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K c d f Cadhe i ge e confers resistance to Cry2A and Cry1C in *Chilo suppressalis*

Za Zha g[1] Xia Te g[2] Weih a Ma[1] Fei Li[3]

Bacillus thuringiensis B C i a a i a ei he a age e f i ec e Re i a ce B i ha bee e ed i a ei ec b he echa i e ib ef hi resistance in rice crop pests remains largely unknown. Cadherin is one of several Bt toxin receptors. At e e e cadhe i ge e *CsCAD1* ha bee d c e ed i he i ed ice e b e *Chilo suppressalis.* We amplified a nearly full-length transcript of another *C. suppressalis* cadhe i ge e *CsCAD2*, and found that it has a different expression pattern to *CsCAD1 CsCAD1* a high e e ed in fifth and sixth instar larvae, especially in the midgut, while the expression levels of *CsCA2* e e e ab i each de e e a age Ne ha ched a ae e e fed ice ea ed i h he i ed siRNA to knockdown either *CsCAD1 CsCAD2* a d he e e fed a ge ic ice e e i g ei he he *Cry2A Cry1C* toxins. The siRNA-treatment groups had lower mortality and higher survival rates than he c g gge i g ha ed ced e e i f *CsCAD1 CsCAD2* i c ea ed e i a ce *Cry2A* a d *Cry1C* We c c de ha *CsCAD1* a d *CsCAD2* i e ac i h B i i *C. suppressalis* a d ha hi i e ac i c d be he echa i de i g B e i a ce i hi i ec

Insecticidal Cry proteins from *Bacillus thuringiensis* (Bt) have been widely used to develop transgenic crops that have become an important part of agricultural pest management[1 3]. Cry toxins are ingested by digestive proteases in the midgut of insects where the activated toxins interact with midgut brush border membrane proteins, including cadherin[4], ABC type C transporters (ABCCs)[5, 6], alkaline phosphatase (ALP)[7, 8], and aminopeptidase N (APN)[8, 9]. Cry toxins are integrated into the membrane, leading to pore formation, cell lysis and insect death[10]. However, the development of resistance to Bt toxins in many pest insects threatens to make transgenic Bt crops redundant[11].

e development of resistance to Bt toxins in insects has been associated with mutation, down-regulation, or deletion, of Bt receptors[2, 12, 13]. Cadherin, a calcium-dependent cell adhesion protein[14], is thought to be one of several such receptor proteins that bind to Cry toxins[15, 16]. e rst such cadherin protein to be identi ed was the *Cry1A* toxin-binding protein in *Manduca sexta*[17], which was then found to be involved in binding Cry toxins in other Lepidopteran, coleopteran and dipteran insects[4, 18 23]. However, the a nity of cadherins for di erent Cry toxins varies in di erent insects. Fox example, some Cry toxins are not lethal to the Coleoptera or Lepidoptera[24, 25].

e striped rice stem borer, *Chilo suppressalis* Walker, is one of the most destructive rice pests in China and other Asian countries. Transgenic rice strains expressing the Cry toxins *Cry2A* and *Cry1C* have been developed to protect rice crops from this notorious pest. However, it is likely that *C. suppressalis* will develop resistance to these toxins once transgenic rice becomes more widely grown. It is, therefore, important to understand the mechanisms that confer resistance to Cry toxins in this species.

A cadherin-like *C. suppressalis* gene (*CsCAD1*, AY118272) has been deposited in the NCBI GenBank[26 28]. We used Rapid-ampli cation of cDNA ends (RACE) to clone another *C. suppressalis* cadherin gene, which we named *CsCAD2*, and investigated the expression of both genes in di erent *C. suppressalis* developmental stages and tissues. We found that knockdown of these genes reduced sensitivity to both *Cry2A* and *Cry1C* in *C. suppressalis*.

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Amplifying the *CsCAD2* ge e We obtained a fragment of a cadherin gene from the transcriptome of *C. suppressalis* and, a er mapping this fragment onto the *C. suppressalis* genome[29], found that this gene had not previously been reported. We named this newly discovered gene *CsCAD2*. By amplifying the transcript with RACE and incorporating information from the *C. suppressalis* genome, we obtained a nearly full-length transcript of *CsCAD2*, including a 5 untranslated coding region (UTR), open reading frame (ORF). e *CsCAD2* ORF was 4,912 bp, encoding 1,493 amino acids. e *CsCAD2* protein sequence had high identity with other insect cadherins, for example 88% with that of *Bombyx mori* and 86% with that of *Plutella xylostella*.

Ge e c ea d h ge e ic a a i f *CsCAD1* a d *CsCAD2* e nucleic acid sequences of *CsCAD1* and *CsCAD2* were aligned with the genome of *C. suppressalis* to obtain the structures of both genes (Fig. 1A). e two genes are located in di erent sca olds of the genome. *CsCAD1* had 41 exons and 40 introns and a length of 44,762 bp, whereas *CsCAD2* had only 24 exons and 23 introns and a length of 316,095 bp. Surprisingly, the rst *CsCAD2* intron was very long; 253,600 bp. Conserved domain analysis indicates that both *CsCAD1* and *CsCAD2* have characteristics that are conserved in other cadherin proteins. *CsCAD1* had eight cadherin repeat domains, two Ca^{2+} binding sites and one trans-membrane region, whereas *CsCAD2* had seven cadherin repeat domains, ve Ca^{2+} binding sites and one trans-membrane region (Fig. 1B). Comparison of *CsCAD1* and *CsCAD2* with nine other cadherin genes of three well-studied insects; *B. mori*, *P. xylostella* and *D. melanogaster*, indicated that *CsCAD1* was conserved in Lepidopteran. However, *CsCAD2*

A

B
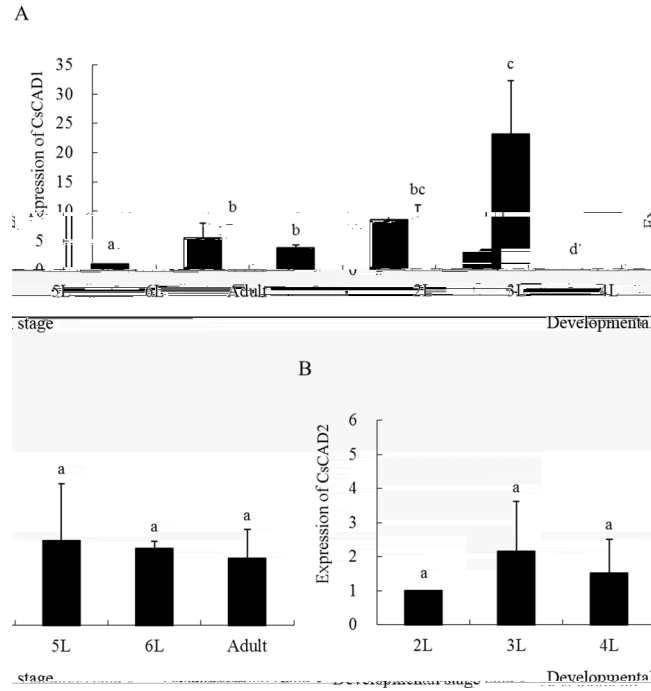
Figure 2. Relative abundance of mRNA of the *Chilo suppressalis* cadherin genes (**A**) *CsCAD1* and (**B**) *CsCAD2* in different larval instars and adults. Results are means ± SE. Bars with the same lowercase letter are not significantly different.

A

B
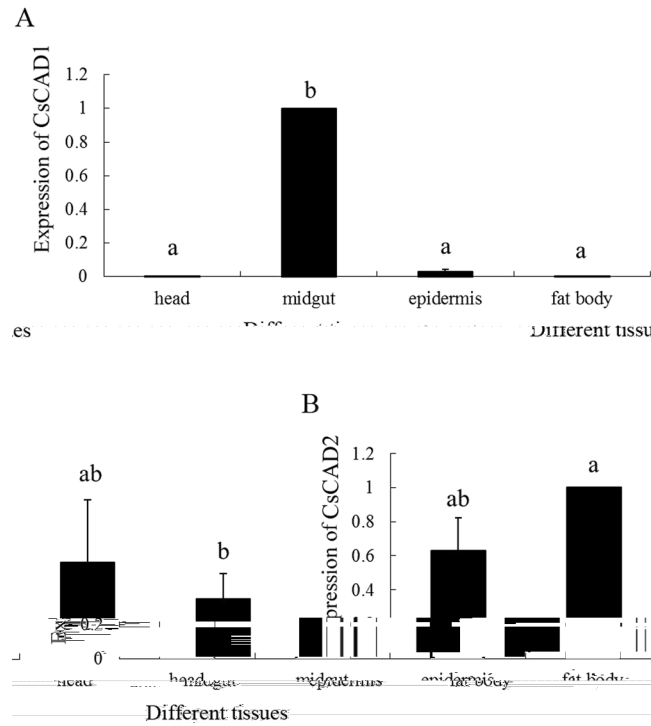
Figure 3. Relative abundance of mRNA of the *Chilo suppressalis* cadherin genes (**A**) *CsCAD1* and (**B**) *CsCAD2* in different body parts. Results are means ± SE. Bars with the same lowercase letter are not significantly different.
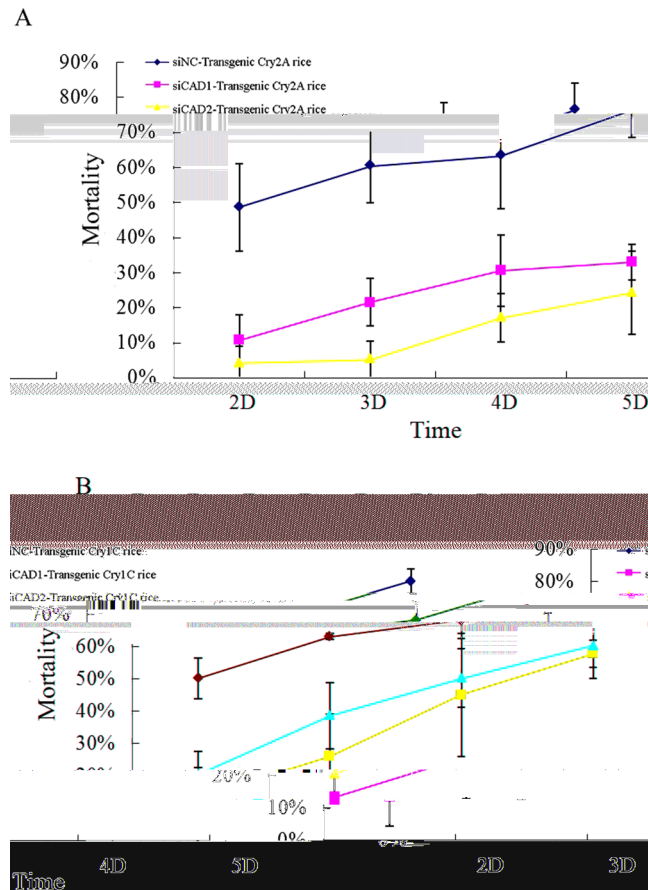
Figure 5. Mortality of *Chilo suppressalis* larvae treated with siRNA designed to silence the cadherin genes *CsCAD1* or *CsCAD2*, a er feeding on transgenic rice expressing (**A**) *Cry2A* or (**B**) *Cry1C*; siNC = negative control, si*CAD1* = *CsCAD1* knockdown treatment group, siCAD2 = *CsCAD2* knockdown treatment group.

 >80% humidity.   e midguts of fourth instar larvae were dissected, immediately frozen in liquid nitrogen and stored at − 70 C until required for RACE.

Two transgenic rice strains, one expressing *Cry2A* and the other *Cry1C*, were used in experiments.   ese strains were derived from the same parental strain, Minghui 63, which was used as the negative control. Rice seeds were soaked for three days and then germinated in petri dishes on wet   lter paper. All rice seeds were kindly provided by Prof. Yong-Jun Lin of Huazhong Agricultural University.

Total mRNA isolation and cDNA synthesis.   Whole bodies of di erent developmental stages of *C. suppressalis* (2nd to 6th instar larvae and adults), and speci c body parts (head, midgut, epidermis, fat body), were   rst homogenized in a tissue grinder. TRIzol reagent (GIBCO, USA) was then used to isolate total mRNA from these samples according to the manufacturer's protocol. Genomic DNA was removed from total RNA with a DNA-free kit (Ambion, USA).   e integrity of the RNA obtained was checked by electrophoresis on a 1.5% agarose gel.   e 260/280 nm absorbance ratios of all RNA samples were between 1.8 and 2.2. First strand cDNA was synthesized using M-MLV reverse transcriptase (Takara, Japan) with Oligo (dT18) as the anchor primer.   e reaction mixtures were incubated at 70 C for 10 min followed by 42 C for one hour and 70 C for 15 min.   e cDNA was stored at − 20 C for further use.

Quantitative real-time PCR.   Quantitative real-time PCR (qPCR) was carried out with a SYBR Premix Ex Taq kit (Takara) using an ABI Prism 7300 (Applied Biosystems, USA) to detect the expression of *CsCAD1* and *CsCAD2* in the midgut of di erent development stages, and in di erent body parts. Primers were designed with Beacon Designer 7 (Table S1) and dissolution curves and gel electrophoresis were used to determine primer speci city.   e ampli cation e ciency of all primers was checked with a cDNA dilution gradient, a er which 2 μL of cDNA template was used in the PCR reaction according to the PCR kit's protocol.   e qPCR began at 95 C for 30 secs, followed by 40 cycles of 95 C for 5 secs, annealing at 60 C for 31 sec, ending with cycles of 95 C for 15 sec, 60 C for 60 sec, and 95 C for 15 sec.   e speci city of the qPCR reactions was monitored with melting curve analysis using SDS so ware (version 1.4) and gel electrophoresis. Ampli cation e ciencies were determined by a series of template dilutions. All experiments were repeated in triplicate.   e raw Ct values were obtained using ABI 7300 SDS so ware (Version 1.4).   e standard Delta-Delta-Ct method was used to analysis the qPCR
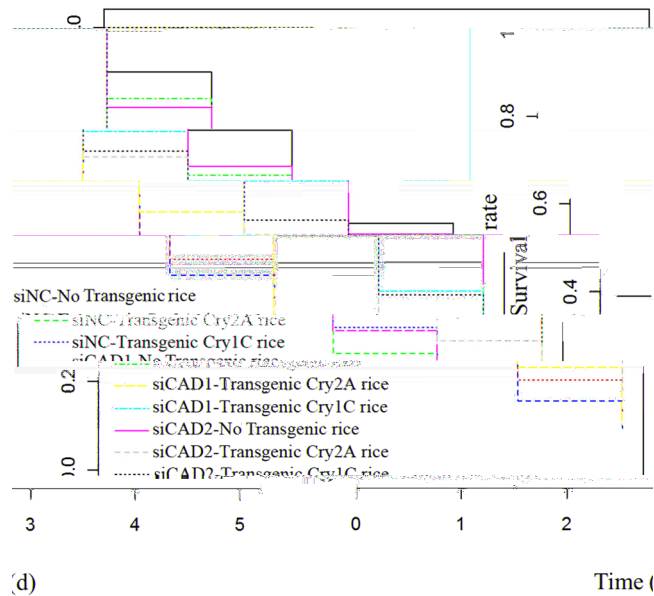
Figure 6. COX proportional hazard models for survival analysis; siNC-non-transgenic rice = negative control group feeding on non-transgenic rice, si*CAD1*-non-transgenic rice = the *CsCAD1* knockdown treatment group feeding on non-transgenic rice, si*CAD2*-non-transgenic rice = the *CsCAD2* knockdown treatment group feeding on non-transgenic rice, siNC-Transgenic Cry2A rice = the negative control group feeding on transgenic rice expressing Cry2A; si*CAD1*-Transgenic Cry2A rice = the *CsCAD1* knockdown group feeding on rice expressing Cry2A; si*CAD2*-Transgenic Cry2A rice = the *CsCAD2* knockdown treatment group feeding on transgenic rice expressing Cry2A, siNC-Transgenic Cry1C rice = negative control group feeding on transgenic rice expressing Cry1C, si*CAD1*-Transgenic Cry1C rice = the *CsCAD1* knockdown treatment group feeding on transgenic rice expressing Cry1C, si*CAD2*-Transgenic Cry1C rice = *CsCAD2* knockdown treatment group feeding on transgenic rice expressing Cry1C.

data. The housekeeping genes E2F and GAPDH (GenBank No.: DQ311161.1 and AB262581.1) were used as the internal controls. Significant differences among multiple means were determined using Tukey's HSD (P < 0.05).

RACE amplification of *CsCAD2*　　Total RNA was extracted from the midgut of the 3rd instar larvae. RACE amplification was carried out with a SMARTer RACE cDNA Amplification Kit (Takara) according to the manufacturer's protocol. Fragments of *CsCAD2* cDNA were obtained from the transcriptome data used in previous studies[33]. The primers CTCATTACCTCCCTCCCACTCGGCAG (5′ RACE) and TGACAATCCACCACATTTCACGCAGG (3′ RACE) were designed, based on the sequences obtained, to amplify the full-length of the *CsCAD2* gene. The end-to-end primers (5′ AAACTTAATAGGCTTACTCGTTCTACC and 3′ GCTGTTCCCTGTCAAATGTCAC) were designed to amplify the full length of the *CsCAD2* gene. PCR products were inserted into vector (Takara, Dalian, China) and sequenced by the Nanjing Genscript Company, China. The transcriptome and genome data[29] were used to obtain the full-length transcript of *CsCAD2*. The resultant sequence was submitted to GenBank (Accession No. JQ747493).

RNA interference of cadherins.　　Two types of siRNA, si*CAD1* and si*CAD2*, were used to silence the *CsCAD1* and *CsCAD2* genes, respectively, and a random sequence siRNA was included as a negative control (siNC) (Table S2). All siRNAs were synthesized by the GenePharma Company. Larvae were treated with siRNAs in petri dishes with wet filter paper on the bottom. The siRNA was smeared onto 4 cm-long sprouts of non-transgenic rice and about 1000 newly hatched larvae were then put in the petri dishes to feed on the treated rice sprouts. Rice sprouts were replaced every 4 hrs for three days. All experiments were conducted at 27 C and were repeated in triplicate so that there about 3000 insects were used per treatment and nearly 10 thousand in total.

Larvae from each treatment were used to assess both susceptibility to Bt transgenic rice and investigate gene expression. Susceptibility to transgenic rice was assessed in a randomly selected group of 90 larvae. These were subdivided into three groups of 30 which were randomly assigned to feed on either transgenic rice expressing *Cry2A* toxin, transgenic rice expressing *Cry1C* toxin, or non-transgenic rice. For another part, to examine the gene expression change, 50 insects were randomly selected at the 2nd day after the insects were fed on transgenic rice. All experiments were repeated in triplicate.

Gene identification and analysis　　Phylogenetic analysis was conducted in MEGA (v6.0) using the Neighbor-joining method with 1000 bootstrap replications. Domain structures of candidate cadherin genes were analyzed using CD Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Signal peptides were predicted with SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and transmembrane domains with TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Cox' proportional hazard model implemented in

Program R (R Core Team R version 3.2.3) was used to analyze changes in survival from the 2nd to 5th day a er larvae had commenced feeding on transgenic rice plants.

Refe e ce

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Author Contributions

Z.Z. analyzed the data, made the figures and tables and wrote the manuscript. X.L.T. carried out experiments and analyzed the data. W.H.M. contributed to the discussion. F.L. designed the project, analyzed the data and also contributed to writing the manuscript.

Additional Information

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