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RNA interference knockdown of aminopeptidase N genes decrease the susceptibility of *Chilo suppressalis* larvae to Cry1Ab/Cry1Ac and Cry1Ca-expressing transgenic rice

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ABSTRACT

Transgenic rice expressing *Bacillus thuringiensis* (Bt) Cry toxins are resistant to lepidopteran pests, such as *Chilo suppressalis*, a major insect pest of rice in Asia. Understanding how these toxins interact with their hosts is crucial to understanding their insecticidal action. In this study, knockdown of two aminopeptidase N genes (APN1 and APN2) by RNA interference resulted in decreased susceptibility of *C. suppressalis* larvae to the Bt rice varieties TT51 (*Cry1Ab* and *Cry1Ac* fusion genes) and T1C-19 (*Cry1Ca*), but not T2A-1 (*Cry2Aa*). This suggests that APN1 and APN2 are receptors for Cry1A and Cry1C toxins in *C. suppressalis*.

1. Introduction

The bacterium *Bacillus thuringiensis* (Bt) produces crystal (Cry) proteins that are toxic to a number of important Lepidopteran, Dipteran and Coleopteran pests (Schnepf et al., 1998; Tabashnik et al., 2008). Cry proteins are active ingredients in Bt sprayable formulations, and *cry* genes have been used to create insect-resistant transgenic crops (Bravo et al., 2011; James, 2015), including transgenic Bt rice which has been widely promoted as a more environmentally friendly and economical alternative to traditional methods of pest control (Bajaj and Mohanty, 2005; Deka and Barthakur, 2010).

The mechanism underlying the toxicity of Cry proteins to lepidopteran larvae has been thoroughly investigated (Adang et al., 2014). Ingested toxin is solubilized and proteolytically activated in the midgut of susceptible insects. The activated toxin then travels to the peritrophic matrix where it binds to specific receptors on the brush border membrane vesicles (BBMVs) of the midgut, resulting in toxin oligomerization and the formation of toxin pores that cause osmotic cell lysis. A substantial number of proteins have been identified as Cry toxin receptors, including cadherin, aminopeptidase N (APN), ATP-binding cassette (ABC) transporters and alkaline phosphatase (ALP) (Bravo et al., 2005; Pigott and Ellar, 2007; Soberon et al., 2009; Pardo-López et al., 2013; Guo et al., 2015).

The striped stem borer, *Chilo suppressalis* (Walker), is a well-known pest of rice crops in China. Transgenic Bt rice lines have been developed that are significantly more resistant to *C. suppressalis* than non-transgenic strains. One such strain was produced by transforming *Cry1A*, *Cry2A* and *Cry1C* genes into the parental cultivar Minghui 63 (MH 63) (Tu et al., 2000; Chen et al., 2005; Tang et al., 2006). Transgenic rice expressing the *Cry1Ab* and *Cry2Ab* genes has also recently been developed (Zhao et al., 2014) and additional new strains of genetically engineered rice will continue to be developed and become commercially available. Although it has been proposed that APN and cadherin-like proteins act as Cry1A binding proteins (Yu et al., 2010; Ma et al., 2012; Wang et al., 2017), the receptors of the Bt toxins Cry2A and Cry1C in *C. suppressalis* remain unknown. (see Table 1).

A number of studies have concluded that APN1 proteins are receptors for Cry toxins in lepidopteran pests (Zhang et al., 2009; Tiewsiri and Wang, 2011; Flores-Escobar et al., 2013; Wei et al., 2016; Wang et al., 2017). The evidence that APN2 is a receptor

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Table 1
Specific primers used in qRT-PCR and RNAi.

Genes	Forward primers (5′–3′)	Reverse primers (5′–3′)	PCR efficiency	Standard curve R^2
qRT-PCR				
CsAPN1	GCAACATTGGCCATCCTGGG	CCGGTACAGAACACATGGCG	97.3%	0.997
CsAPN2	ACTTGCGACTGGTGGGACAA	GCGGAGTCAGTGAGCAAAGC	92.4%	0.993
CsEF-1	TGAACCCCCATACAGCGAATCC	TCTCCGTGCCAACCAGAAATAGG	99.8%	0.992
RNAi				
CsAPN1	atGCGGCCGCATAAATATGACACTGTTAATAGT[a]	atGGTACCCTACTTTAAGAGCACTTTATTA[b]	n.a.	n.a.
CsAPN2	atGCGGCCGCCGACTGCCTTCAATTCAGCTGTCA[a]	atGGTACCGAATGACATTACTTGGGTATCA[b]	n.a.	n.a.

n.a. = not applied.
[a] Underlined sequence indicates the position of the *Not* I endonuclease site.
[b] Underlined sequence indicates the position of the *Kpn* I endonuclease site.

for Cry1A is, however, more equivocal (Nakanishi et al., 2002; Rajagopal et al., 2003). In this study, we test the hypothesis that *C. suppresalis* APN1 (GenBank accession no. JQ747494.1) and APN2 (GenBank accession no. JQ747495.1) are receptors for Cry1A, Cry2Aa and Cry1Ca toxins. RNA interference experiments using dsRNA of the corresponding genes demonstrated that knockdown of *CsAPN1* and *CsAPN2* significantly decreased the susceptibility of *C. suppressalis* larvae to both Cry1Ab/Cry1Ac and Cry1Ca rice lines, but not to Cry2Aa. These results demonstrate that both *C. suppressalis* APN1 and APN2 may function as receptors for both Cry1A and Cry1Ca toxins.

2. Materials and methods

2.1. Insect rearing

C. suppressalis larvae were collected from Dawu County, Hubei Province, China in 2012 and used to found a colony descended that was maintained in our laboratory for 4 years. Larvae were reared on an artificial diet (Han et al., 2012) at 28 ± 1 °C under a 16-h photoperiod and 80% relative humidity.

2.2. RNAi knockdown of CsAPN1 and CsAPN2

A method adapted from Qiu et al. (2015) was used to produce a dsRNA-expressing vector. Briefly, 554 bp (*CsAPN1)* and 519 bp (*CsAPN2)* fragments corresponding to the nucleotides 1738–2291 (*CsAPN1*) and 2623–3141 (*CsAPN2*) were amplified from *C. suppressalis* midgut cDNA and cloned into a pET-2P vector with flanking T7 promoter and T7 terminator sites to produce *Cs*APN1 and *Cs*APN2 dsRNA. The control treatment was EGFP dsRNA produced by a pET2P/*EGFP* recombinant plasmid. Correct inserts of the recombinant plasmids were confirmed by sequencing conducted by the Genscript Biology Company, Nanjing, China. For dsRNA expression, 200 ng of recombinant plasmids were transformed into competent *Escherichia coli* HT115 (DE3) cells. Positive clones were cultured in 100 ml LB medium containing 50 μg/ml kanamycin and induced to generate dsRNA by adding 0.4 mM isopropyl-D-thiogalactoside (IPTG), after which the bacteria were cultured for an additional 4 h at 37 °C. Bacteria were precipitated by centrifugation at 5000 rpm for 10 min and resuspended in 1 ml distilled water. DsRNA was extracted according to the method described by Timmons et al. (2001) and Dong et al. (2016). The size of the resultant dsRNA was checked using 1% agarose gel electrophoresis (data not shown).

2.3. Bioassay

Newly hatched larvae were allowed to feed on non-transgenic rice (MH 63) overlaid with *E. coli* suspension containing either *Cs*APN1, *Cs*APN2, or EGFP, dsRNA, or water, for 48 h at 27 °C, then transferred to either TT51, T2A-1 or T1C-19 transgenic rice plants where they were allowed to continue feeding for another 7 days. A total of 90 larvae were used with three replicates for each treatment. The effectiveness of silencing the target genes was verified using quantitative real-time PCR (qRT-PCR) to measure their expression levels. Briefly, total RNA was extracted from the whole bodies of 15 larvae from each dsRNA treatment group with three replicate samples for each group. qPCR primers were designed using the NCBI profile server (http://www.ncbi.nlm.nih.gov/tools/primer-blast) with the *C. suppressalis* elongation factor-1 (*EF-1*) gene as the internal reference (Zhu et al., 2016). The qPCR protocol used has been described elsewhere (Qiu et al., 2015).

2.4. Data analysis

Gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Differences between treatment means were analyzed using one-way ANOVA implemented in SPSS for Windows (SPSS 18.0, Chicago, IL, USA).

3. Results and discussion

Since APNs were first identified as Cry toxin-binding proteins (Gill et al., 1995; Knight et al., 1995), different APN isoforms have been found act as Cry toxin receptors in more than 20 lepidopteran species (Nakanishi et al., 2002; Herrero et al., 2005; Wang et al., 2005, 2017; Pigott and Ellar, 2007; Angelucci et al., 2008; Simpson et al., 2008).

We used RNAi technology to test the hypothesis that *C. suppressalis* APN1 and APN2 act as receptors for Cry1A, Cry2A and Cry1C toxins. *CsAPN1* and *CsAPN2* transcription levels of the *Cs*APN1 or *Cs*APN2 dsRNAs treatment groups were significantly lower than those of the dsEGFP and water control groups (Fig. 1A). Mortality of the dsAPN1 and dsAPN2 treatment groups following ingestion of Cry1A (or Cry1Ab and Cry1Ac in the case of the TT51 cultivar) were 65.3% and 65.4%, respectively, significantly (ANOVA, $P < 0.05$) lower than those of the water (96.6%) or dsEGFP (90.0%) control groups (Fig. 1B and C). This suggests that the APN1 and APN2 proteins are associated with Cry1A toxicity in *C. suppressalis* larvae. It has been suggested that APN1 proteins act as Cry1Ac receptors in *Trichoplusis ni*, *Manduca sexta* and *Helicoverpa armigera* (Zhang et al., 2009; Tiewsiri and Wang, 2011; Flores-Escobar et al., 2013). More recently, APN1 has been identified as a Cry1Ac receptor in *Helicoverpa zea* (Wei et al., 2016) and RNAi knockdown of *CsAPN1* has been found to reduce the susceptibility of *C. suppressalis* to Cry1Ab (Wang et al., 2017). The evidence that APN2 is also a Cry receptor is, however, equivocal; for example, although APN2 was found to bind Cry1Ac in *H. armigera*, it does not appear to do so in *M. sexta*, *Plutella xylostella* or *Bombyx mori* (Masson et al., 1995; Denolf et al., 1997; Nakanishi et al., 2002; Rajagopal et al., 2003).

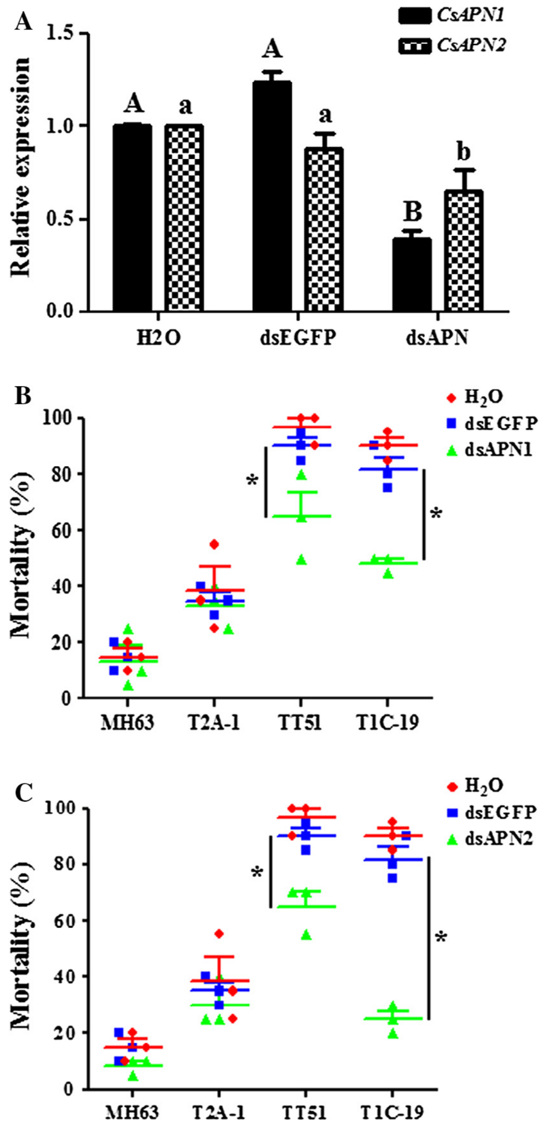

Fig. 1. RNA interference knockdown of *CsAPN* transcripts in *C. suppressalis* larvae and subsequent mortality of larvae after feeding on three transgenic Bt rice lines; TT 51, T1C-19 and T2A-1, and the non-transgenic parental rice cultivar MH 63. (A) Relative expression of *CsAPN* transcripts were determined by qRT-PCR of *C. suppressalis* larvae that had been allowed to feed for 48 h on non-transgenic rice (MH 63) overlaid with either water or dsEGFP as control treatments, or dsRNA targeting *CsAPN1* and *CsAPN1*. The *CsEF-1* reference gene was used to standardize transcription levels. Different letters on top of bars indicate significant differences (ANOVA followed by Tukey's HSD post hoc tests, $P < 0.05$). (B) Larvae were fed on MH 63 overlaid with dsRNA targeting EGFP, *Cs*APN1, or water, before being exposed to transgenic rice. Asterisks indicate significant differences between treatment groups (ANOVA, $P < 0.05$). (C) Larvae were fed on MH 63 overlaid with dsRNA targeting EGFP, *Cs*APN2, or water, before being exposed to transgenic rice. Asterisks indicate significant differences between treatment groups (ANOVA, $P < 0.05$).

Mortality rates after feeding on Cry1C rice were 48.3% and 25.0% in the dsAPN1 and dsAPN2 treatment groups, respectively, compared to 81.6% and 90% for the dsEGFP and water control groups (Fig. 1B and C). This suggests that RNAi knockdown of *CsAPN1* or *CsAPN2* significantly decreased the toxicity of transgenic Cry1C rice. A number of studies indicate that APN1 act as a Cry1C receptor in lepidopteran insects. For example, knockdown of *APN1* expression reduced susceptibility to Cry1Ca toxin in *Spodoptera exigua* larvae, and lack of *APN1* gene expression is associated with Cry1Ca resistance in *S. exigua* (Herrero et al., 2005; Ren et al., 2014). Silencing the *APN1* gene also reduced the susceptibility of *Spodoptera litura* to Cry1C (Rajagopal et al., 2002). Although the results of this study suggest that APN2 is also a potential Cry1Ca receptor in *C. suppressalis* larvae, there is no supporting evidence for this in the literature. Specific glycosylation or sequence attributes may explain this specificity of Cry1Ca for some APN proteins.

When larvae were fed on T2A-1 transgenic rice producing the Cry2A toxin, the mortality of the dsAPN1 and dsAPN2 treatment groups was no different to that of the control groups (Fig. 1). However, both Cry2Aa and Cry2Ab have been successfully incorporated into crop to produce insect-resistant transgenic crops (Chen et al., 2005; Adamczyk et al., 2008). This suggests that additional variables may affect the susceptibility of *C. suppressalis* larvae to the Cry2A toxin. For example, it has recently been found that the ABCA gene mediates the toxicity of the Cry2Ab protein in lepidopteran pest (Tay et al., 2015).

In conclusion, we confirmed that expression of the APN1 and APN2 genes affects the susceptibility of *C. suppressalis* larvae to transgenic rice expressing Cry1A and Cry1C. This suggests that the evolution of cross-resistance between Cry1A and Cry1C in *C. suppressalis* is possible. Other studies have found evidence of cross-resistance between Cry1Ab and Cry1Ca in *S. exigua* larvae with toxin selection (Hernandez-Martinez et al., 2009). The potential shared receptors need to be further investigated to evaluate the risk of cross-resistance between the Cry1A and Cry1C toxins in *C. suppressalis*.

Competing interests

The authors have declared that no competing interest exists.

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