac remain unclear. Although no differences in quinclorac metabolism have been found between resistant and susceptible *Echinochloa spp*, differential absorption, translocation or metabolism have been linked to quinclorac resistance (Lovelace et al., 2007, Yasuor et al., 2012). Differences in the expression of photosynthesis-related genes may contribute to the mechanism of resistance (Gao et al., 2019). More importantly, reduced ethylene production and subsequent cyanide by-product appear to

be the main mechanism of quinclorac resistance (Grossmann & Kwiatkowski, 1995, Abdallah et al., 2006). Studies have shown that ethylene biosynthesis was stimulated to a lesser extent in the resistant biotypes of *E. crusgalli* var. *zelayensis*, which is linked to the reduced expression of three ethylene biosynthesis genes (*EcACS-like*, *EcACS7*, and *EcACO1*) (Gao et al., 2018). In addition, higher detoxification of cyanide conferred by elevated expression of *EcCAS* was found in quinclorac-resistant biotypes (Gao et al., 2017, Ul Haq et al., 2020). However, all of the above studies have focused on auxin-responsive genes and subsequent biochemical changes that are located downstream of auxin signal transduction. Little research has been conducted on the changes in the genes and enzymes involved in auxin homeostasis.

Auxin homeostasis in plants is regulated by auxin synthesis, conjugation (glycosylation and methylation), oxidation, and intercellular and intracellular auxin transport (Qin et al., 2005, Staswick et al., 2005, Tognetti et al., 2010, Rosquete et al., 2012, Zhao et al., 2013, Tanaka et al., 2014, Kasahara, 2016). The primary IAA synthesis pathway is the Trp-dependent pathway, in which the rate-limiting step is the *YUCCA* enzyme, a flavin-containing monooxygenases (Cao et al., 2019). It has been reported that the overexpression of genes from the *YUCCA* genes family leads to increased levels of IAA and results in high-auxin phenotypes in plants (Zhao et al., 2001, Yamamoto et al., 2007). Endogenous auxin pools are also regulated, at least in part, by negative feedback from a group of auxin-inducible *GH3* genes that encode acyl-acid-amido synthetases (Park et al., 2007). Conjugation to sugars is conferred by IAA-glucosyltransferase (IAA-glc synthase) which is a uridine diphosphate glucosyltransferase (*UGT*) (Ostrowski et al., 2015). IAA is predominantly degraded via IAA oxidases (IAAOs) encoded by dioxygenase for auxin oxidation (*DAO*) genes, which convert active IAA into biologically inactive 2-oxoindole-3-acetic acid (OxIAA) (Mellor et al., 2016, Porco et al., 2016). Less specific oxidases, including indole acetic acid oxidase (IAAO) (Tang & Bonner, 1948) and peroxidase (POD), participate in the catabolic processing of IAA (Beffa et al., 1990). All of these activities help maintain optimal endogenous auxin concentrations as part of homeostasis.

To elucidate the role of endogenous auxin pools in susceptibility and resistance

to the SAH quinclorac, the IAA content, expression patterns of genes and enzyme activities involved in auxin homeostasis were explored in quinclorac susceptible and resistant biotypes of *Echinochloa crusgalli* var. *zelayensis*.

2 MATERIALS AND METHODS

2.1 Plant materials

Seeds of sensitive biotypes (JNNX-S: ED_{50} value 36.75 g. ha^{-1}, R.I.=1.00) were collected from Xuanwu District, Nanjing, Jiangsu Province, China (32.04°N,118.88°E) and seeds of resistant biotype (SSXB-R: ED_{50} value 2457.79 g. ha^{-1}, R.I.=66.88) were collected from Songjiang District, Shanghai, China (30.94°N, 121.07°E). Seeds were germinated on a pre-germination plate (22 cm ×22 cm) in a growth chamber at 30/25 °C under a 12 h photoperiod (300 μmol m^{-2} s^{-1}). Twenty germinated seeds of both biotypes were transferred into each floating plate with the bottom of the wells cut. Floating plates were placed in a paper cup containing 300 mL of Kasugai nutrient solution. Plants were placed in a growth chamber under the same conditions as those used for germination (Satoshi et al., 2014). All roots of the seeds were soaked in nutrient solutions for growth. When the plants grew to the 3-leaf stage, they were used for the subsequent experiments. All experiments were repeated twice and three biological replicates for every treatment were applied in each experiments.

2.2 Chemicals and herbicide treatment

Quinclorac (96%, technical grade) was supplied by Sigma-Aldrich Co. Ltd (Shanghai, China). Quinclorac was dissolved in dimethyl sulfoxide to obtain a 100 mM stock solution, which was then added to the nutrient solution to a final concentration of 50 μM.

2.3 IAA extraction and purification

IAA in plants was extracted and purified as described by Yang et al. (2001), with some modifications. Three biological replicates were conducted in parallel, and the leaves and stems from each replicate (contains 20 plants) were cut and mixed before sampling. The samples (200 mg for each) were homogenized in liquid nitrogen and extracted on ice with 2 mL of cold 80% (v/v) methanol with butylated hydroxytoluene (1 mmol·L^{-1}) for 4 h at 4 °C. After centrifugation at 10000 ×g (4 °C) for 10 min, the supernatant was then passed through a C18 Sep-Pak cartridge (Waters, Milford, MA, USA) and prewashed with 2 mL of 80% methanol (v/v). The IAA fractions were eluted with 2 mL of 100% methanol and 2 mL ether from the column successively and the elution was dried under N_2 then dissolved in PBS (0.01 mol·L^{-1} at pH 7.4) containing 0.1% (v/v) Tween 20 and 0.1% (w/v) gelatine for the subsequent analysis by enzyme-linked immunosorbent assay (ELISA).

2.4 Quantification of IAA by ELISA

To determine the IAA content in both biotypes of *E. crusgalli* var. *zelayensis*, foliar samples were harvested at 0 (before quinclorac treatment), 6, 12, and 24 h after treatment (HAT) with quinclorac and IAA extracted, as described in Section 2.3. A commercial ELISA kit (produced by the Phytohormones Research Institute, China Agricultural University) based on an indirect competitive ELISA technique (icEISA) (Yang et al., 2001) was used for IAA content determination. The mouse monoclonal antigen and antibody against IAA and IgG-horseradish peroxidase used in the ELISA were supplied by the kit and the ELISA was performed as described by Yang et al. (2001). Briefly, a 96-well microtitration plate was used for ELISA. Each well was pre-coated with 100 μL coating buffer (1.5 g L^{-1} Na_2CO_3, 2.93 g L^{-1} $NaHCO_3$, and 0.02 g L^{-1} NaN_3, pH 9.6) containing 0.25 μg mL^{-1} antigen against IAA, and then kept at room temperature for 30 min. The plate was then washed four times with PBS (0.1% [v/v] Tween 20, pH 7.4). In each well, 50 μL of either extract or IAA standards (0–100 ng mL^{-1} dilution range), and 20 μg mL^{-1} antibody against IAA were added. The plates were

incubated overnight at 4 °C and washed as described above. One hundred microliters of 1.25 μg mL^{-1} IgG-horseradish peroxidase substrate was added to each well and incubated at 37 °C for 30 min. The plate was then washed as described above, and 100 μL of a coloured solution containing 2 mg mL^{-1} o-phenylenediamine and 0.012% (v/v) H_2O_2 was added to each well. The reaction was stopped by adding 50 μL of 2 mol L^{-1} H_2SO_4 per well when the colour of the standard sample exhibited a fine gradient (from light to deep colour). Absorbance at OD_{490} was measured using an ELISA reader. The IAA content was calculated as described by Weiler et al. (1981).

The changes in IAA content in different aboveground biomass after treatment with quinclorac were expressed as a percentage of the mean value of untreated plants. All treatments had three biological replicates and the experiment was conducted twice. Data from the two repeated experiments were subjected to ANOVA using SPSS version 20 (SPSS, Chicago, IL, USA), and if Levene's test showed no significance ($p>0.05$), the data were pooled for analysis. Significant differences were analysed by SPSS using Duncan's multiple range test.

2.5 Expression pattern of auxin homeostasis-related genes determined by real-time PCR

Eight genes involved in auxin homeostasis were identified in the transcriptome sequencing database of *E. crusgallli* var. *zelayensis* (PRJNA430735) (Gao et al., 2019). *EcYUCCA10* is responsible for auxin synthesis, five genes (*EcGH3.1*, *EcGH3.3*, *EcGH3.5*, *EcGH3.11* and *EcUGT*) are involved in IAA conjugation, and *EcDAO* is involved in auxin oxidation. The *EcActin* gene (GenBank accession number HQ395760) was chosen as the reference gene for Q-PCR (Gao et al., 2017). These sequences were aligned on the NCBI website (http://www.ncbi.nlm.nih.gov/) and used for Q-PCR primer design using the online primer design tool available at http://bioinfo.ut.ee/primer3-0.4.0/. Amplified sequences were blasted to confirm the identity of the gene fragments (Table 1).*Table 1 near here*

The expression profiles of all eight genes in *E. crusgalli* var. *zelayensis* were analyzed by real-time PCR (Q-PCR). Plant samples (mixed 0.1g different plant leaves) of both biotypes were collected at 0 (untreated with quinclorac), 12, and 24 HAT with 50 μM quinclorac. Total RNA was extracted using the RNAsimple Total RNA Kit (TIANGEN, China), according to the manufacturer's instructions. The extracted RNA was transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan). The cDNA samples were diluted to a uniform concentration for Q-PCR. The Q-PCR reactions were conducted using the SYBR® Premix Ex Taq™ II kit (TaKaRa, Otsu, Japan). The reaction system (20 μL) consisted of 10 μL SYBR® Premix Ex Taq™, 2 μL diluted cDNA, 0.4 μL 10 μM primers for each (in Supplements), 0.4 μL Rox, and 6.8 μL ddH_2O. Real-time PCR was performed on an 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.

The threshold values (Ct) were determined automatically using Onbore software. Relative transcript levels were calculated using the ΔΔCt method, with at least six technical and biological replicates as described by Gao et al. (2018). Relative mRNA levels of each gene at different HAT were normalized by dividing by the relative mRNA level of JNNX-S at 0 HAT. All normalized data were subjected to ANOVA followed by Duncan's multiple range test ($P < 0.05$) for the separation of means. The data analysis was performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). Two threshold values, a significant result in the t-test ($P < 0.05$) and the fold change (three-fold) were used to determine up- or down-regulation of gene expression caused by quinclorac. The dissolution curve of the quantitative test showed a single peak, which could be used for Q-PCR.

2.6 Influence of quinclorac on IAAO and POD activity

The activities of IAAO and POD in leaf extracts from both biotypes were evaluated as described by Yuan and Ding (1990) with minor modifications. For IAAO, the samples (0.5 g) were ground on ice with 5 mL of 20 μM phosphate buffer (pH 6.0). The

homogenates were centrifuged at 4,000 × g for 20 min at 4 °C, and the supernatants were transferred into new tubes for crude enzyme preparations. Reaction mixture A (2 mL 200 μg/mL IAA, 1 mL 1 mM $MnCl_2$, 2 ml 1 mM 2,4-dichlorophenol, 5 mL 20 mM phosphate buffer and 1 mL crude enzyme solution or sterilized pure water) was incubated at 30 °C in a water bath for 30 min. Reagent B (0.5 mol/L $FeCl_3$: 35% $HClO_4$=1:50) was also prepared. Four mL of Reagent B was added to each new tube, and 2 mL of mixture A was added. All the tubes were incubated at 30 °C in the dark for 30 min. Finally, the absorbance was measured at 530 nm using a UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve of IAA (0, 2, 5, 10, 15, 20, and 25 μg/mL) was prepared and the OD_{530} values were plotted against the corresponding IAA concentrations. The level of IAAO activity can be expressed as the rate at which it destroys the indole acetic acid (1).

(1) Activity of IAAO ($\mu g\ IAA g^{-1} FW\ h^{-1}$) $= \frac{(C_{CK}-C_T)\times V_T \times V}{W\times t\times V1}$

where C_{ck} is the control treatment, C_t is the quinclorac treatment, W is the sample fresh weight (g), V_T is the total volume of the enzyme solution, V is the total system of the reaction solution in the second operation, V_1 is the volume of the enzyme solution used in the reaction, and t is the treatment time.

For POD activity assays, crude enzyme solution was obtained as described above. The reaction mixture consisted of 200 mL of 0.2 mM phosphate buffer (pH 6.0), 0.112 mL 30% hydrogen peroxide and 0.076 mL guaiacol, which were mixed and stored at 4 ℃. The reaction mixture and 30 μL enzyme solution were reacted for 5 min at room temperature (22–25 ℃) before the OD_{470} value was measured. One unit of POD activity was defined as the increase in optical density value per minute by 0.01 (2).

(2) Activity of POD（u $g^{-1}min^{-1}$) $= \frac{\Delta A_{470}\times V_t}{W\times V_S\times 0.01\times t}$

where ΔA_{470} is the change in absorbance during the reaction, W is the sample fresh weight (g), t is the reaction time (min), V_t is the total volume of the extracted enzyme solution, and V_S is the volume of the enzyme solution added during the measurement.

Three replicates were included for each assay, and each assay was conducted twice. Significant differences in enzyme activities were analysed as described in

Section 2.3 using SPSS V.20.

3 RESULTS

3.1 Influence of quinclorac on the IAA content in *E. crusgalli* var. *zelayensis*

There was no significant difference according to Levene's test analysis; therefore, we pooled the data. IAA content in both biotypes increased over time after treatment with quinclorac, and the resistant biotype displayed less accumulation of IAA in leaf tissues following treatment. At 24 HAT, the IAA contents of JNNX-S was increased from 38.50 ng g^{-1} FW to 81.11 ng g^{-1} FW which was 2.11-fold higher than that at 0 HAT. Meanwhile, the IAA contents of SSXB-R was increased from 30.52 ng g^{-1} FW to 39.91 ng g^{-1} FW which was 1.30-fold higher than that at 0 HAT (Fig. 1).

Fig. 1

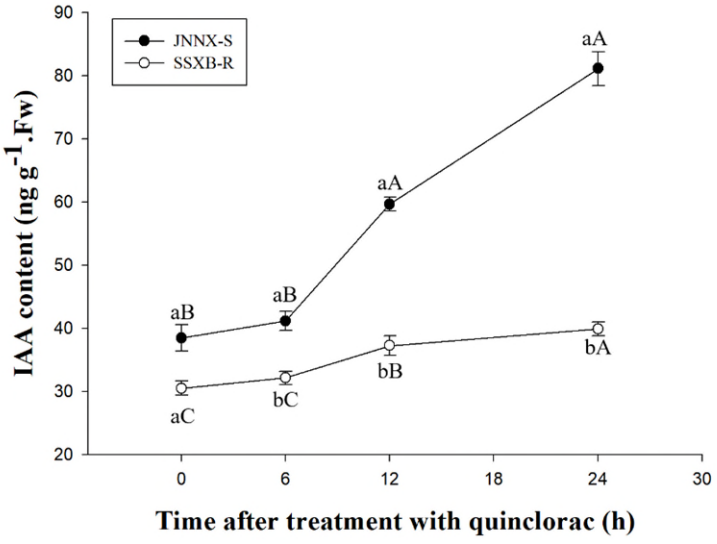

Fig. 1：The change of IAA contents after treatment with quinclorac in leaf extracts from both quinclorac susceptible and -resistant biotypes (JNNX-S and SSXB-R) of *E. crusgalli* var *zelaynsis*. Different letters (A-C) represent the significant difference of the measured data at different sampling time for each biotype after quinclorac treatment; Different letters (a-b) represent the significant difference of the measured data at the same sampling time point for both biotypes.

3.2 Expression patterns of auxin biosynthesis-related genes under quinclorac treatment

The expression of *EcYUCCA10* was up-regulated in both biotypes. At 12 HAT, its expression increased less in SSXB-R and partially decreased in JNNX-S. However, at 24 HAT, its expression in JNNX-S was 2.78-fold higher than that at 0 HAT and was significantly higher than that in the resistant biotypes (Fig 2-a).

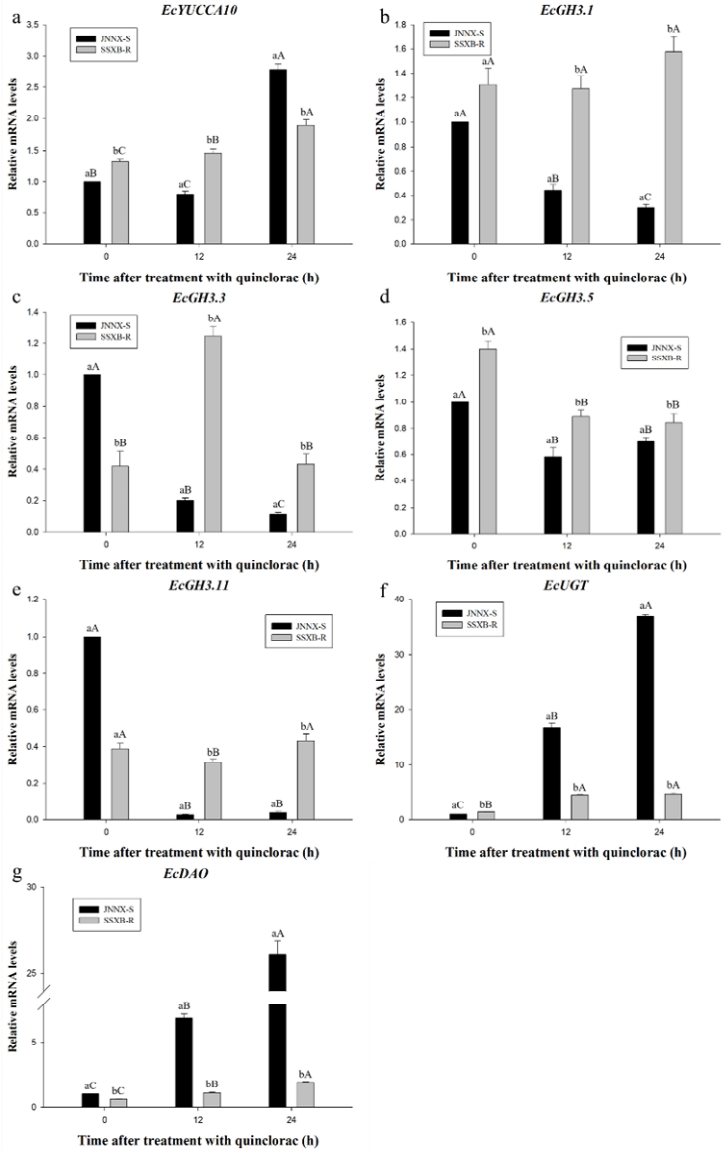

Fig. 2

Fig2-a: The expression pattern of auxin biosynthesis related genes (*YUCCA10*) after treatment with quinclorac in leaf extracts from both quinclorac susceptible and -resistant biotypes (JNNX-S and SSXB-R) of *E. crusgalli* var

zelaynsis. The meanings of the letters (A-C, a-b) are the same as in Fig. 1. Fig.2-b–e: The expression pattern of amino acid conjugation genes (*EcGH3s*) after treatment with quinclorac in leaf extracts from both quinclorac susceptible and -resistant biotypes (JNNX-S and SSXB-R) of *E. crusgalli* var *zelaynsis*. The meanings of the letters (A-C, a-b) are the same as in Fig. 1. Fig.2-f: The expression pattern of amino acid conjugation related genes (*EcUGT*) after treatment with quinclorac in leaf extracts from both quinclorac susceptible and -resistant biotypes (JNNX-S and SSXB-R) of *E. crusgalli* var *zelaynsis*. The meanings of the letters (A-C, a-b) are the same as in Fig. 1. Fig.2-g: The expression pattern of auxin oxidation related genes（*EcDAO*） after treatment with quinclorac in leaf extracts from both quinclorac susceptible and -resistant biotypes (JNNX-S and SSXB-R) of *E. crusgalli* var *zelaynsis*. The meanings of the letters (A-C, a-b) are the same as in Fig. 1.

3.3 Expression patterns of IAA conjugation-related genes under quinclorac treatment.

In the leaves of JNNX-S, the expression of *EcGH3.1*, *EcGH3.3*, *EcGH3.5* and *EcGH3.11* were all decreased significantly at 24 HAT (0.44-, 0.12-, 0.69- and 0.04-fold that at 0 HAT, respectively). However, in SSXB-R, the expression of these genes was not significantly different at 24 HAT, except for *EcGH3.5* (1.21-, 1.03-, 0.60-, and 1.11-fold that at 0 HAT, respectively) (Fig 2-b–e).

The expression of *EcUGT* in both biotypes increased at 12, and 24 HAT. However, in JNNX-S, the expression of *EcUGT* at 24 HAT showed a 36.9-fold increase relative to 0 HAT, while the increase in SSXB-R was only 3.43-fold (Fig 2-f).

3.4 Expression patterns of auxin oxidation-related genes under quinclorac treatment

In the leaves of both biotypes, the expression of *EcDAO* increased at 12, and 24 HAT. However, the expression of *EcDAO* increased more in the sensitive biotypes than in the quinclorac-resistant biotypes. At 24 HAT, the expression of *EcDAO* in JNNX-S (22.9-fold) was much higher than that in SSXB-R (1.91-fold) (Fig 2-g).

3.5 Difference in the activities of IAAO and POD after treatment with quinclorac

The activities of IAAO in the leaves of both biotypes decreased at 24 HAT, but the decrease in IAAO activity in SSXB-R was consistently significantly lower than that in JNNX-S. At 24 HAT, the IAAO activity of JNNX-S decreased by 74% which from 346 μg $IAAg^{-1}$ FW h^{-1} to 90 μg $IAAg^{-1}$ FW h^{-1}. The IAAO activity of SSXB-R decreased by 54% which from 313 μg IAA g^{-1} FW h^{-1} to 144 μg IAA g^{-1} FW h^{-1} (Fig 3-a).

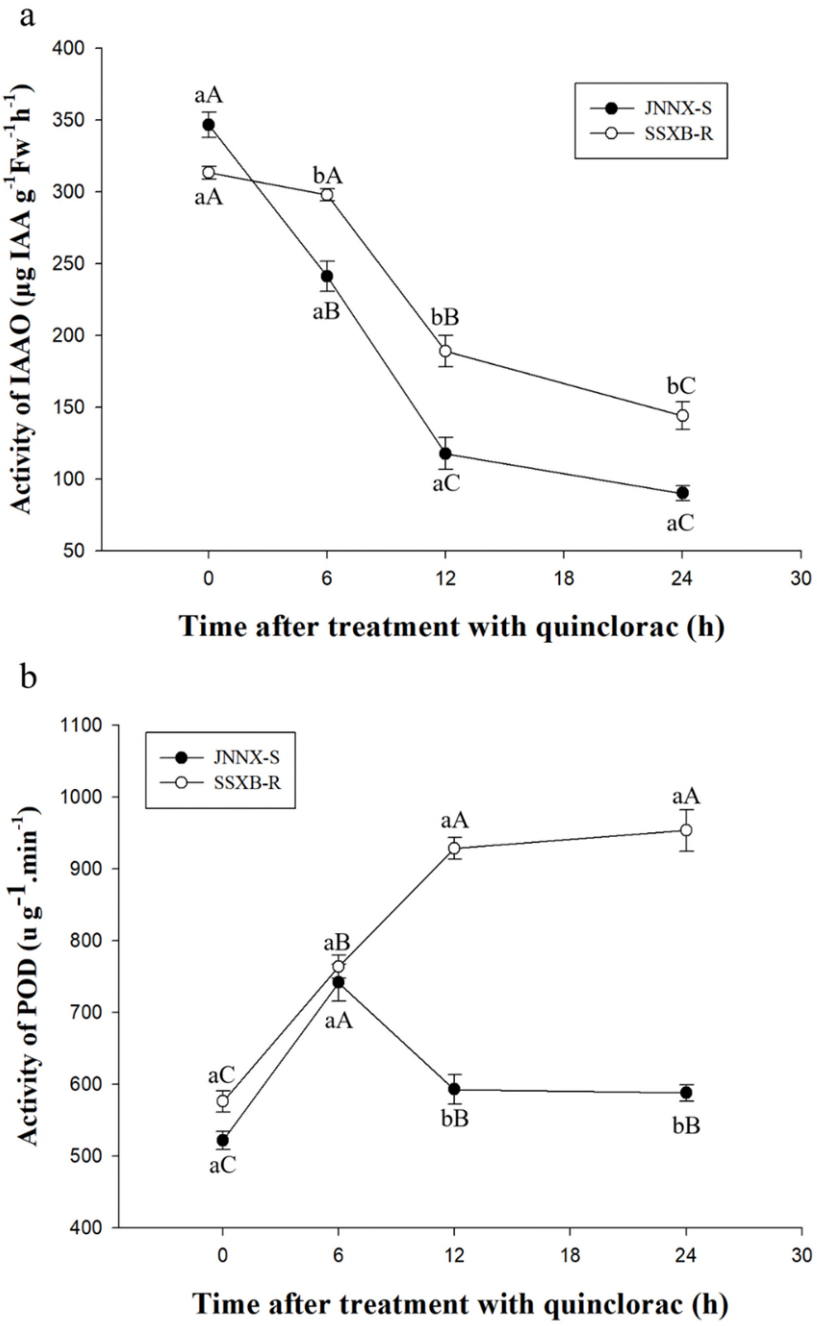

Fig. 3

Fig.3-a: IAAO activity in leaf extracts from both quinclorac susceptible and -resistant biotypes (JNNX-S and SSXB-R) of *E. crusgalli* var *zelaynsis* after treatment with quinclorac. The meanings of the letters (A-C, a-b) are the same as in Fig. 1. Fig.3-b: POD activity under the influence of quinclorac in leaf extracts from both quinclorac susceptible and -resistant biotypes (JNNX-S and SSXB-R) of *E. crusgalli* var *zelaynsis* after treatment with quinclorac. The meanings of the letters (A-C, a-b) are the same as in Fig. 1.

The activity of POD in JNNX-S reached its peak at 6 HAT, increasing from 541 u $g^{-1}min^{-1}$ (0 HAT) to 741 ug^{-1} min^{-1} (6 HAT), and then began to decline and remained at

approximately 590 u $g^{-1}min^{-1}$, whereas in SSXB-R, POD activity at 12 h increased significantly higher than that at 0 h and maintained a high activity until 24 h. At 24 h, POD activity was still at a maximum (from 576 ug^{-1} min^{-1} to 953 ug^{-1} min^{-1}), which was significantly higher than that of JNNX-S. (Fig 3-b).

4 DISCUSSION

Mechanisms of resistance to synthetic auxin herbicides (SAHs) in weeds have rarely been characterised. For target-site-based resistance, it has been reported that an *Arabidopsis AFB5* mutant line is insensitive to picloram (Prigge et al., 2016), but no mutated auxin receptors have been found to be responsible for resistance to SAHs in weeds. However, a mutation in the co-receptor AUX/IAA protein (*KsIAA16*) in *Kochia scoparia* has been shown to confer cross resistance to the SAHs 2,4-D and dicamba (LeClere et al., 2018) and a mutation in the co-receptor *IAA2* in *Sisymbrium orientale* confers resistance to the herbicide 2,4-D (Figueiredo et al., 2022). In terms of non-target site resistance, it has been reported that alterations in the activity of the ABCB class of plasma membrane transport proteins facilitate long-distance transport of 2,4-D, with loss of activity leading to resistance to 2,4-D in *Raphanus raphanistrum* (Goggin et al., 2016). Other potential target sites for SAHs resistance have also been identified (Busi et al., 2018). To the best of our knowledge, reduced absorption/translocation and enhanced degradation of SAHs in resistant weeds are all possible non-target site-based mechanisms for the resistance to 2,4-D in *Papaver rhoeas* (Peterson et al., 2016). Resistance has been also reported in *Conyza canadensis*, *Amaranthus hybridus*, *C. sumatrensis*, *Hirschfeldia incana* and *Parthenium hysterophorus* (Palma-Bautista et al., 2021). All these resistant phenotypes have arisen in eudicot species.

Quinclorac is somewhat unusual for SAHs because it controls some monocot weed species, including *E. crusgalli* var. *zelayensis*. Quinclorac stimulates ethylene production, which can lead to the production of abscisic acid (inhibiting growth) and cause production of cyanide which could be directly toxic. The widespread use of this herbicide has led to resistant biotypes, and the basis of this resistance has been linked

to the decreased expression of genes encoding ACC synthase and ACC oxidase and, consequently reduced ethylene production (Gao et al., 2018). Cyanide is a by-product of ethylene biosynthesis and elevated expression of *EcCAS*, which encodes ß-cyanoalanine synthase (ß-CAS), has also been associated with resistant biotypes of *E. crusgalli* var. *zelayensis.* These resistant plants may metabolise cyanide more effectively (Gao et al., 2019).

All the genes associated with resistance noted above are associated with auxin signaling or downstream of auxin perception. In the present study, we concentrated on upstream events and examined how the endogenous pool of IAA changes in susceptible and resistant biotypes after quinclorac treatment. The free IAA content in the susceptible biotype JNNX-S increased much more than that in the resistant (SSXB-R) biotype when treated with 50 μM quinclorac (Fig. 1). Elevated auxin levels lead to increased ethylene synthesis by stimulating the transcription of genes responsible for ethylene synthesis, such as genes encoding ACS (Stepanova et al., 2007). It is reasonable to hypothesise that regulation of IAA homeostasis is perturbed by SAHs and that these changes might be involved in resistance mechanisms. IAA biosynthesis, conjugation, and oxidation are cooperatively regulated to maintain IAA homeostasis (Zhang & Peer, 2017). Therefore, we measured changes in both of total IAA levels and the expression of representative genes associated with inputs and outputs of the IAA pool.

A key observation was that the elevated level of IAA in the susceptible biotype associated with a far greater increase in IAA after treatment (Fig. 1). The *YUCCA* genes family causes increased levels of IAA, resulting in high auxin phenotypes in plants. It was also observed that in JNNX-S, the expression of *EcYUCCA10* increased more than that in SSXB-R 24 h after treatment, which would lead to more IAA being synthesized (Zhao et al., 2001, Yamamoto et al., 2007).

Another way to increase IAA levels is to reduce losses from the IAA pool. Conjugation of IAA is a major way to deactivate endogenous auxin, principally by conjugation to amino acid by *GH3s* (Westfall et al., 2010), but also by conjugation of sugars by glucosyltransferases (Busi et al., 2018). High levels of auxin can stimulate

the expression of *GH3s* (Staswick et al., 2005), but the expression of *EcGH3.1, EcGH3.3, and EcGH3.11* were all decreased after treatment with quinclorac, whereas the expression of these three genes was unchanged or slightly increased in SSXB-R. These data suggest that the susceptible biotype had a much-reduced ability to conjugate IAA with amino acids after quinclorac treatment compared to SSXB-R.

The IAA-glucosyltransferase encoded by the *EcUGT* gene participates in the reversible conversion of IAA into biologically inactive glucose conjugates (Mateo-Bonmati et al., 2021). The S biotype responded to quinclorac treatment by strong induction of *EcUGT*, whereas expression in the R biotype was mildly increased (Fig. 2-f). Given that IAA levels remained lower in the R biotype, we speculated that *GH3s* played a dominant role in the homeostasis of endogenous IAA. Many *UGTs* have a wide specificity (Jin et al., 2013, Su et al., 2017). It is possible that the *UGT* induced in the S biotype uses quinclorac as substrate, diverting it from the glycosylation of IAA, which may then accumulate. Alternatively, higher IAA levels in the S biotype might stimulate higher *UGT* gene expression.

IAA oxidation is controlled by the dioxygenase *DAO* (Porco et al., 2016). The major IAA catabolite in Arabidopsis is 2-oxoindole-3-acetic acid (oxIAA) (Pencik et al., 2013) which can be further metabolized by conjugation to glucose to form oxIAA-glc by *UDP* glucosyltransferase *UGT74D1* (Tognetti et al., 2010). Catabolism of IAA has been shown to be irreversible (Kowalczyk & Sandberg, 2001), oxIAA has very little biological activity and it is not transported via the polar auxin transport system (Pencik et al., 2013). *EcDAO* was induced after quinclorac treatment with much higher expression in the leaves of JNNX-S than in SSXB-R (Fig. 2-g), which might be a response to the elevated IAA (Fig. 1) or exposure to quinclorac. In contrast to the expression levels of *EcDAO*, the measured biochemical activity of IAA oxidases decreased after treatment in both biotypes, but more acutely in the S biotype (Fig. 3-a). Reduced peroxidases activity was also observed (Fig. 3-b). The greater loss of auxin oxidation activity from both enzyme groups in the S biotype is likely to contribute to the accumulation of free IAA (Fig. 1).

It is well reported that the control of auxin homeostasis is complex given the

many contributory pathways involved. The results reported above indicate that all these pathways are changed by the application of exogenous SAH and quinclorac, resulting in an increase in endogenous IAA levels. This may be explained by the expression patterns of *EcYUCCA10* and the *EcGH3.1, 3.3, 3.5* and *3.11*, which are all consistent with the increase in IAA levels. The expression patterns of *EcUGT* and *EcDAO* are not correlated with the elevated IAA level in the S biotype, but it is known that the activity of *GH3s* is significantly higher than that of *DAO* (Mellor et al., 2016, Porco et al., 2016, Zhang & Peer, 2017) and the results of the present study, under the treatment of quinclorac, are consistent with *GH3s* being a dominant participant in IAA homeostasis. In agreement with the measured increase in IAA in the susceptible biotype, there were greater reductions in the levels of oxidase and peroxidase activities in this line, which promoted IAA accumulation. The novel data presented have illustrated that there are multiple components of the auxin response system which are engaged by treatment with SAH. The resistant biotype maintains low IAA levels by affecting a variety of steps in the auxin signalling system, which collectively could help to minimize ethylene synthesis and the associated accumulation of cyanide.

5 CONCLUSIONS

Fig. 4

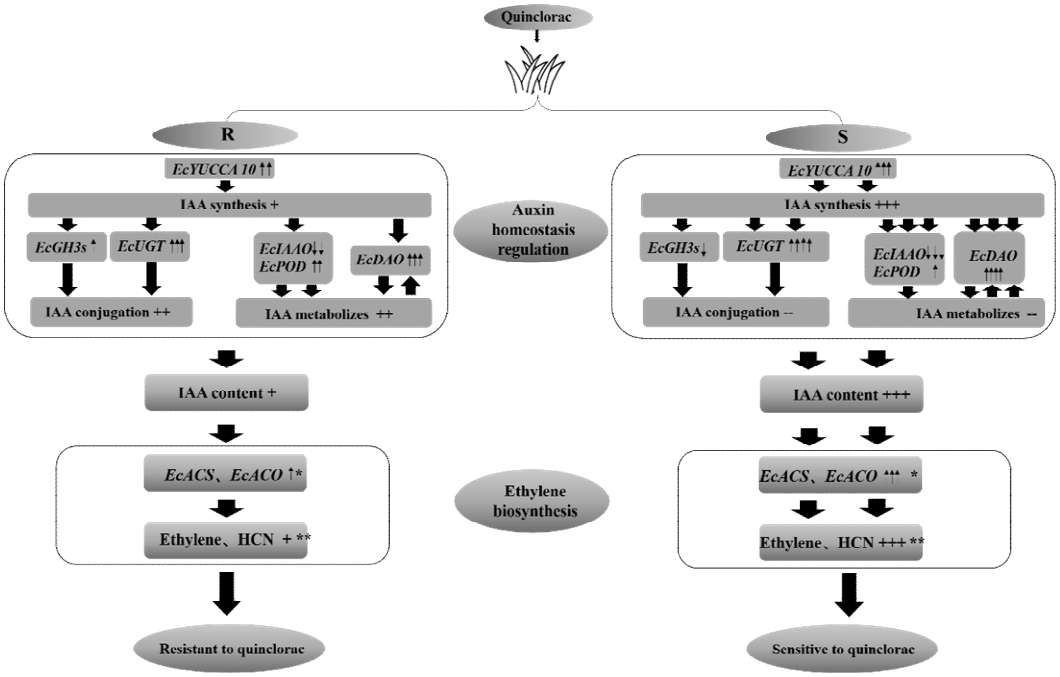

Fig4: Differences in the regulation of auxin homeostasis in resistant and sensitive biotypes. "↑" means up-regulated expression, up-regulated range ：0~50%、51%~150%、151%~500%、≥500%；"↓" means down-regulated expression:0~30%、31%~60%、61%-80%、81%~100%."+" means increase, "-" means decrease, the number of "+" indicates the difference in relative increment, the number of "-" indicates the difference in relative decrease. The number of ⬇ indicates the strength of regulation. *&**: Quoted from previous research results of our laboratory. (Xu et al., 2013, Gao et al., 2018)

Increasing auxin concentrations in plants, especially by the application of SAHs, directly disturbs plant growth or even causes lethal damage (Grossmann, 2007). In *Echinochloa spp.*, damage is largely associated with induced ethylene production, and ethylene biosynthesis enzymes are known to be induced by increases in auxin (Aharoni & Yang, 1983, Hansen & Grossmann, 2000). _ENREF_100Excessive IAA levels were observed in JNNX-S after treatment with quinclorac, whereas in SSXB-R there was less IAA synthesis, enhanced IAA conjugation mediated by *GH3*s, and enhanced IAA oxidation potential contributed by POD and IAA oxidases. Collectively, the data

reported above contribute to a novel understanding of the mechanism of resistance to quinclorac in *E. crusgalli* var. *zelayensis* (Fig.4). Efforts are still needed to elucidate the underlying mechanisms of resistance to synthetic auxin herbicides.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKONWLEDGEMENT

This research was funded by National natural science foundation of China (31871993). The authors thank Jiangyan Xu and Yuan Gao for the previous studies.

AUTHORS' CONTRIBUTIONS

Jun L. was involved in conceptualization and funding acquisition. Zerui Z., Xudong L. and Xukun P. were involved in methodology, investigation, writing – original draft and writing – review and editing. Richard Napier and Liyao D. were involved in formal analysis and visualization.

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