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The midgut cadherin-like gene is not associated with resistance to *Bacillus thuringiensis* toxin Cry1Ac in *Plutella xylostella* (L.)

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ABSTRACT

The Gram-positive bacterium *Bacillus thuringiensis* (Bt) produces Cry toxins that have been used to control important agricultural pests. Evolution of resistance in target pests threatens the effectiveness of these toxins when used either in sprayed biopesticides or in Bt transgenic crops. Although alterations of the midgut cadherin-like receptor can lead to Bt Cry toxin resistance in many insects, whether the cadherin gene is involved in Cry1Ac resistance of *Plutella xylostella* (L.) remains unclear. Here, we present experimental evidence that resistance to Cry1Ac or Bt var. *kurstaki* (Btk) in *P. xylostella* is not due to alterations of the cadherin gene. The bona fide *P. xylostella* cadherin cDNA sequence was cloned and analyzed, and comparisons of the cadherin cDNA sequence among susceptible and resistant *P. xylostella* strains confirmed that Cry1Ac resistance was independent of mutations in this gene. In addition, real-time quantitative PCR (qPCR) indicated that cadherin transcript levels did not significantly differ among susceptible and resistant *P. xylostella* strains. RNA interference (RNAi)-mediated suppression of cadherin gene expression did not affect larval susceptibility to Cry1Ac toxin. Furthermore, genetic linkage assays using four cadherin gDNA allelic biomarkers confirmed that the cadherin gene is not linked to resistance against Cry1Ac in *P. xylostella*. Taken together, our findings demonstrate that Cry1Ac resistance of *P. xylostella* is independent of the cadherin gene.

1. Introduction

The Gram-positive entomopathogen *Bacillus thuringiensis* (Bt) produces insecticidal Cry toxins, which have been used to control insect pests in agriculture, forestry, and public health (Schnepf et al., 1998). Bt Cry toxins are considered useful alternatives to chemical insecticides because they are biodegradable and are safe for humans and for most beneficial organisms (Sanahuja et al., 2011). In 2013, transgenic crops harboring genes that encode Bt toxins (Bt crops) were planted on more than 75 million hectares worldwide (James, 2013). The long-term efficacy of Bt sprays and Bt crops, however, is threatened by the development of Bt resistance, which has been reported in at least seven pest species (Tabashnik et al., 2011, 2013). Clarifying the molecular mechanisms of field-evolved Bt resistance in insect pests could suggest ways to prevent such resistance from developing.

High levels of resistance to Bt Cry toxins are associated with alterations in midgut receptor genes of the resistant insects (Ferré and Van Rie, 2002). The midgut membrane proteins cadherin, aminopeptidase (APN), and alkaline phosphatase (ALP) are major receptors of Bt Cry toxins (Pigott and Ellar, 2007; Bravo et al., 2011). More recently, an ABC transporter protein (ABCC2) has also been proposed as a functional receptor of Bt Cry toxins (Tanaka et al., 2013). Although the molecular mechanism by which the midgut cadherin receptor interacts with Bt toxins is still controversial (Vachon et al., 2012; Pardo-López et al., 2013), the midgut cadherin is pivotal for Bt intoxication in both a pore formation model (Gómez et al., 2014) and a signaling transduction model (Zhang et al., 2006, 2012a). Moreover, high resistance to Bt Cry toxins has been associated with mutations in the midgut cadherin gene that can disrupt cadherin interaction with Bt Cry toxins (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005).

Cadherins with typical extracellular cadherin repeat domains (EC or CR domains) are calcium-dependent membrane glycoproteins that form a large and versatile gene superfamily in a wide variety of organisms (Angst et al., 2001; Wheelock and Johnson, 2003; Hulpiau and van Roy, 2009). In the cadherin superfamily,

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proteins with one or more EC/CR-like domains (domains defined as being non-consecutive or lacking the conserved calcium-binding motifs of protocadherin) are designated as cadherin-like proteins (Hulpiau and van Roy, 2009). Despite their diversity and unique functions, the cadherin-like genes received little attention from insect toxicologists until the first cadherin-like gene was identified to be a midgut functional receptor of Bt Cry1Ab toxin and was subsequently cloned in *Manduca sexta* (Vadlamudi et al., 1993, 1995). Since then, midgut cadherin-like genes involved in insect Bt resistance have been cloned in diverse insects from the Lepidoptera, Coleoptera, and Diptera. These cadherin-like proteins share common structural characteristics including an extracellular domain (generally containing 9–12 cadherin repeats), a membrane-proximal extracellular domain (MPD), a transmembrane domain (TMD), and a cytoplasmic domain (CPD) (Bel and Escriche, 2006; Pigott and Ellar, 2007). To date, insect midgut cadherins have been reported to interact with Bt Cry toxins in at least eight lepidopteran insects, two dipteran insects, and four coleopteran insects (Bravo et al., 2011; Yang et al., 2011; Contreras et al., 2013; Park and Kim, 2013; Ren et al., 2013; Hua et al., 2014).

The diamondback moth, *Plutella xylostella* (L.), is notorious for its ability to develop resistance to many insecticides, and the total management cost for this pest controlling is estimated to be US $4–5 billion annually (Furlong et al., 2013). Moreover, *P. xylostella* was the first insect that was documented to develop high resistance to Bt sprays in the field (Tabashnik et al., 1990). Although recent study has shown that Cry1Ac resistance is associated with a mutation of the ABCC2 gene in *P. xylostella* (Baxter et al., 2011), whether the midgut cadherin-like gene is also involved in Cry1Ac resistance in *P. xylostella* remains unclear. A previous study demonstrated that *cis*-mutations in the cadherin gene were not linked to Cry1Ac resistance in the SC1 and NO-QA strains of *P. xylostella*, however, the involvement of this gene in Cry1Ac resistance cannot be ruled out because changes in the expression of the cadherin gene have not been evaluated (Baxter et al., 2005). Furthermore, cadherin has been proposed as a putative Cry1Ac binding receptor that may be associated with Cry1Ac resistance in the PXR strain of *P. xylostella* (Higuchi et al., 2007). Therefore, because comprehensive and direct functional assays of this gene are lacking, the role of the cadherin gene in Cry1Ac resistance of *P. xylostella* remains controversial. Clarifying whether this gene is involved in Cry1Ac resistance in *P. xylostella* warrants additional study.

In this research, we investigated the potential involvement of midgut cadherin in resistance to Bt Cry1Ac toxin in diverse *P. xylostella* strains. We provide direct evidence that Bt Cry1Ac resistance in *P. xylostella* is independent of the cadherin gene.

2. Materials and methods

2.1. Insect strains

The susceptible DBM1Ac-S and resistant DBM1Ac-R (previously referred to as Cry1Ac-R) strains of *P. xylostella* were originally provided by Drs. J. Z. Zhao and A. Shelton (Cornell University, USA) in 2003. The DBM1Ac-R strain originated from insects with field-evolved resistance to Javelin (Bt var. *kurstaki*) from Loxahatchee (Florida, USA) (Shelton et al., 1993) that were crossed with the DBM1Ac-S (Geneva 88) strain (originated from Geneva, NY, USA) and further selected with Cry1Ac-expressing broccoli (Metz et al., 1995). Resistance to Cry1Ac in DBM1Ac-R is autosomal, incompletely recessive, and mostly monogenic (Tang et al., 1997). The SZ-R (previously referred to as T2-R) and SH-R strains originated from moths collected in China at Shenzhen (2003) and Shanghai (2005), respectively. The SZ-R strain was generated by selection in the laboratory with Cry1Ac protoxin, and the SH-R strain was selected with a Bt *kurstaki* (Btk) formulation (WP with potency of 16,000 IU/mg, provided by Bt Research and Development Centre, Agriculture Science Academy of Hubei Province, China). The DBM1Ac-S strain was kept unselected while the DBM1Ac-R and SZ-R strains have been kept under constant selection with a Cry1Ac protoxin solution, which regularly kills 50–70% of the larvae on sprayed cabbage leaves. The near-isogenic NIL-R strain was constructed when this study carried out, and it was generated by multiple (six times) backcrossing between DBM1Ac-S and DBM1Ac-R and with selection of offspring as for DBM1Ac-R (Zhu et al., 2015). At the time of this study, the resistance ratios (resistant larvae LC_{50} value divided by susceptible larvae LC_{50} value) of DBM1Ac-R (LC_{50} = 3,052.33 μg/ml), NIL-R (LC_{50} = 3,401.51 μg/ml), and SZ-R (LC_{50} = 563.06 μg/ml) to Cry1Ac protoxin were about 3500-, 4000-, and 450-fold compared to the DBM1Ac-S (LC_{50} = 0.86 μg/ml), respectively, and the resistance ratio of SH-R (LC_{50} = 1,323.18 μg/ml) to the Btk formulation was about 1900-fold compared to the DBM1Ac-S (LC_{50} = 0.70 μg/ml). All strains were reared on JingFeng No. 1 cabbage (*Brassica oleracea* var. *capitata*) without exposure to any Bt toxins or chemical pesticides at 25 °C, 65% RH, and a 16D:8L photoperiod. Adults were fed with a 10% sucrose solution.

2.2. Cry1Ac toxin preparation and bioassays

Cry1Ac protoxin was extracted and purified from Bt *kurstaki* strain HD-73 as previously described (Perera et al., 2009). Both purified Cry1Ac protoxin and trypsin-activated toxin were quantified by densitometry as described elsewhere (Crespo et al., 2008). The purified Cry1Ac toxin was solubilized in 50 mM Na_2CO_3 (pH 9.6) and stored at −20 °C until used.

The toxicity of the Cry1Ac toxin or Btk formulation was determined with 72-h bioassays using larvae from five strains of *P. xylostella* and a leaf-dip method as described elsewhere (Yang et al., 2009). Ten third-instar *P. xylostella* larvae were tested for each of seven toxin concentrations, and bioassays were replicated four times. Mortality data were corrected using Abbott's formula (Abbott, 1925), and experiments in which control mortality exceeded 10% were discarded and repeated. The LC_{50} values were calculated by Probit analysis (Finney, 1971).

2.3. cDNA synthesis and gDNA isolation

Fourth-instar larvae from different *P. xylostella* strains were anesthetized on ice for about 15 min before the midgut tissues were dissected in sterile DEPC (diethyl pyrocarbonate)-treated distilled water containing 0.7% NaCl. Total RNA was extracted from a pool of dissected midguts from fourth-instar larvae for each *P. xylostella* strain using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The integrity of the RNA was determined using 1% TBE agarose gel electrophoresis, and the RNA was then quantified with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). For gene cloning, the first-strand cDNA was prepared using 5 μg of total RNA and the PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Dalian, China) following manufacturer's recommendations. For qPCR analysis, the first-strand cDNA was prepared using 1 μg of total RNA with the PrimeScript RT kit (containing gDNA Eraser, Perfect Real Time) (TaKaRa, Dalian, China) following the manufacturer's instructions. The synthesized first-strand cDNA was immediately stored at −20 °C until used.

Genomic DNA (gDNA) was prepared from fourth-instar larvae of DBM1Ac-S and NIL-R strains using a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) with the following slight modification in the pretreatment of samples: the individuals were first well homogenized with an electric pestle in 1.5-ml centrifuge tubes before gDNA was extracted according to the manufacturer's