REPORT

Functional identification of three new genes involved in resistance to Bacillus thuringiensis *in* Plutella xylostella

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ABSTRACT

The widespread and sustained exploitation of the entomopathogen *Bacillus thuringiensis* (Bt) in the control of insects, pests and disease vectors is threatened by the evolution of resistance. In the present work, the construction of subtractive libraries and analysis of gene function by RNA interference-mediated gene silencing (RNAi) were combined to determine the molecular basis of resistance to DiPel® bio-insecticide in the $KarUK_6$ population of the model insect *Plutella xylostella*. DiPel® is the commercial product of its type most used for the control of different insect pests, based on a mixture of spores and insecticidal toxins of Bt subspecies *kurstaki* HD1 strain (Btk-HD1), with a broad action spectrum. A population susceptible to DiPel®, genetically similar to $KarUK_6$, was used as a control. The ontological analysis of the subtractive libraries showed a multitude of altered biological processes in the resistant strain; and gene expression analysis in the presence of DiPel® evidenced the pre-induced nature of the transcriptional response in the $KarUK_6$ strain. By their regulation, three genes stood out: *SDF2L1*, *CDKAL1* and *HEL-1*, the first two had never before been described in invertebrates, whose vertebrate orthologues are critical to regulate cellular homeostasis under different types of stress. Gene silencing via RNAi of all three genes suppressed DiPel® resistance in $KarUK_6$ larvae, demonstrating that these genes or the cellular mechanisms in which they participate are crucial for resistance, and their potential use as targets to minimize the risk of resistance to Bt products and increase their effectiveness. This research granted the 2015 Award of the Cuban National Academy of Sciences.

Keywords: Bacillus thuringiensis, *Plutella xylostella*, Bt-resistance, ABCC2 gene, differential expression, RNAi

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RESUMEN

Identificación funcional de tres nuevos genes involucrados en la resistencia a *Bacillus thuringiensis* en *Plutella xylostella*. La explotación amplia y sostenida del entomopatógeno *Bacillus thuringiensis* (Bt) en el control de insectos plagas y vectores de enfermedades está amenazada por la evolución de la resistencia. En el presente trabajo, se combinaron la construcción de genotecas substractivas y el análisis de función génica mediante ARN interferente (ARNi), para determinar las bases moleculares de la resistencia al bio-insecticida DiPel® en la población $KarUK_6$ del insecto modelo *Plutella xylostella*. DiPel® es el producto comercial de su tipo más empleado para el control de diferentes insectos plagas, basado en una mezcla de esporas y toxinas insecticidas de Bt subespecie *kurstaki* cepa HD1 (Btk-HD1), con amplio espectro de acción. Como control se usó una población susceptible a DiPel®, genéticamente similar a $KarUK_6$. El análisis ontológico de las genotecas substractivas mostró varios procesos biológicos alterados en la cepa resistente; y el análisis de la expresión génica en presencia de DiPel® evidenció una respuesta transcriptional pre-inducida en la cepa $KarUK_6$. Por su regulación, se destacaron tres genes: SDF2L1, CDKAL1 y HEL-1, los dos primeros nunca antes descritos en invertebrados, cuyos ortólogos en vertebrados son claves para regular la homeostasis celular ante diferentes tipos de estrés. El silenciamiento génico vía ARNi de los tres genes suprimió la resistencia a DiPel® en las larvas de $KarUK_6$, demostrando que estos genes o los mecanismos celulares en que participan, son cruciales para la resistencia, y con uso potencial como blancos en estrategias para minimizar el riesgo de resistencia e incrementar la efectividad de productos comerciales basados en Bt. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2015.

Palabras clave: Bacillus thuringiensis, *Plutella xylostella*, resistencia a Bt, gen ABCC2, expresión diferencial, ARNi

*I*ntroduction

The molecular basis of resistance to insecticides based on *B. thuringiensis* has not been fully elucidated yet. The most widespread mechanism of resistance is related to the loss of binding of Bt toxins to their receptors in the insect gut. However, the various genetic factors responding to biotic stress identified in cellular defense to Bt intoxication indicates the complexity of this host-pathogen interaction. DiPel® insecticide of Btk HD1 is the most successful and widespread worldwide commercial product based on *B. thuringiensis* and it is made up of a mixture of spores and insecticidal protein crystals, where 5 different types of toxins coexist with activity against lepidopterans pests: Cry1Aa, Cry1Ab, Cry1Ac, Cry2A and Cry2B. Even so, there are reports in the literature on the evolution of insect-resistance to DiPel®. In the present

work we undertook the task of characterizing molecularly the resistance to DiPel® in a strain of the diamondback moth, *Plutella xylostella*, a cosmopolitan pest and a model insect as well for the study of mechanisms of resistance to insecticides due to its high adaptability to them.

Results

The study used functional genomics methodologies such as Suppression Subtractive Hybridization (SSH) and post-transcriptional silencing via interfering RNA (RNAi). The kinetic analysis of gene expression was performed by real-time PCR. In bioassays, the dose-response relationship was inferred with the PROBIT statistical package. For the analysis of mortality in the RNAi experiments, a generalized linear model and the logit link function were used [1].

The 'Karak' strain of *P. xylostella*, which evolved towards DiPel® resistance under field conditions after several years of exposure to the insecticide, was crossed with the Lab-UK strain (susceptible to DiPel®), and the offspring was subjected to six backcrossing cycles with Lab-UK, selecting each generation with DiPel®. By these means, the $KarUK_6$ strain was obtained, approximately 23 times more resistant to DiPel® than its genetic isolate Lab-UK (Figure). The determination of the average lethal concentration (LC_{50}) of DiPel® or concentration causing death in 50 % of the population, determined in bioassays with larvae of both strains of *P. xylostella*, showed for $KarUK_6$ an LC_{50} 93.1 ppm and 95 % confidence limits of 60.3-144, compared to Lab-UK with LC_{50} 3.97 ppm and 95 % confidence limits of 1.10-14.3.

A total of 134 complementary DNA fragments (cDNA) belonging to $KarUK_6$ larval gut genes, differentially expressed in the presence of DiPel® as compared to Lab-UK, were cloned into the subtractive hybridizations. The ontological analysis distributed the sequences among five different functional categories. The genes involved in the synthesis and folding of proteins (18 %) and the response to oxidative stress (17 %) were the most represented in the subtractive libraries, whereas the genes related to extracellular matrix processes formed the smallest group (7 %). The abundance of gene sequences involved in lipid metabolism (14 %) and transport/energy (11 %) was intermediate. The remaining 33 % corresponded to sequences with unknown function. The sequences were deposited in the GenBank® database (Accession No. HO652045, HO652175 and HO663508) and correspond to the first report of gene sequence with differential expression against DiPel® insecticide in a resistant lepidopteran.

Twelve sequences were selected from those showing a highly significant identity (E value < 10^{-4}) representing each functional category for the analysis of gene expression kinetics in the response to DiPel®. In all cases, except for a sequence with chaperonin homology, basal gene expression was significantly higher in resistant larvae compared to susceptible ones [1]. This shows that the molecular response to DiPel® is preferably pre-induced in the resistant strain of *P. xylostella*, an effect not reported before. The expression kinetics of the 12 genes during the first 24 h of intoxication showed three of them with

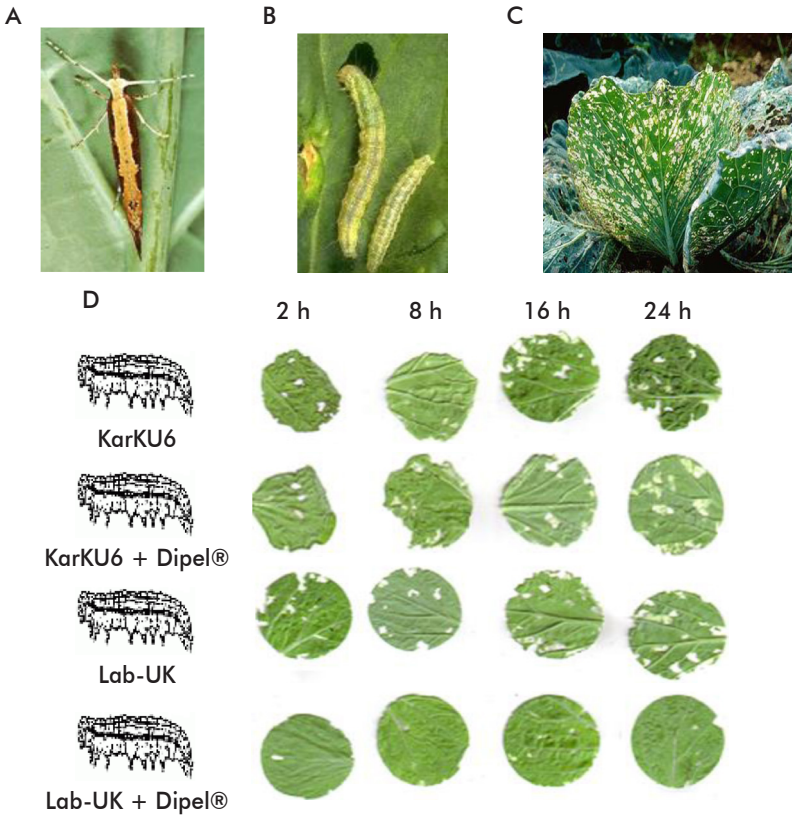

Figure. Incidence of *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) A) Adult stage. B)Larval stage. C) Plants affected by *P. xylostella* L. D) Exposure of larvae of the strains KarUK6 (resistant) and Lab-UK (susceptible) to the action of Dipel® and related damage in leaves of *Brassica pekinensis* cv. 'One Kilo SB' plants.

attractive regulation by their early induction (*SDF2L1* and *CDKAL1*) or by showing the greatest difference in expression levels between $KarUK_6$ and Lab-UK (*HEL-1*) [1], which made us consider the hypothesis of a possible role of these genes in resistance to DiPel® in *P. xylostella*. Then, the complete cDNA sequence of each of these three *P. xylostella* genes was cloned by means of the rapid amplification of cDNA ends procedure (RACE) and sent to GenBank. The sequences showed sizes of 1707, 1111 and 983 bp for *PxCDKAL1* (Accession No. HQ199330), *PxSDF2L1* (Accession No. HQ199329) and *PxHEL-1* (Accession No. HQ199327), respectively. Prediction function and structural domains using the SMART program (http://smart.embl-heidelberg.de/), combined with the analysis of phylogenetic relationships, revealed important features about the molecular function of each of these genes not previously described in *P. xylostella* [1].

Determination of the role of the genes *PxCDKAL1*, *PxSDF2L1* and *PxHEL-1* in the resistance of $KarUK_6$ to DiPel® was carried out by combining the post-transcriptional silencing of gene expression via RNAi with insect bioassays, using different concentrations of the DiPel® insecticide formulation. To this end, fragments of double-stranded RNA (dsRNA) corresponding to each gene were synthesized *in vitro* and were administered orally to third-stage larvae. The effectiveness of RNAi was corroborated 24 h after dsRNA treatment by qRT-PCR using total RNA

1. Ayra-Pardo C, Raymond B, Gulzar A, Rodríguez-Cabrera L, Morán-Bertot I, Crickmore N, *et al*. Novel genetic factors involved in resistance to *Bacillus thuringiensis* in *Plutella xylostella*. Insect Mol Biol. 2015;24(6):589-600.

from the larval gut. The specificity of the RNAi for the target sequence was validated by the administration of non-specific dsRNA from the gene of the enzyme β-D-glucuronidase (Gus) of *Escherichia coli*, which did not cause any alteration in the transcript levels of the three genes selected [1].

Results of bioassays of DiPel®-exposed insects treated with RNAi of *PxSDF2L1*, *PxCDKAL1* and *PxHEL-1*, showed that the administration of control dsRNA significantly increased the mortality of all insects, both resistant and susceptible, as compared to those treated with specific RNAi, with engagement in the expression of *PxSDF2L1* and *PxCDKAL1*. In the case of *PxHEL-1*, a significant increase in the mortality of larvae treated with RNAi was detected exclusively for the $KarUK_6$ strain [1]. No differences in larval mortality rates were observed between buffer-fed control groups or containing nonspecific dsRNA-Gus ($F_{4.21}$ = 1.63, p = 0.20), which indicates that interaction with dsRNA molecules per se does not affect response to DiPel®. Likewise, in the control groups it was verified that the RNAi treatment did not affect the formation of pupae and rise of adults. These results are novel since there were no reports on the participation of the genes *PxSDF2L1*, *PxCDKAL1* and *PxHEL-1* in the resistance to the DiPel® insecticide in the $KarUK_6$ strain of *P. xylostella*.

Conclusions

In summary, our results show a connection between DiPel® resistance and constitutive over-expression in $KarUK_6$ of three new genes of P. *xylostella* that code for homologues of CDKAL1 methyl-thiotransferase, SDF2L1 co-chaperone and metalloproteinase HEL-1, here designated PxCDKAL1, PxSDF2L1 and PxHEL-1, respectively. The suppression of the expression of these genes in the intestine of *P. xylostella* affected in all cases the ability of the larvae to survive the ingestion of the pathogen. The diversity of genes with differential expression in the $KarUK_6$ gut implies that a variety of cellular processes are involved in resistance maintenance, although the genes selected in this study for function analyses correspond to determinants of cellular homeostasis in other pathologies. For example, CDKAL1 is important for assuring the quality of proinsulin in β cells from pancreas, while SDF2L1 is crucial in the innate immunity response in *Arabidopsis thaliana* against bacterial attack, by assisting in the correct folding of type 'LRR' receptors-kinases in the endoplasmic reticulum. HEL-1 metalloprotease belongs to the family of astacins, which have been associated with the formation of extracellular matrix and wound repair [1]. Therefore, the three new genes for *P. xylostella* described in this study are linked to the multitude of factors whose expression changes in resistant insects and are directly involved in determining host susceptibility to *B. thuringiensis*.

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