Down Regulation of a Gene for Cadherin, but Not Alkaline Phosphatase, Associated with Cry1Ab Resistance in the Sugarcane Borer *Diatraea saccharalis*

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Abstract

The sugarcane borer, *Diatraea saccharalis*, is a major target pest of transgenic corn expressing *Bacillus thuringiensis* (Bt) proteins (i.e., Cry1Ab) in South America and the mid-southern region of the United States. Evolution of insecticide resistance in such target pests is a major threat to the durability of transgenic Bt crops. Understanding the pests’ resistance mechanisms will facilitate development of effective strategies for delaying or countering resistance. Alterations in expression of cadherin- and alkaline phosphatase (ALP) have been associated with Bt resistance in several species of pest insects. In this study, neither the activity nor gene regulation of ALP was associated with Cry1Ab resistance in *D. saccharalis*. Total ALP enzymatic activity was similar between Cry1Ab-susceptible (Cry1Ab-SS) and -resistant (Cry1Ab-RR) strains of *D. saccharalis*. In addition, expression levels of three ALP genes were also similar between Cry1Ab-SS and -RR, and cDNA sequences did not differ between susceptible and resistant larvae. In contrast, altered expression of a midgut cadherin (*DsCAD1*) was associated with the Cry1Ab resistance. Whereas cDNA sequences of *DsCAD1* were identical between the two strains, the transcript abundance of *DsCAD1* was significantly lower in Cry1Ab-RR. To verify the involvement of *DsCAD1* in susceptibility to Cry1Ab, RNA interference (RNAi) was employed to knock-down *DsCAD1* expression in the susceptible larvae. Down-regulation of *DsCAD1* expression by RNAi was functionally correlated with a decrease in Cry1Ab susceptibility. These results suggest that down-regulation of *DsCAD1* is associated with resistance to Cry1Ab in *D. saccharalis*.

Introduction

Evolution of insecticide resistance in target pests threatens the durability of transgenic crops expressing toxins from *Bacillus thuringiensis* (Bt). To date, field resistance that resulted in control failures or reduced efficacy of Bt crops has been documented in several target pest species of Bt corn and Bt cotton [1,2,3,4]. Knowledge of Bt resistance mechanism is essential in understanding Bt resistance evolution and for developing effective management strategies. The most common mechanism of Bt resistance in the insect species that have been investigated is cadherin-mediated resistance. Recent studies [5,6] showed that reduced level of membrane-bound ALPs is associated with Cry1Ab-resistant larvae. In contrast, altered expression of a midgut cadherin (*DsCAD1*) was associated with the Cry1Ab resistance. Whereas cDNA sequences of *DsCAD1* were identical between the two strains, the transcript abundance of *DsCAD1* was significantly lower in Cry1Ab-RR. To verify the involvement of *DsCAD1* in susceptibility to Cry1Ab, RNA interference (RNAi) was employed to knock-down *DsCAD1* expression in the susceptible larvae. Down-regulation of *DsCAD1* expression by RNAi was functionally correlated with a decrease in Cry1Ab susceptibility. These results suggest that down-regulation of *DsCAD1* is associated with resistance to Cry1Ab in *D. saccharalis*.

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knock-down cadherin gene expression in Cry1Ab-SS strain, and establish a functional correlation between susceptibility to Bt and reduced cadherin expression.

Materials and Methods

Insect strains

A Cry1Ab-resistant strain (Cry1Ab-RR) of *D. saccharalis* was established from a single two-parent family-line collected in a corn field near Winnboro, Louisiana (Franklin Parish) during 2004 [12]. This resistant strain was shown to carry a major resistance allele identified using a F2 screen method. It has a significant resistance (~100-fold) to purified trypsin-activated Cry1Ab toxin [14], and individuals of the Cry1Ab-RR strain can complete larval development on commercial Bt corn hybrids expressing the Cry1Ab protein. A Cry1Ab-susceptible strain (Cry1Ab-SS) of *D. saccharalis* was also developed from the same location. The Cry1Ab-RR strain had been backcrossed with the Cry1Ab-SS strain and re-selected on Cry1Ab corn leaf tissue for 3-4 times before it was used in this study.

Quantification of alkaline phosphatase (ALP) activities

To examine the ALP activity, 3rd, 4th, or 5th instars from both Cry1Ab-SS and -RR strains of *D. saccharalis* were dissected in cold 0.1 M Tris-HCl buffer (pH 8.0) to obtain entire guts and gut contents. Each gut was homogenized individually with 50 μl of 0.1 M Tris-HCl buffer (pH 8.0) and centrifuged at 10,000×g for 5 min at 4°C. The quantity of protein in homogenate supernatants was determined by the method of Bradford [15] with bovine serum albumin as the standard. Specific ALP enzymatic activities of midgut homogenates were measured with p-nitrophenyl phosphate disodium (pNPP) (Sigma) as substrate using the method described in [16]. The quantity of enzyme producing 1 μmol of chromogenic product p-nitrophenol (p-NP) was used in calculation of the specific ALP activities. For each combination of insect strain and instar, ALP activity was measured from ten reactions (replications) and two readings (sub-samples) for each reaction. Specific ALP activities are presented as means and standard errors of the mean (±SEM). Activity data were analyzed with a two-way analysis of variance (ANOVA) using the GLM procedure [16]. Treatment means were separated using LSMEANS tests at the α = 0.05 level [16].

cDNA library construction

To clone full-length ALP and cadherin cDNAs, cDNA libraries of Cry1Ab-SS and -RR strains of *D. saccharalis* were constructed, sequenced, and subjected to Blast search of GenBank databases as described in [13]. Guts from ten 4th instars of each of the Cry1Ab-SS and -RR strains of *D. saccharalis* were dissected and homogenized using TRIzol reagent (Invitrogen). Total RNA was precipitated from the homogenates according to the manufacturer’s protocols. By using Nuclear Trump RNA purification kit (Clontech), mRNA was purified and used as template for the first strand cDNA synthesis. To ensure an appropriate yield of double strand cDNAs, these cDNAs were synthesized using a combination of primer extension and PCR amplification. For cDNA library construction, cDNAs were ligated into pDNR-LIB vector using Creator SMART cDNA Library Construction kit (Clontech). The ligation products were used to transform TOP10 competent cells (Invitrogen). Approximately 12,500 clones were obtained and sequenced using an ABI 3730XL DNA analyzer. Annotation of the transcriptomes was performed by using BlastX-NR similarity search of GenBank nucleotide database at National Center for Biotechnology Information (NCBI) [17].

Cloning full-length cDNAs coding for three ALPs and one cadherin proteins of *D. saccharalis*

BlastX similarity searches of both the cDNA libraries yielded 30 and three clones that matched the ALP and cadherin genes in GenBank, respectively. By using SeqMan module of the Lasergene (DNASTar), these ALP and cadherin cDNA clones were assembled into cDNAs partially coding for three different ALPs and one cadherin, which were designated DsALP1, DsALP2, DsALP3, and DsCAD1, respectively. Cloning the full-length cDNAs of the DsALPs and DsCAD1 was achieved using the similar procedures described in [18] with some modifications as described in [13]. Briefly, to obtain the full length cDNAs, total RNA was extracted from guts of 3rd instars from Cry1Ab-SS and -RR strains as described above. The concentration of total RNA was determined using NanoDrop spectrophotometer (Thermo Fisher Scientific). The SuperScript First Strand cDNA Synthesis kit (Invitrogen) was used in a reverse transcriptase polymerase chain reaction (RT-PCR) with 5 μg of total RNA and an oligo-dT primer for cDNA synthesis. The template RNA was removed by adding 1 μl RNase H (2 U) after cDNA synthesis.

For cloning the three relatively short DsALPs cDNAs (~1.5 kb), specific primers (Table S1) were directly designed to clone the major portions of cDNAs based on the corresponding sequences from the libraries. These primers were then used to directly amplify cDNA fragments with complete 3′-ends via PCR reactions. The 5′-end of the cDNA for each of the three DsALPs was obtained by using the 5′ rapid amplification of cDNA end (5′ RACE) system (Invitrogen). Based on the cDNA sequence obtained from cDNA libraries, two specific reverse primers for each of the three DsALPs (Table S1) were designed and used in the semi-nested amplifications with a forward abridged anchor primer from 5′-RACE kit. The 5′-end of the cDNA was isolated and C-tailed, and then was cloned into a pGEM-T vector. Plasmid DNAs were prepared and sequenced using an ABI 3730XL DNA analyzer to confirm the full coding sequences of the three DsALPs.

To determine the major cDNA sequence of the relatively long DsCAD1 (~5 kb), four degenerate forward primers (Table S2) were designed based on the conserved regions of 10 cadherin cDNAs previously identified in other lepidopteran species. The amino acid sequences of the four conserved regions of the cadherin genes were ITQRQDYE, LINWNDE, ATDIDGP, and DEDGLHAG. In addition, one specific reverse primer, DsCAD1R0 (Table S2), was also designed based on the partial DsCAD1 sequence from the cDNA libraries. In each PCR reaction, a fragment of expected size was amplified using one degenerate forward and one degenerate/specific reverse primers.

The 3′-end of the cDNA was amplified with a specific forward primer, named DsCAD1F2 (Table S2) along with the oligo-dT primer, while the 5′-end of the cDNA was obtained by using the 5′ RACE system with two specific reverse primers (Table S2) and sequenced following the same protocol described above.

To obtain error-proof full-length cDNAs for the three DsALPs and DsCAD1, a thermal-stable proof-reading Platinum High Fidelity Taq DNA polymerase (Invitrogen) was used in the subsequent PCR reactions. Total RNAs extracted from Cry1Ab-SS and -RR strains were used for synthesizing RT-cDNA. Three pairs of specific primers for the three DsALPs, flanking the 5′-and
3’-untranslated regions (Table S1), were used to re-amplify each of the three cDNAs. To obtain full length of DsCAD1 cDNA, three pairs of primers were designed (Table S2) with an average of 150 bp overlapping. The full-length cDNAs for each gene were re-amplified using the RT-cDNAs from Cry1Ab-SS and -RR strains, respectively. The PCR products were purified using a Quick PCR purification kit (Qiagen) and sequenced from both directions as described above.

Identification and phylogenetic analyses of three DsALPs and DsCAD1

Presence of a signal peptide at the N-terminus of the deduced protein sequence of three DsALPs and DsCAD1 was determined using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP), while molecular weight and protein isoelectric points were predicted using the ExPaSy Compute pI/Mw tool [http://ca.expasy.org/tools/pi_tool.html] [19]. Analysis of deduced protein sequences was conducted in the Myhits server (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Two GPI modification site prediction servers (PredGPI: http://gper2.biocomp.unibo.it/gp/; and GPI-SOM: http://gpi.unibe.ch/) were used to predict the GPI-anchor signal sequence and GPI anchoring sites of the three DsALPs. Presence of N- and O-glycosylation on the predicted protein sequences of three DsALPs and DsCAD1 was tested using the NetNGlyc 1.0 and NetOGlyc 3.1 servers (http://www.cbs.dtu.dk/services), respectively. Sequence-similarity analyses were performed using Blast through the NCBI [17]. Sequence comparisons were conducted using the ClustalW [20]. The Molecular Evolutionary Genetics Analysis (MEGA) (Ver. 4.1) [21] was used to perform multiple-sequence alignments and to examine phylogenetic relationships with the ALP or with the cadherin amino acid sequences of other lepidopteran species available in the GenBank.

Quantitative real-time PCR

Total RNAs from 3rd and 5th instars of the Cry1Ab-SS and -RR strains of D. saccharalis were extracted as described above. Total RNAs then were treated with 2 μl DNaseI (1 mg/ml) (Boehringer Mannheim GmbH) at 37°C for 1 h to remove any residual DNA. For each treatment replication, three guts were pooled and total RNAs were extracted from the pooled samples. Concentrations of total RNAs, as measured with the NanoDrop spectrophotometer, were adjusted to 1 ng/μl. The iScript One-Step RT-PCR Kit with SYBR green (Bio-Rad) was used in a 25 μl reaction of Quantitative real-time PCR (qRT-PCR). For each combination of instar and insect strain, there were three replications in the qRT-PCR analysis. To obtain the absolute mRNA quantities of the three DsALPs and DsCAD1, two qRT-PCR were performed for each gene as described in [13]. The ribosomal 18S gene was used to estimate RNA concentration for each sample. One pair of specific primers, Ds18SF1 and Ds18SR1 (Table S1), was designed to produce amplicons of 139, 145, 143, and 112 bp, respectively. A partial cDNA fragment for each of these four genes was amplified and prepared as internal standards. Serial dilutions of internal standard (0.001, 0.01, 0.1, 1, and 10 pg/μl) were used to establish a standard curve as described above. Five microliters of the internal standard solution or standardized total RNA templates (10 ng/μl) were added into each reaction along with a negative control that contains all components and 5 μl ddH2O to replace the RNA template. Upon completion of the RT-PCR, a dissociation curve analysis was conducted to verify the absence of any nonspecific amplicons. Based on the internal standard curve of the second qRT-PCR, absolute quantities of RT-cDNA of the three DsALPs and DsCAD1 were compared at 3rd and 5th instars between Cry1Ab-SS and -RR strains. There were 3–4 replications for each treatment. The transcript levels were presented as means and standard errors of the mean (±SEM). Results from gene expression assays were analyzed with a three-way (for DsALPs) or a two-way (for DsCAD1) ANOVA using the GLM procedure [16]. Treatment means were separated using LSMEANS tests at the α= 0.05 level [16].

RNA interference of DsCAD1

To examine the functional linkage between the down-regulation of DsCAD1 and Cry1Ab resistance, RNA interference (RNAi) was used first to knock down DsCAD1 in the Cry1Ab-SS strain and then to assess susceptibility change using a bioassay. One pair of DsCAD1-specific primers, both containing the T7 promoter sequence (5’-TAATACGACTCACTATAGGG-3’), was designed to flank to position 488 to 888 of DsCAD1. DsCAD1 cDNA was used as template in PCR reaction to amplify a 401-bp fragment. The expected size of this PCR product was verified on an agarose gel and then used for in vitro transcription of the double-stranded RNA (dsRNA) using the MEGAscript RNAi kit (Ambion) based on the manufacturer’s protocol. After being purified with the Minelute kit (Qiagen), the dsRNA was diluted in an elution solution (ES) (10 mM Tris-Cl with 1 mM EDTA, pH 7) and quantified using Nanodrop spectrophotometer as describe above.

To analyze gene expression of DsCAD1 after RNAi, oral delivery of dsRNA [22,23] was applied as described in [13]. The

Figure 1. Total alkaline phosphatase (ALP) activity of whole midgut (gut tissue plus gut contents in the lumen) in different instars of Cry1Ab-susceptible and resistant strains of D. saccharalis. Bars represent the means and standard errors of ten gut samples from a total of eight different larvae. Mean values in the figure followed by a different letter are significantly different (P<0.05, LSMEANS tests).

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non-invasive oral delivery of dsRNA has recently become a more attractive method in RNAi studies despite reports that it is less effective than dsRNA micro-injection [24,25,26]. To increase RNAi effectiveness and reduce variability in individual responses, a relatively high dose of dsRNA (250 ng) for each 3rd instar was used for dsRNA feeding. Briefly, 3rd instars from the Cry1Ab-SS strain were droplet-fed with 0.5 μl of the ES (control) or 0.5 μl ES containing 250 ng dsRNA (RNAi-treated). After 3 h, the droplet-fed larvae were transferred to individual cups containing 1 g of the meridic diet, and held in an environmental chamber maintained at 25°C, 50% RH, and a photoperiod of 16:8 (L:D) h. At 24 h post feeding, guts were dissected to obtain total RNA, and DsCAD1 transcript levels of RNAi-treated and control larvae were determined by qRT-PCRs as described above.

Cry1Ab susceptibilities of RNAi-treated and control larvae of D. saccharalis were measured using a standard method of diet incorporating Bt toxin described in [14]. A Cry1Ab concentration of 16 μg g−1 diet−1 was used for these tests. Treatment with this concentration was shown previously [14] to cause ~50% and ~8% mortality to larvae from the Cry1Ab-SS and -RR strains, respectively, in 7 days. To examine larval susceptibility to Cry1Ab toxin after RNAi, ES only, or ES containing 50 ng dsRNA was droplet-fed to neonates (<24 h old) of the Cry1Ab-SS strain. The droplet-fed neonates were placed in cells of 128-cell trays (C-D international) individually and rested for 3 h before they were used for bioassays. In the bioassays, approximately one gram of regular diet (non-treated control) or diet treated with purified trypsin-activated Cry1Ab toxin at the

![Figure 2. Deduced amino acid sequences of three alkaline phosphatase isoforms (DsALP1, DsALP2, and DsALP3) in D. saccharalis aligned by ClustalW. The predicted signal peptide sequences are single-underlined. Potential N-glycosylation sites are double-underlined. Alkaline phosphatase active domains are indicated inside the rectangle. Predicted GPI anchor sites are indicated by filled-black triangles.](https://doi.org/10.1371/journal.pone.0025783.g002)
concentration of 16 μg g⁻¹ diet was dispensed into each cell of the 128-cell trays (C–D International). One larva was then placed on the diet surface in each cell. The bioassay trays were held in an environmental chamber maintained as described above. Larval mortality was checked after 7 days. The 7-d mortality data were corrected based on the mortality observed on the control diet [27]. There were three replications for each treatment with 48 larvae in each replicate. The corrected mortalities were presented as means and standard errors of the mean (±SEM). A one-way ANOVA was performed to determine treatment differences at $\alpha = 0.05$ level [16].

**Results**

Enzymatic and molecular comparisons of three DsALP proteins, cDNAs and gene transcripts

Activities of ALPs from Cry1Ab-SS and -RR strains of *D. saccharalis* were not significantly different at each instar ($F = 0.01$;
A: DsALP1

- Cry1Ab-resistant
- Cry1Ab-susceptible

ALP mRNA per total RNA (pg/µg)

3rd instar 5th instar

B: DsALP2

- Cry1Ab-resistant
- Cry1Ab-susceptible

ALP mRNA per total RNA (pg/µg)

3rd instar 5th instar

C: DsALP3

- Cry1Ab-resistant
- Cry1Ab-susceptible

ALP mRNA per total RNA (pg/µg)

3rd instar 5th instar
Figure 4. Gene expression levels of three alkaline phosphatase (ALP) genes (A: DsALP1, B: DsALP2, and C: DsALP3) of 3rd and 5th instars of Cry1Ab-susceptible and -resistant strains of D. saccharalis. Absolute transcript abundance (ALP mRNA per total RNA (pg/mg)) was determined using qRT-PCR with SYBR. Bars represent the means and standard errors of 4 total RNA samples each containing a pool of total RNAs from three larvae. Mean values in the three figures followed by a different letter are significantly different (P<0.05, LSMEANS tests). doi:10.1371/journal.pone.0025783.g004
Cadherin-Mediated Bt Resistance in D. saccharalis

1 MMEVVKLAAL LLALVAVK TQVVDHCAFMA VAIPIPRPTPE LPEFDFDGLS WEDRQLLLPPP
61 VREEVCMGLY QRQINVGQIIQ YIMEEIEEGD VIYIAKLNYQG NATPTIGVFL IQGYFDMLGA
121 EIRRIPELDN WYVLVTQOR DYTQMMNYL QEMIRIPGET VAGVGVTLVIV NIDDNPPIIH
181 ALEPCVVPEL GDARLTCEVY EVTDREDGIES TRFMTEVEILS DRDNMEIFYM KGEYNSADWO
241 TMSMTGQITF ELNFETKLRLT SFTVVAKDL FPNMDFSLRV RVENVEHRPP RWYVEIFAVQO
301 FDEKTKONFEF VRAIDAEIO DKRIDIYLYL IDPEDDFPSIE PNYDELGGAT LFVAPIRDRD
361 LLERLFLRTLI VAHVIDNNSL ATEANIVIVI NDINDQPFPK LRIKHYIEIE EETPVTLNFG
421 PDDGGFDHDL GENARYTVEL RDVSPPPGAAS AFATSPYDEGY RQQTIFMSTI NHAMLYEYVP
481 EFQINVLELV ATDRNNSSEFV GVARLYIDLI NWDNELPIFG ETAYSVSNEA TVEKDFFWGT
541 VLATDRDIDD TVVHSMLGNA GDLLRIDEES GNIVYYKIDDA FDKHRQNILF VQPRADDTLI
601 TLDGRTHTTT TQLVHLEDV NNTPTTPLRP RASPSVEENV PEGYVVEDNL RATDPDPTTH
661 LFVEINWDES WATKOGRPTT VDEYVGCVAIL KTEYVDGNTG SAIGIEIEVRQ IRPVDTIDFE
721 EFELYLISIR VRDINTVGYD EDDDEAIFTWN IIDDMDNPPPI FSEGSELEQSM RVREMSATGT
781 VIGSSLLATDI DGPLYNNVHY SIKPINETPD GLVKKIDLISG QLTVDADEAI DADVPPRHYL
841 YYEVTSADRC LEECCPCPDCH HFNTTGYIAI EIIIDTNKFP EERTDLFRTV VPWRENETSNG
901 YEIEQIFASD LDRDELYHQV RQINYAANNP RLRAFFDIDL DTGMLFVNTY TDELVLRDRDG
961 EPTHTIFISL IDNFFTAGDG RNNQSTTIVE IVLLDVNDNA PELPEGLSWS ISEAIPGEGEL
1021 VGDIVFAPDI DEPGTENRSV GYSVLNMDTD RDIIILPILFD TYLIEHDEGN RARLRTIQL
1081 RGYWGDMHIIK IAKFDHGVEP MHSDMWYPVE IRPYNFHPDV FVFPRPGATI RFRATERANAM
1141 NLLLTADGNV LERLSATDED GLHAGRVTFS IFGDSAIEH FTITNGENS ATLLLSKVFPD
1201 DNVMEFQITI RATDGGTEPH SRHTDSTIRA LFVTTGLEPI FNQESITVPF TEGPIGLEES
1261 HQLPLKDPDK NYYCCTCIDN IFYRIAGGTSE YFHPHDPSVS NRLTLARELD QEOSRHTITIV
1321 IATNSPTAS GTPLDGTTLT VTNYVEIENP RRIFERKLYT AGISVLDTIO REEVTVAQARH
1381 SLGDTITYAI MDMSMGGSS LNAVAGSAFL LHIFSTGVLTL NMQPTANNHG MFQFDVTATD
1441 TANGTGRAQ VQILISSQQR VVFVFVDSLD EVVKYSDFIT DIFSAAFMT CNIDQVLPAS
1501 DDQGIATDDK TEVRAHFIRD NVPVEAFEIE FLGRDGTLLL QIQOLWNEHS LVQDLCSTGP
1561 GSVDNHTAQI TTVVLAALSA LLAPLCVILEL ITRICKRAL NRMKMAMSLT KEGSVDNGLN
1621 RNSGITPGYNIK HTAEGSNPIY NETLKAHPFD ALDSASNDSD LIGIEDLPQF GNDFFPPGDN
1681 NSLQGIMGDQ DTSTHRNNF GFKTSPFSPE FTKNFGR
Discussion

The ALPs (EC 3.1.3.1) are mainly localized in microvilli of columnar cells and the midgut epithelium cells of insects [31,32]. Insect ALPs have been proposed to function in active absorption of metabolites and transport processes as well as to participate in cell adhesion and differentiation in some cases [32,33]. The ALPs can be grouped into those that are soluble or membrane-bound [32,34,35], and members from both groups are found in larval midgut epithelium cells. The two ALP groups are believed to have different functions in vivo due to the differences in enzymatic activity and the structure of the sugar side chain [36]. Membrane-bound ALPs are thought to be involved with digestion and absorption of nutrients, whereas soluble-ALPs may play a role in the regulation of ionic balance [32,35]. Based on the lepidopteran ALP sequences available in GenBank, seven out of the 13 ALPs identified in four other lepidopteran species have a predicted GPI-anchoring site in their deduced protein sequences. Among those, all seven ALPs with a GPI-anchoring site were membrane-bound [35,37,38]. The GPI sequence signatures of DsALP1 and DsALP3 identified in D. saccharalis suggest that these two DsALPs likely be membrane-bound, whereas the DsALP2 (without the possible GPI-anchoring site) is likely a soluble form. However, the phylogenetic analysis revealed that DsALP2 was grouped with GPI-anchored ALPs that were considered as Bt toxin binding receptors for B. mori, B. mandarina, H. virescens, and H. armigera [35,37,38].

In a previous study, interactions between Cry1Ac toxin and ALPs resulted in a decreased ALP enzymatic activity in Manduca sexta [39]. Several studies have demonstrated that membrane-bound ALPs in several lepidopteran species can act as Cry toxins binding proteins [5,37,40,41]. In addition, recent studies have shown that reduced ALP gene expression is associated with Bt resistance in three major pests targeted by Bt crops [5,6]. Results of this study did not show any reduction in expression of ALP activity in the Cry1Ac-RR strain of D. saccharalis compared to the Cry1Ac-SS larvae, although there was a trend toward increased transcription of the three DsALPs in the Cry1Ac-RR strain relative to the Cry1Ac-SS strain for both instars tested. Further, cDNA sequences of the three DsALP genes were identical between the Cry1Ac-SS and -RR strains. These data, together with similar ALP enzymatic activity data, suggest that the Cry1Ac resistance of D. saccharalis is not associated with mutations or reduced expression in ALP genes. It is possible that use of whole gut tissue in this study, instead of brush border membrane vesicles [6], might obscure the down-regulation of the ALPs. However, the
detection of down-regulation of both DsCAD1 from this study and APNs [13] in the same tissue indirectly validated our ALP data. Results of this study suggest that the Cry1Ab resistance in *D. saccharalis* is likely associated with the reduction in gene expression of DsCAD1. The cadherin-like protein identified from *D. saccharalis* demonstrated a relatively high similarity to other members of the cadherin super-family in lepidopteran species, indicating that the cadherin-like protein from *D. saccharalis* may share structures, functions, and consequently specificity for Cry1A toxins with other insects. Several previous reports showed that Cry1A resistance in several other lepidopteran species was associated with mutations of the cadherin genes resulting in either deletions (e.g., in *H. armigera* and *O. nubilalis*) [42,43,44] or premature stop codons (e.g., in *H. virescens*, *P. gossypiella*, and *O. nubilalis*) [44,45,46]. In addition, single amino acid mutations in the toxin-binding region of a cadherin protein in *H. virescens* caused a substantial decrease in toxin binding [47]. These results indicate that such single amino acid mutations or deletions can lead to high levels of Bt resistance in lepidopteran species. However, in the current study, we found no differences in the sequence of the cadherin cDNAs between the Cry1Ab-SS and -RR strains of *D. saccharalis*. Numerous studies have suggested that APNs are receptors of Bt Cry toxins and are involved in Bt resistance in many insect species [48,49,50,51,52]. As observed for the cadherin gene, our previous study [13] also showed that cDNAs of three APN genes were identical between the Cry1Ab-SS and -RR strains of *D. saccharalis*, but the expression levels of all the three APN genes were significantly reduced in the resistant strain compared to those of the susceptible strain. Taken together, these results suggest that the reduction in expression of both the cadherin and APNs is associated with the Cry1Ab resistance in *D. saccharalis*. However, our finding does not exclude involvement of other genes in the resistance. Recently, we conducted a microarray analysis of 7,145 genes, which revealed 273 significantly up-regulated and 111 significantly down-regulated genes in Cry1Ab-RR strain. Our microarray analysis provided some interesting data, especially the up-regulation of large portion of metabolic or catalytic activity related genes in the Bt resistant strain (manuscript is in preparation). Future studies will focus on comparative characterizations of those differentially expressed genes and establish a linkage between gene regulation and Cry1Ab resistance in *D. saccharalis*.

**Supporting Information**

Table S1 Sequences of primers used in cDNA cloning and quantitative reverse transcriptase polymerase chain
reaction (qRT-PCR) for characterization of three midgut alkaline phosphatase genes from Cry1Ab-susceptible and -resistant strains of D. saccharalis.

(DOC)

Table S2 Sequences of primers used in cDNA cloning, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and double-stranded RNA (dsRNA) synthesis for characterization of a midgut cadherin gene from Cry1Ab-susceptible and -resistant strains of D. saccharalis.

(DOC)

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Conceived and designed the experiments: YH YCZ JO CH BRI FH. Performed the experiments: YH YCZ. Analyzed the data: YH YCZ. Contributed reagents/materials/analysis tools: YCZ FH BRI JO CH CA R. Wrote the paper: YH YCZ FH.
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