Science Bulletin xxx (xxxx) xxx

Contents lists available at ScienceDirect

Science Bulletin

journal homepage: www.elsevier.com/locate/scib

Science Bulletin
www.scibull.com

Short Communication

Resurrecting the past: ancestral *Bacillus thuringiensis* pesticidal proteins reveal broad-spectrum insecticidal activity and protein engineering hotspots

Kui Wang [a,b,c], Ben Raymond [d], Jingan Zhang [a], Xiaolong Hu [a], Beibei Cao [a,b,c], Min Liao [a,c], Jie Zhang [a,b], Haiqun Cao [a,c], Changlong Shu [b,*]

[a] School of Plant Protection, Anhui Agricultural University, Hefei 230036, China
[b] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[c] Key Laboratory of Agri-products Quality and Biosafety (Anhui Agricultural University), Ministry of Education, Hefei 230036, China
[d] Department of Ecology and Conservation, University of Exeter, Penryn TR10 9FE, UK

ARTICLE INFO

Article history:
Received 23 April 2025
Received in revised form 17 July 2025
Accepted 5 September 2025
Available online xxxx

Bacillus thuringiensis (Bt) toxins are important means of insect pest management, particularly in genetically modified crops. Their widespread use has led to the evolution of resistance in many insects, creating the need to identify novel proteins with improved activity [1]. Different biotechnological methods have been used to explore novel toxin sequences, including random mutagenesis [2] and phage-assisted continuous evolution to select for effective toxins *in vitro* [3].

Here, we used ancestral sequence reconstruction (ASR) to explore ancient extinct insecticidal toxins as a source of novel functional proteins. ASR infers the likely path of evolution to identify putative ancestral sequences. Models of enzyme evolution propose that ancestral enzymes were generalists, able to interact with a wider range of substrates, and the generalist properties of ancient enzymes have produced several biotechnological applications [4]. In pest management, identifying broad-spectrum toxins that can kill a broad range of hosts is a key challenge.

The pesticidal proteins of Bt are obligate virulence factors, shaped by host selective pressure over millions of years. Coevolutionary theory suggests that ancient toxins may not necessarily be active against modern insects. For example, in one scenario, broad selective sweeps may cause “general resistance” to pathogen genotypes, in a process termed “arms race dynamics” [5,6]. This general resistance is long-lasting on an evolutionary timescale, and pathogen responses to this resistance are slow [5]. General resistance, therefore, implies that ancestral toxins will be less effective than modern variants [7].

Alternatively, if pathogen resistance is costly to maintain, then we may see fluctuating selective pressure on host/pathogen interactions [7–10]. In this scenario, resistance is not long-lasting, and ancient proteins could be effective against modern insects. Understanding which evolutionary model might apply to the evolution of Bt toxins is important. For example, if fluctuating dynamics are prevalent, then ancient toxins may be a valuable source of variants that can overcome modern resistance or extend host range. Finally, the sequence of effective ancient toxins can be exploited to inform the design of synthetic variants.

Here, we investigated ASR as a method for exploring functional sequence variation in Cry toxins with a view to identifying novel variants and to test the hypothesis that ancient toxins have a broader host range than modern proteins. Second, we test whether assays of ancestral toxins support a model of stable general resistance or fluctuating selection pressure. Finally, if ancient toxins can kill modern insects, we aimed to identify the residues responsible for activity and test if these residues can be used to re-engineer modern variants with improved characteristics. We focus on the Bt toxin Cry2 because small changes in sequence can have significant impacts on host range [11]. Cry2 toxins comprise a well-characterized family that displays a diverse toxicity spectrum against four different insect Orders.

We reconstructed the phylogeny of the *cry2A* family and predicted amino acid sequences at seven ancestral nodes, which were cloned and expressed in *Escherichia coli* (Anc0-Anc6) (Fig. 1a, Fig. S1 online). For these seven ancestral sequences characterized

---

[*] Corresponding author.
E-mail address: shuchanglong@caas.cn (C. Shu).

https://doi.org/10.1016/j.scib.2025.09.018
2095-9273/

Please cite this article as: K. Wang, B. Raymond, J. Zhang et al., Resurrecting the past: ancestral *Bacillus thuringiensis* pesticidal proteins reveal broad-spectrum insecticidal activity and protein engineering hotspots, Science Bulletin, https://doi.org/10.1016/j.scib.2025.09.018

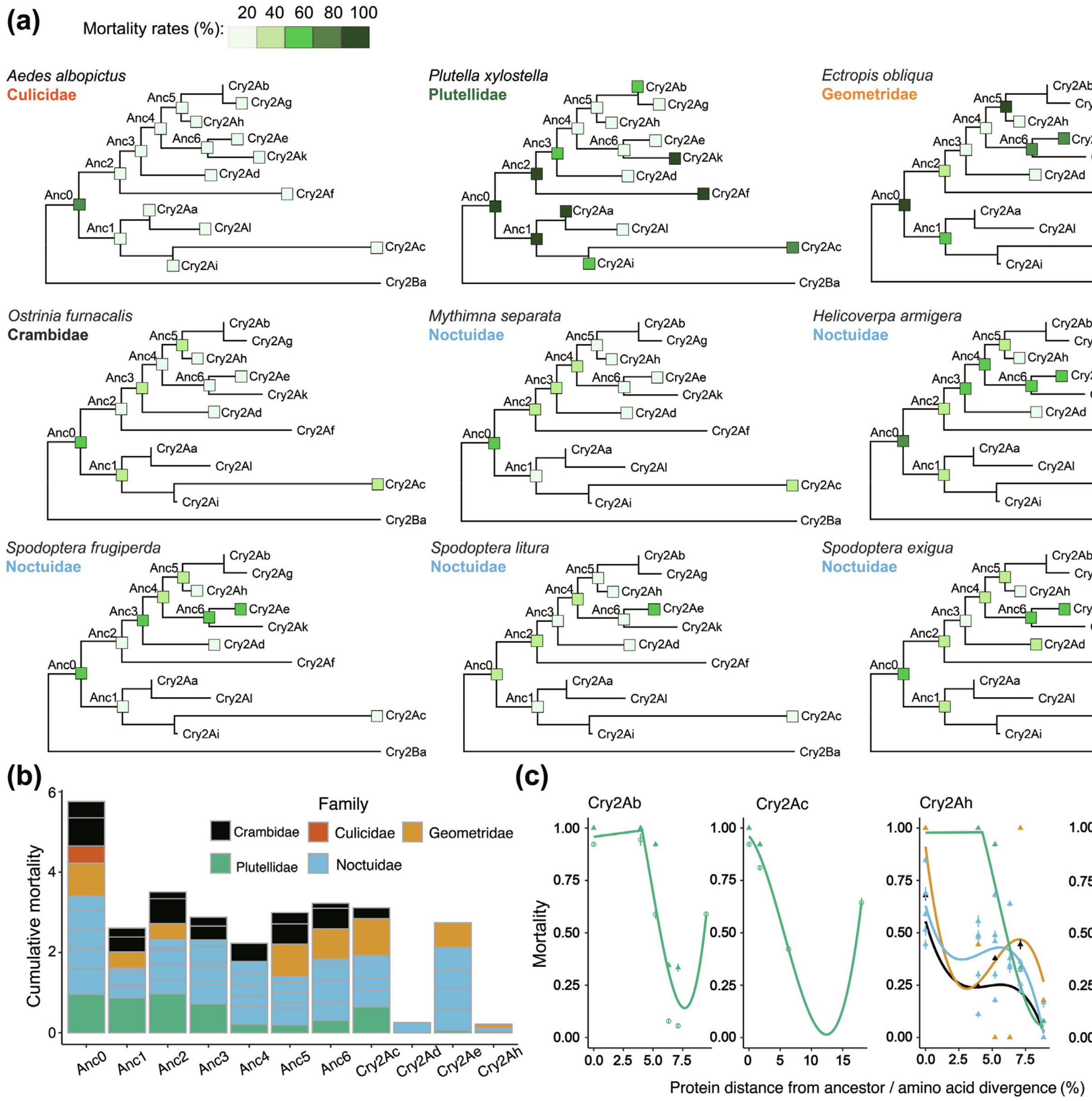

Fig. 1. Fluctuating efficacy of ancient, reconstructed Bt Cry2 insecticidal proteins and modern proteins across ten insect species. (a) We have mapped the toxicity of proteins across the maximum likelihood phylogeny of protein sequences for one mosquito species *Ae. albopictus* and nine commercially significant Lepidopteran species. We show the mortality of *Ae. albopictus* and *P. xylostella* at toxin doses of 100 ng and 10 μg/mL, respectively. Mortality data for all other species were totaled across 10 and 100 μg/mL. (b) Variation in host range across insect families for ancient and modern Cry2 toxins the bar plots show the total cumulative (proportional) mortality for all bioassay data (three replicate assays at 100 and 10 μg /mL). Species-level data are separated by grey outlines. (c) We examined how protein toxicity in modern Lepidoptera evolved by using amino acid divergence from the Cry2 ancestor (Anc0) to represent toxin age. Most toxins did not show a steady decline in efficacy over time (arms race dynamics); instead, efficacy fluctuated with protein age, supported by significant non-linear terms in all models (Table S3 online). Data show mean (±SEM) mortality from three replicates; error bars are hidden if smaller than data points. Lines represent back-transformed statistical models. While species/genus explained some variation, we present a conservative family-level summary. Filled triangles indicate doses of 100 μg/mL, hollow circles 10 μg/mL. Colour coding follows panel (b). Plots of Cry2Ah and Cry2Ae assay show only 100 μg/mL (lower dose was qualitatively similar). Cry2Ai toxicity data were fitted as an intermediate node in the Cry2Ac lineage.

in this work, more than 95 % of the ancestor sites exhibited posterior probabilities greater than 90 % (Fig. S2 online). The reconstructed sequences exhibit amino acid identities ranging from 79.30 % to 98.26 %, relative to contemporary Cry2A proteins (Table S1 online). We assayed pesticidal activity against nine Lepidoptera insects, *Helicoverpa armigera, Chilo suppressalis*, *Plutella xylostella, Ostrinia furnacalis*, *Ectropis obliqua, Mythimna separata, Spodoptera exigua*, *S. frugiperda*, *S. litura,* and the mosquito *Aedes albopictus.* Note that the low expression of some modern toxins limited bioassay coverage in some cases (Fig. 1a). All reconstructed toxins were able to kill some insect hosts. As predicted, the most ancient Cry2A protein, the root node Anc0, was a broad-spectrum toxin and was active against *Ae. albopictus* and all Lepidoptera (Fig. 1a, b, Table S2 online). No other protein could kill this Dipteran, although activity against other *Aedes* mosquitoes is known from some extant Cry2A proteins [12]. The remaining ancestral toxins had broad activity across the Lepidoptera, while three of the four modern toxins had a narrower host range (Fig. 1a, b).

In order to explore if Bt toxins are shaped by fluctuating selection, we tested for non-linear changes in toxicity against toxin age, where we had data from three or more nodes within the Cry2 phylogeny (Fig. 1c). We used sequence divergence from the root node to represent toxin age. Full statistics are presented in Table S3 (online). Assays of *P. xylostella* against Cry2Ab and Cry2Ac lineages were consistent with fluctuating selection pressure (Fig. 1c, cubic term Cry2Ab, quadratic term Cry2Ac, $P < 0.0001$). When we examined toxin efficacy in the Cry2Ae and Cry2Ah lineages across all the assayed Lepidoptera, we found evidence for fluctuating selection within the two modern "macro-moth" families (Noctuidae, Geometridae) (Fig. 1c, cubic distance X family interaction $P < 0.0001$). Simple linear declines in toxin efficacy were observed in *P. xylostella* (Plutellidae) and *O. furnacalis* (Crambidae); this pattern is not predicted by arms-race dynamics (which predicts that ancient toxins will be less effective) [7] but is consistent with the recent evolution of resistance and a shift to narrower host-range toxins. Since the macro-moths (Macroheterocera) are an abundant and speciose taxon within Lepidoptera [13], we speculate that Bt toxin activity within the Cry2 family may have been shaped by this insect group in particular. One explanation for the weaker activity of toxins of intermediate age is that errors in reconstructing sequences meant that this group was less active than the root Anc0. However, the high activity of the ancient toxins illustrates the power of this method in terms of exploring novel functional variation in toxin sequence (Fig. 1).

The ancestral proteins exhibited a typical three-domain Cry protein structure (Fig. 2a). The root mean square deviation (RMSD) of the Anc0 protein's structural similarity with Cry2Aa (PDB: 1I5P) was 0.168 Å. We hypothesized that the toxicity of ancestral variants can help in understanding the molecular basis of activity. Previous studies demonstrated that Domain I consists of a seven α-helix bundle that plays a key role in membrane insertion, toxin oligomerization, and pore formation. Domain II is a beta-prism formed by three anti-parallel β-sheets arranged around a hydrophobic core, with exposed loop regions responsible for receptor recognition. Domain III adopts a β-sandwich structure composed of two anti-parallel β-sheets. Both domains II and III contribute to insect specificity through specific interactions with distinct gut proteins in target insects [14]. Here, we looked at Cry2Ae and Cry2Ah, which are potential candidates for insect biocontrol [15,16]. Both these toxins had limited efficacy against *M. separata* and *E. obliqua,* and we tested whether we could re-engineer these toxins to improve efficacy and host range based on our reconstruction of ancient variants. The ancestral root Anc0 has 79 amino acids diverging from Cry2Ae and Cry2Ah, with 33 residues potentially associated with toxicity (Figs. S3–S6 online). Our assays indicated that introducing ancestral residues (I318, S337, N361, E385, A460) into Cry2Ae and Cry2Ah resulted in increased insecticidal activity ($\chi^2 = 342$, $df = 6$, $P < 0.0001$ and $\chi^2 = 384$, $df = 6$, $P < 0.0001$ respectively). Increased activity was primarily seen in Cry2Ae against *M. separata* and in Cry2Ah against *E. obliqua* (Fig. 2b, c), although several Cry2Ae mutants had improved activity in multiple hosts (protein X host interaction, $\chi^2 = 290$, $df = 18$, $P < 0.0001$). None of these mutants, however, recovered the full host range of the root Anc0 toxin. These five sites were

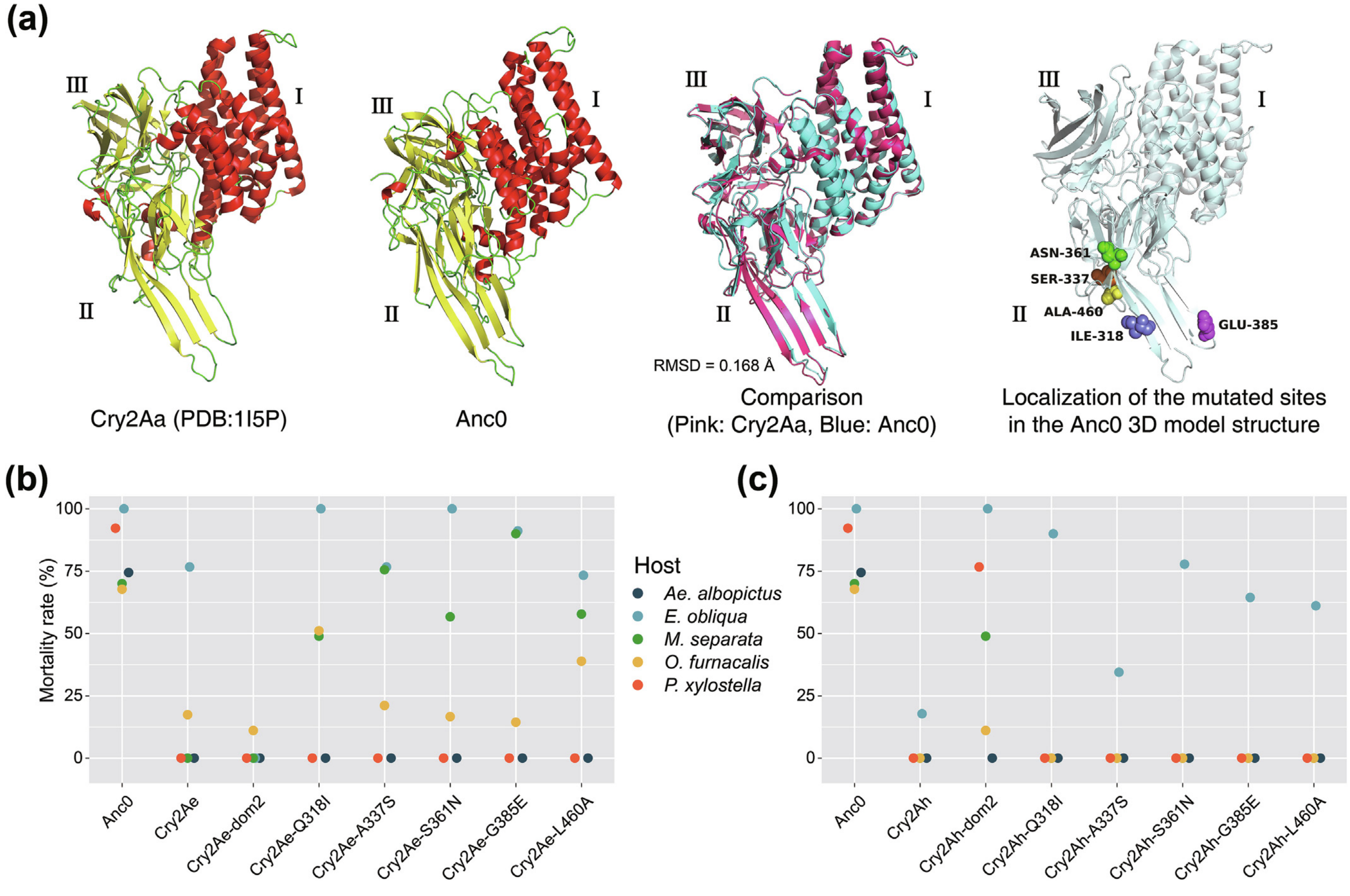

Fig. 2. Exploring the basis of toxicity in ancient Cry2 pesticidal proteins. (a) Comparison of 3D-structure of Cry2Aa and Anc0; the 3D-structure of Anc0 was predicted by AlphaFold3 (https://alphafoldserver.com/). Anc0 residues engineered into modern toxins are shown in the far-right structure. The mutated sites (Ile-318, Ser-337, Asn-361, and Glu-385) were all located within β-folded sheet regions, and Ala-460 was located in a loop region. Toxicity of mutated Cry2A proteins against a panel of insect species, including (b) Cry2Ae protein and (c) Cry2Ah proteins. Bioassays were conducted using a dose of 100 μg/mL for all Lepidoptera species, except for *P. xylostella*, which was tested at 10 μg/mL. In contrast, *Ae. albopictus* was evaluated at a dose of 100 ng/mL. The presented data represent the average mortality across three replicates, with $n = 30$ insects per replicate. For comparison, we have re-plotted the toxicity data for Anc0 and native proteins; Cry2Ae-dom2 and Cry2Ah-dom2 are mosaics of modern proteins that incorporate the entire domain II of Anc0.

all located in Domain II, which is associated with the specificity of Cry proteins [11]. Notably, replacing the entire domain II of Cry2Ae with that of Anc0 decreased toxicity relative to the native Cry2Ae ($z = -7.82$, $P < 0.0001$), indicating that a residue level investigation does have value. Electrostatic and hydrophobicity analyses combined with MD simulations suggest that these mutations modulate receptor binding by altering surface properties and hydrogen-bond networks (Figs. S7–S14 online). And we propose a "Goldilocks principle" of toxin dynamics: excessive rigidity leads to dysfunction, over-stabilization reduces specificity, whereas ancestral intermediate dynamics enable broad-spectrum activity through controlled flexibility. The insecticidal efficacy of Bt proteins depends on the precise tuning of structural dynamics, with ancestral domain configurations being optimally suited for recognizing a diverse range of targets.

In consideration of the broad application prospects of ancestral proteins, particularly Anc0, we evaluated the alkali solubility of Anc0 and its potential risk to *Apis mellifera* L. workers. Compared with Cry2Aa, Anc0 exhibited significantly enhanced solubility in a weakly alkaline buffer at pH 10 (Fig. S15, Table S4 online). A dietary exposure experiment conducted on adult workers under controlled laboratory conditions showed that the average survival rate of bees exposed to 10 μg/mL of Anc0, the negative control, and the solvent control for 48 h remained above 66 %. In contrast, 70 % of the bees exposed to 175 μg/mL dimethoate (positive control) died during the testing period (Table S5 online). These data suggested that Anc0 has good potential for application in insect control.

In conclusion, we demonstrated that the ancient Bt Cry2A pesticidal proteins commonly have a greater host range than modern variants. Within the limits of the study, we found evidence of protein toxicity decreasing and increasing over evolutionary time, in support of a model of fluctuating selection dynamics between Bt and its hosts. This suggests that other ancient proteins may be a rich source of novel variants for the management of insects. Despite the ongoing debate regarding the accuracy of reconstructed proteins [17], this study showcases the potential of ancestral reconstruction as a tool for designing novel pesticidal proteins with desirable properties.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (32102295).

Author contributions

Kui Wang, Jie Zhang, and Changlong Shu conceptualized the study. Jingan Zhang and Xiaolong Hu performed all experimental work. Ben Raymond, Beibei Cao, and Min Liao conducted the formal analysis. Kui Wang, Ben Raymond, and Changlong Shu interpreted the results and wrote the paper. Jie Zhang and Haiqun Cao supervised the project. Kui Wang was responsible for funding acquisition.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scib.2025.09.018.

References

[1] Melo AL, Soccol VT, Soccol CR. *Bacillus thuringiensis*: mechanism of action, resistance, and new applications: a review. Crit Rev Biotechnol 2016;36:317–26.

[2] Yamamoto T. Engineering of *Bacillus thuringiensis* insecticidal proteins. J Pestic Sci 2022;47:47–58.

[3] Badran AH, Guzov VM, Huai Q, et al. Continuous evolution of *Bacillus thuringiensis* toxins overcomes insect resistance. Nature 2016;533:58–63.

[4] Jensen RA. Enzyme recruitment in the evolution of new function. Annu Rev Microbiol 1976;30:409–25.

[5] Mundt CC. Durable resistance: a key to sustainable management of pathogens and pests. Infect Genet Evol 2014;27:446–55.

[6] Ishibashi K, Kezuka Y, Kobayashi C, et al. Structural basis for the recognition-evasion arms race between Tomato mosaic virus and the resistance gene Tm-1. Proc Natl Acad Sci USA 2014;111:3486–95.

[7] Koskella B, Parr N. The evolution of bacterial resistance against bacteriophages in the horse chestnut phyllosphere is general across both space and time. Philos Trans R Soc Lond B Biol Sci 2015;370:20140297.

[8] Gaba S, Ebert D. Time-shift experiments as a tool to study antagonistic coevolution. Trends Ecol Evol 2009;24:226–32.

[9] Bitter MC, Berardi S, Oken H, et al. Continuously fluctuating selection reveals fine granularity of adaptation. Nature 2024;634:389–96.

[10] Sasaki A. Host-parasite coevolution in a multilocus gene-for-gene system. Proc Biol Sci 2000;267:2183–8.

[11] Shu C, Zhou J, Crickmore N, et al. *In vitro* template-change PCR to create single crossover libraries: a case study with *B. thuringiensis* Cry2A toxins. Sci Rep 2016;6:23536.

[12] Goje LJ, Elmi ED, Bracuti A, et al. Identification of *Aedes aegypti* specificity motifs in the N-terminus of the *Bacillus thuringiensis* Cry2Aa pesticidal protein. J Invertebr Pathol 2020;174:107423.

[13] Kawahara AY, Plotkin D, Espeland M, et al. Phylogenomics reveals the evolutionary timing and pattern of butterflies and moths. Proc Natl Acad Sci USA 2019;116:22657–63.

[14] Bravo A, Gill SS, Soberón M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 2007;49:423–35.

[15] Gouffon C, Van Vliet A, Van Rie J, et al. Binding sites for *Bacillus thuringiensis* Cry2Ae toxin on heliothine brush border membrane vesicles are not shared with Cry1A, Cry1F, or Vip3A toxin. Appl Environ Microbiol 2011;77:3182–8.

[16] Li S, Wang Z, Zhou Y, et al. Expression of *cry2Ah1* and two domain II mutants in transgenic tobacco confers high resistance to susceptible and Cry1Ac-resistant cotton bollworm. Sci Rep 2018;8:508.

[17] Gumulya Y, Gillam EM. Exploring the past and the future of protein evolution with ancestral sequence reconstruction: the 'retro' approach to protein engineering. Biochem J 2017;474:1–19.