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Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins

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ABSTRACT

Bacillus thuringiensis Vip3 proteins are synthesized and secreted during the vegetative growth phase. They are activated by gut proteases, recognize and bind to midgut receptors, form pores and lyse cells. We tested the susceptibility to Vip3Aa and Vip3Ca of Cry1A-, Cry2A-, Dipel- and Vip3-resistant insect colonies from different species to determine whether resistance to other insecticidal proteins confers cross-resistance to Vip3 proteins. As expected, the colonies resistant to Cry1A proteins, Dipel (*Helicoverpa armigera*, *Trichoplusia ni*, *Ostrinia furnacalis* and *Plodia interpunctella*) or Cry2Ab (*H. armigera* and *T. ni*) were not cross-resistant to Vip3 proteins. In contrast, *H. armigera* colonies resistant to Vip3Aa or Vip3Aa/Cry2Ab showed cross-resistance to the Vip3Ca protein. Moreover, the Vip3Ca protein was highly toxic to *O. furnacalis* (LC_{50} not significantly different from that of Cry1Ab), whereas the Vip3Aa protein only showed moderate growth inhibition at the highest concentration tested (100 μg/g of diet). These results extend the cross-resistance studies between Vip3 and Cry proteins, show for the first time cross-resistance between proteins within the Vip3 subfamily, and points to *O. furnacalis* as a target for the Vip3Ca protein.

1. Introduction

Vip3 insecticidal proteins are synthesized by *Bacillus thuringiensis* (Bt) during the vegetative growth phase and are active against lepidopteran insects (Chakroun et al., 2016a; Estruch et al., 1996). Vip3 proteins are classified into three protein subfamilies based on their amino acid sequence identity: Vip3A, Vip3B, and Vip3C (Crickmore et al., 2013). Most studies on the insecticidal activity of Vip3 proteins have been performed on the Vip3A protein subfamily, in particular, with the Vip3Aa protein. Vip3Ca was discovered more recently and show some toxic effect against some lepidopteran species (Palma et al., 2012; Gomis-Cebolla et al., 2017). In contrast to Vip3Ca, Vip3A proteins have a broad insecticidal spectrum against lepidopteran pests (Chakroun et al., 2016a). The fact that the insecticidal spectrum and the mode of action of the Vip3 proteins differ from that of the Cry1 and Cry2 proteins, makes Vip3 proteins good candidates to be used in combinations with Cry proteins in Insect Resistance Management (IRM) programs.

The mode of action of Vip3 proteins (Vip3A and Vip3C) shares some similarities to that of the Cry proteins in that they are synthesized in the form of protoxins, which are further processed by midgut proteases rendering the active toxin (Estruch et al., 1996; Yu et al., 1997; Lee et al., 2003; Chakroun et al., 2012; Caccia et al., 2014; Gomis-Cebolla et al., 2017). The activated toxins bind to specific receptors in the midgut membrane leading to the disruption of the midgut epithelial cells and eventual death of the larva. The Vip3 receptors are not shared by Cry proteins (Lee et al., 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun et al., 2014; Gomis-Cebolla et al., 2017). However, it has been recently shown that the Vip3Aa and Vip3Ca proteins compete for shared binding sites (Gomis-Cebolla et al.,

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2017).

In agriculture worldwide, repeated applications of *Bt* sprays and widespread adoption of *Bt*-crops (transgenic crops protected from insects by the expression of *cry* or/and *vip3* genes) have led to resistance (Ferré and Van Rie, 2002; Ferré et al., 2008, Tabashnik, 2015; Tabashnik et al., 2009). Therefore, in this arms race against insects, it is necessary to keep exploring the potential of new insecticidal proteins for pest control and, at the same time, to test for their compatibility in combinations with other proteins in terms of cross-resistance. Although cross-resistance studies have been performed with Cry1- and Cry2-resistant colonies against Vip3Aa (Jackson et al., 2007; Fang et al., 2007; Anilkumar et al., 2008; Vélez et al., 2013; Huang et al., 2014; Qian et al., 2015; Welch et al., 2015; Horikoshi et al., 2016), and with Vip3Aa-resistant colonies against Cry1A proteins (Mahon et al., 2012; Chackroun et al., 2016b; Pickett et al., 2017), cross-resistance to Vip3Ca has never been tested.

In this study, we tested the susceptibility of Cry1-, Cry2- and Dipel-resistant colonies from four insect species (*Trichoplusia ni*, *Plodia interpunctella*, *Helicoverpa armigera* and *Ostrinia furnacalis)* to the Vip3Aa and Vip3Ca proteins and compared the results to the non-selected controls. In addition, we tested two Vip3Aa-resistant colonies from *H. armigera* for cross-resistance to Vip3Ca.

2. Materials and methods

2.1. Insect colonies

2.1.1. Insect rearing of T. ni strains

Three *T. ni* strains were used to examine their response to Vip3Aa and Vip3Ca. The *T. ni* Cornell laboratory strain (Wang et al., 2007) was used as the susceptible control strain. The two resistant strains were a Cry1Ac-resistant strain, GLEN-Cry1Ac-BCS (Wang et al., 2007), and a Cry2Ab-resistant strain, GLEN-Cry2Ab-BCS (Song et al., 2015). Both of the resistant *T. ni* strains were near-isogenic to the susceptible Cornell strain and the resistance is fixed (i.e., they were homozygous for the resistance genes). The *T. ni* colonies were maintained on artificial diet without exposure to Bt toxins (Bell et al., 1981).

2.1.2. Insect rearing of H. armigera strains

Five *H. armigera* strains were used to determine their response to Vip3Ca. The *H. armigera* susceptible colony, GR, was used as a control (Mahon et al., 2007). The *H. armigera* homozygous resistant colonies ISOC8, (Cry1Ac), Sp15 (Cry2Ab resistant) and Sp85 (Vip3A resistant) were established from lab selection (ISOC8) and positive F_2 tests in 2002 (Sp15) and 2010, (Sp85) respectively. The *H. armigera* Cry2Ab/Vip3A resistant strain was established by reciprocal crosses placing male pupae from one strain with female pupae from the other in cages (Walsh et al., 2014). All of the resistant strains were repeatedly outcrossed to a susceptible colony and reselected with the appropriate toxin(s). The Cry2Ab resistant line Sp15 carries an ABCA2 transporter mutation that confers the phenotype (Tay et al., 2015), but the mechanism of resistance is unknown for ISOC8 (Cry1Ac) and Sp85 (Vip3A). The rearing methods used to maintain *H. armigera* were modified from those described by Teakle and Jensen (1985).

2.1.3. Insect rearing of P. interpunctella strains

Two *P. interpunctella* strains were used to characterize their response to Vip3Aa. The *P. interpunctella* susceptible colony, EP, was obtained from a grain storage bin and has been maintained in the laboratory on cracked wheat diet (Oppert et al., 2010). The resistant colony EP-Dpl500 was selected from the parental EP, with 500 mg Dipel (Bt subspecies *kurstaki*, strain HD-1) per kg diet, gradually increasing the dose to 10,000 mg/kg, the maintenance dose for this resistant colony.

2.1.4. Insect rearing of O. furnacalis strains

Two strains of *O. furnacalis*, a Bt susceptible strain and a Cry1Ab-resistant strain were established in the laboratory. The Bt susceptible strain was collected from the field and had been reared using standard rearing techniques without exposure to any insecticide before bioassays were conducted (Song et al., 1999). The Cry1Ab-resistant strain was selected from the Bt susceptible strain by exposure to trypsin-activated Cry1Ab. The Cry1Ab-resistant strain was initially exposed throughout larval development to Cry1Ab in the artificial diet (2.5 ng of toxin/g diet). The toxin concentration was increased in succeeding generations to target 40–70% mortality in the exposed insects. After 51 generations, larvae were reared on diet containing 400 ng of toxin/g diet. The Cry1Ab-selected colony had developed > 100-fold resistance to Cry1Ab after 35 generations (Xu et al., 2010).

2.2. Source and expression of Vip3 proteins for cross-resistance assays

Vip3Aa (NCBI accession No AAW65132) was overexpressed in recombinant *Escherichia coli* BL21 carrying the *vip3Aa16* gene (Abdelkefi-Mersati et al., 2009). The Vip3Ca protein (NCBI accession No AEE98106) was prepared from recombinant *E. coli* WK6 carrying the *vip3Ca2* gene (Palma et al., 2012).

The Vip3Aa protein was expressed following the conditions described by Chakroun et al., 2012. For the Vip3Ca protein, a single colony was inoculated in 7 ml of LB medium containing 100 μg/ml ampicillin and grown overnight at 37 °C and 180 rpm. A 1/100 dilution of the culture in 700 ml LB medium containing 100 μg/ml ampicillin was further incubated at 37 °C and 180 rpm. The culture was induced with 1 mM IPTG at an OD of 0.7 and it was grown overnight at 37 °C and 200 rpm. Cells were collected at 6000 g for 15 min at 4 °C. The pellet was weighed and suspended in 3 ml lysis buffer (PBS, pH 8.0, containing 3 mg/ml lysozyme, 10 μg/ml DNase, and 100 μM PMSF) per gram of pellet. The sample was incubated at 37 °C for 30 min and then sonicated on ice applying two 1 min pulses at 70 W at a constant duty cycle, separated by a 10-s cooling period on ice. Then, the insoluble material was separated by centrifugation at 16,000 g for 15 min at 4 °C and the soluble cellular fraction sequentially filtered through sterile 0.45 μm and 0.22 μm cellulose acetate filters.

2.3. Purification of Vip3 proteins for cross-resistance assays

Vip3 proteins used for dose-response assays for the *T. ni*, *H. armigera* and *O. furnacalis* colonies were purified by isoelectric point precipitation (IPP) (Chackroun et al., 2012; Gomis-Cebolla et al., 2017). The pH of the lysate was lowered with acetic acid to pH 5.5 for Vip3Aa and pH 5.95 for Vip3Ca. The pellets were recovered by centrifugation at 16,000 g for 10 min and then dissolved in 20 mM Tris, 150 mM NaCl, pH 9, and dialyzed against the same buffer overnight. The Vip3Aa protein used for dose-response assays for the *P. interpunctella* colonies was purified by immobilized metal ion absorption chromatography (IMAC) on a Hi-Trap chelating HP column (GE Healthcare) charged with Ni^{2+} (Fig. 1A) (Chackroun et al., 2012). The purified proteins were frozen at −80 °C and then lyophilized. The concentration of the Vip3 proteins purified by IPP was estimated by densitometry after SDS-PAGE separation. The concentration of the Vip3Aa purified by Hi-Trap chelating HP column was measured by the method of Bradford (Bradford et al., 1976). In both methods, bovine serum albumin (BSA) was used as standard. The purity of the Vip3 proteins was analyzed by SDS-PAGE (Fig. 1).

2.4. Insect toxicity assays

2.4.1. Dose-response assays for the susceptible and resistant T. ni strains

Examination of *T. ni* strains for their susceptibilities to Vip3Aa and Vip3Ca were conducted using the surface contamination method (Kain et al., 2004). Briefly, an aliquot of 200 μl of Vip3Aa or Vip3Ca solution was spread on the surface of diet in 30-ml cups (surface area is approximately 7 cm^2) and 10 neonate larvae were placed in each cup that

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