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A novel *EcGH3* gene with a different expression pattern in quinclorac-resistant and susceptible barnyardgrass (*Echinochloa crus-galli*)

Gang Li [a], Ming Fei Xu [a], Li Ping Chen [a], Lei Ming Cai [a], Lian Yang Bai [b], Chang Xing Wu [a,*]

[a] State Key Lab Breeding Base for Zhejiang Sustainable Plant Pest Control, Zhejiang Province Key Laboratory for Pesticide Residue Detection and Control, Institute of Quality and Standard for Agro-products, Zhejiang Academy of Agricultural Sciences, 198 Shiqiao Road, Hangzhou 310021, China
[b] Institute of Plant Protection, Hunan Academy of Agricultural Sciences, Changsha 410125, China

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ABSTRACT

Some plant *GH3* genes are specifically induced by auxin within minutes and play important roles in plant growth and development. A *GH3* cDNA was isolated from barnyardgrass. The gene, designated *EcGH3.1* (Genebank Number: JN241678), has a full-length 1839 bp open reading frame predicted to encode a 67.82 kDa protein. Sequence alignment showed that *EcGH3.1* is a *GH3* homolog. Its transcript level in leaves and roots of quinclorac-susceptible (S) biotypes was higher than that in quinclorac-resistant (R) biotypes. After exposure to quinclorac in 30 min, *EcGH3.1* expression was obviously decreased in leaves of S biotypes, but markedly increased in leaves of the R plants. *EcGH3.1* with its different expression patterns can be considered a marker and applied to distinguish R and S biotypes. The results of this study also provide basic information for further research of the function of *EcGH3* in barnyardgrass under herbicide stress.

1. Introduction

Barnyardgrass is a widespread and important agricultural weed species that infests rice, maize, and soybean fields. Its control is mainly dependent on herbicides. To date, 43 biotypes have been reported for this species that show resistance to seven different chemical classes of herbicides, namely, synthetic auxins, acetolactate synthase (ALS) inhibitors, acetyl coenzyme A carboxylase (ACCase) inhibitors, long-chain fatty-acid inhibitors, lipid inhibitors, microtubule inhibitors, and photosystem II inhibitors. In paddy fields in China, barnyardgrass has evolved resistance to the first five of the above-listed herbicides (Heap, 2015).

Quinclorac, a synthetic auxin, has been applied widely and frequently for control of barnyardgrass in China since 1990. Rice farmers began reporting failure of barnyardgrass control in 2000. Quinclorac-resistant barnyardgrass is now a problem in some rice farming in China (Li et al. 2003; Dong et al. 2005; Wu et al. 2007). However, the molecular mechanism of barnyardgrass resistance to quinclorac remains uncertain.

In a study of the resistance mechanism, we identified a novel gene belonging to the Gretchen Hagen 3 (*GH3*) family. Through examination of the literature, we found that the first plant *GH3* gene was identified in soybean (*Glycine max*) treated with 2,4-dichlorophenoxyacetic acid (2,4-D) (Hagen et al. 1984). Most studies of *GH3* homologs have focused on model plants such as *Arabidopsis thaliana* in which the *GH3* genes are represented by a multigene family consisting of 20 members (Hagen and Guilfoyle 2002) and classified into three groups (Staswick et al. 2002).

GH3 proteins function to maintain auxin homeostasis through adenylate synthetases with respect to plant hormones (Staswick et al. 2002) and amido synthetases that synthesize IAA–amino acid conjugates (Staswick et al. 2005). For example, in *Arabidopsis*, approximately 90% of IAA is in inactive forms conjugated via amide linkages to peptides or amino acids, approximately 10% is in inactive forms conjugated via ester linkages to sugars, and approximately 1% is present as free IAA (Woodward and Bartel 2005). Six GH3 enzymes from *Arabidopsis*, including GH3.2, GH3.3, GH3.4, GH3.5, and GH3.6, and GH3.17, conjugate IAA to multiple amino acids, such as alanine, leucine, aspartate, and glutamate, through amide bonds in vitro (Staswick et al. 2002, 2005). GH3.11 (JAR1) synthesizes adenylation-jasmonic acid conjugates to form the active isoleucine conjugate (Staswick et al. 2002). WES1 (GH3.5) can adenylate not only IAA but also SA (Staswick et al. 2005). Other auxin substrates for GH3 enzymes include phenylacetic acid, indole-3-butyric acid, indole-3-pyruvic acid, and a-naphthaleneacetic acid. Moreover, the auxin-like herbicide 2,4-D and benzoic acids (e.g., dicamba) are not substrates for GH3 enzymes (Staswick et al. 2005) but can induce *GH3.3* expression (Gleason et al. 2011). Thus, *GH3* genes play important roles in plant growth, development, and

* Corresponding author.
E-mail address: wucx@mail.zaas.ac.cn (C.X. Wu).

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environmental responses. *A. thaliana AtGH3.6* (*DFL1*) and *AtGH3.2* (*YDK1*) have been shown to negatively regulate shoot and hypocotyl cell elongation and lateral root formation (Nakazawa et al. 2001; Takase et al. 2004). *AtGH3-12* (*PBS3*) is known to regulate stress-induced SA metabolism and plant defense responses (Jagadeeswaran et al. 2007; Okrent et al. 2009). A ripening-related GH3 protein has been shown to be involved in the regulation of endogenous IAA concentrations in grape berries (*Vitis vinifera* L.) (Böttcher et al. 2011). Furthermore, differential expression of two *GH3* genes has also been reported in a stage- and tissue-specific manner during fruit development in longan (*Dimocarpus longan*) (Kuang et al. 2011).

Accurate and timely diagnosis of weed resistance levels is the first step to weed management and weed population mitigation. The classical approach to determining resistance is through a whole-plant bioassay; however, this approach is not suitable for large-scale testing. Therefore, simpler and quicker screening methods are needed for identification of weed resistance and control, and also for research purposes. Some relatively quick assays have been developed, such as the Petri-dish bioassay of seed germination (Bechie et al. 1990), agar-based seedling assays (Kaundun et al. 2011), leaf disk assays (Gerwick et al. 1993), and pollen germination test (Letouze and Gasquez 2000). DNA-based methods, including DNA sequencing (Powless and Yu 2010) and single nucleotide polymorphism assay (Guttieri et al. 1992) have also been applied to determine the resistance of weeds to herbicides.

To our knowledge, there have been no reports on *GH3* genes from barnyardgrass or on RNA expression-based assays for resistance to herbicides. Here, we report a novel *GH3* gene from barnyardgrass and describe its expression pattern. This study involved identification of the *EcGH3.1* gene from resistant (R) biotypes, analysis of transcript levels of the gene in R and sensitive (S) biotypes, and analysis of their phylogenetic relationships. We propose a new screening method for herbicide resistance in barnyardgrass based on the different expression patterns of *EcGH3.1* in R and S biotype seedlings. Our study also provides more information on the function of the *GH3* gene in plant responses to stress induced by the herbicide quinclorac.

2. Materials and methods

2.1. Echinochloa crus-galli material

Seed samples from barnyardgrass populations were collected in September 2012 from separate rice fields in Shaoxing Country of Zhejiang Province in China. Since the 1980s, these rice fields had been treated every year with quinclorac to control barnyardgrass. In the summer of 2013, seed samples were sown into 15-cm-diameter plastic pots containing potting mix (50% peat and 50% river sand) and grown in a glasshouse with day/night temperatures of 32/25 °C under natural sunlight. Plants were watered and fertilized regularly. After 7 days, seedlings with 3–4 leaves were separately transplanted and isolated to ensure self-pollination. Seeds from each selfed plant were harvested separately. In the summer of 2014, seeds from each selfed parental plant were sown and the seedlings used for a resistance assay. Seedlings with 2–3 leaves were sprayed with quinclorac at the normal field rate of 300 g of active ingredient ha^{-1}. We performed three replicate treatments with 20 plants in each replicate. The test was repeated twice. After treatment, plants were identified as R biotypes if they survived and as S biotypes if they died. The remaining seeds from the same single plants were then identified as R and S biotypes and grown to the 2–3 leaf stage for molecular analysis.

2.2. Amplification of the central region of the EcGH3.1 sequence

Leaves from six identified R plants were used to extract total RNA using a TransZol reagent kit (TransGen Biotech, China). First-strand cDNA was synthesized from 1 μg of total RNA using RNaseH-M-MLV reverse transcriptase (TransGen Biotech, China). The central region of the *EcGH3.1* sequence was amplified from the cDNA using two pairs of degenerate primers (Table 1), which were designed based on the highly conserved regions of *GH3* determined by alignment of homologous sequences (Table 2). The amplification product of the first round of PCR (primers: *EcGH3.1*-F1 and *EcGH3.1*-R1) was used as the template for the second round of PCR (primers: *EcGH3.1*-F2 and *EcGH3.1*-R2). The amplification regime consisted of an initial denaturation step of 98 °C for 2 min, followed by 35 cycles of 98 °C for 10 s, 57 °C for 5 s, and 72 °C for 48 s, and finally an extension step of 72 °C for 10 min. The PCR fragment was separated, purified, and cloned into a pEASY-Blunt cloning vector (TransGen Biotech, China). Five inserts were sequenced and then analyzed using DNASTAR Lasergene v7.1 software (DNASTAR, USA).

All primers were designed using Primer Premier 5.0 (Premier, Canada) and Oligo 6.0 (Cascade, USA) software. Synthesis and DNA sequencing were performed by Sangon Biotech Co. Ltd. (Shanghai, China).

Table 1
Sequences of PCR primers used for cDNA isolation and real-time PCR.

Primer type	Primer name	Sequence
Degenerate primers		
	EcGH3.1-F	CBTAYACYATVATGCCHAACATG
	EcGH3.1-R	CCBCTKGARATKGCRTARTCCAT
RACE primers		
	EcGH3.1-5'RACE-R1	CAGCACCGCTCCAGCGTCTCATCATC
	EcGH3.1-5'RACE-R2	CCGTCTTGTCGGACTCGATGGACAGC
	EcGH3.1-3'RACE-F1	TGCCTAACATGGGCTACTTCGAGTTCCTC
	EcGH3.1-3'RACE-F2	GGCGCCGCAGTTCAGGTTCGT
Verification primers		
	F1	CCCGTCCCCTCCTCTGCTGCTA
	R1	CTGCTCCCAGTGGAGCTGGAGGAA
	F2	CTCCTGTTCCATTCCATCGGCACC
	R2	GTACGGGTCGTAGGGACGGTTCTTG
	F3	CTGATGCCCACCATCAAGGAGGAGC
	R3	CCACGGGGAGGAACTCGAAGTAGC
	F4	CGGGGCTGGACAAGGGCAAGG
	R4	CCATGTTGGGCATGATGGTGTAGGAG
	F5	CCATGTACGCCTCCTCCGAGTGCTA
	R5	CGACGAAAGTGGCAGTATTCTACTACCTG
	F6	GGTCTCCTACACCATCATGCCCAACA
	R6	GGCACCTTGTACTGGTTGATGGACGC
	F7	CAGCAATAAGCACCTACTCATCAC
	R7	CTTGGCATACAAACTCTAGCACTG
Real-time PCR primers		
	Ec-actin(HQ395760)-F	GTGCTGTTCCAGCCATCGTTCAT
	Ec-actin(HQ395760)-R	CTCCTTGCTCATACGGTCAGCAATA
	EcGH3.1-Realtime-F	CGCCGCAGTTCAGGTTCGTGC
	EcGH3.1-Realtime-R	CTCCGCCTCGTCCGTCTTGTC

2.3. Rapid amplification of cDNA ends (RACE)

The central region of *EcGH3.1* was extended in both the 5′ and 3′ directions by RACE-PCR using a GeneRacer kit (Invitrogen, US). The primer EcGH3.1-5'RACE-R1 (Table 1) and the 5′ RACE outer primer provided in the GeneRacer kit were combined for the first round of 5'RACE with the following conditions: 94 °C for 2 min, followed by five cycles of 98 °C for 10 s and 72 °C for 60 s; five cycles of 98 °C for 10 s and 70 °C

Table 2
EcGH3.1 homologs used to design the degenerate PCR primers.

Homolog number	Source species	GenBank accession no.
1	*Arabidopsis thaliana*	NM119902
2	*Ricinus communis*	XM002524901
3	*Nicotiana tabacum*	AF123503
4	*Capsicum chinense*	AY525089
5	*Vitis vinifera*	XM002271216
6	*Oryza sativa* 'Indica'	EF103572
7	*Zea mays*	NM001158280