Continuous evolution of *Bacillus thuringiensis* toxins overcomes insect resistance

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The expression of insecticidal proteins from *B. thuringiensis* (Bt toxins) in crops has proved to be a valuable strategy for agricultural pest management1. Bt-toxin-producing crops have been widely adopted in agriculture with substantial economic and environmental benefits2, and have increased global agricultural productivity by an estimated US$78 billion from 1996 to 2013 (ref. 3). Unfortunately, Bt toxin resistance has evolved among insect pests and threatens the continued success of this strategy for pest control4. While resistance management strategies have been developed, including the use of multiple Bt toxins and preserving susceptible alleles in insect populations, the evolution of insect resistance to Bt toxins remains the most serious current threat to sustaining the gains offered by transgenic crops5.

Bt toxins interact with protein receptors on the surface of insect midgut cells, leading to pore formation in the cell membrane and cell death5. Bt toxin resistance is commonly associated with the mutation, downregulation, or deletion of these receptors2. We hypothesized that it might be possible to overcome Bt toxin resistance by evolving novel Bt toxins that bind with high affinity to new gut cell receptor proteins in insects. If successful, such an approach has the potential to alter toxin specificity, improve toxin potency, and bypass receptor-related resistance mechanisms.

Here we use phage-assisted continuous evolution (PACE) to rapidly evolve Bt toxins through more than 500 generations of mutation, selection, and replication to bind a new receptor expressed on the surface of insect midgut cells. PACE-derived Bt toxins bind the new receptor with high affinity and specificity, induce target receptor-dependent lysis of insect cells, and enhance the insecticidal activity against both sensitive and Bt-resistant insect larvae up to 335-fold. Collectively, these results establish an approach to overcoming Bt toxin resistance and provide a new platform for the rapid evolution of other protein-binding biomolecules.

Development of protein-binding PACE

PACE has mediated the rapid laboratory evolution of diverse protein classes including polymerases, proteases, and genome-editing proteins, yielding variants with highly altered activities and specificities6–12. While PACE has not been previously used to evolve protein-binding activity, we speculated that the bacterial two-hybrid system13 could serve as the basis of a protein-binding PACE selection (Fig. 1a). Target binding results in localization of RNA polymerase upstream of a reporter gene, initiating gene expression. To adapt this system into a protein-binding selection for PACE, we envisioned that protein:target binding could instead activate the expression of the filamentous bacteriophage gene III, which is required for the infectivity of progeny phage6 (Fig. 1b).

To maximize the sensitivity of the bacterial two-hybrid, we extensively optimized parameters including (1) transcriptional activation and DNA-binding domains, (2) protein expression level, (3) interaction binding affinity, (4) DNA-binding domain multivalency state, (5) reporter gene ribosome-binding site, (6) operator–promoter distance, (7) RNA polymerase–promoter affinity, and (8) DNA-binding domain–bait linker length. While the previously described bacterial two-hybrid system yielded a 17-fold increase in transcriptional activation using a model high-affinity interaction (HA4 monobody binding to the SH2 domain of ABL1 kinase)14, our optimized system enhanced transcriptional activation >200-fold using the same interaction (Extended Data Figs 1–3). This system consists of the *Escherichia coli* RNA polymerase omega subunit (RpoZ) as the activation domain, the 434 phage cI repressor as the DNA-binding domain, and an optimized P_{lacZ}–derived promoter (P_{lacZ-opt}) to drive reporter transcription. Together, these results extend and improve previously described bacterial systems15 that transduce protein-target binding into gene expression in a manner that can be tuned by the researcher.

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The HA4 monobody binds to the SH2 domain of ABL1 kinase (Kd = 7 nM)\(^\text{14}\). The mutant HA4 Y87A monobody binds the ABL1 SH2 domain with 100- to 1,000-fold weaker affinity\(^\text{14}\). Whereas wild-type HA4 monobody fused to RpoZ in the presence of 434cI–SH2 resulted in potent transcriptional activation in our optimized bacterial two-hybrid system, transcriptional activation using HA4\(_{Y87A}\) was negligible (Fig. 2a). Similarly, selection phage expressing the rpoZ–HA4 fusion robustly propagate using host cell strains carrying accessory plasmids expressing the 434cI–SH2 fusion, whereas a selection phage encoding rpoZ–HA4\(_{Y87A}\) did not support phage propagation. These findings demonstrate that the Y87A mutant HA4 monobody does not support gene III expression or phage propagation (Extended Data Fig. 3).

To validate protein-binding PACE, we challenged the system to evolve a functional SH2-binding monobody starting from the HA4\(_{Y87A}\) mutant. To revert the HA4\(_{Y87A}\) mutant back to a Tyr87 protein requires a strategy to overcome Bt toxicity, we sought to evolve Cry1Ac, a widely used Bt toxin, to bind TnCAD, an insect cell membrane cadherin-like receptor from cabbage looper (\(T. \text{ni}\)) that is not natively bound by wild-type Cry1Ac (see Supplementary Discussion). \(T. \text{ni}\) has developed Bt resistance in agricultural settings and has been widely studied for insect resistance to Bt toxins\(^\text{2}\).

Previous studies of Cry1Ac binding to cadherin-like receptor proteins from Lepidoptera identified multiple putative toxin binding regions (TBRs) in the cadherin\(^\text{16,17}\). The homologous region of the TBR in TnCAD differs from that of cadherin-like proteins from other lepidopteran species at seven amino-acid positions (Extended Data Fig. 4). To create an evolutionary stepping-stone from cadherin-like proteins that bind Cry1Ac to TnCAD, three residues (F1433, S1436, and A1437) from the TBR of four other lepidopteran species\(^\text{18–21}\) were introduced into TnCAD, resulting in an artificial receptor fragment designated TnTBR3 (Extended Data Fig. 4). We constructed accessory plasmids expressing various TnTBR3 fragments fused to 434cI and assessed transcriptional activation levels in the presence of various domains of Cry1Ac fused to RpoZ (Extended Data Fig. 4). Only Cry1Ac containing three domains of the active toxin (residues 1–609) showed weak binding activity for TnTBR3 fragment 3 (TnTBR3-F3) (Extended Data Fig. 4). A selection phage carrying the rpoZ–Cry1Ac fusion gene replicated ~100-fold in a host strain carrying the TnTBR3-F3 accessory plasmid after propagation overnight, whereas a control selection phage lacking the rpoZ–Cry1Ac fusion did not replicate (Extended Data Fig. 4). These observations identified TnTBR3-F3 as a promising evolutionary stepping-stone to serve as a starting target for continuous evolution in PACE.

### Evolution of Cry1Ac to bind TnCAD

We performed 528 h of PACE on Cry1Ac in four segments while varying mutagenesis levels and selection stringency (Fig. 3a). For the first two segments (0–144 h and 144–276 h), the accessory plasmid expressed the TnTBR3-F3 stepping-stone target fused to 434cI. For the final two segments of PACE (276–396 h and 396–528 h), the accessory plasmid expressed the TnCAD-F3 final target fused to 434cI. To enhance mutagenesis, we used the moderate-potency mutagenesis plasmid MP4 (ref. 12) during PACE for binding to TnTBR3-F3 (PACE segments 1 and 2) in an effort to decrease the likelihood of accessing early mutations that could impair essential features of Cry1Ac beyond target receptor binding. During the final two PACE segments for binding to TnCAD-F3 (PACE segments 3 and 4) we used MP6, which induces a greater mutation rate and broader mutational spectrum than MP4 (ref. 12), as phage washout consistently occurred during TnCAD-F3 PACE attempts with MP4, suggesting that higher levels of mutagenesis were required to access rare Cry1Ac mutational combinations that conferred binding to the final TnCAD-F3 target. We increased selection stringency during PACE by increasing lagoon flow rates and reducing the number of TnTBR3-F3 or TnCAD-F3 fragments participating in Cry1Ac variant recognition (Fig. 2a and Extended Data Fig. 3). Phage surviving 528 h of PACE experienced on average 511 generations of mutagenic replication under selection conditions\(^\text{5}\).

Sequencing of individual clones at the end of the first PACE segment (144 h; four copies of TnTBR3-F3 per \(p_{\text{bacZ, opt}}\) promoter) revealed a strong consensus of two coding mutations in Cry1Ac, and one coding
Amarin, or enhanced mutagenesis with genetic drift (mutagenesis plasmid induced with arabinose in addition to an initial period of zero selection stringency), then selected for binding to the ABL1 SH2 target protein. A combination of drift and enhanced mutagenesis during PACE (green line) resulted in the evolution of Tyr and Trp residues at position 87, either of which restores SH2-binding activity, while no mutagenesis (red line) or enhanced mutagenesis without drift (blue line) resulted in phage washout. Error bars in a, s.d. of at least three independent biological replicates.

Figure 2 | Protein-binding PACE selection development and stringency modulation. a, The relationship between target protein multivalency and transcriptional output measured by luciferase expression. The number of ABL1 SH2 domains available to bind the HA4 monobody was modulated by varying the 434cl DNA-binding domain multivalency state (1 ×, 2 ×, 4 ×, or 6 × SH2). ‘No operator’ indicates a scrambled 434cl operator control accessory plasmid. b, During PACE, the inactive monobody mutant HA4Y87A was subjected to no mutagenesis (mutagenesis plasmid induced with arabinose in addition to an initial period of zero selection stringency), then selected for binding to the ABL1 SH2 target protein. c, The combination of drift and enhanced mutagenesis during PACE (green line) resulted in the evolution of Tyr and Trp residues at position 87, either of which restores SH2-binding activity, while no mutagenesis (red line) or enhanced mutagenesis without drift (blue line) resulted in phage washout. Error bars in a, s.d. of at least three independent biological replicates.

Characterization of evolved Cry1Ac variants

DNA sequencing of individual clones surviving 528 h of PACE revealed several consensus genotypes carrying up to 16 mutations per clone out of 22 consensus mutations, most of which localize to domain II, the predicted cadherin-binding domain of Cry1Ac (Extended Data Figs 4 and 5). To illuminate the evolution trajectories en route to TnCAD-F3 binding activity, we analysed all lagoon samples, by high-throughput DNA sequencing using both shorter-read (Illumina) and longer-read (Pacific Biosciences) methods (Extended Data Fig. 6). These efforts yielded seminal, highly functional new variants.

On the basis of our mutational analysis, we designed and synthesized consensus Cry1Ac variants containing the most commonly observed mutations (Fig. 4a, b). Purified activated Cry1Ac variants encoding PACE-derived consensus mutations bind strongly (Kd = 18–34 nM) to a TnCAD fragment containing the TBR (TnCAD-FL; Extended Data Fig. 4) by ForteBio bio-layer interferometry analysis, with evolved Cry1Ac variants C03 and C05 exhibiting the highest binding affinities (Fig. 4a and Supplementary Table 1). In contrast, wild-type Cry1Ac exhibited no significant affinity for TnCAD-FL (Kd > 1 mM) under the same conditions. These results together establish the ability of protein-binding PACE to rapidly evolve extensively mutated proteins with high target affinity.

Cry1Ac is proteolytically activated in the insect midgut2. The evolved consensus mutants, however, exhibited extensive proteolysis by trypsin under conditions in which the wild-type Cry1Ac was cleanly cleaved into its active form (Fig. 4c). Thermal melt studies confirmed this reduced stability (consensus variants: melting temperature Tm = ~45 °C; wild-type Cry1Ac: Tm = 71 °C; Supplementary Table 1). Despite this lower stability, trypsin-activated consensus variants robustly killed Sf9 cells expressing TnCAD, whereas wild-type Cry1Ac did not exhibit toxicity (Fig. 4d). Moreover, these evolved consensus Cry1Ac mutants showed insecticidal activity in T. ni larvae, although they were less potent than wild-type Cry1Ac (Fig. 4e).

We hypothesized that a subset of the consensus mutations were impairing apparent toxin potency against insect larvae by decreasing Cry1Ac stability and thus promoting degradation in insect gut. We generated Cry1Ac variants containing combinatorial reversions of the identified consensus mutations (Fig. 4b and Supplementary Table 1) and identified mutations D384Y and S404C, two mutations that arose early during PACE against the TnTBR3 stepping-stone target (Figs 3d, e and 5a), as the source of reduced protein stability. Variants lacking these two mutations, but containing the other seven consensus C05 mutations, exhibited greatly improved stability (Tm = ~60 °C). Variants lacking D384Y and S404C also exhibited proteolytic resistance similar to that of wild-type Cry1Ac, while retaining high binding affinity to TnCAD-FL (Kd = 11–41 nM) (Fig. 5a, b and Supplementary Table 1).

We assayed the toxicity of two evolved consensus Cry1Ac variants (C05 and C03) and three stabilized evolved consensus Cry1Ac variants (C05s, C03s, and A01s) lacking D384Y and S404C to cultured Sf9 insect cells expressing an ABCC2 receptor (positive control) or TnCAD. The stabilized evolved Cry1Ac variants retain their ability to bind to the ABCC2 receptor, while acquiring the ability to potently kill Sf9 cells expressing TnCAD, in contrast to the ability of wild-type Cry1Ac to only kill cells expressing the ABCC2 receptor, but not cells expressing TnCAD (Fig. 5c).
In vivo activity of evolved Cry1Ac variants

Finally, we assayed the insecticidal activity of the stabilized evolved Cry1Ac variants against Cry1Ac-sensitive *T. ni* larvae when added to their diet. Consistent with the *in vitro* results, the stabilized evolved Cry1Ac variants exhibited substantially increased toxicity to *T. ni* larvae compared with that of the consensus-evolved Cry1Ac mutants before stabilization (Fig. 5d). Interestingly, the stabilized evolved Cry1Ac variants also exhibited insecticidal potency against susceptible *T. ni* up to fourfold higher than that of wild-type Cry1Ac, suggesting that the evolved affinity of the toxins to a new receptor may augment their insecticidal potency, even against insects susceptible to wild-type Cry1Ac. These results also suggest that the evolution of Bt toxins that recognize novel receptors could expand the range of insects that can be targeted by Bt toxins, consistent with previous *in vitro* studies on designing Bt toxin derivatives.

Next we evaluated the insecticidal activity of the stabilized evolved Cry1Ac variants against Cry1Ac-resistant *T. ni* larvae. *T. ni* resistance to Cry1Ac has been genetically mapped to the ABCG2 transporter gene and downregulation of expression of APN1 (refs 25, 26), and is known to be independent of alteration of the cadherin-like receptor27. In this study, we also confirmed that wild-type Cry1Ac does not bind the TBR in TnCAD (see above), consistent with the previous finding that Cry1Ac does not bind TnCAD in *T. ni* midgut cell membranes25,27,28. Indeed, we observed a 1,000-fold lower potency of wild-type Cry1Ac against a Cry1Ac-resistant *T. ni* strain than the potency of wild-type Cry1Ac against susceptible *T. ni* (Fig. 5e). Compared with wild-type Cry1Ac, stabilized evolved Cry1Ac variants C05s, C03s, and A01s showed dramatically improved activity against Cry1Ac-resistant *T. ni*, with median lethal concentration (*LC₅₀*) values up to 335-fold lower than wild-type Cry1Ac (Fig. 5e and Extended Data Table 1).
Bt crystals; T, trypsin-treated.

(SDS–PAGE) analysis of Cry1Ac variants after trypsin digestion, revealing the first segment of PACE. S404C double mutant (DM) that enabled TnTBR3-F3 recognition during a

Figure 4

resistant variants greatly outperform wild-type Cry1Ac toxin in killing Bt toxin-sensitive larvae compared with wild-type Cry1Ac. Stabilized evolved variants moderately enhance mortality in Cry1Ac-resistant variants, or stabilized evolved variants were added to the diets of Cry1Ac-resistant larvae. Error bars, s.d. of at least three independent biological replicates. d. Insect larvae diet bioassays using wild-type and evolved consensus Cry1Ac variants, showing the loss of evolved Cry1Ac potency in insect larvae arising from impaired stability.

Importantly, these evolved and stabilized Cry1Ac variants showed similar toxicity in Bt-resistant T. ni (LC50 = 0.15 ppm) as that of wild-type Cry1Ac in susceptible larvae (LC50 = 0.04 ppm) (Fig. 5e and Extended Data Table 1). Taken together, these results establish that the evolution of novel receptor binding among Bt toxins can overcome Bt toxin resistance in an agricultural pest.

To characterize the species profile of their insecticidal activity, we tested the evolved Cry1Ac variants in diet bioassays against 11 additional agricultural pests: a lepidopteran related to T. ni (Chrysodeixis includens, soybean looper) that encodes a cadherin-like receptor highly homologous to ToCAD, eight more distantly related lepidopteran pests, and three non-lepidopteran pests (Extended Data Figs 7 and 8). As expected, the stabilized evolved Cry1Ac variants were more potent than wild-type Cry1Ac against C. includes, and comparably potent as wild-type Cry1Ac against the other pests assayed (Extended Data Fig. 7). These results further support the mechanism of action of the PACE-evolved Bt toxins as binding to the cadherin receptor in T. ni and the closely related cadherin receptor in C. includes. Notably, the evolved Bt toxins did not acquire new activity against species lacking a receptor homologous to ToCAD. Taken together, these findings demonstrate that an evolved Bt toxin that binds a novel target can potentially kill closely related insect pest species, while maintaining a similar overall insect spectrum as the parental Bt toxin.

Discussion

Protein-binding PACE rapidly discovered variants of Cry1Ac that bind with high affinity to the novel receptor ToCAD. Perhaps unsurprisingly, we observed a moderate reduction in stability of the evolved variants compared with wild-type Cry1Ac, as stability was not an implicit requirement of the selection. The two mutations that reduced Cry1Ac stability (D384Y and S404C) arose within the first few days of PACE on the stepping-stone target TnTBR3-F3 and were inherited by virtually all subsequent evolved variants (Fig. 3e). It is tempting to speculate that these mutations broadened the substrate scope of Cry1Ac binding to enable downstream protein evolution, at the expense of stability, but were not required once affinity for ToCAD-F3 evolved. Additional affinity measurements of reverted consensus mutations reveal the key roles of E461K, N463S, and S582L, which evolved in quick succession during the third PACE segment (Fig. 3e), consistent with their contri-
bution to TnCAD binding. All three mutations lie on the same face of Cry1Ac (Extended Data Fig. 5c), albeit in different domains, suggestive of potential direct interaction with the cadherin receptor.

Collectively, our findings establish that the laboratory evolution of novel or enhanced Bt toxin–receptor interactions can overcome insect resistance to Bt toxins. This strategy complements existing approaches to limit the incidence of Bt toxin resistance. The ‘gene pyramiding’ strategy for resistance management, for example, requires the availability of multiple effective toxins with different binding sites in target insects. The refuge strategy necessitates that the resistance is a recessive trait and requires compliance by growers. The engineering of Bt toxins to eliminate the reliance on cadherin receptor interaction for toxin oligomerization has been shown to enhance toxicity against resistant strains of several insects, but also reduces the insecticidal potency of the toxins against sensitive insects and may broaden the target specificity of the toxin.

The approach established here enables targeting of a Bt-resistant pest through the evolution of high-affinity Bt toxin variants that bind a specific target insect protein. In principle, this strategy should be applicable to target a variety of insect pests. While the evolution of insect resistance to an evolved Bt toxin is a likely possibility, this work has the potential to provide access to many new Bt toxins that, individually or in combination, may manage resistance and extend the effectiveness of this important approach to pest control. We also envision that this system may be used to explore potential resistance mechanisms by evolving the receptor in the presence of a Bt toxin, analogous to the recent use of PACE to identify protease inhibitor drug resistance mechanisms. Finally, we note that the ability of protein-binding PACE to rapidly evolve novel protein–protein interactions may prove useful in the discovery or improvement of protein therapeutics.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Prado, J. R. et al. Genetically engineered crops: from idea to product. Annu. Rev. Plant Biol. 65, 769–790 (2014).
2. Pardo-López, L., Soberón, M. & Bravo, A. Bacillus thuringiensis insecticidal crystal protein: mode of action, insect resistance and consequences for crop protection. FEBS Microbiol. Rev. 37, 3–22 (2013).
3. James, C. Global Status of Commercialized Biotech/GM Crops: 2014. ISAAA Brief No. 49 (International Service for the Agro-biotechnologies, 2014).
4. Tabashnik, B. E., Brévart, T. & Carrière, Y. Insect resistance to Bt crops: lessons from the first billion acres. Nature Biotechnol. 31, 510–521 (2013).
5. Adang, M. J., Crickmore, N. & Jurat-Fuentes, J. L. Diversity of Bacillus thuringiensis crystal toxins and mechanism of action. Adv. Insect Physiol. 47, 39–87 (2014).
6. Esvelt, K. M., Carlson, J. C. & Liu, D. R. A system for the continuous directed evolution of biomolecules. Nature 472, 499–503 (2011).
7. Dickinson, B. C., Leconte, A. M., Allen, B., Esvelt, K. M. & Liu, D. R. Experimental interrogation of the path dependence and stochasticity of protein evolution using phage-assisted continuous evolution. Proc. Natl Acad. Sci. USA 110, 9007–9012 (2013).
8. Leconte, A. M. et al. A population-based experimental model for protein evolution: effects of mutation rate and selection stringency on evolutionary outcomes. Biochemistry 52, 1490–1499 (2013).
9. Carlson, J. C., Badran, A. H., Guggiana-Nilo, D. A. & Liu, D. R. Negative selection and stringency modulation in phage-assisted continuous evolution. Nature Chem. Biol. 10, 216–222 (2014).
10. Dickinson, B. C., Packer, M. S., Badran, A. H. & Liu, D. R. A system for the continuous directed evolution of proteases rapidly reveals drug-resistance mutations. Nature Commun. 5, 5352 (2014).
11. Hubbard, B. P. et al. Continuous directed evolution of DNA-binding proteins to improve TALEN specificity. Nature Methods 12, 939–942 (2015).
12. Barbi, A. H. & Liu, D. R. Development of potent in vivo mutagenesis plasmids with broad mutational spectra. Nature Commun. 6, 8425 (2015).
13. Dove, S. L. & Hochschild, A. Conversion of the omega subunit of Escherichia coli RNA polymerase into a transcriptional activator or an activation target. Genes Dev. 12, 745–754 (1998).
METHODS

No statistical methods were used to predetermine sample size. The in vivo experiments were blinded and randomized.

General methods. PCR was performed using PfuTurbo Cx Hotstart DNA polymerase (Agilent Technologies), VeraSeq Ultra DNA polymerase (Enzymics), or Phusion H Hot Start DNA Polymerase (Life Technologies). Water was purified using a MilliQ water purification system (Millipore). Plasmids and selection phages were constructed using USER cloning (New England Biolabs). Genes were codon-optimized and gblocks were cloned into expression vectors (Integrated DNA Technologies) or amplified by PCR from native sources. CrylAc was amplified by PCR from the B. thuringiensis strain Bt_B107284 and cloned into the Bt expression vector pMON101647 using Hot Fusion to generate the expression plasmid pMON133051, which served as a template for amplifying CrylAc fragments for constructing PACE vectors. The toxin-binding region from T. ni cadherin (A1133–T1582, AEA29692.10) was fused to TnCAD-FL, synthesized using 45–60-base oligonucleotides (Integrated DNA Technologies) by overlap extension PCR using KOD Hot Start DNA polymerase (EMD Millipore). The synthetic wild-type TnCAD-FL template was used to generate the TnTBRS-FL fragment via site-directed mutagenesis using the QuikChange II kit according to the manufacturer’s instructions (Agilent Technologies). DNA vector amplification was performed using NEB Turbo or DH5α cells (New England Biolabs).

Electrocompetent strain preparation. The previously described strains S1030 (ref. 9) or S2060 (ref. 11) were used in all luciferase and plaque assays, as well as in PACE experiments. The glycerol stock of either strain was used to seed a 2-ml overnight culture using 2xYT media (United States Biological) supplemented with 10 μg/ml tetracycline (Sigma Aldrich), 50 μg/ml streptomycin (Sigma Aldrich), 10 μg/ml fluconazole (TCI America), and 10 μg/ml amphotericin B (TCI America) in a 37°C shaker at 230 r.p.m. The saturated culture was diluted 1,000-fold in 50 ml of the same supplemented media and grown under identical conditions until it reached mid-log-phase (absorbance at 600 nm (A600nm) = 0.5–0.8). Once the appropriate A600nm was reached, the cells were pelleted in a 50-ml conical tube (VWR) centrifuged at 10,000g for 5 min at 4°C. The supernatant was immediately decanted and the interior of the tube was wiped with a few Kimwipes (Kimberly-Clark) to remove residual media and salts. The cells were resuspended in 25 ml of pre-chilled, sterile filtered 10% glycerol in MilliQ purified water using a pipette to quickly break up the pellet. The cells were centrifuged and washed an additional three times. After the last centrifugation step, the interior of the tube was wiped with a few Kimwipes to remove residual glycerol solution. The pellet was resuspended in as little volume as possible, typically ~150 μl, and split into 10 μl aliquots for storage. Cells were flash frozen in a liquid N2 bath, then quickly transferred to −80°C for extended storage. Electrocompetent S1030 or S2060 cells produced by this method typically yielded 107–108 colonies per microgram plasmid DNA and enable the simultaneous electroporation of up to three plasmids carrying orthogonal origins of replication and antibiotic resistance cassettes to yield transformants containing all plasmids.

General USER cloning. All PACE-related plasmids and phage materials were constructed via USER cloning33 (see Extended Data Table 2). Briefly, primers were designed to include a single internal deoxyuracil base 15–20 bases from the 5’ end of the primer, specifying this region as the ‘USER junction’. Criteria for design of the USER junction were: it should contain minimal secondary structures, have 45°C < Tm < 70°C, and begin with a deoxyadenosine and end with a deoxythymine (to be replaced by deoxyuridine). The USER junction specifies the homology required for correct assembly. We note that PfuTurbo Cx Hotstart DNA polymerase (Agilent Technologies), VeraSeq Ultra DNA polymerase (Enzymics), or Phusion H Hot Start DNA Polymerase (Life Technologies) are able to use primers carrying deoxyuracil bases, whereas some other polymerases undergo a phenomenon known as PCR poisoning and do not extend the primer.

All PCR products were purified using a MinElute PCR Purification Kit (Qiagen) to 10 μl final volume and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). For assembly, PCR products carrying complementary USER junctions were mixed in an equimolar ratio (up to 1 pmol each) in a 10 μl reaction containing 15 units DpnI (New England Biolabs), 0.75 units USER (Usaric-Specific Excision Reagent) enzyme (Endonuclease VIII and Usaric-DNA Glycolysase, NEB), 50 μM potassium acetate, 20 μM Tris-acetate, 10 mM magnesium acetate, 100 μg/ml BSA at pH 7.9 (1 × CutSmart Buffer, New England Biolabs). The reactions were incubated at 37°C for 45 min, followed by heating to 95°C and slow cooling to 22°C at 0.1°C s−1 in a temperature-controlled block. The hybridized constructs were directly used for heat-shock transformation of chemically competent NEB Turbo E. coli cells according to the manufacturer’s instructions. Transformants were selected on 1.8% agar-2xYT plates supplemented with the appropriate antibiotic(s).

For selection phage cloning, the hybridized constructs were purified using EconoSpin purification columns (Epoch Life Sciences), eluted using 25 μl 10% glycerol, and transformed into electrocompetent S2060 cells carrying the phage-responsive accessory plasmid pJC175e, which produces functional pIII in response to phage infection (this strain is henceforth referred to as S2208). After recovery for 3–4 h at 37°C using 2xYT (United States Biological) media, the culture was centrifuged and the supernatant was purified using a 0.22 μm PVDF UltraTurrax centrifugal filter (Millipore). The supernatant was diluted serially in 100-fold units, and plated on 2xYT agar plates containing the appropriate antibiotic to an A600nm of 0.6–0.9. The phage supernatant was diluted serially in three, 100-fold increments to yield four total samples (undiluted, 101-, 102-, and 103-fold diluted) to be used for infections. For each sample, 150 μl of cells were added to 10 μl of phage that had been filtered using a 0.22 μm PVDF UltraTurrax centrifugal filter (Millipore). Within 1–2 min of infection, 1 ml of warm (~55°C) top agar (7 g l−1 bacteriological agar in 2xYT) was added to the phage/cell mixture, mixed by pipetting up and down once, and plated onto quartered plates that had been previously poured with 2 ml of bottom agar (18 g l−1 bacteriological agar in 2xYT) in each quadrant. The plates were then grown overnight at 37°C before plaques could be observed.

PACE. Host cell cultures, lagoons, media, and the PACE apparatus were as previously described4. Recombined selection phage harbouring gene III (rSP) will poison a PACE experiment by outcompeting the evolving selection phage. We have previously noted that the likelihood of rSP occurrence in a selection phage stock increases with extended standing culture growth during the initial selection phage stock preparation. To reduce the likelihood of rSP formation, all selection phages were repurified before any continuous evolution experiments. Briefly, selection phages were plated on S2208 cells. A single plaque was picked into 2 ml 2xYT (United States Biological) supplemented with the appropriate antibiotics and grown until the culture reached mid-log-phase (A600nm = 0.5–0.8). The culture was centrifuged using a tabletop centrifuge for 2 min at 10,000g, followed by supernatant filtration using a 0.22 μm PVDF UltraTurrax centrifugal filter (Millipore). This overnight growth period routinely yields titres of 106–108 plaque-forming units per millilitre and was found to minimize the occurrence of rSP during PACE experiments.

To prepare the PACE strain, the accessory plasmid and MP were co-transformed into electrocompetent S1030 cells (see above) and recovered using Davis rich media6 (DRM) to ensure MP repression. Transformations were plated on 1.8% agar-2xYT containing 50 μg ml−1 carbenicillin, 40 μg ml−1 chloramphenicol, 10 μg ml−1 fluconazole, 10 μg ml−1 amphotericin B, 100 μM glucose (United States Biological) and grown for 12–18 h in a 37°C incubator. After overnight growth, four single colonies were picked and resuspended in DRM, then serially diluted and plated on 1.8% agar-2xYT containing 50 μg ml−1 carbenicillin, 40 μg ml−1 chloramphenicol, 10 μg ml−1 fluconazole, 10 μg ml−1 amphotericin B, and either 100 μM glucose or 100 μM arabinose (Gold Biotechnology) and grown for 12–18 h in a 37°C incubator. Concomitant with this plating step, the dilution series was used to inoculate liquid cultures in DRM supplemented with 50 μg ml−1 carbenicillin, 40 μg ml−1 chloramphenicol, 10 μg ml−1 tetracycline, 50 μg ml−1 streptomycin, 10 μg ml−1 fluconazole, 10 μg ml−1 amphotericin B and grown for 12–18 h in a 37°C shaker at 230 r.p.m. After confirmation of arabinose sensitivity using the plate assay, cultures of the serially diluted colonies still in log-phase growth were used to seed a 25-ml starter culture for the PACE chemostat.

The recovered log-phase donor strain (A600nm = 0.5–0.8), the 25-ml culture was added directly to 175 ml of fresh DRM in the chemostat. The chemostat culture was maintained at 200 ml and grown at a dilution rate of 1.5–1.6 volumes per hour as previously described5. Lagoons flowing from the chemostats were maintained at 40 ml, and diluted as described for each experiment. Lagoons were supplemented with 25 mM arabinose to induce the MP for 8–16 h before infection with packaged selection phage. Samples were taken at the indicated time points, centrifuged at 10,000g for 2 min, then filtered with a 0.2 μm filter and stored overnight at 4°C. Phage aliquots were titre by plaque assay on S2208 cells (total phage titre) and S1030 or S2060 cells (rSP titre) for all time points. For selection phage cloning, the basal mutation rate of replicating filamentous phage in E. coli (7.2 × 10−12 substitutions per base pair per generation) is sufficient to generate all possible single but not double mutants of a given gene in a 40-ml lagoon after one generation of phage replication. For the 2,139-base-pair ropZ-CrylAc target, a basal mutation rate of 7.2 × 10−12 substitutions per base pair per generation applied to 2 × 1010 copies of the gene (a single generation)
in a 40-ml lagoon yields ~3.1 × 10^7 base substitutions, easily enough to cover all 6,417 single point mutants but not all double mutants. Arabinose induction of our first-generation mutagenesis plasmid, MP1, increased the phage mutation rate by ~100-fold, resulting in 7.2 × 10^−5 substitutions per base pair per generation, yielding ~3.1 × 10^7 substitutions spread over 2.1 × 10^4 copies of the gene after a single generation. This enhanced mutation rate is sufficient to cover all possible single mutants (6.4 × 10^6 possibilities) and double mutants (4.1 × 10^7 possibilities), but no triple mutants (2.6 × 10^11 possibilities) after a single phage generation. Our second-generation mutagenesis plasmid successfully amplified the improved MP6 system, which increases the phage mutation rate by an additional 100-fold compared with MP1, resulting in 7.2 × 10^−5 substitutions per base pair per generation, yielding ~3.1 × 10^7 substitutions spread over 2.1 × 10^4 copies of the gene after a single generation. This elevated mutation rate is sufficient to cover all possible single mutants (6.4 × 10^6 possibilities), double mutants (4.1 × 10^7 possibilities), and many triple mutants (2.6 × 10^11 possibilities) after a single phage generation.

**Luciferase assays.** Complementary plasmids were co-transformed with an accessory plasmid of interest into electrophoretic SI030 (ref. 9) or S2060 (ref. 11) cells and plated onto 1.8% agar-2×YT plates with 50 μg ml^−1 carbenicillin and 100 μg ml^−1 spectinomycin. After overnight growth at 37°C, single colonies were each picked into 2 ml DRM supplemented with 50 μg ml^−1 carbenicillin, 100 μg ml^−1 spectinomycin, 10 μg ml^−1 tetracycline, 50 μg ml^−1 streptomycin, 10 μg ml^−1 fluconazole, 10 μg ml^−1 amphotericin B and grown for 12–18 h in a 37°C shaker at 230 rpm. After overnight growth, cultures were diluted 1,000-fold in a 96-well deep well plate containing 500 μl DRM with 50 μg ml^−1 carbenicillin, 100 μg ml^−1 spectinomycin, and the indicated arabinose, isopropyl-β-D-thiogalactoside (IPTG), or anhydrotrehaline (ATc) concentration to induce protein expression from either the accessory plasmid or complementary plasmid. Constitutive accessory plasmids and complementary plasmids were used where no inducer concentration is given. After growth with shaking at 37°C for 4–5 h, 150 μl of each culture was transferred to a 96-well black wall, clear bottom plate (Costar), and the A_{S000 nm} and luminescence for each well was measured on an Infinite M1000 Pro microplate reader (Tecan). The A_{S000 nm} of a well containing only media was subtracted from all sample wells to obtain a corrected A_{S000 nm} value for each well. The raw luminescence value for each well was then divided by that well’s corrected A_{S000 nm} value to obtain the luminescence value normalized to cell density. Each variant was assayed in at least biological triplicate, and the error bars shown reflect the standard deviations of the independent measurements.

**High-throughput sequencing and oligotyping analysis.** Raw reads have been deposited in the NCBI Sequence Read Archive under accession number PRJNA293870, and all custom scripts used in analysis are available at http://github.com/MonsantoCo/BdadEAtAL2015. Illumina reads obtained from each time point were mapped to the SP055-rpoZ-cMyc-Cry1Ac1-d123 reference sequence using bowtie version 2.1.0 (ref. 34), and the resulting SAM files were combined into a single BAM file using samtools version 0.1.19 (ref. 35). This BAM file was used as input to freebayes version 0.9.21-12-g29e653a^36 to call single nucleotide polymorphisms, using the command ‘freebayes–use-best-n-allleses–1-pooled–continuous–use-reference–alleles–theta 500000000–min-allele-frequency 0.01–region SP055-rpoZ-cMyc-Cry1Ac1-d123-2833-4971’. The analysis is encapsulated in the PRResequencing_Barcode.1 workflow provided by PacBio. Polymerase reads with quality score lower than 0.80 (defined by the PacBio scoring algorithm) or reads-of-inserts were obtained by calling consensus of subreads generated under identical conditions two additional times. The washed spore/crystal pellet from each 1-ml culture was solubilized in the 96-well plate using 300 μl of solubilization buffer composed of 50 mM CAPS, pH 11, and 10 mM DTT, then incubated while shaking at room temperature (21°C) for 1 h. The insoluble debris was pelleted by centrifugation at 3,200 g for 15 min at 4°C, and 0.1 ml of the supernatant was transferred to a sterile U-bottom 96-well plate. To each well, 10 μl of 0.2 mg ml^−1 trypsin in 1 M Tris-HCl pH 7.5 was added. The mixture was incubated at 37°C for 2 h while shaking at 150 rpm, followed by quenching using 2 μl 0.1 M PMSE. The solution was filtered using a Millipore multiscreen plate with a 0.22 μm membrane. Protein stability was assessed by SDS-PAGE and quantified using spot densitometry. Proteins purified using this protocol were tested in downstream insect cell assays.

**Secondary Bt toxin purification and analysis.** Bt glycerol stocks described above were used for large-scale protein expression and purification. A 2-ml starter culture of Bt medium supplemented with 5 μg ml^−1 chloramphenicol was inoculated from the glycerol stocks and grown overnight at 280 rpm in a 28°C shaker. The following day, the saturated culture was transferred into 500 ml complete C2 medium containing 5 μg ml^−1 chloramphenicol in a 21 baffled flask and grown for an additional 72 h at 26°C while shaking at 280 rpm. Sporulation and crystal formation in the culture was verified by optical microscopy of a 2-μl aliquot of the saturated Bt culture. Upon confirmation of crystals, the partly lysed sporulated cells were harvested by centrifugation at 10,000 g for 12 min at 4°C. The pellet was then resuspended in 100 ml TX wash buffer composed of 10 mM Tris-HCl, pH 7.5, and 0.005% Triton X-100 supplemented with 0.1 mM PMSE, 25 μg per milliliter Benzonase (Sigma-Aldrich), and 2 mM MgCl2, incubated at room temperature (21°C) for 30–60 min (with vigorous vortexing every 10 min), then centrifuged at 3,200 g for 15 min at 4°C. The resulting pellet was resuspended and centrifuged under identical conditions two additional times.

The washed spore/crystal pellet was solubilized in 120 ml 50 mM CAPS, pH 11, 10 mM DTT at room temperature for 1 h while shaking at 130 rpm. The solubilized protein was separated from the insoluble debris by centrifugation at 35,000 g for 20 min at 4°C. The supernatant was transferred to a fresh flask, and then supplemented with 10 μl 0.2 mg ml^−1 trypsin in 1 M Tris-HCl at pH 7.5. The mixture was incubated at 30°C for 2–6 h with shaking at 150 rpm. and trypsinization was terminated with 100 μl of 1 M Tris-HCl, pH 8.5 supplemented with 1 mM PMSF, 2 μg per milliliter Benzonase (Sigma-Aldrich), and 2 mM MgCl2, incubated at room temperature (21°C) for 30–60 min (with vigorous vortexing every 10 min), then centrifuged at 3,200 g for 15 min at 4°C. The resulting pellet was resuspended and centrifuged under identical conditions two additional times.

The washed spore/crystal pellet was solubilized in 120 ml 50 mM CAPS, pH 11, 10 mM DTT at room temperature for 1 h while shaking at 130 rpm. The solubilized protein was separated from the insoluble debris by centrifugation at 35,000 g for 20 min at 4°C. The supernatant was transferred to a fresh flask, and then supplemented with 10 μl 0.2 mg ml^−1 trypsin in 1 M Tris-HCl at pH 7.5. The mixture was incubated at 30°C for 2–6 h with shaking at 150 rpm. and trypsinization was terminated with 100 μl of 1 M Tris-HCl, pH 8.5 supplemented with 1 mM PMSF, 2 μg per milliliter Benzonase (Sigma-Aldrich), and 2 mM MgCl2, incubated at room temperature (21°C) for 30–60 min (with vigorous vortexing every 10 min), then centrifuged at 3,200 g for 15 min at 4°C. The resulting pellet was resuspended and centrifuged under identical conditions two additional times.
**T. ni receptor fragment expression and purification.** Custom expression vectors pMON251427 and IS0008 (the same as pMON251427 but with wild-type TnCAD) were used to express 6xHis-TnTBR3-FL and 6xHis-TnCAD-FL fragments in *E. coli*. Both vectors contain an amino (N)-terminal MBP-TVMV protease cleavage site tag and a carboxy (C)-terminal 6× histidine tag flanking the receptor fragment of interest, with the open reading frame driven by the T7 promoter. Expression vectors were transformed into commercial BL21 (DE3) competent cells (Life Technologies) that had been previously transformed with TVMV protease expression vector (pMON101695; encodes constitutive TVMV protease from a pACYC184 (New England Biolabs) backbone). A single colony was inoculated in 2 ml of Luria-Bertani (LB) media supplemented with 50 μg ml⁻¹ kanamycin and 25 μg ml⁻¹ chloramphenicol, and grown at 37 °C for 4 h to generate a starter culture, which was used to prepare glycerol stocks and stored at −80 °C for the future protein expression. A second starter culture was inoculated using the BL21 (DE3) strain glycerol stocks in 2 ml of LB media supplemented with 50 μg ml⁻¹ kanamycin and 25 μg ml⁻¹ chloramphenicol and grown in a 25 °C shaker (280 rpm) for 15 h. The culture was transferred into 500 ml of Terrific Broth medium (24 g⁻¹ yeast extract, 12 g⁻¹ tryptone, and 5 g⁻¹ glucose) supplemented with 50 μg ml⁻¹ kanamycin and 25 μg ml⁻¹ chloramphenicol, and grown at 37 °C for 4 h at 280 rpm, then transferred to 15 °C and grown for an additional 48 h after supplementation with IPTG to a final concentration of 0.1 mM.

The cells were harvested by centrifugation at 10.000g for 12 min at 4 °C. The bacterial cell pellet was resuspended in affinity buffer A (25 mM Tris–HCl at pH 8.0, 0.5 M NaCl, 15 mM imidazole, and 0.2 mM CaCl₂) containing 125 units per millilitre of Benzonase (EMD Millipore), 10,000 units per millilitre of chicken egg white lysozyme (Sigma Aldrich) and 1× BugBuster (Novagen). The cell slurry was incubated at room temperature for 15 min, followed by sonication using a Cell Disruptor W-0375 (Heat Systems-Ultrasonics) at 45% Duty Cycle (output number 5) for 30 s with 60 s rests for a total of three cycles. The cell lysate was centrifuged at 35,000g for 20 min at 4 °C. The supernatant was loaded onto a 5-ml Ni-NTA column that had been pre-equilibrated with affinity buffer A. After extensive washing with affinity buffer A, the receptor fragment was eluted with the affinity buffer B (25 mM Tris–HCl at pH 8.0, 0.1 M NaCl, 250 mM imidazole, 0.2 mM CaCl₂). Fractions containing the receptor fragment were pooled, concentrated and loaded on a Hiloand Superdex 200 gel filtration column using an ÄKTA chromatography system (GE Healthcare). The column was pre-equilibrated and run with 25 mM Tris–HCl at pH 8.0, 0.1 M NaCl, 0.2 mM CaCl₂. Dimer and monomer peaks of the *T. ni* TBR3 and CAD fractions were collected separately and concentrated to 1–2 mg ml⁻¹. Only TnTBR3 and TnCAD monomers were used for Cry1Ac binding studies.

**Fluorescence thermal shift assays.** All assays were performed using a BioRad CFX96 real-time PCR thermal cycler, enabling thermal manipulations and dye fluorescence detection. The fluorescence sensitive dye SYPRO orange (Life Technologies, S7020) was added at a 5× concentration in all assays. The temperature was increased by 0.5 °C each cycle over a temperature range of 25–90 °C. Assay reactions were performed in 96-well white PCR plates (Bio-Rad, number 3591,010). A total of 5 μl of the cell lysate was denatured at 95 °C for 1 min and at 72 °C for 20 min. The fluorescence was measured on a CLARIOstar microplate reader (BMG Labtech) every minute for 20 min at 90 °C.

**Insect cell assays.** Sf9 cells (Life Technologies) were plated in SF-900 III SFM (Life Technologies) at a density of 50,000 cells per well in a 96-well optical bottom black plate (Nunc, Thermo Scientific). The cells were incubated at 27 °C overnight to allow for adherence to the plate, and confirmed to be free of mycoplasma contamination using a MycoAlert™ Mycoplasma Detection Kit (Lonza). After overnight incubation, the medium was aspirated from the cells and 100 μl of p3 or p4 generation (third or fourth generation of baculovirus amplification in Sf9 cells after initial transfection with plasmid) recombinant baculovirus encoding each receptor diluted in SFM was added to each well. The plates were kept in a humidified environment to prevent evaporation and incubated at 27 °C for 48 h. Receptor expression was confirmed by western blotting. Toxins were diluted to the same protein concentration in 25 mM sodium carbonate at pH 11, supplemented with 1 mM β-mercaptoethanol, followed by an additional tenfold dilution in unsupplemented Grace’s insect media with 2 μM SYTOX green nucleic acid stain (Life Technologies, S7020). The media was removed from the wells without disturbing the attached cells, and the diluted toxins or buffer controls were added to respective wells. The fluorescence was measured on a CLARIOstar microplate reader (BMG Labtech) after incubation for 4 h. The fluorescence intensity of control cells expressing β-glucuronidase (GUS) was subtracted from wells expressing the variable receptor fragments with or without toxins. Replicates were averaged and signal was plotted for each toxin condition.

**Primary insect diet bioassays.** Insect diet bioassays using the evolved consensus Cry1Ac variants were performed as previously described. Briefly, 200 μl of artificial diet in 96-well plates were overlaid with 20 ml aliquots of toxin Bt spore/crystal or Bt crystal suspension, dried, after which wells were infested with neonate insect eggs suspended in 0.2% agar, dried again, sealed with Mylar sheets, and incubated at 20 °C, 60% relative humidity, in complete darkness for 5 days. The plates were scored for larval mortality and growth. Each assay was performed in three independent biological replicates with eight insects per replicate.

**Secondary insect diet bioassays.** An inbred Bt-susceptible laboratory strain of *T. ni* (designated the Cornell strain) and a Cry1Ac-resistant strain nearly isogenic to the Cornell strain, GLEN-Cry1Ac–BCS, were maintained on a wheat germ-based artificial diet at 27 °C with 50% humidity and a photoperiod of 16 h light and 8 h dark. Diet surface overlay bioassays were conducted to determine the insecticidal activity of the toxins in the susceptible and Cry1Ac-resistant *T. ni*, as previously described. Briefly, 200 μl of each toxin dose solution was spread on the surface of 5 ml of artificial diet in 30-ml plastic rearing cups (diet surface area was ~7 cm²). Ten randomly selected neonatal larvae were placed into each rearing cup after the toxin solution had dried. For each bioassay, seven to eight concentrations of the toxins were used and each treatment included five replicates (50 larvae in total per concentration). Larval growth inhibition (neonates that did not reach second instar after 4 days) and mortality were recorded after 4 days of feeding. The observed larval growth inhibition and mortality were corrected using Abbott’s formula. Median inhibitory concentration (IC₅₀) and LC₅₀ values and their 95% confidence intervals were calculated by probit analysis using the computer program POLO (LeOra Software).

32. Fu, C., Donovan, W. P., Shikapwashya-Hasser, O., Ye, X. & Cole, R. H. Hot Fusion: an efficient method to clone multiple DNA fragments as well as inverted repeats without ligase. *PLoS ONE* 9, e115318 (2014).
33. Lund, A. M. *et al.* A versatile system for USER cloning-based assembly of expression vectors for mammalian cell engineering. *PLoS ONE* 9, e96693 (2014).
34. Langmead, B. & Salzberg, S. L. *Fast* gapped-read alignment with Bowtie 2. *Nature Methods* 9, 357–359 (2012).
35. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 28, 2078–2079 (2009).
36. Garrison, E. & Marth, G. Haplotype-based variant detection from short-read sequencing. Preprint at http://dx.doi.org/10.1101/1207. 3907053 (2012).
37. Chaisson, M. J. & Tesler, G. Mapping single molecule sequencing reads using basic local alignment with successive refinement (BLASR): application and theory. *BMC Bioinformatics* 13, 238 (2012).
38. Tan, Y. & Donovan, W. P. Deletion of aprA and nprA genes for alkaline protease A and neutral protease A from *Bacillus thuringiensis* var. kurstaki. *J. Biotechnol.* 67–72 (2001).
39. Donovan, W. P. *et al.* Amino acid sequence and entomocidal activity of the P2 crystal protein. An insect toxin from *Bacillus thuringiensis vari. kurstaki*. *J. Biol. Chem.* 263, 561–567 (1988).
40. Nallamsetty, S. *et al.* Efficient site-specific processing of fusion proteins by tobacco vein mottling virus protease in vivo and in vitro. *Protein Expr. Purif.* 38, 108–115 (2004).
41. Baum, J. A. *et al.* Cotton plants expressing a hemipteran-active *Bacillus thuringiensis* cry toxin crystal protein impact the development and survival of *Lygus hesperus* (Hemiptera: Miridae) nymphs. *J. Econ. Entomol.* 105, 616–624 (2012).
42. Kain, W. C. *et al.* Inheritance of resistance to *Bacillus thuringiensis* Cry1Ac toxin in a greenhouse-derived strain of cabbage looper (*Leipodoptera: Noctuidae*). *J. Econ. Entomol.* 97, 2073–2078 (2004).
43. Abbott, W. S. *A method of computing the effectiveness of an insecticide*. 1925. *J. Am. Mosq. Control Assoc.* 3, 302–303 (1987).
Extended Data Figure 1 | Bacterial two-hybrid component validation and optimization. a, Plasmids encoding an IPTG-inducible λcI–SH2 cassette (‘DBD’) and an ATc-inducible activator-HA4 cassette (‘activator’) were co-transformed into the E. coli S1030 host strain and induced using either or both small molecules. T4 AsiA-mediated transcriptional activation required low-level expression of the σ70 (R541C/F563Y/L607P) mutant to alleviate AsiA toxicity. Use of RpoZ as the activation domain showed the greatest degree of transcriptional activation (~17-fold).

b, DNA-binding domain variation shows that multivalent phage repressors yield a greater degree of transcriptional activation than the monomeric zinc finger Zif268.

c, Transcriptional activation from a combination of the λcI DNA binding domain and RpoZ transcriptional activator was evaluated using several previously evolved protein–protein interactions involving either monobodies or DARPins, showing the generality of binding interaction detection. Error bars, s.d. of at least three independent biological replicates.
Extended Data Figure 2 | Optimization of the $\text{P}_{\text{lacZ}}$ promoter for improved sensitivity and dynamic range. a, Promoter and DNA-binding domain combinations tested during $\text{P}_{\text{lacZ}}$ optimization, showing uninduced and induced levels of absorbance-normalized luminescence. The SH2/HA4 interaction pair was used in all cases. The fold activation in each case was calculated as the ratio of the induced and uninduced luciferase expression signals. b, Graphical representation of the data in a, showing the wide distribution of promoter background levels and degrees of transcriptional activation. In a and b, the red and green dots indicate the starting ($\text{P}_{\text{lac62}}$) and final ($\text{P}_{\text{lacZ-opt}}$) promoter/DNA-binding domain combinations, respectively. Each data point in b reflects the average of at least three independent biological replicates.
Extended Data Figure 3 | Bacterial two-hybrid optimization. a, Inducer titration of the interacting fusion proteins driving the two-hybrid system. The black and green lines represent the uninduced (0 μM IPTG) and induced (1 μM IPTG) levels of IPTG-inducible 434cI–SH2 expression, while ATc induces expression of the rpoZ–HA4 cassette. In subsequent graphs and assays, the expression level resulting from the IPTG-inducible Plac promoter was measured by western blot and approximated using a constitutive promoter to reduce experimental variability. b, Degree of transcriptional activation using HA4 monobody mutants correlated with known binding affinities. The highest levels of activation resulted from \( K_d \) = low nanomolar affinities, while weak affinities in the \( K_d \) = low micromolar range could still be detected. c, Relationship between DNA-binding domain multivalency state (monomeric, dimeric, or tetrameric DNA-binding domain fused to the SH2 domain) and transcriptional activation resulting from the SH2/HA4 interaction, with higher multivalency states yielding greater activation levels. d, RBS modification enables robust modulation of the relative activation levels from the PlacZ-opt promoter using the SH2/HA4 interaction. e, Operator–promoter binding site spacing strongly affects transcriptional activation levels; 434cI binding at 61 base pairs upstream of the PlacZ-opt promoter resulted in the most robust activation. f, Linker extension to include one, two, or three G4S motifs result in reduced activation levels using the SH2/HA4 interacting pair. g, Phage plaque formation as a function of target protein multivalency. ’No operator’ indicates a scrambled 434cI operator control accessory plasmid; ’phage control’ indicates an accessory plasmid in which the phage shock promoter (activated by phage infection) drives gene III expression. h, Co-crystal structure of the ABL1 SH2 (blue) bound to the HA4 monobody (red), highlighting the interaction of HA4 Y87 (red spheres) with key residues of the phosphotyrosine-binding pocket (blue spheres) of the SH2 domain (Protein Data Bank accession number 3K2M). The phosphate ion is shown in orange at the interaction interface. i, Apparent binding activity of mutants of the HA4 monobody at position 87. Tyrosine, tryptophan, and phenylalanine are tolerated at position 87 and enable protein–protein interaction by bacterial two-hybrid assay. Error bars, s.d. of at least three independent biological replicates.
Extended Data Figure 4 | Choice of Cry1Ac and TnTBR3 fragments used in PACE. a, Protein sequence alignment of known Cry1Ac-binding motifs from cadherin receptors in several lepidopteran species, as well as the cadherin receptor from T. ni (TnCAD). The toxin-binding region (TBR; shown in red) of the known Cry1Ac-binding motifs differs from TnCAD at seven positions (shown in blue). Mutation of three residues in the TnCAD TBR (M1433F, L1436S, and D1437A) to resemble the corresponding positions of the cadherin-receptor TBRs yielded the evolutionary stepping-stone target TnTBR3. b, Schematic representations of the Cry1Ac and T. ni TBR3/CAD full-length receptors and fragments tested in this study. The red stars in the TnTBR3 variants represent the three mutations introduced into TnCAD to generate TnTBR3. c, Transcriptional activation assay using Cry1Ac and TnTBR3 fragments shows that the greatest degree of transcriptional activation resulted from full-length Cry1Ac together with TBR3 fragment 3 (TnTBR3-F3). RpoZ–Cry1Ac and 434cI–TnTBR3 fusions were used in all cases. d, Overnight phage enrichment assays using selection phages that encode either kanamycin resistance (KanR) only or KanR together with RpoZ–Cry1Ac. Compared with the KanR-only selection phage, the RpoZ–Cry1Ac selection phage enriches >26,000-fold overnight. e, Continuous propagation assays in the PACE format using either the KanR-only selection phage or the RpoZ–Cry1Ac selection phage show that the moderate affinity of Cry1Ac for TnTBR3 allows phage propagation at low flow rates (≤1.5 lagoon volumes per hour).
Extended Data Figure 5 | Single-clone sequencing and evolved Cry1Ac characterization after PACE using the bacterial two-hybrid luminescence reporter. a, Coding mutations of the tested RpoZ–Cry1Ac clones at the end of each of the four segments of PACE. Consensus mutations are coloured according to the segment in which they became highly enriched in the population (Fig. 3a). Mutations coloured in black were observed at low abundance (≤5% of sequenced clones). b, Mutational dissection of the consensus mutations from the first segment of PACE reveals the requirement for both D384Y and S404C to achieve high-level transcriptional activation using the TnTBR3-F3 target. Mutations listed in red occurred in the RpoZ activation domain, whereas mutations listed in blue occurred in the Cry1Ac domain. Error bars, s.d. of at least three independent biological replicates. c, Structure of wild-type Cry1Ac (Protein Data Bank accession number 4ARX) showing the positions of the evolved consensus mutations. The colours correspond to the PACE segments shown in Fig. 3 during which the mutations became highly abundant.
Extended Data Figure 6 | High-throughput DNA sequencing of PACE Cry1Ac selection phage libraries. The number of reads mapped to the wild-type rpoZ–Cry1Ac reference sequence using (a) Pacific Biosciences (PacBio) or (b) Illumina sequencing. Time points are coloured according to the corresponding segment of the PACE experiment (Fig. 3a).

c, In general, most PacBio reads aligned to the wt rpoZ–Cry1Ac reference sequence were found to cluster around ~2,200 base pairs, corresponding to the size of the full-length fusion gene and indicating high-quality sequencing reads. d, Illumina high-throughput sequencing yielded several high-quality single nucleotide polymorphisms across all time points. The corresponding mutations are shown in e.
Insect diet bioassay activity of PACE-evolved Cry1Ac variants against various agricultural pests. Two consensus and three stabilized PACE-evolved Cry1Ac variants were tested for activity in eleven pests: a, *Chrysodeixis includens* (soybean looper); b, *Heliothis virescens* (tobacco budworm); c, *Helicoverpa zea* (corn earworm); d, *Plutella xylostella* (diamondback moth); e, *Agrotis ipsilon* (black cutworm); f, *Spodoptera frugiperda* (fall armyworm); g, *Anticarsia gemmatalis* (velvetbean caterpillar); h, *Diatraea saccharalis* (sugarcane borer); *Spodoptera eridania* (southern armyworm); *Leptinotarsa decemlineata* (Colorado potato beetle); and *Lygus lineolaris* (tarnished plant bug). Stabilized variants showed enhanced activity in *C. includens* and *H. virescens* compared with wild-type Cry1Ac, and comparable activity to wild-type Cry1Ac in *H. zea*, *P. xylostella*, *A. ipsilon*, *S. frugiperda*, *A. gemmatalis*, and *D. saccharalis*. No activity was observed for any of the Cry1Ac variants at any tested dose for *S. eridania*, *L. decemlineata*, or *L. lineolaris*. No insect larvae mortality was observed for *S. frugiperda*, although high toxin doses greatly stunted growth.
Extended Data Figure 8 | Comparison of cadherin receptor sequence identity. The percentage sequence identity using the full-length cadherin receptor (a) or fragment used for directed evolution experiments (b) for insects tested in Extended Data Fig. 7. Numbers in parentheses denote the number of identical amino acids between the two receptors. In general, mortality and stunting data from diet bioassays correlate with cadherin receptor sequence identity.
### Extended Data Table 1  | Insect bioassays against susceptible and resistant *T. ni*

| Toxin          | LC₅₀ (ppm) | 95% CL | Slope | SE  | Relative potency (%) |
|----------------|------------|--------|-------|-----|----------------------|
| **Mortality**  |            |        |       |     |                      |
| Cry1Ac         | 0.009      | 0.019 - 0.059 | 2.54 | 0.26 | 100                  |
| Cry1Ac-C03     | 0.793      | 0.505 - 1.082  | 2.84 | 0.41 | 5                    |
| Cry1Ac-C05     | 0.715      | 0.407 - 1.176  | 1.78 | 0.22 | 5                    |
| Cry1Ac-C03s    | 0.018      | 0.014 - 0.020  | 4.68 | 0.75 | 217                  |
| Cry1Ac-C05s    | 0.035      | 0.026 - 0.045  | 3.59 | 0.41 | 111                  |
| Cry1Ac-A01s    | 0.021      | 0.015 - 0.024  | 4.82 | 1.09 | 166                  |
| **Growth inhibition** |        |        |       |     |                      |
| Cry1Ac         | 0.019      | 0.011 - 0.007  | 3.09 | 0.39 | 100                  |
| Cry1Ac-C03     | 0.136      | 0.110 - 0.160  | 4.00 | 0.62 | 14                   |
| Cry1Ac-C05     | 0.217      | 0.167 - 0.288  | 2.59 | 0.32 | 9                    |
| Cry1Ac-C03s    | 0.007      | 0.003 - 0.010  | 3.65 | 0.61 | 271                  |
| Cry1Ac-C05s    | 0.016      | 0.014 - 0.018  | 5.53 | 0.82 | 119                  |
| Cry1Ac-A01s    | 0.005      | 0.004 - 0.006  | 4.92 | 0.9  | 380                  |
| **Resistant T. ni** |        |        |       |     |                      |
| Toxin          | IC₅₀ (ppm) | 95% CL  | Slope | SE  | Relative potency (%) |
|----------------|-----------|---------|-------|-----|----------------------|
| **Mortality**  |            |         |       |     |                      |
| Cry1Ac         | 51.229    | 9.299 - 90.241 | 1.89 | 0.36 | 100                  |
| Cry1Ac-C03     | 408.713   | 263.629 - 660.973 | 0.81 | 0.1  | 13                   |
| Cry1Ac-C05     | 235.698   | 79.467 - 510.323 | 1.12 | 0.15 | 22                   |
| Cry1Ac-C03s    | 1.841     | 1.380 - 2.312  | 2.25 | 0.28 | 2780                 |
| Cry1Ac-C05s    | 1.938     | 1.550 - 2.352  | 2.55 | 0.29 | 2643                 |
| Cry1Ac-A01s    | 0.153     | 0.046 - 0.299  | 2.01 | 0.22 | 33483                |
| **Growth inhibition** |        |         |       |     |                      |
| Cry1Ac         | 23.402    | 4.587 - 46.512 | 1.49 | 0.25 | 100                  |
| Cry1Ac-C03     | 56.626    | 40.600 - 75.685 | 1.84 | 0.21 | 41                   |
| Cry1Ac-C05     | 47.232    | 20.256 - 90.729 | 1.16 | 0.12 | 50                   |
| Cry1Ac-C03s    | 0.733     | 0.515 - 0.949  | 2.06 | 0.28 | 3190                 |
| Cry1Ac-C05s    | 1.116     | 0.797 - 1.484  | 2.19 | 0.23 | 2097                 |
| Cry1Ac-A01s    | 0.083     | 0.061 - 0.104  | 2.57 | 0.38 | 28193                |

The LC₅₀ and IC₅₀ values were determined using seven to eight concentrations of the indicated toxins in an insect diet surface overlay bioassay using either Cry1Ac-susceptible or Cry1Ac-resistant *T. ni* neonatal larvae. Each toxin concentration was tested in five replicates, each of which contained ten randomly selected neonatal larvae. In each case, the 95% confidence interval (95% CI), the slope of the best fit (Slope) and the standard error (SE) is given. The relative potency (%) has been normalized to the activity of wild-type Cry1Ac for each case.
Extended Data Table 2  |  Plasmids used in this work

| Plasmid name | Class (spec) | Origin of replication | ORF 1 | ORF 2 | ORF 3 | Figures |
|--------------|-------------|-----------------------|-------|-------|-------|---------|
| pAB3005      | CP (spec)   | SC101                  | flh    | gaa    | -     | -       |
| pAB3006      | AP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3007      | AP (spec)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3008      | AP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3009      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3010      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3011      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3012      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3013      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3014      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3015      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3016      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3017      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3018      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3019      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3020      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3021      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3022      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3023      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3024      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3025      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3026      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3027      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3028      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |
| pAB3029      | CP (cont)   | SC101                  | gaa    | tet    | -     | -       |

Each plasmid is defined by the plasmid class, antibiotic resistance, origin of replication, and promoter/gene combinations describing the relevant open reading frames carried by the plasmid. Relevant figures where these materials were used are given in each case.