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Protein complexes from edible mushrooms as a sustainable potato protection against coleopteran pests

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Summary

Protein complexes from edible oyster mushrooms (*Pleurotus* sp.) composed of pleurotolysin A2 (PlyA2) and pleurotolysin B (PlyB) exert toxicity in feeding tests against Colorado potato beetle (CPB) larvae, acting through the interaction with insect-specific membrane sphingolipid. Here we present a new strategy for crop protection, based on *in planta* production of PlyA2/PlyB protein complexes, and we exemplify this strategy in construction of transgenic potato plants of cv Désirée. The transgenics in which PlyA2 was directed to the vacuole and PlyB to the endoplasmic reticulum are effectively protected from infestation by CPB larvae without impacting plant performance. These transgenic plants showed a pronounced effect on larval feeding rate, the larvae feeding on transgenic plants being on average five to six folds lighter than larvae feeding on controls. Further, only a fraction (11%–37%) of the larvae that fed on transgenic potato plants completed their life cycle and developed into adult beetles. Moreover, gene expression analysis of CPB larvae exposed to PlyA2/PlyB complexes revealed the response indicative of a general stress status of larvae and no evidence of possibility of developing resistance due to the functional inactivation of PlyA2/PlyB sphingolipid receptors.

Introduction

Improved protection of crops against insect pests is an urgent need of modern agriculture. New approaches should avoid the introduction of novel toxins to the environment, and strategies that will be rapidly overcome by the rapid evolution of resistant populations are also undesirable. The Colorado potato beetle (*Leptinotarsa decemlineata*; CPB) is one of the most important potato pests, causing high economic losses all over the world despite it is being controlled by chemical pesticides. According to FAO data for 2021 (FAOSTAT, 2023), on a global scale, 18.1 million hectares were planted with potato, yielding on average 20.7 tons/hectare. The annual potato yield reaches 376 million of tons and is worth approximately 90 billion USD. According to Oerke (2006), without appropriate potato crop protection, the annual losses due to CPB and other insect potato pests would reach up to 70%, which could result in the economic damage of 63 billion USD.

The protection of crops against CPB is currently managed through the use of foliar synthetic insecticides that can have harmful effects on the environment and on human health. The usual alternative to chemical insecticides comprises the use of sprays with different formulations based on proteinaceous endotoxins from *Bacillus thuringiensis subsp. tenebrionis* (Bt toxins), agents based on Spinosad, neem extract or natural pyrethrin (Göldel *et al.*, 2020). Genetically transformed potatoes expressing Bt toxins Cry1Ia1 and Cry3Aa were also introduced as an alternative to chemical insecticides in 1996; however, due to various problems associated with the emergence of resistance, changes in the marketing orientations of biotech companies and consumer aversion, these potatoes did not persist on the market (Cooper *et al.*, 2004). Testing of new varieties of Bt potatoes is currently in course in China, where in some parts of the country CPB still holds the status of a quarantine pest, and where the production of transgenic varieties is seen as one of the most efficient solutions for the long-term reduction of crop damage caused by CPB (Mi *et al.*, 2015). Among the latest advancements in pest control, pesticides based on RNAi mode of action (Mateos Fernández *et al.*, 2022), offer another biotechnological solution for control of CPB (Petek *et al.*, 2020).

The CPB has been quickly developing resistance against different insecticides (Alyokhin *et al.*, 2008; Huseth and Groves, 2014), and the mechanisms of its counter response against natural defence compounds produced in potato when exposed to the insect were studied on several levels. It was shown that larvae adapt to protease inhibitors produced in potato leaves through substitution of inhibitor-sensitive digestive cysteine proteases with a resistant set (Gruden *et al.*, 2003, 2004), and can also adjust the set of other proteins active in the gut, including other digestive enzymes, depending on the diet which the larvae are feeding on (Petek *et al.*, 2012, 2014). The full potential for plasticity of the beetle was also confirmed with the first draft of its genome determined (Schoville *et al.*, 2018). Thus, it is important to address this issue with any new pesticide being developed.

For the discovery and development of new, environmentally and human-friendly biopesticides for CPB control, lipid-binding

proteins belonging to the aegerolysin family and deriving from edible oyster mushrooms (*Pleurotus sp*.) have recently gained significant interest (Berne *et al*., 2009; Butala *et al*., 2017; Kraševec and Skočaj, 2022; Novak *et al*., 2015; Panevska *et al*., 2021a). The common high-affinity membrane lipid receptor (K_D ~ 1–10 nM) for these proteins is ceramide phosphoethanolamine (CPE) (Bhat *et al*., 2015; Milijaš Jotić *et al*., 2021; Panevska *et al*., 2019a), which is also the major sphingolipid in invertebrate (in particular insect) cell membranes (Panevska *et al*., 2019b). We have recently evaluated the insecticidal potential of the aegerolysin proteins deriving from *Pleurotus* mushrooms, namely ostreolysin A6 (OlyA6), pleurotolysin A2 (PlyA2) and erylysin A (EryA) in concert with their protein partner pleurotolysin B (PlyB), which has a 'membrane-attack-complex/ perforin' (MACPF) domain and is produced by *P. ostreatus*. When added to insect diet, OlyA6/PlyB, PlyA2/PlyB or EryA/PlyB complexes selectively killed larvae and adults of two coleopteran pests that belong to the family Chrysomelidae, the CPB and the western corn rootworm (*Diabrotica v. virgifera*; WCR) (Panevska *et al*., 2019a). The acidic milieu in the midgut of these species allows optimal conditions for binding of these insecticidal proteinaceous complexes and is probably responsible for their specificity towards CPB and WCR (Berne *et al*., 2005). The death of the insect is a consequence of the formation of bi-component transmembrane pores in the epithelial cells of their midgut upon binding of aegerolysin proteins to their lipid target, the CPE (Milijaš Jotić *et al*., 2021). The toxicities of these protein complexes against CPB were shown to be even greater than that of the only aegerolysin-based bioinsecticidal Bt protein complex Cry34Ab1/-Cry35Ab1 (Panevska *et al*., 2019a; Schlotter and Storer, 2009), that was genetically introduced into corn hybrids in 2005 as tool for the control of WCR larvae (Devos *et al*., 2012; Narva *et al*., 2013). These toxicity data from the feeding assays strongly suggest the use of OlyA6/PlyB, PlyA2/PlyB or EryA/PlyB cytolytic complexes for the development of genetically modified crops for the control of WCR and CPB.

Here we describe the strategy for producing the bioinsecticidal aegerolysin-based complex PlyA2/PlyB in potato plants and show that the developed transgenic potatoes are resistant to CPB. We also checked the physiology of larvae when exposed to these proteins and we show they are severely stressed, while no mechanism leading to resistance was detected.

Results

Strategy for expression of PlyA2 and PlyB in potato

To generate potato plants that produce sufficient amounts of both PlyA2 and PlyB to protect the plant and yet not to cause significant impact on its performance, we first designed *in planta* expression strategy. We first performed testing of several promoters for their applicability (Figure S1). Considering the previously described requirements for optimal promoter strength, and potential silencing in case the strongest constitutive promoter CaMV35S drives expression (Mishiba *et al*., 2005; Rajeevkumar *et al*., 2015; Weinhold *et al*., 2013; Yamasaki *et al*., 2011) we selected *PcUBI* and CsVMV promoters for further experiments.

We optimized the codon usage for protein production in potato. Additionally, in order to minimize the risk of protein degradation, we eliminated predicted SUMOlyation and ubiquitination sites of lysine at positions 52 and 85 in one of the constructs (Figure 1a,b top). To determine the most efficient way to compartmentalize PlyA2 and PlyB, we tested targeting to three different subcellular compartments: the endoplasmic reticulum, the vacuole and the cytosol. We chose vacuole due to its acidic environment and protein storage characteristics and used localization signal KISIA, the C-terminal vacuolar-sorting sequence from the amaranth 11S storage globulin (Marin Viegas *et al*., 2017; Petruccelli *et al*., 2007). Cytosol was chosen because of its neutral pH environment and its high capacity to accumulate heterogeneous proteins (De Jaeger *et al*., 1999; Ligaba-Osena *et al*., 2017), while endoplasmic reticulum was chosen due to its high protein production, low proteolytic activity (Benchabane *et al*., 2008; Ligaba-Osena *et al*., 2017; Sainsbury *et al*., 2008) and neutral pH value (Martinière *et al*., 2013; Paroutis *et al*., 2004). Signal sequence KDEL was used to target proteins to endoplasmic reticulum (Dobhal *et al*., 2013; Song *et al*., 2018). Furthermore, the localization signal had to be fused at a specific terminus for the two proteins to fold correctly. Based on previous results revealing that N-terminal tags prevent the interaction of *Pleurotus* aegerolysins with lipid membranes (Lukoyanova *et al*., 2015; Ota *et al*., 2013; Skočaj *et al*., 2014), and due to the fact that, in the pore complex, PlyB interacts with its aegerolysin membrane-binding partner through its C-terminus (Lukoyanova *et al*., 2015), KISIA was fused to PlyA2 at the C-terminus in one of the constructs (Figure 1b bottom), while KDEL was fused to PlyB at the N-terminus in all constructs (Figure 1b). The other two constructs had PlyA2 targeted to cytosol (Figure 1b top and middle). The PlyA2 and PlyB transcriptional units were transcribed in the same forward direction. Moreover, we tested the expression of the two proteins encoded in a bicistronic construct containing a self-cleaving peptide (LP4/F2A; Wang *et al*., 2015; Sun *et al*., 2021) with the modified PlyA2 construct (Figure 1b top).

Besides binding to CPE, *Pleurotus* aegerolysins PlyA2 can interact with moderate affinity with mammalian-specific membrane sphingolipid, sphingomyelin in complex with cholesterol (Panevska *et al*., 2019a), and can cause haemolysis of red blood cells when combined with PlyB. This feature was used to monitor the PlyA2/PlyB expression in transient transformation of *Nicotiana benthamiana* plants. All transcriptional units (CsVMV::$PlyA2_{ARG}$_LP4/F2A_$PlyB_{KDEL}$_NOST (Arg), CsVMV::$PlyA2_{cyto}$_35ST_PcUBI::$PlyB_{KDEL}$_NOST (Cyto) and CsVMV::$PlyA2_{KISIA}$_35ST_-PcUBI::$PlyB_{KDEL}$_NOST (KISIA) (Figure 1b)) were functional *in planta* (Table S1), thus we proceeded with stable transformation of *Solanum tuberosum* cv. Désirée with all three of them.

Transgenic potatoes targeting PlyA2 in vacuole and PlyB in endoplasmatic reticulum show best protection against CPB larvae

We generated several lines per each designed construct. Six lines, whose phenotype did not visibly differ from control, non-transgenic Désirée plants (NT), were chosen for further experiments (Figure S2). The screening tests of different transgenic lines for their effect on CPB mortality and development were performed on excised potato leaf discs. Exposure of the CPB larvae to the leaf discs of four transgenic lines (Cyto L1, Cyto L9, KISIA L1, Arg L2) significantly reduced the larval weight gain (Figure S3a,b). The lower weight gain of CPB larvae was the result

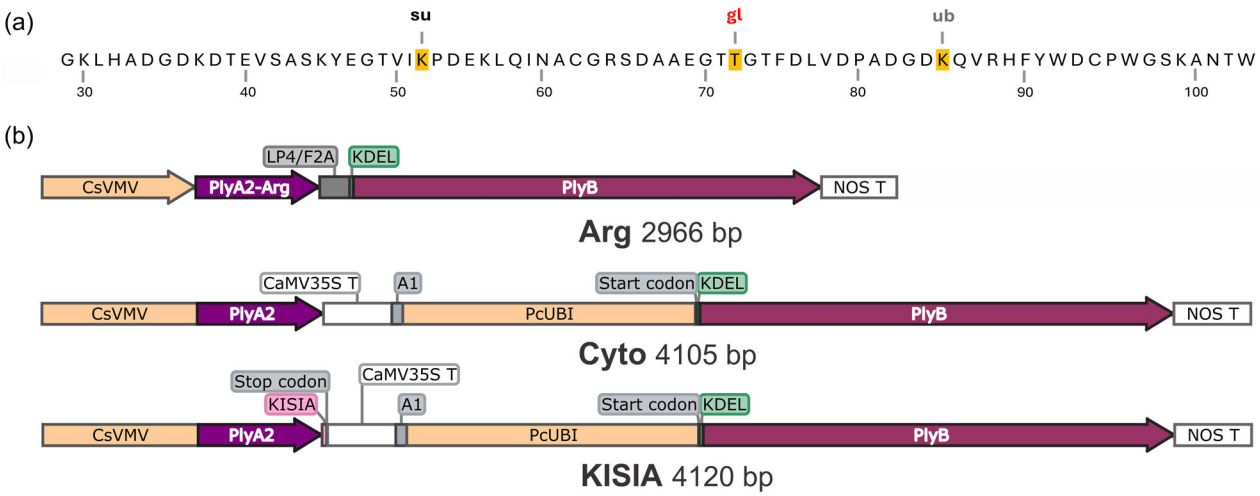

Figure 1 Design of constructs for potato transformation. (a) Prediction of post-translational modifications (PTMs) of PlyA2 using a deep-learning framework MusiteDeep. The program detected SUMOlyation at position K52 and ubiquitination at position K85. Sites with confidence scores higher than 0.5 are predicted and highlighted in orange as PTM sites. (b) Design of constructs used to produce Arg, Cyto and KISIA transgenic lines. Transgenic Arg lines have modified PlyA2 protein and allow polycistronic expression mediated by a self-cleaving peptide. Transgenic Cyto lines contain the genes in two different transcriptional units and are targeting PlyB in endoplasmic reticulum (ER) and PlyA2 in cytoplasm or vacuole in case of transgenic KISIA lines. LP4/F2A – self-cleaving peptide, CsVMV – CsVMV promoter, Pc-UBI – PcUBI promoter. PlyA2, PlyB – optimized coding sequences of both aegerolysins, CaMV35S T – CaMV35S terminator, NOS-T – NOS terminator, KDEL – ER localization signal, KISIA – vacuole localization signal, A1 – linker sequence.

of a significant reduction of their feeding rate, when fed by leaf discs of transgenic lines. The largest reduction of feeding rate was observed in KISIA L1, where a significantly lower feeding rate was measured in comparison to other transgenic lines (with the exception of Arg L2) (Figure S3c). However, despite observed significant differences in CPB larval feeding rate and weight gain between transformed lines and NT plants, the mortality of CPB larvae fed on leaf discs of transgenic plants was increased but did not significantly differ from the mortality of larvae fed on leaf discs of NT plants (Figure S3d).

The leaf discs from transgenic and NT plants were tested for haemolytic activity when the discs were excised from plants and after 24 h in assay. The haemolysis, indicating the presence of functional PlyA2/PlyB constructs, was observed in extracts obtained from fresh leaf discs but not in 24-h-old leaf discs (Table S2). The PlyA2/PlyB complexes are not stable in detached plant parts, indicating that the exposure of larvae to the toxin was not equal for the whole test period, which influenced the performance outcome of screening experiment.

Potato plants producing insecticidal PlyA2/PlyB complexes affect larval growth and survival, and delay beetle development

In order to estimate which parameters in CPB development are affected, the most promising transgenic line from *in vitro* tests was subjected to more detailed feeding trials on intact, living plants. KISIA L1 was chosen, as the fresh leaf disc extracts of KISIA L1 showed higher haemolytic activity (Table S2). In addition, the KISIA L1 caused an insignificantly higher feeding inhibition of the CPB larvae compared to Arg L2, and significantly higher feeding inhibition compared to Arg L9 and all Cyto lines (Figure S3a–c). An additional transgenic line KISIA L6, which was obtained later due to the delay in regeneration stage during stable transformation, was included in the experiments to generate transformation event independent data.

The estimated amount of the proteins accumulating in leaves of KISIA L1 and L6 plants was 20 ng and 54 ng for PlyA2 along with 265 ng and 100 ng for PlyB per 1 mg of leaf material respectively (Figure 2b). As seen in Figure 2a, KISIA L1 and L6 plants were only moderately damaged by CPB while NT plants were close to be defoliated after 1 week of feeding. Moreover, NT plants had to be replaced with fresh ones two times during the 2-month span of the experiment, due to extensive damage of plants.

Feeding of CPB neonate larvae on transgenic lines significantly affected their survival ($\chi^2 = 29$; $P < 0.0001$) during the first 8 days of bioassay duration (Figure 2c). Dunn's post-test has shown that feeding on KISIA L1 significantly increased CPB larvae mortality. The hazard ratio was insignificantly higher in the KISIA L1 (2.61), compared to KISIA L6 group (1.71). CPB larvae feeding on transgenic plants for 10 days had significantly lower weights (Kruskal–Wallis statistic = 175, $P < 0.0001$; Figure 2d), indicating sublethal effects in the sense of decreased larval feeding and potentially other physiological effects. Feeding on transgenic plants also significantly reduced CPB adult emergence. Namely, five-fold less beetles emerged in cages with KISIA L1 plants (11% of the initial larvae developed into adults) or two-fold in KISIA L6 (37% of the initial larvae developed into adults) compared to control cages, where on average 65% of the initial larvae emerged as adult beetles (Figure 3a). CPB test subjects which survived feeding on transgenic plants developed significantly slower compared to larvae feeding on NT plants (Kruskal–Wallis statistic = 70.2, $P < 0.0001$, Figure 2e). In control treatment the complete lifecycle (i.e. four larval stages, prepupa, pupa, metamorphosis and imago emergence) took on average 29 ± 1 days. Imago emergence was delayed by ca. 20 or 10 days after feeding on KISIA L1 (49 ± 3) or KISIA L6 (37 ± 1) respectively (Figure 2e).

Feeding of CPB larvae on selected transgenic lines did not have an effect on their fertility as all successfully eclosed and emerged

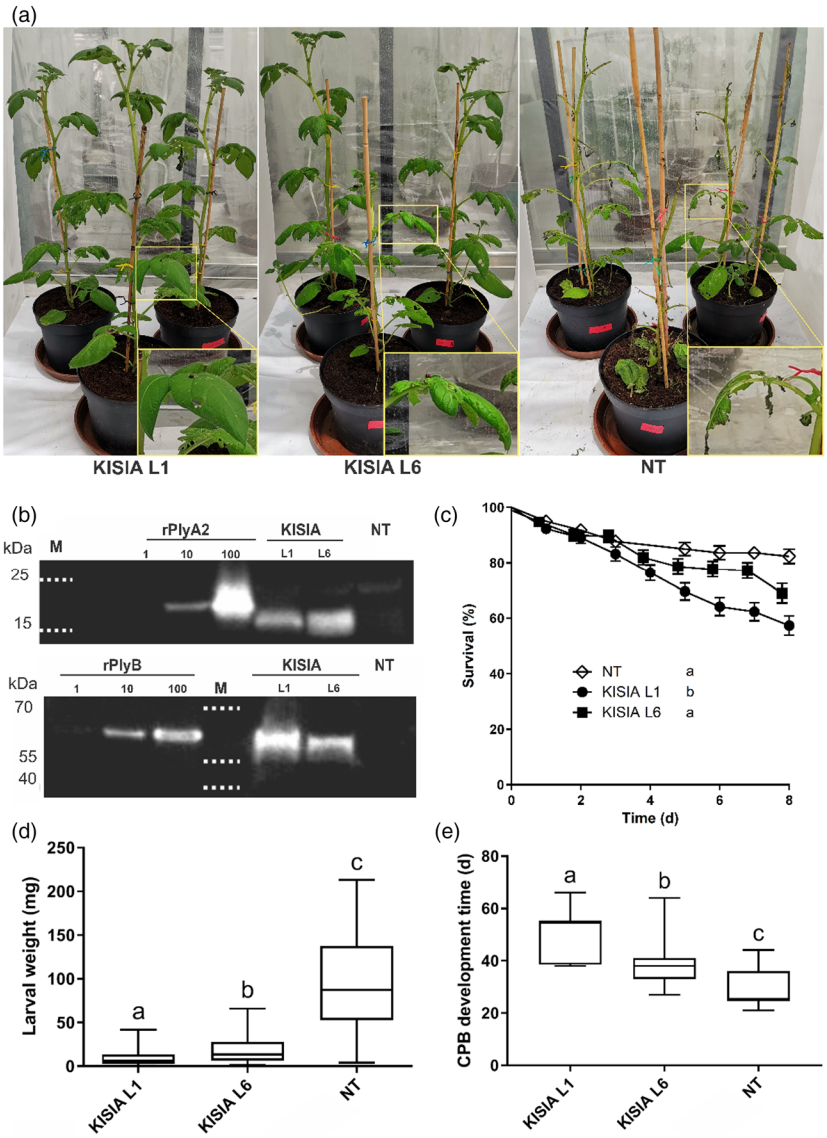

Figure 2 Transgenic KISIA L1 and L6 potato plants are effectively protected from CPB. (a) Phenotypes of KISIA and non-transformed (NT) plants after 1 week feeding of 20 CPB larvae per plant. (b) Quantification of PlyA2 and PlyB proteins in transgenic lines KISIA L1, KISIA L6 and NT plants. Recombinant proteins PlyA2 (rPlyA2) and PlyB (rPlyB) were applied as dilution series 1, 10 and 100 ng respectively. Dashed lines indicate individual bands of the protein standard (M). (c) Colorado potato beetle larval survival curves in the first 8 days of the experiment, (d) larval weights assessed on day 10, (e) development time following up to 66 days feeding on transgenic potato plants (KISIA L1 and L6) or NT plants. Different lowercase letters above bars denote significant differences between treatments in graphs c, d and e ($P < 0.05$). Data points are nudged ±0.2 units to prevent overlapping in graph c.

females laid eggs. There were also no significant effects on fecundity assessed as average number of eggs per egg cluster, number of egg clusters per female and number of eggs per female (Table S3).

Phenotype of transgenic plants is not affected by PlyA2/PlyB production

To check if there is any growth penalty on KISIA plants that produce and accumulate PlyA2/PlyB complexes, we observed the aboveground phenotype and tuber yield. The aboveground phenotype did not differ between non-transgenic and transgenic plants (Figure 3b). Tuber formation trials were conducted for the transgenic KISIA lines and NT plants to compare yields. Tubers, gathered from 3-month-old plants had comparable number and weight between non-transgenic and transgenic plants (Figure 3c-e).

RNA-Seq unveils the molecular signature of severe stress in surviving CPB larvae

We further checked for any potential adaptive response in the guts of CPB larvae surviving after feeding for 1 or 5 days on potato leaves soaked in PlyA2/PlyB solution. Only a few genes were differentially expressed 1-day post-treatment (DPT), many of which are linked to either transcriptional or translational processes (Figure 4). The transcriptional response of genes initially regulated at 1 DPT was found to be intensified in the larvae that survived until 5 DPT. Additionally, a broader range of genes, associated with diverse stress-related processes, were

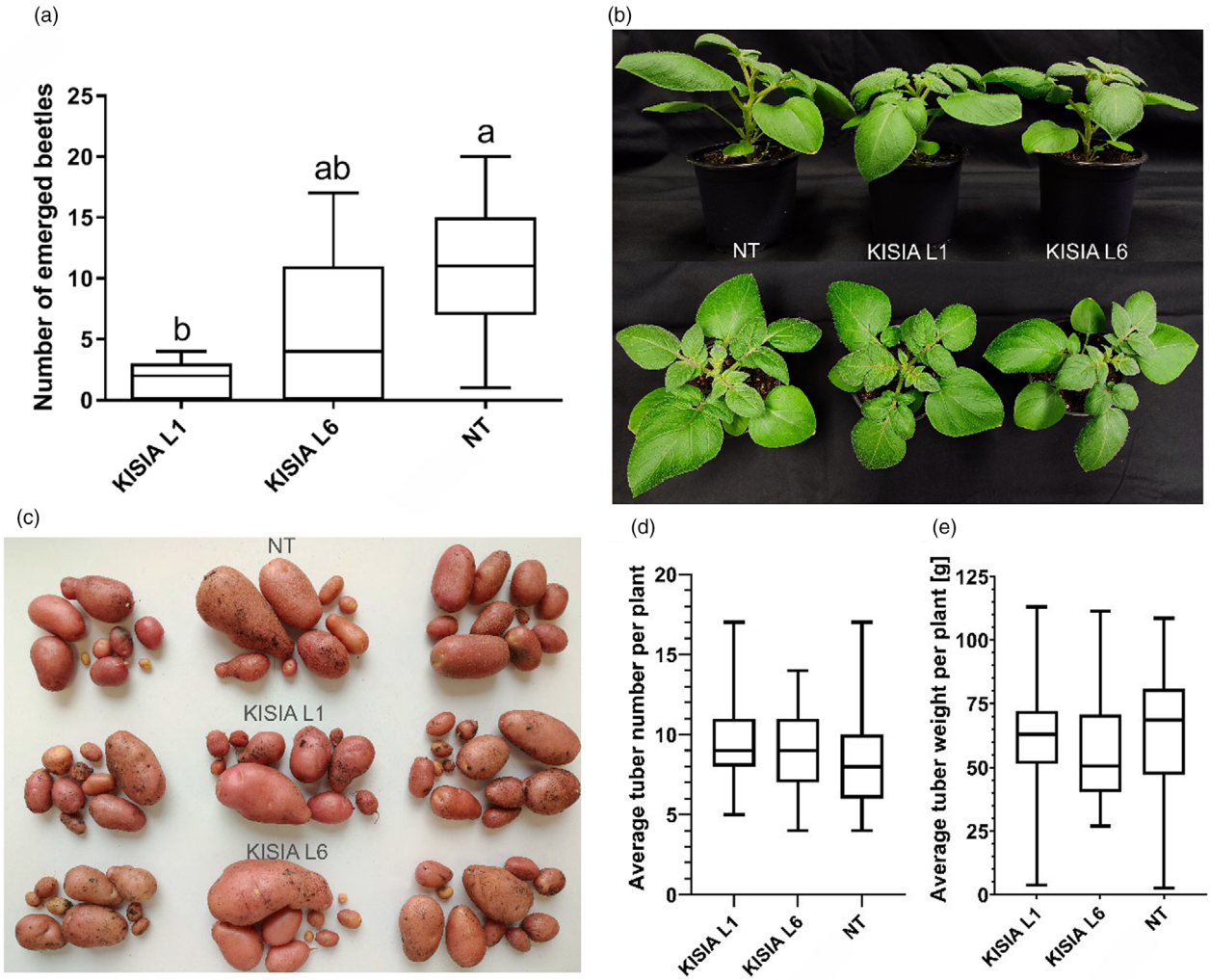

Figure 3 Transgenic KISIA L1 and L6 potato plants show no adverse effects on plant and tuber development. (a) Average number of successfully metamorphosed and emerged beetles feeding on transgenic potato plants (KISIA L1 and L6) or NT plants. (b) Aboveground comparison between control NT plants and transgenic KISIA L1 and KISIA L6 plants. (c) Three examples of tuber yield per plant in KISIA and NT plants. (d) Average tuber number per plant of transgenic lines and NT plants. (e) Average tuber weight [g] per plant of transgenic lines and NT plants. Different lowercase letters above bars denote significant differences between treatments in a ($P < 0.05$; $N = 11$). Number of tubers per plant and average tuber weight did not differ significantly between treatments in d and e ($P = 0.7$ and $P = 0.4$; $N = 25$). NT – non-transformed plants.

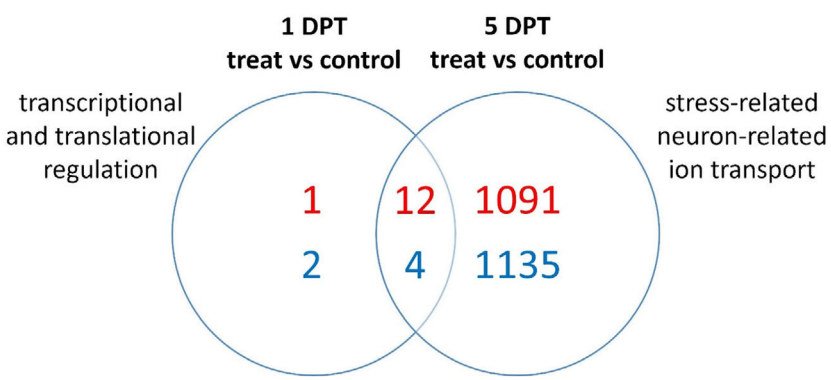

Figure 4 Venn diagram of differentially expressed genes (false discovery rate adjusted *P*-value <0.05 and $|\log_2 FC| > 1$) at 1 and 5 days post-treatment (DPT) for CPB larvae fed on potato leaves soaked in PlyA2/PlyB solution (treat) compared to control larvae fed on leaves soaked in the buffer only. Red – number of upregulated genes, blue – number of downregulated genes. Functions for most prevalent regulated genes groups are displayed on left for 1 DPT and on the right for 5 DPT.

also regulated at this later time point (Supplementary file S1), indicating a sustained elevated general stress status in these larvae. Gene set enrichment analysis revealed that, in contrast to 1 DPT, several gene sets were significantly enriched with regulated genes at 5 DPT, including ion transporter and neuron-related gene sets (Table S5). Looking at individual genes belonging to these sets, we identified seven glutamate receptor genes and five calcium channel genes that were upregulated, and four potassium channel genes that were downregulated. Additional functional annotations of these genes indicate that these glutamate receptors and potassium channels are associated with neuronal functions. Additionally, several stress-responsive cytochromes P450 (CYP) genes were regulated at 5 DPT. Among these, the most upregulated (logFC >2) were two CYP9E2-like genes, a CYP4C3-like and a CYP6A13 gene (File S1).

We further specifically inspected individual genes annotated as being part of sphingolipid metabolism. Several beta glucosidases as well as saposins, all known to be involved in degradation of sphingolipids were identified as down-regulated in CPB larvae 5 DPT. At the same time, transcription of genes coding for enzymes involved in synthesis of sphingolipids was downregulated, confirming that the observed response is indicative of a general stress status of larvae (Table S5).

Discussion

Improved crop protection against pest insects like CPB is crucial in modern agriculture, without relying on costly and ecotoxic chemicals. Despite public concerns, consumption of genetically modified (GM) crops has shown no harm to health (Ala-Kokko *et al.*, 2021; Burachik, 2010; Mahaffey *et al.*, 2016; National Academies of Sciences, 2016) and can provide means for introduction of environmentally friendly crop protection strategies.

Protein complexes from edible oyster mushrooms have been recently found to strongly interact with the invertebrate-specific membrane sphingolipid, the CPE (Bhat *et al.*, 2015). This interaction is followed by the formation of transmembrane lytic pore complexes, which are responsible for the specific insecticidal activity against CPB or WCR larvae treated with these proteins in feeding assays (Panevska *et al.*, 2019a). In this work, we present the strategy of production of potato plants transformed with the aegerolysin-based insecticidal complex PlyA2/PlyB, and we demonstrate that these plants are resistant against CPB.

Pleurotus aegerolysins/MACPF complexes are stable in a wide pH-range (3.2–9.2) (Berne *et al.*, 2005). To distribute the burden of protein accumulation to different subcellular compartments (Glick, 1995) and to minimize the likelihood of protein interaction in plant cells, we chose different localization signals, thus the recombinant version of PlyA2 was targeted to the vacuole or to the cytosol, while PlyB was targeted to endoplasmic reticulum. The amount of PlyA2 targeted to vacuole was similar to the studies of Marin Viegas *et al.* (2015) and Ocampo *et al.* (2016). PlyB had higher yields than PlyA2, which is consistent with several reports on better accumulation and stability of recombinant proteins in the endoplasmic reticulum of different plant species (Choi *et al.*, 2014; Lee *et al.*, 2015; Ma *et al.*, 2005; Sharma *et al.*, 2008; Virdi *et al.*, 2013). Importantly, targeting of PlyA2 and PlyB proteins in different subcellular compartments did not alter the aboveground phenotype, and tuber production of GM potato plants (Figure 3).

The exposure of second instar (L2) CPB larvae to leaf discs excised from the leaves of transgenic potato traits expressing PlyA2/PlyB complexes (*in vitro* screening tests) allowed us to choose the best performing trait, in our case the KISIA plants. These were further analysed in a series of *in vivo* tests *via* the transfer of newly hatched CPB neonate larvae onto intact, growing KISIA plants. The bioassays showed a decreased feeding and a decreased growth rate in comparison to the larvae fed on control plants. Based on calculations from our experiments, the average consumption of KISIA L1 was 9.2 mg/larva/day, which is comparable to the best preforming transgenic potato plants expressing the *BT-Cry3* gene (Mi *et al.*, 2015). The mortality rate of the neonate larvae fed on KISIA L1 plants, that accumulated the highest amounts of PlyB, was significantly higher than control (Figure 2c). It is also noteworthy that the cessation of CPB larvae feeding enabled the KISIA plants to remain almost unaffected, while the control ones were heavily damaged by the larvae (Figure 2a).

Continuous exposure to transgenic plant tissue can result in differential effects on adult beetle mating and oviposition behaviour (Nault, 2001). In our study, lower biomass accumulation of CPB larvae fed on transgenic lines resulted in a delay in their development to the pupal stage, and in the lower success rate in eclosion and emergence of adult beetles. These results are similar to those reported for CPB larvae feeding on Bt Cry1Ia1- or Cry3A-transformed potato (Cooper *et al.*, 2007). However, emerged imago females exhibited the same level of fecundity as females derived from larvae fed with control plants, similar to results of Cloutier *et al.* (2000), who exposed CPB beetles to oryzacystatin I- (cysteine proteinase inhibitor) transformed potato plants. These findings might reflect the fact that in our study, the emerged beetles were fed on non-transgenic potato plants, since we aimed to determine whether feeding larvae with GM plant tissue could had long-term effects on their reproductive potential.

The CPB has been quickly developing resistance against different Bt toxins, and has also developed cross-resistance and multi-resistance against different insecticides (Alyokhin *et al.*, 2008; Balaško Kadoić *et al.*, 2020; Cingel *et al.*, 2016; Huseth and Groves, 2014). Reduced binding of insecticidal Bt toxins to their protein receptors as a result of mutation of the protein receptor gene is the major mechanism of field-evolved resistance to these toxins (de Almeida Melo *et al.*, 2016; Ferré and van Rie, 2002). The PlyA2/PlyB complexes specifically target an evolutionary conserved insect-specific membrane lipid component as a receptor, and not pest membrane proteins which are prone to mutations, as is the case for Bt toxins (Pigott and Ellar, 2007). Therefore, we hypothesized that the onset of the insect resistance against PlyA2/PlyB insecticidal protein complexes could be significantly delayed or even prevented. Indeed, the changes in gut transcriptome of CPB larvae fed with PlyA2/PlyB at both time points (1 and 5 DPT) suggest that these larvae do not compensate for sphingolipid functional inactivation (Figure 4). This makes the adaptation less likely to develop for PlyA2/PlyB complexes. Considering the expression of other genes in PlyA2/PlyB-fed larvae, the downregulation of hormone biosynthesis and lipid metabolism resembles the transcriptional responses observed in insect treatment with insecticidal Bt Cry proteins (Jin *et al.*, 2022; Zhao *et al.*, 2019). This might be a signature of a stress-related response associated with gut damage and/or decrease in metabolic activity.

Conclusion

Our combined data strongly endorse the use of PlyA2/PlyB protein complexes for the development of genetically modified crops for the efficient control of CPB and other susceptible insects, for example, the western corn rootworm. Such genetically modified crops might represent an innovative way to counter pests, along with possibly less chances for resistance emergence as the aegerolysin lipid receptor, ceramide phosphoethanolamine, is an essential component of insect cell membranes. It is also noteworthy that these protein complexes, which derive from edible mushrooms, show no or very low toxicity to dipterans, lepidopterans, and other non-target arthropod or mammalian species (Panevska *et al*., 2019b, 2021b), are immediately degraded by mammalian digestive enzymes (Landi *et al*., 2022), and present no hazard for the environment due to their proteinaceous nature and thus lower environmental persistence. Therefore, the introduction and large-scale production of potato plants transformed with insecticidal PlyA2/PlyB protein complexes might not only provide savings through reduced insecticide use, but also offer better and more sustainable CPB management.

Experimental procedures

Plant material

Potato (*Solanum tuberosum* L.) cultivar Désirée and derived transgenic lines (see below) were grown in stem node tissue cultures in MS30 medium under controlled environmental conditions (22 ± 2 °C in the light and 19 ± 2 °C in the dark with 70–90 μmol/m^2/s^2 radiation (OSRAM L 58 W/77 FLUORA lamps, Germany)) and a 16-h photoperiod as described in Lukan *et al*. (2022a). Two weeks after node segmentation they were planted into 0, 5-L pots with soil and kept in growth chambers under controlled conditions as described in Lukan *et al*. (2023). For bioassays, 3-week-old plants were transferred to bigger 3-L pots and grown in a growth chamber under controlled environmental conditions of 22 ± 1 °C, 77% relative humidity and a photoperiod of 14 h/10 h light/day. All CPB *in vivo* bioassays with transgenic and NT plants were performed under semi-controlled conditions in a greenhouse: natural lighting, 20 ± 3.9 °C/17 ± 2.2 °C day/night temperature and 60 ± 17.2/73 ± 7.2% relative humidity day/night. For phenotype assessment plants were grown in 0, 5-L pots for 3 weeks and then transferred to bigger 3-L pots and grown in a growth chamber as described in Lukan *et al*. (2023). The tobacco species *N. benthamiana* plants were grown from seeds as previously described in Lukan *et al*. (2018).

Constructs for luciferase assay

Petroselinum crispum ubiquitin promoter (PcUBI) was amplified from pDe_ScCas9 (Lukan *et al*., 2022b) using primers Ubqp_F: 5′ **CACC**AAAAATTACGGATATGAATATAGGCATATCCG 3′ and Ubqp_R: 5′ GCTGCACATACATAACATATCAAGATCAG 3′ and cloned into pENTR (pENTR™/D-TOPO® Cloning Kit, Invitrogen™). Cassava vein mosaic virus (CsVMV) promoter, cauliflower mosaic virus 35S promoter (CaMV35S) and *Arabidopsis* actin promoter (*ACT2*) were ordered as synthetic constructs in pENTR at ATG-biosynthetics GmbH (Germany). LR reaction, using Gateway™ LR Clonase™ II enzyme mix (Invitrogen™) was performed to recombine the promoter sequences into the pGWB435 plasmid, containing the luciferase reporter (Nakagawa *et al*., 2007). Constructs were transformed in *E. coli* One Shot™ TOP10 cells (Invitrogen™) and were confirmed by colony PCR using KAPA Taq PCR Kits (Kapa Biosystems) and the primers reported in Table S6a. For further verification, plasmids were isolated from positive colonies using GeneElute Plasmid Miniprep Kit (Sigma) and analysed by SANGER sequencing (Eurofins Genomics service provider) using primers in Table S6a. All steps were performed according to provider's instructions.

Transient transformation of *N. benthamiana*

Constructs were introduced into homemade electrocompetent *Agrobacterium tumefaciens* GV3101 and LBA4404 as described in Lukan *et al*. (2022b). Transformed cells were grown overnight at 28 °C in yeast extract and beef liquid medium with corresponding antibiotics by shaking at 225 rpm. After centrifugation, the cell pellet was resuspended in agroinfiltration solution (10 mM $MgCl_2$, 10 mM MES, 0.15 mM acetosyringone) with the culture OD_{600} adjusted to 0.5 for prepared constructs and to 0.6 for the p19 silencing suppressor (kindly provided by prof. Jacek Hennig, Polish Academy of Sciences, Poland) and mixed in 1:1 volume ratio. The agroinfiltration solution was incubated for 2 h at room temperature by shaking at 225 rpm in the dark, before agroinfiltrating the bottom of three fully developed leaves of 3- to 4-week-old *N. benthamiana* plants.

Luciferase assay for promoter selection

Luciferase assay was performed on 0.5-cm-diameter leaf discs (bottom side up) sampled from transiently-transformed *N. benthamiana* plants 4 days post infiltration in 96-well microtitre plates containing 35 μM D-luciferin in Murashige and Skoog liquid media. Luminescence was measured in 10-min intervals for 20 h using Centro LB963 Luminometer (Berthold Technologies, Germany). Non-infiltrated *N. benthamiana* leaf discs were used as a control. Luminescence was measured in 45 leaf discs per construct and normalized to the control.

Constructs for stable transformation

The nucleotide sequences of *plyA2* and *plyB* genes from *Pleurotus sp*. were codon-optimized to increase gene expression level and translational efficiency by using IDT codon optimization tool (IDT Codon Optimization Tool (idtdna.com)).

Using the MusiteDeep (www.musite.net) tool, we identified lysine residues at potential sumoylation sites in PlyA2 and replaced them with arginine residues. The modified protein structure was modelled via the SWISS-MODEL platform and subsequently visualized using UCSF Chimera to evaluate potential effects on PlyA2 structure and function.

Three different constructs were used for stable transformation of potato. The first one was a construct with self-cleaving peptide (F2A) (Wang *et al*., 2015) together with LP4 linker peptide (Sun *et al*., 2017) positioned between PlyA2 and PlyB which were under the control of CsVMV promoter and NOS terminator (Figure 1b, top). The construct was synthesized and assembled in a cloning vector pGH by ATG-biosynthetics (www.atg-biosynthetics.com), with *I-SceI* (NEB) restriction sites on both ends. After restriction with *I-SceI* according to provider's instructions, the construct was purified using Wizard® SV Gel and PCR Clean-Up system (Promega) and inserted into pCAMBIA_ASX (Lukan *et al*., 2018) plant expression vector by NEBuilder® HiFi DNA Assembly (NEB) reaction according to Lukan

et al. (2018). The second construct comprised two transcriptional units assembled in forward direction. The first transcriptional unit contains the coding sequence for the PlyA2 protein under the control of CsVMV promoter, amplified from pENTR, and CaMV35S terminator, amplified from pJCV52 vector (Karimi *et al*., 2002). The second transcriptional unit contains the coding sequence for the PlyB protein with ER localization signal (KDEL) under the control of PcUBI promoter, amplified from pDe_Sc-Cas9 vector (Lukan *et al*., 2022b), and NOS terminator, amplified from pTA7002 (Aoyama and Chua, 1997) (Figure 1b, middle). The third construct differed from the second one only in the PlyA2 localization signal, which has a vacuole localization signal (KISIA) (Figure 1b, bottom). The second and the third constructs were assembled by PlantX-tender cloning strategy (Lukan *et al*., 2018) into pCAMBIA_ASX plant expression vector using primers reported in Table S7a,b. All constructs were transformed in *E. coli* One Shot™ TOP10 cells (Invitrogen™) and were confirmed by colony PCR using KAPA Taq PCR Kits (Kapa Biosystems) using primers reported in Table S6b. For further verification, plasmids were isolated from positive colonies using GeneElute Plasmid Miniprep Kit (Sigma) and analysed by SANGER sequencing (Eurofins Genomics service provider) using primers in Table S6b. All steps were performed according to provider's instructions.

Stable transformation

Constructs in *A. tumefaciens* strain LBA4404 which demonstrated haemolytic activity in transiently transformed *N. benthamiana* plants were used for the generation of transgenic cv. Désirée lines as described before (Lukan *et al*., 2022b). The plantlets were grown and sub-cultured in MS30 medium with hygromycin selection (final concentration 20 mg/L), under controlled environmental conditions as described above.

Protein extraction

Protein extraction (for further Western blot and haemolytic assays) was performed from leaves (200 mg) of transgenic and non-transgenic potatoes as well as from transiently transformed *N. benthamiana*. Samples were homogenized in liquid nitrogen using mortar and pestle. Four hundred μL of extraction buffer (10 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 1 mM 1,4-dithiothreitol (DTT) (Sigma-Aldrich, USA), 0.5% Igepal CA-630 (Sigma-Aldrich, USA) and EDTA-free proteinase inhibitor cocktail (Roche, Germany) was added to the leaf material (200 mg). Samples were vortexed for 30 s, and placed on ice for 10 min, mixing several times during incubation. Samples were then centrifuged at 17,000 × *g* for 30 min at 4 °C, the supernatant was centrifuged again under the same conditions to remove cell debris and stored at −80 °C prior to use.

Haemolytic activity

Haemolytic activity of extracts obtained from leaves of non-transgenic and transgenic plants (see Chapter 'Protein extraction') was measured by a turbidimetric method as described previously (Sepčić *et al*., 2003). Bovine erythrocytes were centrifuged from freshly collected citrated blood and washed three times with 140 mM NaCl, 20 mM Tris–HCl buffer, pH 8.0. Typically, 100 μL of leaf extracts were combined with 100 μL of erythrocyte suspension with an initial absorbance (at 650 nm) of 0.5. The decrease of absorbance was monitored for 20 min using a Kinetic Microplate Reader (Dynex Technologies, USA) in order to define the time needed for 50% haemolysis (t_{50}). All experiments were performed at 25 °C and repeated three times.

Western blot

Western blot analysis of leaf extracts was performed to detect and quantitatively measure the amount of target proteins in extracts obtained from leaves from non-transgenic and transgenic plants. First, we ran SDS-PAGE gel with 20 μL of leaf extracts and with different concentrations of recombinant PlyA2 and PlyB as positive controls. Then, the gel was blotted onto a polyvinylidene difluoride (PVDF) membrane that was briefly pre-soaked in 100% methanol. After blotting, the membrane was rinsed in phosphate-buffered saline pH 7.4 (PBS) and placed in a blocking solution (5% skim milk in Tris buffered saline containing 0.1% Tween 20; TBST) to prevent nonspecific binding. After 1 h at room temperature, the membrane was rinsed with TBST three times for 5 min with gentle shaking at room temperature. The membrane was transferred to a primary antibody solution (polyclonal rabbit anti-native ostreolysin for detection of PlyA2 or mouse IgG anti-PlyB for detection of PlyB, 1: 1000 in 5% skim milk/TBST) and shaken for 2 h at room temperature or overnight at 4 °C. The membrane was washed 3 times with TBST for 5 min, and the secondary antibody solution (goat anti-rabbit for PlyA2 or goat anti-mouse for PlyB, conjugated with horse peroxidase, 1:10.000 in 5% skim milk/TBST) was added for 2 h at room temperature. The membrane was rinsed three times with TBST for 5 min and three times with TBS for 5 min. The membrane was immersed in ECL Prime Western Blotting Detection Reagent (Fisher Scientific, Switzerland) and the result was analysed using Chemiluminescence detector (Syngene, UK). The amount of proteins was quantified with ImageJ software (National Institutes of Health (NIH), US) by comparing the band intensity of the leaf samples to different concentrations of recombinant proteins loaded onto a gel.

Phenotyping

The analysis was done on 25 plants per lines KISIA and NT. The upper green plant parts and tubers of the transgenic lines were compared with non-transgenic ones to ensure that the transgenic lines have comparable growth, tuber number and tuber size. The upper phenotype was evaluated visually by comparing plant height as well as the overall morphology. Tubers were harvested, the number of tubers was counted for each individual plant and tubers were weighed for each individual plant. A one-way ANOVA parametric test was used to determine statistical significance between plants with regard to tuber production.

Colorado potato beetle toxicity tests

Toxicity tests of transgenic plants to CPB were performed at the Agricultural Institute of Slovenia. The CPB larvae were obtained from laboratory colony established from adults collected from untreated potato fields in Central Slovenia in June 2021.

The *in vitro* screening tests of different transgenic lines for their effect on CPB mortality and development were performed on potato leaf discs excised from 3- to 4-week-old potted plants from tissue culture. A single leaf disc and a L2 CPB larva (4 days old) were placed in each well of a six-well plate. Every second day (or daily, in case the entire leaf disc was consumed) a fresh potato leaf disc of the relevant transgenic line or non-transformed (NT) plant was provided. Three replicate six-well plates per treatment were performed, and the experiment was repeated two times independently, giving a total of 36 larvae of each group per

treatment. The mortality of CPB larvae was monitored daily over 5 days. Larval weight change was calculated according to the weights of the larvae recorded on days 1, 5 and 10. In addition, the feeding rate of CPB larvae was evaluated using a five-class system, according to the percentage of the surface of the leaf disc eaten: class 0–0-5%, class 1–5-25%, class 2–25-50%, class 3–50-90% and class 4–90-100% leaf disc eaten.

In vivo bioassays with transgenic plants were performed on 2-month-old potted plants obtained from tissue culture. Each bioassay consisted of three plants per treatment (two transgenic lines and NT), which were individually placed in custom-made aluminium cages covered with insect netting (1-mm mesh). On each plant, 20 neonate larvae (1–2 days old) were placed on the adaxial side of the leaf. Bioassays ran over a period of approximately 2 months. During the first month, the focus was on observing the direct effects of feeding transgenic plants on CPB larvae (e.g. larval weight and mortality). In this period, the experiment was observed daily and CPB larvae were counted regularly. Eight days after the start of the experiment, the number of larvae in the control treatment decreased as they began to pupate in the soil. Therefore, we analysed larval mortality data only up to day 8 post-exposure to avoid overestimating the mortality of the control treatment. The effect of the different treatments on larval weight was determined on day 10 by weighing each live input larva. The experiment continued until day 30 for the subjects to complete their life cycle, migrate into the soil and pupate. The second phase of the bioassays focused on the emergence rate of CPB imagos and determination of fertility and fecundity-related parameters of female CPB imagos. Emergence of new beetles (imago emergence) was monitored over a period of 1 month after the emergence of the first beetle for each individual plant. The newly emerged beetles were removed from the emergence cages and transferred to new insect cages where intact NT plants were provided. To estimate fecundity and fertility of emerged CPB beetles, these cages were observed daily for newly deposited egg clusters; these were removed from the leaves to count eggs per every cluster. The bioassay was repeated independently four times.

Insect survival data was analysed using Kaplan–Meier survival analysis. When comparing multiple survival curves, the significance threshold level was adjusted using the Bonferroni method (the Bonferroni corrected significance threshold is calculated as $P_{Bon} = P/K$, where $P = 0.05$ and K is the number of pairwise comparisons). From the survival curve data 'Hazard ratio', defined as the slope of the survival curve and a measure of how rapidly the subjects died, was calculated. The hazard ratio was computed for each treatment and compared to the negative control. The effects of feeding on transgenic potato plants on CPB larval weight changes, feeding rate, number of emerged beetles and fecundity-related parameters were first checked for normality of distribution by D'Agostino–Pearson omnibus K2 test. When the data followed a normal distribution, it was analysed by one-way analysis of variance. In case if significance was observed in ANOVA, individual treatment combinations were subjected to Tukey's multiple comparison post-test (Raju, 2005). When data were not normally distributed it was analysed by Kruskal–Wallis test followed by Dunn's multiple comparison post-test (Motulsky, 1995). The difference was considered significant at P levels lower than 0.05 ($P < 0.05$). The number of biological replicates (N) is indicated in the figure or table captions. Unless stated otherwise data presented are mean values ± standard error (SE). Data were analysed using GraphPad Prism (GraphPad Software; GraphPad Software, San Diego, CA).

Colorado potato beetle gene expression analysis

Potato leaf discs were soaked in PlyA2/PlyB solution (500 μg/mL PlyA2 and 40 μg/mL PlyB) or TRIS buffer (control), and second instar (L2) CPB larvae (4 days old) were fed on treated leaf discs as described in Panevska *et al*. (2019a). Briefly, six fresh potato leaf discs were soaked in PlyA2/PlyB solution and another six in control buffer. One L2 CPB larva was placed on each leaf disc. Fresh treated leaf discs were provided when consumed. After 1 and 5 days of feeding, whole larvae were collected and rapidly frozen in liquid nitrogen, yielding four biological replicates per group at both time points. RNA extraction from individual larvae and DNase treatment were performed as described previously (Petek *et al*., 2020), followed by cleaning using RNeasy MinElute columns (QIAGEN). Illumina library prep without strand specificity and paired-end sequencing on a NovaSeq6000 platform was performed by Novogene Ltd. Sequencing reads were quality controlled using FastQC (Andrews, 2010). The reference CPB genome Ldec 2.0 (Schoville *et al*., 2018) fasta and gff files were downloaded from NCBI. The gff file was converted to gtf format using gffread v0.11.6 (Pertea and Pertea, 2020). Alignment of RNA-Seq reads to the genome was performed using STAR v2.7.5c (Dobin *et al*., 2013) allowing for only unique read mappings (--outFilterMultimapNmax 1). Differential expression analysis was performed in the R environment using packages edgeR and limma (Law *et al*., 2018). Samples SEP128 and SEP040 were identified as outliers in the MDS plot and therefore excluded from analysis. Genes with counts below 50 in less than four samples were filtered prior to statistical analysis. Gene set enrichment analysis was performed using GSEA (Subramanian *et al*., 2005) and gene sets were defined by GO term gene annotations obtained from the EggNOG database v.5.0 (Huerta-Cepas *et al*., 2019). The raw sequencing data is available on the GEO repository under accession GSE237648.

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Conflict of interest

None of the authors have a conflict of interest to disclose.

Data availability

The data that support the findings of this study are openly available in GEO at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE237648, reference number GSE237648.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Haemolytic activity of leaf extracts after transient transformation of *N. benthamina* plants with Cyto, KISIA and Arg constructs.
Table S2. Haemolytic activity of tested leaf discs from transgenic and control potato plants.
Table S3. Effect of CPB larval feeding on transgenic potato plants on fertility and fecundity-related parameters of successfully metamorphosed adult females.
Table S4. Differentially expressed Colorado potato genes at 1 day-post-treatment with PlyA2/PlyB.
Table S5. GO-based gene sets enriched for differentially expressed CPB genes.
Table S6. Oligonucleotides used for bacterial transformation conformation and Sanger sequencing.
Table S7. Oligonucleotides used for the amplification of Level 0 subunits by PCR.
Figure S1. Comparison of strengths of selected promoters.
Figure S2. Transgenic potato lines accumulating PlyA2 and PlyB.
Figure S3. Screening CPB larvae feeding assays on PlyA2/PlyB producing plants.
File S1. Results of RNAseq analysis https://doi.org/10.5281/zenodo.10728905.