Research Article

Received: 19 August 2013 Revised: 22 October 2013 Accepted article published: 29 November 2013 Published online in Wiley Online Library: 2 January 2014

(wileyonlinelibrary.com) DOI 10.1002/ps.3694

The basis for rootstock resilient to *Capnodis* species: screening for genes encoding δ-endotoxins from *Bacillus thuringiensis*

Galina Gindin,[a] Zvi Mendel,[a] Bella Levitin,[b] Pradeep Kumar,[b] Tal Levi,[b] Preeti Shahi,[b] Vadim Khasdan,[b] Dan Weinthal,[b] Tatiana Kuznetsova,[a] Monica Einav,[b] Ariel Kushmaro,[c,d,e] Alex Protasov,[a] Arieh Zaritsky[b] and Eitan Ben-Dov[d,f*]

Abstract:

BACKGROUND: Conventional methods often fail to control the flatheaded borers *Capnodis* spp., major pests of stone fruit trees; the larvae are protected from insecticides and predation because they feed deep in the roots. A potential solution is transgenic trees producing in their roots toxic compounds such as Cry proteins of *Bacillus thuringiensis* (*Bt*).

RESULTS: Toxicities against *Capnodis* larvae were demonstrated by exploiting a recently designed artificial larval diet and an available collection of field isolated *Bt*. An isolate of *Bt tenebrionis* (*Btt*) from commercial bioinsecticide (Novodor) displayed LC_{50} and LC_{95} values of 3.2 and 164 mg g^{-1}, respectively, against neonates of *Capnodis tenebrionis*, whereas values of the most toxic field isolate K-7 were 1.9 and 25.6 mg g^{-1} respectively. Weights of surviving larvae after 1 month on diets containing low concentrations of K-7 (0.1–1.0 mg g^{-1}) were lower than on *Btt* or untreated larvae. K-7 was also toxic against larvae of *C. cariosa* and *C. miliaris* and found to harbour genes encoding Cry9Ea-like and Cry23Aa/Cry37Aa binary toxins.

CONCLUSION: Larvae of *Capnodis* spp. are susceptible to *Bt* Cry toxins. Expressing *cry* genes active against these pests thus seems a feasible solution towards production of transgenic rootstock trees resilient to the pest.

Keywords: biological control; integrated pest management; *Capnodis* spp; *cry* genes; *Bacillus thuringiensis* toxins; rootstock

1 INTRODUCTION

Flatheaded borers *Capnodis* spp. (Coleoptera: Buprestidae) inflict serious damage to fruit and ornamental trees around the Mediterranean, in southern Europe and in western Asia. Three species cause the most serious agricultural damage in the Mediterranean and the Middle East: *C. tenebrionis* and *C. carbonaria* attack stonefruit (almond, apricot, cherry, nectarine, peach and plum) trees,[1,2] and *C. cariosa* affect pistachio plantations in Eastern Asia, especially in Turkey and Iran.[3,4] Adults feed on the twig cortex, and the larvae feed in the roots. Female *Capnodis* lay ∼2000 eggs in the soil over several months, and the neonates penetrate the roots. Development of larvae and pupae may last 6–14 months. Being inside the root tissues, the advanced immature stages are protected from insecticides and predation.[5,6] A single larva of *C. tenebrionis* can destroy a young tree, and a few may kill an adult tree.[7]

Natural occurrence of *Capnodis* enemies is rare and ineffective:[8] entomopathogenic nematodes and parasitoids targeting the larvae have been tried, but success is marginal. Growers mostly rely on non-specific synthetic insecticides such as organophosphates and carbamates or systemic insecticides such as neonicotinoids.[1,9] Continuous exposure to pesticides leads to occurrence of highly resistant *Capnodis* populations.[10] Moreover, none of the routinely used rootstock is sufficiently resilient to *Capnodis* spp.[11,12] In spite of intensive applications of insecticides, significant economic losses are still recognised.[13] The need for safe and effective management options for this pest is thus urgent and critical. Entomopathogenic nematodes and fungi were recently tested as control means against larvae[14–16] and in commercial orchards,[17]

* *Correspondence to: Eitan Ben-Dov, Department of Life Sciences, Achva Academic College, MP Shikmim 79800, Israel. E-mail: etn@bgu.ac.il*

a *Department of Entomology, Agricultural Research Organisation, The Volcani Centre, Bet Dagan, Israel*

b *Department of Life Sciences, Ben-Gurion University of the Negev, Be'er-Sheva, Israel*

c *Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Be'er-Sheva, Israel*

d *National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Be'er-Sheva, Israel*

e *School of Materials Science and Engineering, Nanyang Technological University, Singapore*

f *Department of Life Sciences, Achva Academic College, MP Shikmim, Israel*

but their use is still limited. The question whether δ-endotoxins of *Bacillus thuringiensis* (*Bt*) are toxic to larvae of *Capnodis* and thereby may be used in the management of these beetles is explored here.

The entomopathogenic bacterium *Bt* was first discovered over a century ago[18,19] and has now become the leading biological insecticide used commercially to control insects.[20] It is a gram-positive, aerobic, endospore-forming saprophyte species, naturally occurring in various soil and aquatic habitats. Various subspecies are recognised by their ability to produce large quantities of insect larvicidal Cry (for crystal) and Cyt (for cytolytic) δ-endotoxins assembled as parasporal bodies.[21] These insecticidal crystal proteins (ICPs), synthesised during sporulation, are tightly packed by hydrophobic bonds and disulfide bridges. The crystals are ingested by the larvae and solubilised in the insect midgut, and the proteolitically activated ICPs insert into the apical microvilli membranes. The high potencies and specificities of ICPs have spurred their use as natural control agents against insect pests in agriculture, forestry and human health.[22]

Efficient screening of entomopathogenic microorganisms active against the flatheaded borers *Capnodis* spp. has been hampered by lack of artificial diet. The only biological agents known so far against *C. tenebrionis*, the fungi *Beauveria bassiana* and *Metarhizium anisopliae*, have been tested by immersing neonate larvae (24 h old) individually for 10 s in a spore suspension (10^8 conidia mL^{-1}) and then transferring them onto a cut surface piece of an apricot branch.[15] The recently developed diet[23] enables proper growth of neonate larvae and hence appropriate bioassays. Here, for the first time, the potential of *Bt* to control *Capnodis* spp. is demonstrated.

As the first step towards production of transgenic rootstocks of stone fruit trees to control *C. tenebrionis*, the authors screened a collection of field isolated *Bt* for *cry* or *cyt* genes encoding anti-coleopteran-active toxins. Distribution of such genes in *Bt* field isolates depends on geographic habitats and the genetic varieties of the isolates, as well as on the number, quality and type of the primers used.[24–31]

2 MATERIAL AND METHODS

2.1 Growth of *B. thuringiensis* cultures

Bt subsp. *tenebrionis* (*Btt*) from Novodor (Valent BioSciences) and the screened *Bt* field strains (isolated from soil and different insect cadavers, but not of the *Capnodis* spp.) were cultivated in liquid media – LB for isolating total DNA and nutrient sporulation medium for bioassays. The latter includes nutrient broth (8 g L^{-1}), Tryptone (10 g L^{-1}), NaCl (5 g L^{-1}) and 1 mL of an autoclaved sporulation salt solution (0.14 M $CaCl_2$, 0.20 M $MgCl_2$ and 0.01 M $MnCl_2$).[32] Cultures were vigorously aerated (250 rpm) in a gyratory incubator (30 °C).

For PCR analyses, total DNA was extracted from pure 16–18 h cultures (in LB) using an UltraClean Microbial DNA Isolation kit (MoBio Laboratories, Solana Beach, CA) according to the manufacturer's instructions and stored at −20 °C.

For bioassays, growth (in sporulation medium) was extended for at least 72 h, appearance of spores and parasporal inclusions was ascertained by phase-contrast microscopy and the cultures were harvested by centrifugation. Protein content was determined[33] after alkaline hydrolysis, and dry weight was measured in vacuum-oven-dried pellets (at 70 °C for 24 h).

2.2 PCR analysis

Pairs of universal primers (Uns) for each of 14 *cry* and *cyt2Ca* homology groups encoding anti-coleopteran toxins (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/) were designed (Table 1) to amplify a specific fragment by simultaneous alignment to genes in that group using the Amplify 1.0 program (Bill Engels, University of Wisconsin, Madison, WI).

DNA from each strain was amplified using a TGradient thermocycler (Biometra, Göttingen, Germany). The reaction mixture included 12.5 mL of ReddyMix PCR Master Mix (Thermo Fisher Scientific Inc., Surrey, UK), 1 μL of 10 mM of each primer (forward and reverse), 1 μL of 2.5 mg mL^{-1} bovine serum albumin, 1–2 μL of the sample total DNA (50–100 ng μL^{-1}) and water, for a total volume of 25 μL. An initial denaturation hot start of 4 min at 95 °C was followed by 30 cycles of the following incubation pattern: 94 °C for 30 s, 48–54 °C for 30 s and 72 °C for 30–60 s. Each experiment was associated with two kinds of control: negative (without DNA template) and positive, for *cry3* with a template from *Btt* of a commercial formulation (Novodor, Valent BioSciences) and for *cry7* and *cry8* with a template from *Bt kumamotoensis* HD-867.

2.3 Cloning *cry23*/*cry37* binary toxin genes for expression and analysis of Cry23

The binary toxin genes *cry23Aa*/*cry37Aa* were cloned for a high expression level under strong tandem promoters of *cyt1Aa*.[34] The genes were amplified from isolate K-7 with the primer pair GATCCATGGGAATTATTAATATCCAAGATG (*cry23Aa*-F-*Nco*I) and TTATGCTGGAGTCAAGGAATACTTAATTGTC (*cry37Aa*-R), and the promoter was amplified from strain 4Q2-72 with GATTGAAAGCTTGAGAAAGGTAATAGAGATG (P*cyt*-F-*Hin*dIII) and GTTCCATGGATAAACAACTCCTTAAGTTAATTAG (P*cyt*-R-*Nco*I). The amplicons (529 bp of *Pcyt1Aa* and 1190 bp of *cry23*/*cry37*) were digested appropriately for ligation by *Nco*I, and for subsequent insertion into *Hin*dIII–*Sma*I-digested pHT315. The recombinant plasmid was electroporated into *Escherichia coli* DH5α using a Bio-Rad mini apparatus set, isolated, sequenced and electroporated into the acrystalliferous strain JPS78/11 of *Bt* subsp. *israelensis*. Transformants were screened on LB plates containing 20 μg mL^{-1} of erythromycin at 30 °C.

Putative Cry23Aa was isolated from SDS-PAGE and subjected to total tryptic digestion, and the ensuing peptides were analysed by means of nano-LC-MS/MS with the LTQ-Orbitrap (Thermo Fisher, Bremen, Germany) and identified by Discoverer software v.1.3.

2.4 Rearing *Capnodis* species for bioassays

About 500 adult *C. tenebrionis* were collected in apricot, plum or peach orchards, whereas *C. cariosa* and *C. miliaris*, found in fewer numbers, several dozen, were collected in small stands of *Pistaciae lentiscus* and *Salix* sp. respectively. Old beetles were identified by their dull mandibles and discarded.[1] The adult beetles were fed with host plant twigs over a period of several weeks. Females were reared as described in Gindin *et al.*[23] Eggs were incubated at 28 °C, and newly hatched larvae were used for bioassays.

2.5 Bioassay procedure

The artificial diets were prepared in three parts as described in Gindin *et al.*,[23] with 5% w/w of host plant cortex. Suspensions of spores and crystals of *Bt* isolates were incorporated into the second diet portions that were added to the first and third portions.

Table 1. Characteristics of universal primers for anti-coleopteran-active gene groups *cry3*, *cry7*, *cry8*, *cry14*, *cry18*, *cry22*, *cry23*, *cry26*, *cry28*, *cry34*, *cry35*, *cry36*, *cry38*, *cry43* and *cyt2Ca*

Primer sequences[a]	Targeted genes	Product size (bp)	Reference
Un3(d) – CGTTATCGCAGAGAGATGACATTAAC	*cry3Aa*, *-Ba*, *-Bb*, *-Ca*	589–604	25
Un3(r) – CATCTGTTGTTTCTGGAGGCAAT			
Un7,8(d) – AAGCAGTGAATGCCTTGTTTAC	*cry7Aa*, *-Ab*; *cry8Aa*, *-Ba*, *-Ca*	420–423	25
Un7,8(r) – CTTCTAAACCTTGACTACTT			
Un8(d) – AACTTAGTGGAATGCCTATC	*cry8Aa*, *-Ba*, *-Bb*, *-Bc*, *-Ca*, *-Da*, *-Db*, *-Ea*, *-Fa*, *-Ga*	860–878	Present study
Un8(r) – TTATATACGTAAGGAATGGACTGT			
Un14(d) – CCTAAAGGTGGAAGTGGATACGCT	*cry14Aa*	361	Present study
Un14(r) – ATTTCCCCGTGCTTCCCTTTAC			
Un14(d2) – TGCGTTGGTTGATACAGCTGGAGA	*cry14Aa*	410	Present study
Un14(r2) – CAGTACCTGACCACTGTGCATCTA			
Un18(d) – AAGGGAATGGACAGAATGGAAAG	*cry18Aa*, *-Ba*, *-Ca*	462	Present study
Un18(r) – CGTAAAAAAGTTAAATGAAGCGTG			
Un18(d) – AAGGGAATGGACAGAATGGAAAG	*cry18Aa*, *-Ba*, *-Ca*	762	Present study
Un18(r2) – CCCTCATTCACCTTATTATCCCC			
Un22(d) – TTTCATAGAGGATCAATTGG	*cry22Aa*, *-Ab*, *-Ba*	698–734	Present study
Un22(r) – ATTGTTTTTTCATCACTTTC			
Un23(d) – GTGAAAGCCGGCACCTCAATAAGT	*cry23Aa*	293	Present study
Un23(r) – GCTGCAATAAGCGCACCATCT			
Un26(d) – CGCGCTGTTCAATTATCAAGTGC	*cry26Aa*	362	29
Un26(r) – ATATGGAAAGAAAAGGCGTGTGGA			
Un28(d) – GTATTGGACCGAGGAGATGAAAGT	*cry28Aa*	466	29
Un28(r) – GTACGGCAAAGCGACAGAACA			
Un34(d) – AGGTTGATATTTATGTCAGC	*cry34Aa*, *-Ab*, *-Ac*	649–651	Present study
Un34(r) – ATCAATAGGAAATAAAAACCA			
Un35(d) – GATGATTCAGGTGTTAGTTTAATG	*cry35Aa*, *-Ab*, *-Ac*	364	Present study
Un35(r) – GTGGGAGTTGAATTGTTTGTACAG			
Un36(d) – GATGTGGTTGCCAGCAAGGTAA	*cry36Aa*	554	Present study
Un36(r) – AACTCGACCATTTCCTCGATTCCC			
Un38(d) – TTCTACTCCCACACGTTCTG	*cry38Aa*	741	Present study
Un38(r) – TCAATGGTTCCATCAGCTAACA			
Un43(d) – CTTTACAGTCCCAATAAGTATCC	*cry43Aa*, *-Ba*	842	Present study
Un43(r) – GTATAAATTCCTCTCGTAAGC			
cyt2Ca(d) – TCGCAAGAAAGCGAACGATGGA	*cyt2Ca*	298	Present study
cyt2Ca(r) – TTCTAGGTAAGTGACGTGGCGATT			

[a] (d) and (r), direct and reverse primers respectively.

For efficient initial screening with *C. tenebrionis* larvae, 3–4 *Bt* isolates were grouped; 4 mL of each bacterial suspension was added to the diet for the bioassay (a total of about 10^9 spores g^{-1} diet). This procedure made it possible to screen more than 40 field isolates. Then, the 19 isolates of the most toxic combinations were separately bioassayed at the same single dosage (see Table 2). The seven most toxic isolates among the latter were then separately subjected to dose–response assays. A range of 5–6 concentrations between 0.1 and 5.0 mg total protein g^{-1} diet was used to test each isolate. For each protein concentration, 27–30 neonates (according to their availability in the rearing system) were placed in 50 mm diameter petri dishes (three neonates in each dish) containing 10–12 g of diet. Each isolate was assayed in 3–4 independent tests.

The dishes were kept at 28 °C, and larval survival was monitored. Some of the killed larvae could not be detected because of their minute size; others changed their colour from yellowish white to black. Thus, mortality was calculated by subtracting the number of surviving larvae from their initial number. In several tests, the weight of the larvae was measured. In these cases, the surviving one-week-old larvae were reared individually under the same conditions on the same diet, but with crumble-structure diet.[23] Four weeks later, the weights of the surviving larvae were recorded.

The toxicity of the most active isolate (K-7) was tested against three *Capnodis* species (*C. tenebrionis*, *C. cariosa* and *C. miliaris*) and compared with that of the available *Btt* strain in a diet with 1.5 mg total protein g^{-1} diet, corresponding to its LC_{50} against *C. tenebrionis*. Peach cortex was substituted in the artificial diet for pistachio *Pistacia vera* and willow *Salix nigra* cortex for *C. cariosa* and *C. miliaris* respectively.

The data of 3–4 bioassays for each isolate were pooled and evaluated with the EPA Probit Analysis program v.1.4 (USEPA 1988), and results are presented as LC_{50} and LC_{95} (concentrations that kill 50 and 95% of the exposed population respectively), with their upper and lower confidence limits.[35] The differences between treatments were considered significant when the 95% confidence limits of the dose–response curves of the tested insecticides did not overlap. The effect of *Bt*-containing diets on different *Capnodis* spp. was analysed by ANOVA. If differences between treatments were significant, Tukey's test was applied for multiple comparisons between means.

Table 2. Initial screening of the toxicities against *C. tenebrionis* larvae of individual *Bt* isolates at 1.0×10^9 spores in 1 g of diet (arranged according to percentage mortality after 2 weeks)

Isolate	Mortality[a] (% dead larvae) 1 week	2 weeks
Control (water)	13.9	13.9
U-29	47.6	52.4
K-11	57.1	60
K-39	42.9	61.9
U-53	47.6	66.7
K-3	58.3	66.7
R-36	66.7	66.7
U-17	40.0	73.3
U-3	52.4	74.1
K-5	41.7	75
Btt	70.0	76.7
K-41	52.4	76.9
K-10	42.9	79.2
K-30	77.8	80.0
U-30	73.3	80.1
U-12	42.9	81.0
U-13	76.2	100
U-16	61.9	100
U-40	62.8	100
K-4	85.2	100
K-7	85.7	100

[a] Mortality in control – average of six bioassays (total 180 larvae). Each isolate was assayed in 9–10 dishes with three larvae per dish.

3 RESULTS

To pinpoint toxic field isolates with genes that may be responsible for toxicity against *Capnodis*, 28 of the 215 field isolates with profiles containing *cry7*/*cry8* genes[25] were selected for two parallel screening procedures, PCR analyses and bioassays respectively.

3.1 Toxicity of *Bt* isolates to *Capnodis tenebrionis* larvae

The initial combinatorial screening pointed to 19 isolates and *Btt* which were bioassayed separately (Table 2). The most toxic 7, as well as the commercial strain, were rigorously bioassayed with protein concentrations ranging from 0.1 to 5.0 mg total protein g^{-1} diet (Table 3).

The only commercial *Btt* (Novodor), known to be toxic to larvae of certain coleopteran species,[22] displayed LC_{50} and LC_{95} values of 3.2 and 164 mg g^{-1} respectively against neonates of *C. tenbrionis* (Table 3). This is the first case of a commercially available and new isolates of *Bt* being toxic to larvae of the flatheaded borer *Capnodis sensu lato*.

Toxicity of K-7 towards *C. tenbrionis* (LC_{50}/LC_{95} of 1.9/25.6 mg g^{-1}) was the highest among the tested field isolates, but it differed significantly from that of the commercial *Btt* (LC_{50}/LC_{95} of 3.2/164.3 mg g^{-1}). The toxicities of U-13 and K-4 were similar to the toxicity of *Btt*, whereas those of K-30 and R-36 were very low (Table 3).

Surviving larvae after 1 month on diets containing low sublethal concentrations of bacteria (0.1 and 1 mg protein g^{-1} diet) were weighed (Table 4; Fig. 1). The average weight on K-7 was significantly lower than on *Btt* or unexposed control (ANOVA: $F = 3.07$; df = 6, 148; $P = 0.007$), and on U-16 (with intermediate toxicities) the gained weights were intermediate.

Table 3. Toxicities of the most active *Bt* isolates against *C. tenebrionis* larvae[a]

Isolate	LC_{50} (95% CI)[b]	LC_{95} (95% CI)	χ^2	Slope ± SD
U-13	3.2 (1.6–6.4)	134.8 (35.6–4 924)	0.405	1.01 ± 0.25
U-16	5.1 (2.5–16.9)	459.5 (68.0–575 228)	1.556	0.84 ± 0.25
U-40	4.0 (2.2–8.1)	364.8 (72.6–37 381)	0.133	0.84 ± 0.21
K-4	4.7 (2.7–8.9)	222.9 (58.2–7165)	0.411	0.98 ± 0.23
K –7	1.9 (1.4–2.5)	25.6 (15.1–61.0)	3.042	1.47 ± 0.19
K-30	11.5 (4.4–801)	3 312 (158.8–∞)	0.061	0.67 ± 0.26
R-36	37.1[c]	378 190[c]	0.512	[d]
Btt	3.2 (1.9–5.6)	164.3 (49.9–2 296)	4.039	0.97 ± 0.19

[a] A total of 400–550 neonates (total used in 3–4 independent tests) were used for each of the tested isolates.
[b] LC values (mg protein g^{-1} diet) were determined after rearing for 1 week on diets containing bacterial isolates; df = 5.
[c] EC fiducial limits cannot be computed.
[d] Slope is not significantly different from zero.

Table 4. Survival and weights of *C. tenebrionis* larvae in the presence of low *Bt* concentrations

Isolate	Protein concentration (mg g^{-1})	Mortality (%)	Larval weight (mg ± SD)[a]
K-7	0.1	40	103.2 ± 70.9 x
	1.0	43.3	74.5 ± 49.5 x
U-16	0.1	13.3	118.4 ± 56.5 xy
	1.0	16.7	113.5 ± 76.0 xy
Btt	0.1	21	141.4 ± 49.2 y
	1.0	36.7	135.8 ± 48.6 y
Control	0	6.7	145.4 ± 89.1 y

[a] An average from 17–30 neonates after 1 month rearing at 28 °C. Different letters within a column indicate significant difference ($P < 0.01$).

3.2 Toxicities of *Btt* and K-7: comparison between three *Capnodis* spp.

Mortalities of the most active isolate K-7 and the available *Btt* strain against *C. tenebrionis*, *C. cariosa* and *C. miliaris* (with 1.5 mg *Bt* g^{-1} total protein) resemble each other (Fig. 2). Maximum mortality was reached after 2 weeks. Mortalities to all tested *Capnodis* species were significantly higher than those of corresponding control diets ($F = 8.94$; df = 2, 27; $P = 0.001$ for *C. cariosa*; $F = 5.24$; df = 2, 12; $P = 0.02$ for *C. militaris*; $F = 5.28$; df = 2, 27; $P = 0.01$ for *C. tenebrionis*). Larvae of *C. cariosa* seem to be slightly more susceptible to both tested isolates than to the other two species, but the differences were not significant. In the case of *C. cariosa* alone, efficacy of K-7 was significantly higher than that of the commercial *Btt* strain (Fig. 2).

3.3 PCR screening and analysis (Table 5)

Of the 28 isolates yielding appropriate amplicons with Un7,8(d)/Un7,8(r),[25] the most toxic 7 (Tables 2 and 3) were further screened with designed universal primers (Table 1) to reveal additional *cry* and *cyt* encoding anti-coleopteran-active toxins. Five suspicious genes were thus found (Table 5).

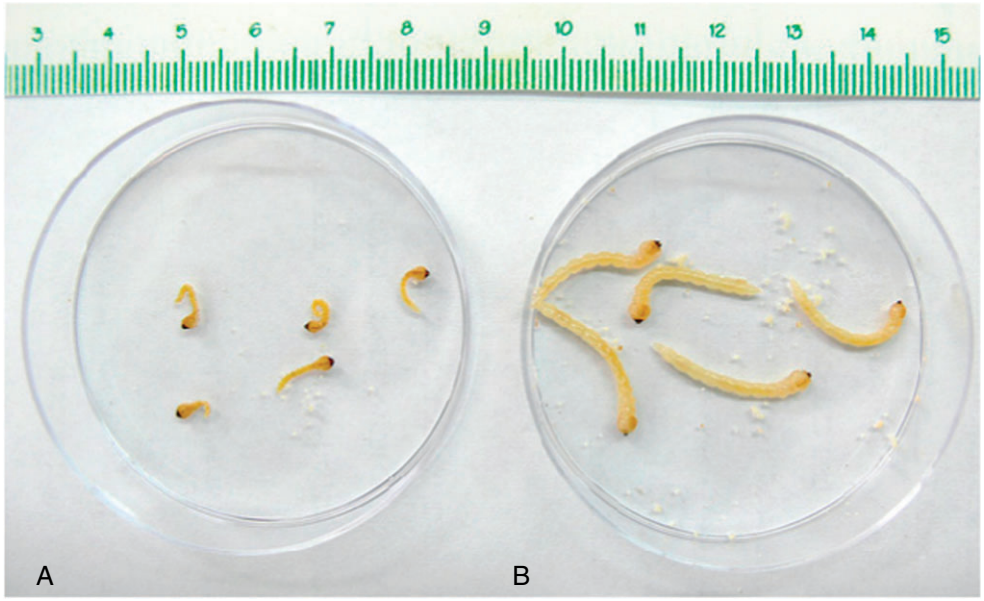

Figure 1. Effect of *Bt* isolate K-7 on growth of *C. tenebrionis* larvae. (A) Neonates were exposed to 1 mg of total protein of sporulated culture in 1 g of diet, and pictures were taken after 2 weeks at 28 °C. (B) Neonate developed in untreated diet.

Table 5. Screening of the *Bt* field isolates using universal primers for anti-coleopteran-active gene groups

Bt field isolate	*cry*-type gene profile identified previously[a]	*cry* gene identified in present study[b]
K-7	+	*cry23Aa* (100%, 259/259), *cry9Ea* (82%, 93/114)
U-16	+, *cry1Aa, -Ab, -Ac, -Da, cry2Aa, -Ab, cry9Aa, -Ba*	*cry1Db* (86%, 330/384)
U-40	+, *cry1Aa, -Ab, -Ca, -Da, cry2Ab, cry9Ea*	–
U-13	+, *cry2Ab*	*cry9Ea* (88%, 328/372)
K-4	+	*cry9Ea* (98%, 328/334)
K-30	+	*cry8La* (89%, 343/385)
R-36	+	*cry8Ia* (94%, 223/236)

[a] From Ben-Dov *et al.*;[26] +, positive with universal primers Un7,8(d) and Un7,8(r).
[b] *cry23Aa* was amplified with Un23 pair of primers; *cry9Ea*, *cry1Db*, *cry8La* and *cry8Ia* were amplified with Un7/8; in parentheses, percentage homology to *cry* of sequenced amplicons and absolute number of identical bases; –, no amplicon produced with universal primers used from Table 1.

The DNA of *isolate K-7* as a template was positive with two pairs of primers, Un7/8 and Un23 (Table 1); the amplicon of the former displayed 82% identity to *cry9Ea*, and that of the latter was identical to *cry23Aa* (AF038048).

The genes *cry23Aa* and *cry37Aa* are arranged in an operon and encode a binary-like toxin Cry23Aa/Cry37Aa (of 29/14 kDa);[36] they were amplified together, inserted for expression under the strong *cyt1Aa* tandem promoters[34] into shuttle vector pHT315[37] and sequenced (KF501394). The first gene in the operon is identical to *cry23Aa* (AF038048), whereas there is a single base discrepancy between the second gene and *cry37Aa* (AF038049) – C rather than T, resulting in a neutral change A117V, both non-polar amino acids. The genes are separated by a stretch of 29 bases (TAAATAACAAAAAAGGAAGGTTGATAAAA)[36] that includes the ribosome-binding site (RBS) for *cry37Aa*.

Putative Cry23Aa (of 267 amino acids) isolated from SDS-PAGE was subjected to total tryptic digestion; nine of the ten ensuing peptides, with 224 amino acids, were identical to those of Cry23Aa. The missing tryptic peptide (of 43 amino acids, position 103–145) is too long for analysis by this method; it lacks a chymotrypsin digestion site.

DNA of isolates U-16 and U-40 amplified appropriate fragments with Un7,8 but not with any other pair of the designed primers (Table 1). The amplicon from U-16 was 86% homologous to *cry1Db*. Both isolates, highly toxic against *C. tenebrionis* (Tables 2 and 3), are known to contain different combinations of genes from the *cry1*, *cry2* and *cry9* groups (Table 5).

The sequence of the amplicon obtained from DNA of isolate R-36 with Un7/8 displayed 94% identity with *cry8Ia*. The whole gene was amplified, cloned, sequenced (JX282317) and defined as a new gene, named *cry8Ra1*.[38]

4 DISCUSSION AND CONCLUSION

Toxicity of *Bt*-ICPs to flatheaded borers is demonstrated here for the first time by exploiting a recently designed artificial diet for larvae of three *Capnodis* species[23] and an available collection of *Bt* field isolates.[25]

ICPs of *Bt* are classified to three toxicity levels: high, when LC_{50} values are in the 0.01–0.10 μg mL^{-1} range, medium, in the 0.10–10 μg mL^{-1} range, and low, in the 10–1000 μg mL^{-1} range.[39] Toxicities of anti-coleopteran Crys are slightly lower and more variable than those of anti-lepidopteran toxins; the Crys are usually assayed *on* the leaf surface, whereas the present bioassays were performed *in* artificial diet. For example, the median LC_{50} of purified Cry3 toxins spread on leaf is 0.18 μg cm^{-2}; by applying the consistent 1:30 ratio between surface:incorporation

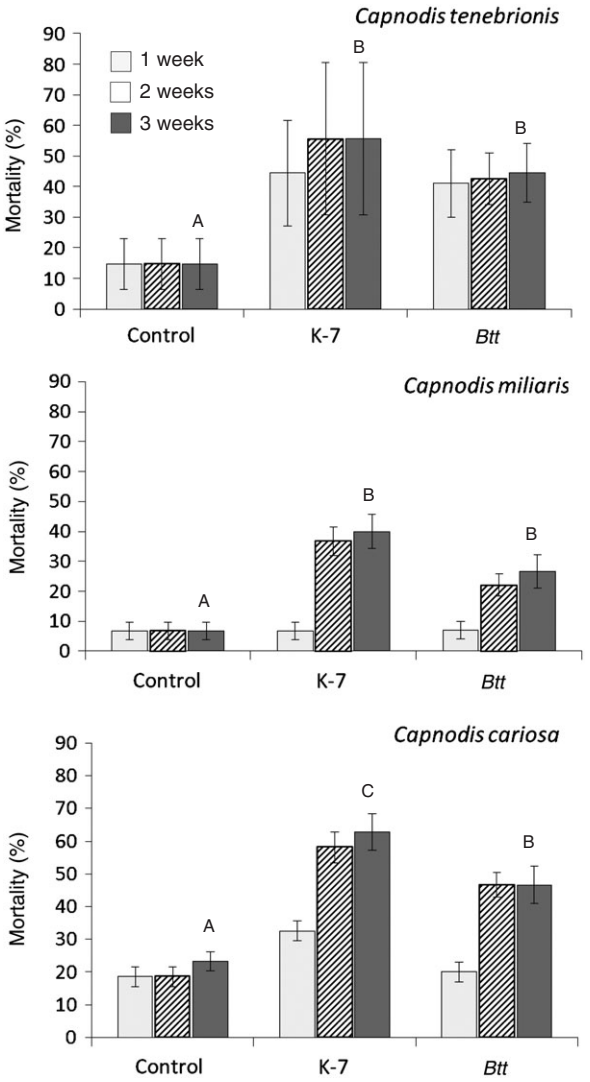

Figure 2. Efficacy of *Bt* isolates against three *Capnodis* species (average ± SD). Neonates were exposed to 1.5 mg of total *Bt* protein in 1 g of diet at 28 °C. Different letters, with each subfigure, indicate significant difference between means of the cumulative mortalities after 3 weeks of exposure to the respective diets ($P < 0.05$).

assays of lepidopteran values to coleopteran surface LC_{50} values, coleopteran purified toxins are calculated to be active in the 5 μg mL^{-1} range in diet incorporation assays.[39] LC_{50} values of the present *Bt* isolates are much lower – 1.9 mg g^{-1} for the most toxic K-7 (Table 3), but they were determined in units of *total protein*. This value is about 40% lower than LC_{50} (3.2 mg g^{-1}) of *Btt* under the same conditions; thus, the authors have identified a field *Bt* that is at least as toxic as a commercial strain that contains *cry3Aa* as its major ICP.[22] Moreover, unlike *cry3Aa* of *Btt*, toxins of K-7 inhibited growth of *C. tenebrionis* larvae at low, sublethal concentration (0.1 mg g^{-1}). It is therefore suggested that isolate K-7 displays a commercial potential.

The *cry23Aa*/*cry37Aa* operon, encoding the binary toxin with the intercistronic 29 bases that contain an RBS, was isolated from K-7 and deposited at the NCBI (KF501394). Both genes are identical to previously published sequences of this region (AF038048 and AF038049), excluding one base in *cry37Aa*.

A 'wild-type *Bt* strain B-21365' (from the Agricultural Research Service Culture Collection, USA) expressing *cry23Aa*/*cry37Aa* was found to be toxic against the weevil *Diaprepes abbreviates*, with LC_{50} to neonates of lyophilised pellets of 258.3 μg mL^{-1}.[40] *D. abbreviates* has become one of the most damaging insect pests of citrus and nursery crops in Florida. Larvae of the weevil feed on roots; hence, a transgenic approach that uses a genetically engineered citrus rootstock to express an ICP active against this pest is a plausible solution.[40] Transgenic corn plants expressing either *cry3Bb1*[41] or the binary toxin *cry34Ab*/*cry35Ab*[42] were protected from another root-feeding beetle, the western corn rootworm, *Diabrotica virgifera virgifera*. Toxicity of *Bt* B-21365 expressing *cry23Aa*/*cry37Aa* was evaluated ($LC_{50} = 6.30$ μg spore–crystal mixture μL^{-1} flour disc) against red flour beetle, *Tribolium castaneum*, a pest of stored grain, cereal products and peanuts.[43]

Both polypeptides of the Cry23Aa/Cry37Aa binary toxin are required for toxicity: Cry23Aa is homologous to the dipteran-active Mtx 2/3 proteins of *Bacillus sphaericus*, whereas Cry37Aa is not related to the other *Bt* crystal proteins.[44] The crystal structure of this complex consists mainly of β-strands.[45] Cry37Aa may facilitate binding of the channel-forming Cry23Aa, which has an elongated structure dominated by antiparallel β-sheets that is capable of forming channels in planar lipid bilayers, and has a structure and shape reminiscent of domains 2–4 of the toxin proaerolysin of *Aeromonas hydrophilia*.[46] Both genes are potential candidates in pyramiding expression with other gene(s) in stone-fruit rootstocks for efficient control of *Capnodis* larvae.

Target specificities of Cry and Cyt toxins, many of which are also synergistic among themselves, are occasionally breached:[39] Cry1Ab, Cry1Ba, Cry1Ia, Cry10Aa, Cry51Aa, Cyt1Aa and Cyt1Ba are active against larvae of at least two orders, including Coleoptera. For example, Cry9 toxins are mainly Lepidoptera active, but Cry9Da is toxic also against scarabs.[47,48] Combinations of *cry* genes, including *cry9* (Table 5), may contribute to the toxicities against *C. tenebrionis* of the five (U-13, U-16, U-40, K4 and K7) most active of the present seven isolates tested (Table 3).

Cry8-type proteins are toxic to larvae of a number of coleopteran pests, especially certain species of scarabs,[49–51] but with narrow target spectra: Cry8Ca2 against *Anomala exoleta* and the related species *A. corpulenta*,[52] and Cry8Ea1, Cry8Fa1 and Cry8Ga1 against *Holotrichia parallela* and *H. oblita*.[53] The present authors revealed in isolate K-30 an amplicon with 89% homology to *cry8La*, and a new *cry8Ra* gene (JX282317) in isolate R-36, which displays low toxicity against *C. tenebrionis* (Table 3), but its toxicity spectrum must still be determined.

Capnodis species are among the most destructive pests of stone fruits, a growing industry in the Mediterranean and southern Europe. Semiochemical-based control, monitoring strategies and various biological control methods, practised against several major pests in agriculture and forestry, are unworkable where wood- and root-boring beetles are concerned. Among the major avenues to promote ecologically safe technologies to cope with *Caponodis*, development of rootstocks displaying antagonism against these borers seems to be the most promising and optimal solution. Grafting rootstock for resilience is a classical strategy in agriculture to cope with soilborne pests and diseases or adverse soil conditions. This approach is widely used in stone fruit cultivation against nematodes.[54] Almond rootstocks or rootstock hybrids with almond 'blood' were traditionally utilised to gain resilience against *C. tenebrionis*,[55] but this has been achieved by the drought tolerance of the plants and not through the high level of cyanogenic compounds in the root cortex of the tree.[12]

The study reported here demonstrates the high susceptibility of *Capnodis* young larvae to *Bt* toxins, suggests that cloning of the toxin-encoding genes for expression in elite rootstock would be useful to gain *Capnodis*-resilient germplasm and opens the way for such a radical solution to the problem.

ACKNOWLEDGEMENTS

This investigation was supported by grant No. 131-1481-10 of the Chief Scientist, Israel Ministry of Agriculture (to GG, ZM, EBD and AZ) and a scholarship (for PK) from the Council for Higher Education (VATAT) of Israel.

REFERENCES

1 Ben-Yehuda S, Assael F and Mendel Z, Improved chemical control of *Capnodis tenebrionis* L. and *C. carbonaria* Klug (Coleoptera: Buprestidae) in stone-fruit plantations in Israel. *Phytoparasitica* **28**:27–41 (2000).
2 Bonsignore CP and Vacante V, Il problema di *Capnodis tenebrionis* (Linnaeus) nei fruttiferi. *Protezione Colture* **5**:18–25 (2009).
3 Karadag S, Mart C and Can C, Species belonging to the family *Buprestidae* in pistachio orchards and some biological properties of *Capnodis cariosa* (Haus.). *Acta Hort (ISHS)* **726**:545–550 (2006).
4 Farivar-Mehin H, Biological study of the pistachio root beetle, *Capnodis cariosa hauseri. Proc 2nd Int Symp – Pistachios and Almonds*, 24–29 August, Davis, CA (1997).
5 Rivnay E, Physiological and ecological studies on the species of *Capnodis*, in Palestine (Col., Buprestidae): I. Studies on the eggs. *Bull Entomol Res* **35**:235–242 (1944).
6 Rivnay E, Ecological and physiological studies on *Capnodis* spp. (Col., Buprestidae) in Palestine: III. Studies on the adult. *Bull Entomol Res* **37**:273–280 (1946).
7 Rivnay E, Physiological and ecological studies on the species of *Capnodis*, in Palestine (Col., Buprestidae): II. Studies on the larvae. *Bull Entomol Res* **36**:103–119 (1945).
8 Marannino P and de Lillo E, The peach flatheaded rootborer, *Capnodis tenebrionis* (L.), and its enemies. *IOBC/WPRS Bull* **30**:197–200 (2007).
9 Garrido A, Bioecology of *Capnodis tenebrionis* L. (Coleop.: Buprestidae) and approaches to its control. *Boletin Servicio Defensa Plagas Inspeccion Fitopatologica* **10**:205–221 (1984).
10 Ben-Yehuda S, Assael F and Mendel Z, Recent outbreaks of phloem- and wood-boring insects in deciduous orchards in Israel. *Phytoparasitica* **25**:163–164 (1997).
11 Salazar DM, Miro M and Garcia S, Rootstocks for dry region apricot tree faced with *Capnodis tenebrionis* L. *Acta Hort* **293**:401–404 (1991).
12 Mendel Z, Assael F and Ben-Yehuda S, Host selection and root colonization by two stone-fruit tree borers (Coleoptera: Buprestidae) and their relation to level of cyanogenic compounds. *Annu J Entomol* **96**:127–134 (2003).
13 Mendel Z, *Capnodis tenebrionis* and *C. carbonaria*, in *Plant Pests of the Middle East*, ed. by Appelbaum SW and Gerson UA. Hebrew University of Jerusalem, Jerusalem, Israel (e-book: www.agric.huj.il/mepests) (2002).
14 Marannino P, Tarasco E and de Lillo E, Biological notes on larval hatching in *Capnodis tenebrionis* (L.) (Coleoptera Buprestidae) and evaluation of entomopathogenic nematodes in controlling neonate larvae. *Redia* **86**:101–106 (2004).
15 Marannino P, Santiago-Álvarez C, de Lillo E and Quesada-Moraga E, A new bioassay method reveals pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against early stages of *Capnodis tenebrionis* (Coleoptera; Buprestidae). *J Invertebr Pathol* **93**:210–213 (2006).
16 Garcia del Pino F and Morton A, Efficacy of entomopathogenic nematodes against neonate larvae of *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) in laboratory trials. *Biocontrol* **50**:307–316 (2005).
17 de Altube MDM, Strauch O, de Castro GF and Pena AM, Control of the flat-headed root borer *Capnodis tenebrionis* (Linne) (Coleoptera: Buprestidae) with the entomopathogenic nematode *Steinernema carpocapsae* (Weiser) (Nematoda: Steinernematidae) in a chitosan formulation in apricot orchards. *Biocontrol* **53**:531–539 (2008).
18 Ishiwata S, On a kind of severe flacherie (sotto disease) (in Japanese). *Dainihon Sanshi Kaiho* **9**:1–5 (1901).
19 Berliner E, Über die Schlaffsucht der Mehlmottenraupe. *Z Gesamte Getreidewesen* **3**:63–70 (1911).
20 de Maagd RA, Bravo A and Crickmore N, How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet* **17**:193–199 (2001).
21 Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J *et al.*, *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**:775–806 (1998).
22 Sanahuja G, Banakar R, Twyman RM, Capell T and Christou P, *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnol J* **9**:283–300 (2011).
23 Gindin G, Kuznetsowa T, Protasov A, Ben Yehuda S and Mendel Z, Artificial diet for two flat headed borers, *Capnodis* spp. (Coleoptera: Buprestidae). *Eur J Entomol* **106**:573–581 (2009).
24 Asokan R, Swamy HMM, Thimmegowda GG and Mahmood R, Diversity analysis and characterization of Coleoptera-, Hemiptera- and Nematode-active *cry* genes in native isolates of *Bacillus thuringiensis*. *Ann Microbiol*, DOI: 10.1007/s13213-013-0636-7 (2013).
25 Ben-Dov E, Zaritsky A, Dahan E, Barak Z, Sinai R, Manasherob R *et al.*, Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. *Appl Environ Microbiol* **63**:4883–4890 (1997).
26 Ben-Dov E, Manasherob R, Zaritsky A, Barak Z and Margalith Y, PCR analysis of *cry7* genes in *Bacillus thuringiensis* by the five conserved blocks of toxins. *Curr Microbiol* **42**:96–99 (2001).
27 Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A *et al.*, Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Appl Environ Microbiol* **64**:4965–4972 (1998).
28 Jua'rez-Pe'rez VM, Ferrandis MD and Frutos R, PCR-based approach for detection of novel *Bacillus thuringiensis cry* genes. *Appl Environ Microbiol* **63**:2997–3002 (1997).
29 Ejiofor A and Johnson T, Physiological and molecular detection of crystalliferous *Bacillus thuringiensis* strains from habitats in the South Central United States. *J Ind Microbiol Biotechnol* **28**:284–290 (2002).
30 Nazarian A, Jahangiri R, Jouzani GS, Seifinejad A, Soheilivand S, Bagheri O *et al.*, Coleopteran specific and putative novel *cry* genes in Iranian native *Bacillus thuringiensis* collection. *J Invertebr Pathol* **102**:101–109 (2009).
31 Tamez-Guerra P, Iracheta MM, Pereyra-Alférez B, Galán-Wong LJ, Gomez-Flores R, Tamez-Guerra RS *et al.*, Characterization of Mexican *Bacillus thuringiensis* strains toxic for lepidopteran and coleopteran larvae. *J Invertebr Pathol* **86**:7–18 (2004).
32 Marquez LM, Helmann JD, Ferrari E, Parker HM, Ordal GW and Chamberlin MJ, Studies of s^D-dependent functions in *Bacillus subtilis*. *J Bacteriol* **172**:3435–3443 (1990).
33 Lowry OH, Rosebrough NJ, Farr AL and Randall RL, Protein measurement with the Folin Phenol reagent. *J Biol Chem* **193**:265–275 (1951).
34 Margalith Y and Ben-Dov E, Biological control by *Bacillus thuringiensis* subsp. *israelensis*, in *Insect Pest Management: Techniques for Environmental Protection*, ed. by Rechcigl JE and Rechcigl NA. CRC Press, Boca Raton, FL, pp. 243–301 (2000).
35 Finney DJ, The comparison of effectiveness, in *Probit Analysis*, 3rd edition. Cambridge University Press, Cambridge, UK, pp. 100–124 (1971).
36 Donovan WP, Slaney AC and Donovan JC, *Bacillus thuringiensis* CryET33 and CryET34 compositions and uses thereof. US Patent 549839, Monsanto Technology LLC (2002).
37 Arantes O and Lereclus D, Construction of cloning vectors for *Bacillus thuringiensis*. *Gene* **108**:115–119 (1991).
38 Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van-Rie J, Lereclus D *et al.*, Bacillus thuringiensis *Toxin Gene Nomenclature*. [Online]. (1998). Available: www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/ [22 October 2013].
39 van Frankenhuyzen K, Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. *J Invertebr Pathol* **114**:76–85 (2013).
40 Weathersbee AA, Lapointe SL and Shatters RG, Activity of *Bacillus thuringiensis* isolates against *Diaprepes abbreviatus* (Coleoptera: Curculionidae). *Fla Entomol* **89**:441–448 (2006).
41 Al-Deeb M and Wilde G, Effect of *Bt* corn expressing the Cry3Bb1 toxin on western corn rootworm (Coleoptera: Chrysomelidae) biology. *J Kansas Entomol Soc* **78**:142–152 (2005).

42 Moellenbeck DJ, Peters ML, Bing JW, Rouse JR, Higgins LS, Sims L *et al.*, Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms. *Nat Biotechnol* **19**:668–672 (2001).

43 Contreras E, Rausell C and Real MD, Proteome response of *Tribolium castaneum* larvae to *Bacillus thuringiensis* toxin producing strains. *PLoS ONE* **8**:e55330 (2013).

44 de Maagd RA, Bravo A, Berry C, Crickmore N and Schnepf HE, Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. *Annu Rev Genet* **37**:409–433 (2003).

45 Rydel T, Sharamitaro J, Brown GR, Gouzov V, Seale J, Sturman E *et al.*, The crystal structure of a coleopteran insect-active binary *B.t.* protein toxin complex at 2.5 Å resolution. *Annu Meet Am Crystallogr Assoc*, Los Angeles, CA (2001).

46 Parker MW, Buckley JT, Postma JPM, Tucker AD, Leonard K, Pattus F *et al.*, Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* **367**:292–295 (1994).

47 Asano S, Identification of *cry* gene from *Bacillus thuringiensis* by PCR and isolation of unique insecticidal bacteria. *Mem Fac Agric Hokkaido Univ* **19**:529–563 (1996).

48 Iizuka T, Sasaki J, Asano S and Yamamoto T, Screening and cloning of novel toxin genes from *Bacillus thuringiensis* strains encoding a highly scarabecidal protein. *Proc 29th Annual Meet SIP and 3rd Int Colloq* – Bacillus thuringiensis, Cordoba, Spain, p. 38 (1996).

49 Ohba M, A unique isolate of *Bacillus thuringiensis* serover *japonensis* with a high larvicidal activity specific for scrabaeid beetles. *Lett Appl Microbiol* **14**:54–57 (1992).

50 Asano S, Yamashita C, Iizuka T, Takeuchi K, Yamanaka S, Cerf D *et al.*, A strain of *Bacillus thuringiensis* subsp. *galleriae* containing a novel *cry8* gene highly toxic to *Anomala cuprea* (Coleoptera: Scarabaeidae). *Biol Control* **28**:191–196 (2003).

51 Yamaguchi T, Sahara K, Bando H and Asano S, Discovery of a novel *Bacillus thuringiensis* Cry8D protein and the unique toxicity of the Cry8D-class proteins against scarab beetles. *J Invertebr Pathol* **99**:257–262 (2008).

52 Huang D-F, Zhang J, Song F-P and Lang Z-H, Microbial control and biotechnology research on *Bacillus thuringiensis* in China. *J Invertebr Pathol* **95**:175–180 (2007).

53 Yu H, Zhang J, Huang D, Gao J and Song F, Characterization of *Bacillus thuringiensis* strain 185 toxic to the Asia cockchafer: *Holotrichia parallela*. *Curr Microbiol* **53**:13–17 (2006).

54 Lu ZX, Reighard GL, Nyczepir AP, Beckman TG and Ramming DW, Inheritance of resistance to root-knot nematodes in peach rootstocks. *Acta Hort* **465**:111–116 (1998).

55 Mulas M, Almond genetic resources and resistance to *Capnodis tenebrionis*. *Acta Hort* **373**:41–48 (1994).