JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Inhibition of the Growth of Colorado Potato Beetle Larvae by Macrocypins, Protease Inhibitors from the Parasol Mushroom

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Supporting Information

ABSTRACT: Proteins from higher fungi have attracted interest because of their exceptional characteristics. Macrocypins, cysteine protease inhibitors from the parasol mushroom *Macrolepiota procera*, were evaluated for their adverse effects and their mode of action on the major potato pest Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say). They were shown to reduce larval growth when expressed in potato or when their recombinant analogues were added to the diet. Macrocypins target a specific set of digestive cysteine proteases, intestains. Additionally, protein−protein interaction analysis revealed potential targets among other digestive enzymes and proteins related to development and primary metabolism. No effect of dietary macrocypins on gene expression of known adaptation-related digestive enzymes was observed in CPB guts. Macrocypins are the first fungal protease inhibitors to be reported as having a negative effect on growth and development of CPB larvae and could also be evaluated as control agents for other pests.

KEYWORDS: *Colorado potato beetle, transgenic potato, protease inhibitor, macrocypin, intestain, adaptation, crop protection, mushroom*

INTRODUCTION

Herbivorous insects depend on breakdown of ingested proteins for normal growth, development, and fertility. In plant leaves a specific defense response against insect damage leads to increased levels of protease inhibitors. However, their effects on herbivore development or mortality are often relatively minor or even absent as insects overcome the plant defense and compensate for the lost digestive proteolytic activity. As has been shown for several coleopterans, this is achieved by overexpression of digestive proteases and/or by changing the composition of digestive proteolytic enzymes to one containing those that are insensitive to or can inactivate the ingested protease inhibitors.[1−9] This is also the case in Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) larvae, in which cysteine proteases constitute the predominant digestive proteolytic activity[10,11] and are complemented by aspartic and serine proteases.[3,12,13] The digestive cysteine proteases, intestains, are highly diverse at the gene level and have been classified into six groups (intestains A, B, C, D, E, and F) on the basis of the available cDNA clones.[3,4,14] At the biochemical level, however, only three groups of intestains (intestains 1, 2, and 3), each with distinct inhibitory profiles and substrate specificities, have been isolated and characterized, of which intestain 3 is able to inactivate a model cystatin.[5] Expression of different intestains is differentially regulated in guts during adaptation to the plant defense, enabling CPB larvae to develop and grow.[3−5,11,15] In addition to intestains, expression of other digestive enzymes, such as serine proteases, pectinases, and cellulases, is induced during long-term adaptation of CPB larvae to potato defenses.[3] Furthermore, as in other studied coleopterans, expression of several other genes involved in metabolism, structure, development, and stress-response is modulated for reallocation of resources into adaptation to plant defense.[3,6,9]

Because proteolysis is essential for nutrient acquisition by herbivores, inhibition of digestive proteases is a promising means of insect pest control if designed appropriately. Several protease inhibitors of plant and animal origin have been investigated for protection of potato against CPB, although with limited success, either because of relatively fast adaptation of CPB larvae or because of low expression levels in plants. Considerable inhibitory activity against digestive proteases extracted from larval guts has been shown for oryzacystatin I,[16] soybean cysteine protease inhibitors,[17,18] the proregion of papaya proteinase IV,[19] engineered cystatins from barley[20] and tomato,[21] human stefin A,[16] the third domain of human kininogen, MHC class II-associated p41 Ii fragment, and equistatin from sea anemone.[15] In most of the studies, however, proteases were extracted from larvae without fully activated adaptive mechanisms against protease inhibitors in the diet. Thus, feeding assays with protease inhibitor-coated potato leaves showed decreased weight gain and/or increased mortality of larvae, but for equistatin[15] and oryzacystatin I[10] the effect was limited to younger larvae and was displayed as decreased growth rate and leaf consumption for a hybrid plant

Received: August 14, 2013
Revised: November 20, 2013
Accepted: December 3, 2013
Published: December 3, 2013

aspartic and cysteine protease inhibitor[22] and for soybean cysteine protease inhibitors.[18] The effects were, for a variety of reasons, even less pronounced when protease inhibitors were expressed in transgenic potato, including oryzacystatin I,[23] sea anemone equistatin,[24] tomato cathepsin D inhibitor,[25] locust serine protease inhibitors,[26] and barley cystatin.[20]

Colorado potato beetle has adapted rapidly to pest control strategies and is a major threat to potato production worldwide, so there is a growing demand for the development of new strategies for its control.[27] One possible source of diverse proteins that has not been widely considered for plant protection against insects is mushrooms.[28] As several mushrooms are not eaten by insects, they constitute a potential source of phytoprotective proteins, such as protease inhibitors, lectins, and lysins. Furthermore, the aqueous extracts of several mushrooms that are nontoxic for humans exhibit strong insecticidal properties, which strengthens their value as a source of candidate insecticides.[29,30]

A new type of cysteine protease inhibitor from edible mushrooms has been described and a new family of protease inhibitors called mycocypins established, which includes clitocypin from *Clitocybe nebularis*[31] and macrocypins (Mcp) from *Macrolepiota procera*.[32] A further study, using three recombinant macrocypins, Mcp1, Mcp3, and Mcp4, showed that their inhibitory profiles differ from those of other known families of cysteine protease inhibitors. Macrocypins are strong inhibitors of papain-like cysteine proteases of animal and plant origin. They are very strong inhibitors of cathepsins L and V and weaker inhibitors of cathepsins B and H. A second reactive site enables mycocypins also to inhibit the asparaginyl endopeptidase (legumain) and, in the case of Mcp4, the serine protease trypsin. Macrocypins contain a β-trefoil fold with functionally versatile loops and exhibit exceptional resistance to exposure to high temperature and extreme pH.[32,33]

We have investigated the potential of macrocypins for plant protection, together with the underlying mode of action, and the potential ability of CPB larvae to adapt to macrocypins in the diet. A macrocypin encoding gene (*mcp4*) was overexpressed in potato (*Solanum tuberosum* L. cv. Désirée), and the transgenic plants were evaluated for the level of protection afforded against CPB. Feeding trials with recombinant macrocypins (Mcp1, Mcp3, Mcp4) were carried out to evaluate their individual effects, because slightly different inhibitory profiles have been reported for each.[32] The mechanism of action of macrocypins in CPB guts and the molecular response of larvae feeding on macrocypin-producing plants was investigated.

MATERIALS AND METHODS

Construction of Binary Vectors and Plant Transformation. Macrocypin 4 (GenBank accession no. FJ495248.1)[32] was chosen for in planta testing of insecticidal activity. Because the codon usage in donor organism differs from that in potato, the coding sequence of the *mcp4a* gene was adjusted. The synthetic gene (GenScript, Piscataway, NJ, USA) was cloned into Gateway compatible vector pMDC32 with a hygromycin resistance gene[34] through pDONR entry vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The new vector was named pMDC32::Mcp4. Plasmids were transferred to ElectroMAX *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen) by electroporation following the manufacturer's recommendations. Potato plants (*S. tuberosum* L. cv. Désirée) were transformed by *A. tumefaciens* strain LBA4404 containing pMDC32::Mcp4 vector as previously described.[35]

Western Blot Analysis. Expression of Mcp4 in transgenic plants at the protein level was analyzed by immunoblot analysis. Leaf tissue (200 mg) was homogenized using Tissuelyser (Qiagen, Hilden, Germany), resuspended in 500 μL of buffer (50 mM Tris-HCl, pH 6.8, with 15 mM DTT) and, after 30 min of incubation on ice, insoluble material was removed by centrifugation. The supernatant (30 μL) was separated in a 12% SDS-PAGE and transferred onto a PVDF membrane Immobilon-P (Millipore, Bedford, MA, USA). Membranes were blocked (10 mM Tris-HCl, pH 7.4, 150 mM NaCl with 5% fat-free milk) and incubated overnight at 4 °C with rabbit antiserum against Mcp (Biogenes, Berlin, Germany) diluted 1:10000. The membrane was washed with TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) at 25 °C, incubated with goat anti-rabbit secondary antibodies (Dianova, Hamburg, Germany) diluted 1:20000, and washed with TBST. The immunoreactive bands were visualized on Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) using the chemiluminescence detection assay Lumi Light^{plus} (Roche Applied Science, Mannheim, Germany).

Feeding Assay with Transgenic Potato Lines. Selected transgenic potato lines A2 and E2 were propagated in vitro and transferred to pots. Nontransgenic (nt) plants were used as a control. Plants were grown in a growth chamber with a 16:8 h light/dark photoperiod at 21 ± 2 °C during the day and 18 °C at night. All treatments were performed on detached fully grown potato plants planted in 0.4% water agar that were replaced daily.[11] Feeding experiments were carried out in a constant-environment chamber at 28 °C in the dark. CPB eggs were provided by French Agricultural Research, Inc., Lamberton, MN, USA. Larvae were hatched on tested plants, and on the third day 20 randomly selected larvae were used for each treatment. One treatment was conducted on a nontransgenic plant and two on transgenic potato plants. CPB eggs and larvae were assigned randomly to each treatment. For each treatment all larvae were put on one plant in one Petri dish. Third- and fourth-instar larvae consumed more than one plant daily; therefore, more plants were provided. In total 20−30 plants were used per treatment. Larval weight and survival rate were measured from the third day after hatching until larvae started to pupate. Weight of individual larvae was recorded daily. Survival rate was calculated as the number of live larvae on a given day divided by the number of larvae on the first day. In a separate experiment, larval guts were sampled as described,[3] after 8 days of feeding, for gene expression analysis. Midguts from two or three larval specimens from the same feeding group were pooled to give six samples of similar mass. Due to accelerated larval weight gain and to stabilize the variance, data were transformed using logarithmic transformation. Data were evaluated statistically using Student's t test by comparing weights of larvae reared on nontransgenic potato line and larvae reared on transgenic potato lines for each time point. Additionally, two-way ANOVA was performed in the R statistical environment using time point and plant genotype (nt, A2, and E2) as factors. Both factors were statistically significant ($p < 0.001$), whereas the interaction between the factors was not significant ($p = 0.34$). To evaluate differences in larval weight when reared on different plant genotypes, a post hoc HSD test (from the R package 'agricolae') was performed at $\alpha = 0.001$.

Feeding Assay with Potato Leaves Coated with Recombinant Protein. Recombinant macrocypins were expressed in *Escherichia coli* and purified as described.[32] Feeding assays with recombinant proteins were performed in a manner similar to the feeding assays, using transgenic plants, with the difference that nontransgenic potato leaves were coated with recombinant proteins and were replaced daily by fresh ones. Leaves were cut from the plant and soaked in solutions of individual recombinant Mcp1, Mcp3, and Mcp4 or bovine serum albumin (BSA) as a control (3 mg/mL each) for 10 min before being placed in Petri dishes. The amount of added protein was approximately 0.03% of leaf weight. CPB eggs for this experiment were collected in the field. Sixteen CPB larvae were used in each feeding assay that was performed at 28 °C in the dark. Starting with 3-day-old larvae, their weight and survival rate were measured daily. Data were evaluated statistically using Student's t test. Additionally, two-way ANOVA was performed as described above

using time point and leaf treatment with recombinant protein (control, Mcp1, Mcp3, and Mcp4) as factors. Both factors were statistically significant ($p < 0.001$), whereas the interaction between the factors was not significant ($p = 0.32$). The HSD post hoc test for differences in treatment was performed at $\alpha = 0.001$.

Measurements of Activity of Larval Digestive Enzymes. CPB larvae (fourth instar) were collected from potato plants grown in the field. Larval guts were excised and homogenized in liquid nitrogen. The crude protein extract was fractionated by gel filtration (Sepharose S100 equilibrated in 20 mM Tris-HCl, pH 7.5, 0.3 M NaCl), and enzymatic activity was measured in separate fractions. Fractionation was repeated to obtain enough material for assays of different digestive enzymes and their inhibition by macrocypins. Individual recombinant macrocypins Mcp1, Mcp3, and Mcp4 were always added at 10 μM final concentration.

Proteolytic activity against Z-Phe-Arg-*p*Na, Z-Arg-Arg-*p*NA (both Bachem, Bubendorf, Switzerland) and *p*Glu-Phe-Leu-*p*NA (Sigma, St. Louis, MO, USA) was assayed with and without the addition of individual recombinant macrocypins at 10 μM or of synthetic cysteine protease inhibitor E-64 (Peptide Institute Inc., Osaka, Japan) at 5 μM final concentration in 0.1 M MES buffer, pH 6.5, with 5 mM DTT. After 10 min of incubation at room temperature, the substrate was added to 150 μM final concentration and the absorbance at 405 nm (A_{405}) measured at different time points. The serine protease activity was determined against Boc-Gly-Arg-Arg-MCA (Bachem) in 0.1 M Tris-HCl buffer, pH 8.8, in the presence of 5 μM E-64 as described,[3] and the inhibitory effects of macrocypins (10 μM), chymostatin (100 μM) (Sigma), aprotinin (10 μM) (Roche Applied Science, Mannheim, Germany), and Pefabloc (AEBSF; 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) (5 mM) (Roche Applied Science) were analyzed. The activity of aspartic proteases was assayed in the presence of 5 μM E-64 and 10 μM aprotinin in citric acid phosphate buffer, pH 5.4, using substrate MOCAc-Ala-Pro-Ala-Lys-Phe-Phe-Arg-Leu-Lys-(Dnp)-NH_2 (Peptide Institute Inc.) at 5 μM final concentration. Pepstatin (6 μM) (Peptide Institute Inc.) was used to confirm aspartic protease activity. General proteolytic activity was determined against azocasein (Sigma) in 0.1 M MES buffer, pH 6.5, with 5 mM DTT as described,[36] and inhibitory effects of macrocypins (Mcp1, Mcp3, Mcp4) and E-64 were analyzed.

Proteolytic activities in fractions were assayed by gelatin zymography performed in 0.1 M MES, pH 6.5, with 5 mM DTT as described.[37]

Cellulolytic activity was assayed in gel filtration fractions using Azo-CM-cellulose (Sigma) in 0.1 M sodium acetate at pH 4.5 and 5.4 with 10 μM BSA, following the Megazyme (Bray, Ireland) assay procedure for endo-1,4-β-D-glucanases. When Mcps were included in the assay, they were incubated with the samples for 10 min at 40 °C prior to addition of substrate.

Polygalacturonase activity was measured in fractions using 1% polygalacturonic acid in citric acid phosphate buffer, pH 6, with 10 μM BSA at 30 °C in the presence or absence of macrocypins. The enzymatic activity was evaluated by determination of reducing sugars with 3-methyl-2-benzothiazolinonehydrazone (MBTH) as described.[38]

Xylanolytic activity was measured in fractions using EnzChek Ultra Xylanase Assay Kit (Molecular Probes, Eugene, OR, USA) following the manufacturer's recommendations, in 0.1 M sodium acetate buffer, pH 4.5, or in 0.1 M MES buffer, pH 6.5, both in the presence and in the absence of individual macrocypins.

Screening for Macrocypin Interacting Proteins in Larval Gut. Macrocypin affinity chromatography was performed using Mcp1 coupled to a monolithic support through the glutaraldehyde spacer as described.[39] Crude protein extract was prepared from homogenized gut tissue of CPB larvae (fourth instar) collected from potato plants grown in the field, cleared by centrifugation at 4 °C for 15 min at 16000*g* and applied to gel filtration chromatography (Sepharose S100 equilibrated in 20 mM Tris-HCl, pH 7.5, 0.3 M NaCl). Fractions containing high levels of protein (as determined by measuring absorbance at 280 nm) or proteolytic activity (as measured with synthetic peptides) were pooled and applied to the macrocypin affinity chromatography. The fraction at the peak of enzymatic activity was excluded from pooling and analyzed separately using the same procedure. The column was washed, and bound proteins were eluted by lowering the pH as described.[39] Eluted proteins were analyzed by SDS-PAGE, and individual bands were excised and, after in-gel trypsin digestion, identified by peptide mass fingerprinting using mass spectrometry (ESI-MS/MS). The procedure was repeated twice on the same CPB gut extract, only the second time the selected fractions from gel filtration were pooled together and applied to the affinity chromatography column and the macrocypin-bound proteins eluted were reapplied to the column prior to mass spectrometry analysis. Database searches were performed by Mascot in-house server using MS/MS Ion Search. All protein hits of insect origin covered by at least two peptides were considered putative Mcp1-binding proteins. In addition, for each intestain-derived peptide, all possible encoding genes were identified using tblastx, and peptides were categorized to intestain groups. The specificity of all intestain peptides was verified by inspection of alignments to available CPB intestain sequences. Because all peptides were group-specific, the number of peptides for each intestain group was calculated as a sum of all peptides categorized to each group.

Cystatin C Cleavage Assay. Inhibition by macrocypins of intestains that cleave the human cystatin C N-terminus was analyzed. Recombinant human cystatin C (22 μg) was incubated with the fraction at the peak of proteolytic activity from gel filtration diluted in 0.1 M sodium acetate buffer, pH 5.5, with or without the addition of individual macrocypins at 10 μM final concentration. After 4 h of incubation at 37 °C, the reactions were analyzed by isoelectric focusing using the Phast System (Pharmacia, Uppsala, Sweden) as described.[40]

RNA Extraction and cDNA Synthesis. Leaf and stem tissue (100 mg) was collected and stored at −80 °C prior to homogenization by Tissuelyser (Qiagen), and RNA was isolated using innuPREP Plant RNA kit (Analytik Jena, Jena, Germany). To monitor gene expression profiling in CPB larvae fed transgenic potato leaves, RNA was isolated from their midguts at day 8 of the feeding trial as described.[3] Midguts from two or three larval specimens from the same group were pooled to give six biological samples.

RNA integrity, purity, and concentration were determined using gel electrophoresis and spectrophotometer NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA). Samples were further treated with DNase (Invitrogen), and the effectiveness of the treatment was checked by qPCR of RNA samples without prior reverse transcription. cDNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) in a 25 μL reaction volume as instructed by the manufacturer.

Gene Expression Analyses. Levels of macrocypin expression in generated transgenic lines were examined by RT-qPCR using custom-designed primers and probe for *mcp4* (Applied Biosystems) and cytochrome oxidase (*cox*) as a reference gene.[41] Samples were analyzed using AgPath-ID One-Step RT-PCR Kit (Ambion, Austin, TX, USA) in 5 μL reaction settings, with 30 min at 48 °C for reverse transcription, followed by universal PCR conditions on ABI 7900HT (Applied Biosystems). For the newly designed *mcp4* qPCR assay the linear range and amplification efficiency were first determined over four 10-fold serial dilutions of plasmid containing macrocypin DNA. Amplification efficiency was calculated from log−linear regression curves of Cq (quantitation cycle) values against dilution factors or input DNA copy numbers (for the reference material). The slope of the log−linear regression curve gave the amplification efficiency as $10^{(-1/\text{slope})}$ and its percentage as $[10^{(-1/\text{slope})} - 1] \times 100$.

For gene expression profiling in CPB larvae midguts, novel RT-qPCR assays were designed using PrimerExpress 2.0 software (Applied Biosystems) to specifically amplify intestains A (IntA), intestains B (IntB), cellulase GH48-1, and cellulase GH48-2, and their efficiency was evaluated as described for *mcp4* assay. For analysis of expression of intestains C, D, and E and serine protease S1A-1, previously designed assays were applied.[3] Analyses were performed using TaqMan Universal Master Mix (Applied Biosystems) for IntA, IntB, cellulase GH48-1, and cellulase GH48-2, and PowerSYBR Green PCR Master Mix (Applied Biosystems) was used for other amplicons. Tests were

carried out in 5 μL reaction settings, using universal PCR conditions on ABI 7900HT (Applied Biosystems). Melting curve analysis was applied to all reactions based on SYBR Green to control primer dimer formation and ensure homogeneity of the reaction product. In the testing phase primer concentrations were optimized to eliminate primer dimer formation if needed. Specificity of intestain amplicons was tested using plasmids containing cDNA as a template. Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems) was used as a reference gene.

The gene expression quantitation and quality control system was used as described.[42] Amplification efficiency and linear range of amplification were followed for each amplicon on each plate by analyzing one pooled sample (which contained a mixture of randomly chosen samples) in five dilution steps of cDNA with two replicate wells per dilution step (range of cDNA dilutions: $10-10^5$). Each sample was analyzed in two replicates of two 10-fold dilutions to check for the presence of inhibitors in the sample. The standard curve quantitation approach was applied, and the reference gene was used for normalization of gene expression in each sample. For every gene, the limit of detection (LOD) was determined from the standard curve. If the determined Cq value of a sample was below the LOD, the sample's copy number was assigned the LOD copy number. Statistical significance of differences in gene expression was calculated using Student's *t* test.

RESULTS AND DISCUSSION

Transformation and Molecular Characterization of Transgenic Lines Expressing Macrocypin 4. To analyze the potential of macrocypins in plant protection against Colorado potato beetle, Mcp4 was chosen for in planta testing because of its ability to inhibit both cysteine and serine proteases of mammalian origin.[32] After transformation with *A. tumefaciens* carrying pMDC32::Mcp4, 15 plants were selected for analysis. All tested transgenic potato lines expressed *mcp4*, as determined by RT-qPCR, but at different levels of transcript (Figure 1A). Although showing high RNA expression of the transgene, line D2 was not used in further analysis due to an altered phenotype showing slow growth rate. Selected lines (A1, A2, D3, E2, E3, E4) that showed no apparent morphological alterations or growth retardation were further analyzed for accumulation of Mcp4 protein. As for the transcript levels, 19 kDa Mcp4 protein was detected in all lines analyzed, but levels varied (Figure 1B). The highest protein accumulation was achieved in lines A1, A2, and E2, in which macrocypin constituted approximately 0.01% of leaf weight.

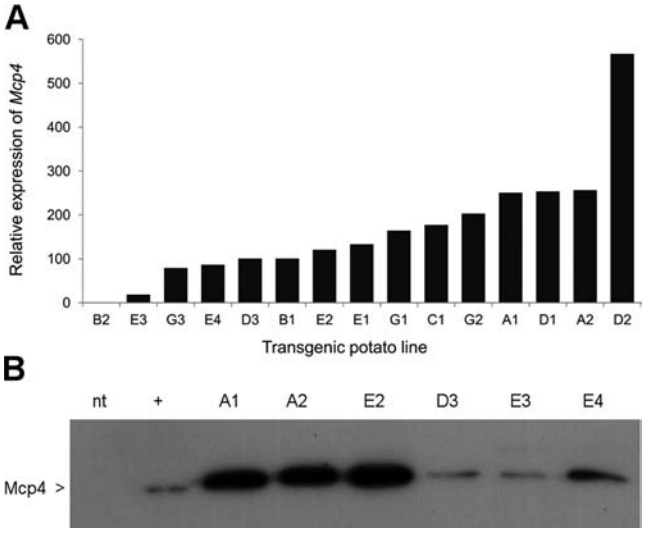

Figure 1. Expression of macrocypin 4 in different lines of transgenic potato (*Solanum tuberosum* cv. Désirée): (A) expression of *mcp4* transcript in different lines of transgenic potato relative to transcript abundance in the lowest expressing line B2; (B) anti-Mcp immunoblot analysis of crude protein leaf extracts of selected independent transgenic potato lines (A1, A2, E2, D3, E3, E4). nt, nontransgenic control; +, recombinant Mcp4 (10 ng) as positive control.

Growth and Survival of Larvae Fed Macrocypin-Expressing Transgenic Potato Lines. In feeding assays, CPB larvae were fed two selected transgenic potato lines expressing Mcp4, A2, and E2 as well as nontransgenic counterpart plants. Larvae were hatched on transgenic potato lines, but only the macrocypin-mediated effect was monitored because fresh plants were supplied daily to avoid the induction of an adaptive response in larval gut to endogenous potato defense compounds induced by larval feeding. Larvae fed transgenic potato exhibited significantly reduced weight compared to larvae fed control plants (Figure 2A), and the latter achieved maximum weight and entered the prepupal stage at least 1 day sooner (on the eighth day for larvae growing on nt plants). Maximum weight for larvae grown on transgenic plants was achieved on day 9 for A2 and on day 10 for E2. Two-way ANOVA showed significant differences between weights of larvae reared on both transgenic lines compared to larvae reared on nontransgenic control. In addition, larval survival rate was reduced on transgenic line A2 (Figure 2B).

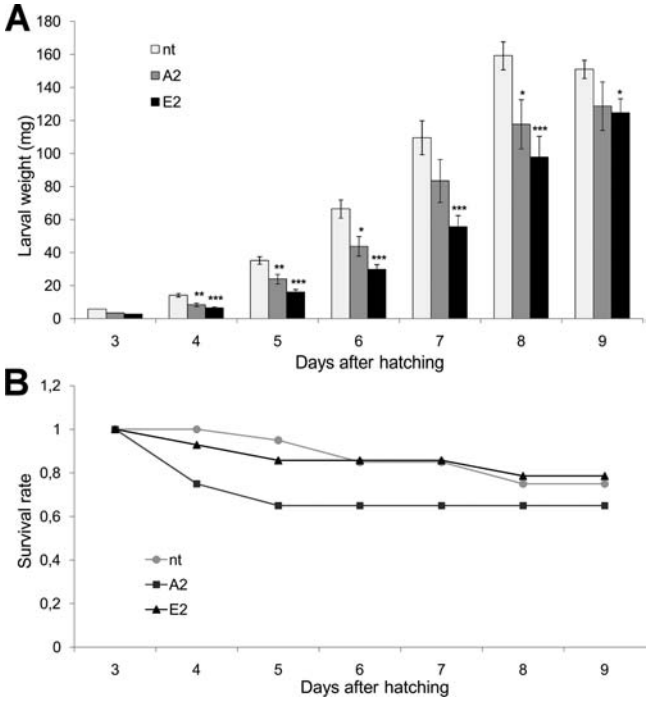

Figure 2. CPB larval growth and survival in feeding bioassays with transgenic potato leaves: average weight (A) and survival rate (B) of Colorado potato beetle larvae fed on nontransgenic control (nt) and independent transgenic potato lines (A2, E2) expressing Mcp4. Error bars indicate standard error; asterisks indicate statistically significant differences between control and test groups determined by Student's *t* test (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

The presence of Mcp4 in larval diet caused a delay in their development, a phenomenon noted in CPB larvae fed transgenic potato expressing other types of protease inhibitors,

including barley cystatin,[20] tomato cathepsin D inhibitor,[25] and locust proteinase inhibitors.[26] Increased mortality has also been observed for larvae fed potato expressing oryzacystatin I.[23] The relatively low impact of dietary inhibitors on larval survival may be the result not only of larval adaptation but also of the insufficient amount of the inhibitor in the diet for complete inhibition of targeted proteases because, in the above trial with oryzacystatin I, the negative effect was stronger with lines expressing higher levels of the inhibitor. A concentration-dependent effect was also observed when CPB larvae fed potato leaves supplemented with E-64 exhibited reduced growth rate and survival and delay in development with increasing E-64 concentration.[43]

Growth and Survival of Larvae Fed Macrocypin-Coated Leaves. In the second series of feeding assays larvae were fed leaves coated with recombinant proteins rMcp1 and rMcp3 besides rMcp4. Those fed leaves coated with rMcp1 showed significantly lower growth (Figure 3); weight gain of larvae fed leaves coated with rMcp3 and rMcp4 did not differ significantly from the control group, whereas maximum weight of larvae was delayed in larvae fed rMcp4 (for 1 day) and rMcp1 (for 2 days). The survival rate did not differ significantly among control and test groups (not shown). Two-way ANOVA showed significant differences between weights of larvae reared on Mcp1-coated leaves compared to larvae reared on control leaves.

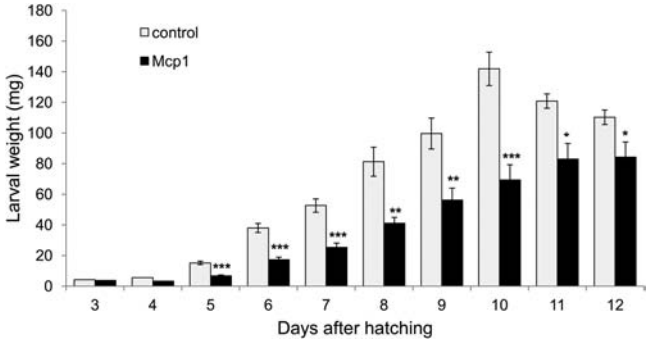

Figure 3. CPB larval growth in a feeding bioassay with potato leaves coated with recombinant Mcp1. Error bars indicate standard error; asterisks indicate statistically significant differences between control and test group determined by Student's *t* test (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

Surprisingly, rMcp1 showed a greater inhibitory effect than rMcp4. All three proteins were at ~0.03% of fresh inhibitor-coated leaf weight, and the heterologously expressed Mcp4 in transgenic potato at ~0.01% of fresh potato leaf weight, the latter being similar to the amount reported for oryzacystatin I expressed in potato.[23] The difference in effectiveness of Mcp1 and Mcp4 is therefore unlikely to be due to differences in the amount of protease inhibitor in the diet. The age at which larvae were first exposed to the protease inhibitor in diet could explain the apparently smaller effect of rMcp4, where 3-day-old larvae were first exposed, compared to exposure to transgenic Mcp4 from hatching onward. Similarly, oryzacystatin-coated leaves showed a significant negative effect on growth only when young CPB larvae were exposed (first and second instars).[10] This age-dependent effect of dietary protease inhibitors could be a general phenomenon, as it has been observed in other coleopterans including southern corn rootworm (*Diabrotica undecimpunctata*).[9] Additionally, the difference in effectiveness of Mcp1 and Mcp4 could also be the result of slight differences in intestain specificity or interaction with other proteins involved in protein digestion or adaptation response.

Screening for Potential Macrocypin Targets in CPB Larvae Guts. Affinity chromatography was used to search for macrocypin targets in guts of CPB larvae grown in the field and thus fully adapted to defense compounds induced in potato leaves upon feeding. Several proteins bound to rMcp1 in two independent assays (Table 1). In both assays the identified targets included two glycoside hydrolases of family 48, a diapause protein 1 (DP1), and several proteases from intestain A and B groups (Table 1). Best protein coverage was obtained for glycoside hydrolase GH48-2, followed closely by DP1. Other potential protein targets that bind to rMcp1 were identified in only one of the assay repetitions. These include other enzymes involved in digestion or oxidative stress response: intestains from groups D, E, and F, endopolygalactur-

Table 1. Potential Targets of Macrocypins in Colorado Potato Beetle Larval Guts

protein accession no.	identified protein	no. of unique peptides (first expt)	no. of unique peptides (second expt)
CAA53691	diapause protein 1	20	27
ADU33353	glycoside hydrolase family protein 48 GH48-2	20	22
ADU33352	glycoside hydrolase family protein 48 GH48-1	10	15
AAN77409, AAN77410, AAN77411, AAN77412, AAS20591, AAS20592	digestive cysteine proteinase intestains B	4	6
AAN77406, AAN77407, AAN77408, AAS20588, AAS20589, AAS20590	digestive cysteine proteinase intestains A	4	3
ABM55480, ABM55481	digestive cysteine proteinase intestains E	NA[a]	5
ABM55484, ABM55485, ABM55486, ABM55488, ABM55491, ABM55492, ABM55495	digestive cysteine proteinase intestains D	NA	3
ABM55487, ABM55489, ABM55490	digestive cysteine proteinase intestains F	NA	3
ABK20176	putative glutathione *S*-transferase GST3	NA	12
ABK20175	putative glutathione *S*-transferase GST1	NA	3
XP_966308	cyclophilin-like (*Tribolium castaneum*)	3	NA
ADU33363	endopolygalacturonase GH28Pect-9	NA	2
XP_001354162	GA16624_arginine-kinase-like (*Drosophila pseudoobscura pseudoobscura*)	2	NA
AF167313	arginine kinase (*Carcinus maenas*)	2	NA

[a]No unique peptides detected.

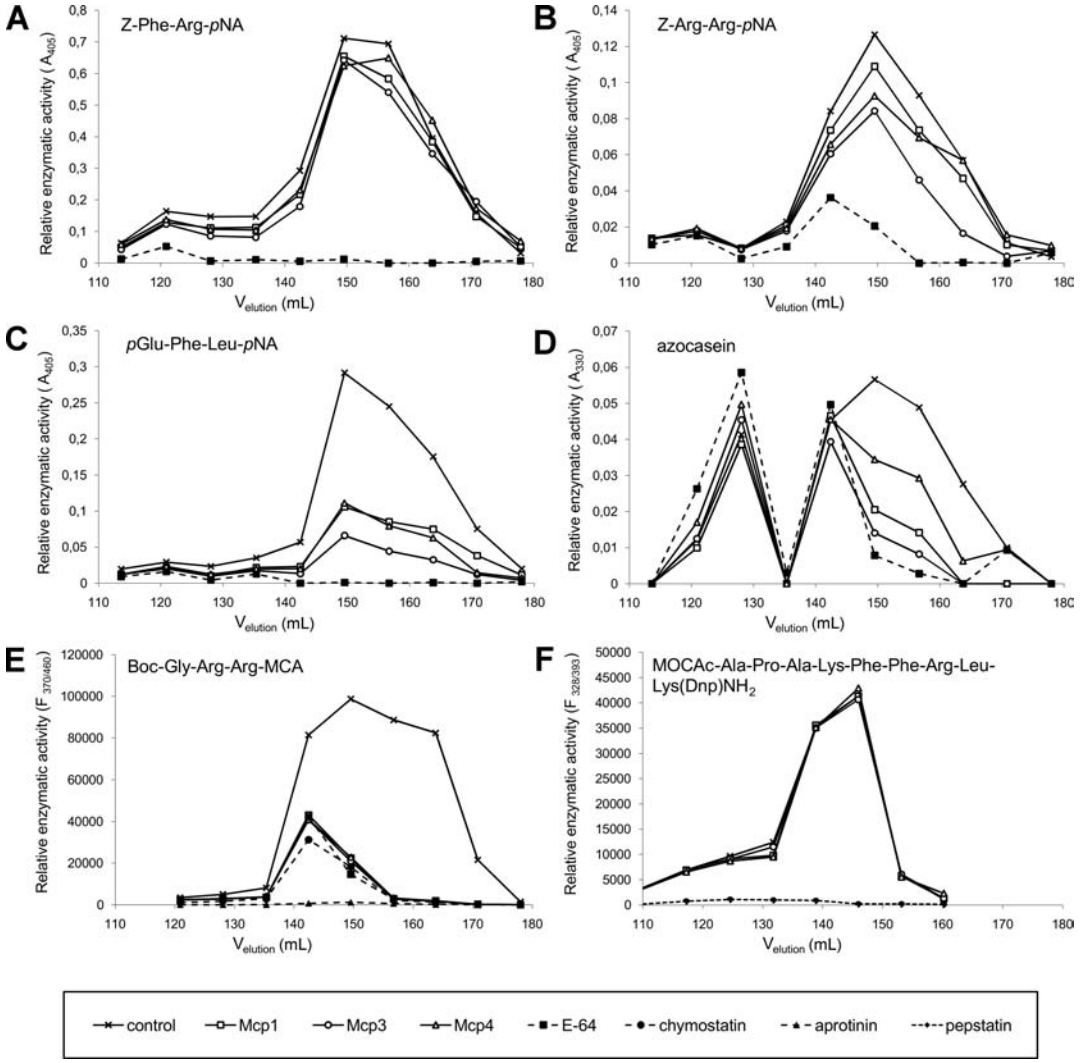

Figure 4. Inhibitory activity of macrocypins against digestive proteases from CPB larvae guts: proteolytic activity in gel filtration fractions against different substrates with and without the addition of individual recombinant macrocypins (Mcp1, Mcp3, and Mcp4) and/or other indicated class specific protease inhibitors. The legend at the bottom applies to all panels. In control reactions measuring hydrolysis of (A) Z-Phe-Arg-*p*Na, (B) Z-Arg-Arg-*p*NA, (C) *p*Glu-Phe-Leu-*p*NA, and (D) azocasein no inhibitor was added. (E) Hydrolysis of Boc-Gly-Arg-Arg-MCA was assayed with individual macrocypins, chymostatin or aprotinin, each in the presence of E-64 to detect serine protease activity. Control reactions contained no added inhibitors and indicate the activity of serine and cysteine proteases. (F) All reactions for hydrolysis of MOCAc-Ala-Pro-Ala-Lys-Phe-Phe-Arg-Leu-Lys(Dnp)-NH_2 included E-64 and aprotinin to detect aspartic protease activity.

onase GH28Pect-9, two glutathione *S*-transferases, and two arginine kinases (Table 1).

The suspected interaction of macrocypin with digestive cysteine proteases intestains was confirmed by affinity chromatography. Identified peptides corresponding to distinct intestain groups cover more than one individual protein because the protein sequences are highly conserved within the groups. In the intestain A group the identified peptides match three sequences from the database (IntA2, IntA26, and IntA27); three proteins were identified also in the intestain B group (IntB11, IntB12, and IntB4). In the intestain D group, identified peptides cover two or more intestain D representatives, whereas all three intestain F peptides (new classification according to Sainsbury et al.[14]) are specific for one protein formerly known as IntD9. In the intestain E group the identified peptides cover two known sequences (IntE2 and IntE3). A similar affinity chromatography approach, using tomato cystatin SICYS8 and its variants, indicated that the set of intestains targeted by these inhibitors differs from that targeted by macrocypins, which bound intestains B, C, D, E, and F but not intestain A.[14]

A level of macrocypin action not mediated through inhibition of digestive enzymes but possibly targeted toward larval response to nutrient stress was indicated by the affinity of rMcp1 for diapause protein 1 (DP1) as well as for putative glutathione *S*-transferases (GST1 and GST3), cyclophilin-like protein, and putative arginine kinases. Biological relevance of macrocypin binding to these proteins would have to be confirmed by further experiments; however, some indications of their effectiveness can be found in published data. DP1 is an arylphorin-type storage hexamer that accumulates in the hemolymph of last-instar larvae and probably serves as a source of amino acids during nonfeeding periods. However, it could also have a developmental role, because its expression was shown to be suppressed by a juvenile hormone analogue, pyriproxyfen.[44] α-Arylphorin, isolated from *Manduca sexta* pupae, has been shown to stimulate midgut stem cell proliferation in primary CPB midgut cell cultures, and its

addition to an artificial diet increased the growth rate of CPB larvae.[45] In addition to an indirect correlation through juvenile hormone binding proteins identified as responsive in CPB adaptation to plant defense compounds,[3] involvement of arylphorins in adaptation to antinutritive compounds is also suggested by their presence in insect guts, as has been shown for beetle *Tribolium castaneum*[46] and caterpillar *Helicoverpa armigera*.[47]

Macrocypins Affect a Specific Group of CPB Digestive Enzymes. Because several digestive enzymes were identified as potential targets of macrocypins in CPB gut, we investigated their mode of action through inhibition of the activity of digestive enzymes extracted from adapted larvae. Proteolytic activity was analyzed with emphasis on cysteine proteases, because they constitute the predominant digestive proteolytic activity in CPB larvae[11,15] and macrocypins are cysteine protease inhibitors.[32] In addition, inhibition of serine and aspartic protease activities was assayed as well as of total proteolytic activity in the larval gut extract. Furthermore, the potential for inhibition of cellulolytic, xylanolytic, and pectinolytic activities by macrocypins was studied.

Gut proteolytic activity against substrates Z-Phe-Arg-*p*Na, Z-Arg-Arg-*p*NA, and *p*Glu-Phe-Leu-*p*NA was assayed in gel filtration fractions, with and without the addition of individual recombinant macrocypins (Mcp1, Mcp3, Mcp4). Hydrolysis of substrate Z-Phe-Arg-*p*Na, which is cleaved by cysteine and serine proteases, was completely inhibited by E-64 at both pH 6.5 and 8, whereas Pefabloc at pH 8 showed approximately 30% inhibition at the peak of Z-Phe-Arg-*p*Na-hydrolyzing activity (not shown), confirming the predominant cysteine protease activity in larval guts against Z-Phe-Arg-*p*Na. Macrocypins had no inhibitory effect against this activity (Figure 4A). On the other hand, they exhibited weak inhibition of the activity against Z-Arg-Arg-*p*NA (Figure 4B), which has been associated with cathepsin B-like activity and cystatin-insensitive cysteine proteases in the CPB larval gut.[15,21] Apart from E-64, this type of activity from CPB guts is inhibited only by the animal-derived kininogens, Ii fragment and equistatin,[15] and a few engineered cystatin variants based on a tomato multicystatin.[21] Digestion of *p*Glu-Phe-Leu-*p*NA was strongly inhibited by all of the macrocypins tested (Figure 4C). Intestain 3, which hydrolyzes *p*Glu-Phe-Leu-*p*NA, is the cysteine protease that inactivates a model cystatin by cleaving its N-terminal region and is induced in guts of CPB larvae in their adaptation to high levels of endogenous protease inhibitors in potato leaves.[5] The fraction showing the highest activity against the *p*Glu-Phe-Leu-*p*NA substrate was further analyzed for cystatin C N-terminal cleavage. The cleavage of cystatin C by digestive proteases of this fraction was confirmed, but was not inhibited by any of the macrocypins tested (Mcp1, Mcp3, or Mcp4) (Figure 5). Affinity chromatography revealed that intestains A, B, and, potentially, also D, E, and F (identified only in one of the pull-down experiments) interact with rMcp1, but we cannot speculate further which of these correspond to the intestain fraction inhibited by macrocypins. Whereas intestains A, C, and D are induced in response to high levels of endogenous potato protease inhibitors in diet, the expression of intestains B and E remains unchanged.[3,4]

A pattern of inhibition similar to that seen in macrocypin inhibition of the hydrolysis of *p*Glu-Phe-Leu-*p*NA was also observed with the general proteolytic substrate azocasein (Figure 4D), indicating that, at pH 6.5, the activity against *p*Glu-Phe-Leu-*p*NA is the dominant proteolytic activity in guts

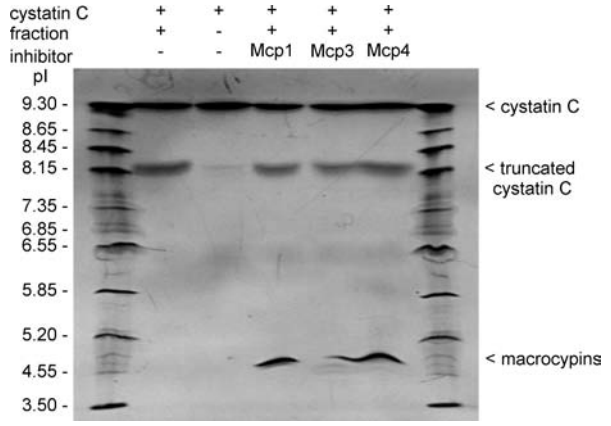

Figure 5. Isoelectric focusing analysis of cystatin C cleavage by a *p*Glu-Phe-Leu-*p*NA-hydrolyzing fraction in the presence of individual macrocypins. First and last lanes contain p*I* marker, in other lanes the symbols + (contains) and − (does not contain) show the content of cystatin C, *p*Glu-Phe-Leu-*p*NA active fraction (corresponding to fraction 164 mL in Figures 4 and 6) or recombinant macrocypins (Mcp1, Mcp3, or Mcp4).

that is inhibited by macrocypins. Inhibition of the *p*Glu-Phe-Leu-*p*NA-hydrolyzing activity by macrocypins and lack of inhibition of the model cystatin cleavage indicated a complexity of proteases in this fraction higher than previously anticipated.[5] The number of different proteases present in separated fractions of the adapted larval guts was estimated by analyzing, by gelatin zymography, selected fractions from gel filtration. Numerous bands proteolytically active at pH 6.5 in the presence of DTT were identified, confirming the profusion of cysteine proteases in adapted larval guts (Figure 6).

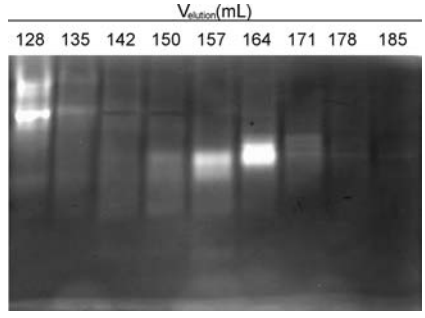

Figure 6. Gelatin zymography of fractionated CPB larval gut protein extract. The same fractions as in Figure 4 were analyzed.

Furthermore, resistance to proteolytic cleavage of the recombinant macrocypins by CPB digestive proteases was observed. Fractions corresponding to the peak of proteolytic activity from gel filtration, which were used in the cystatin C cleavage assay, did not change the isoelectric focusing patterns of any of the macrocypins after a 4 h incubation at 37 °C or after a 24 h incubation at room temperature at pH 6.5 in the presence of DTT (not shown).

Proteases of catalytic types other than cysteine proteases are present in CPB larval guts and could contribute to adaptation to dietary cysteine protease inhibitors. Therefore, the potential of macrocypins to inhibit serine and aspartic proteases was also assessed. Chymotrypsin-like serine protease activity was previously detected in larval guts, the optimal substrate being determined to be Boc-Gly-Arg-Arg-MCA with a pH optimum

at pH 8.8 and was effectively inhibited by chymostatin and aprotinin.[3] The inhibition of this activity by selected inhibitors was assayed in the presence of E-64 (Figure 4E), because the substrate is also cleaved by cysteine proteases. The serine protease activity detected in this study was only weakly inhibited by chymostatin (inhibitor of chymotrypsin but not of trypsin) with approximately 75% residual activity only in the fraction showing highest activity in contrast to a previous study in which <10% residual activity was detected under similar conditions.[3] Furthermore, it was completely inhibited by aprotinin (inhibitor of trypsin, chymotrypsin, elastase, kallikreins, etc.), again in contrast to a previous study, in which approximately 25% residual activity was reported under similar conditions.[3] This serine protease activity was not affected by any of the macrocypins (Figure 4E). The novel serine protease activity described here differs in type from that published previously,[3] emphasizing the complexity of the digestive system of CPB larvae and suggesting a possible population-specific adaptive response. Furthermore, the aspartic proteolytic activity that was assayed in the presence of E-64 and aprotinin using the substrate MOCAc-Ala-Pro-Ala-Lys-Phe-Phe-Arg-Leu-Lys(Dnp)-NH_2 was completely inhibited by pepstatin but was unaffected by macrocypins (Figure 4F).

Glycoside hydrolases were found to be potential binding partners of macrocypins in CPB larval guts (Table 1). Macrocypins are not glycosylated and possess the β-trefoil fold known to be involved in the inhibition of multiple types of hydrolytic enzymes, including proteases and glycosidases.[48] It is therefore possible that macrocypins interact with glycosidases in vivo, and the inhibition of glycolytic activity by macrocypins was examined at respective optimal pH conditions. Several different substrates were included in the analysis as it is currently not possible to assign function based on sequence alone for this group of enzymes. Cellulase activity was assayed against azo-CM-cellulose, polygalacturonase (pectinase) activity against polygalacturonic acid, and xylanase activity against a fluorogenic substrate, and no inhibition by macrocypins was detected in the larval gut extracts, even though rMcp1 binds to glycoside hydrolases GH48-1 and GH48-2 and potentially also to endopolygalacturonase GH28Pect-9. This could mean that (i) the activity of glycoside hydrolases is not affected by macrocypin binding; (ii) the enzyme inhibition assays performed in selected conditions in vitro did not adequately reflect the in vivo conditions; or (iii) enzymes corresponding to GH48-1, GH48-2, and GH28Pect-9 do not degrade the selected substrates.

Absence of Effect by Macrocypins on Gene Expression of Known Adaptation-Related Digestive Enzymes in CPB Guts. Affinity chromatography and enzyme inhibition assays showed that macrocypin present in larval diet inhibits distinct digestive cysteine proteases (Table 1; Figure 4). CPB larvae adapt to exposure to plant inhibitors in the diet by changing the expression profile of digestive enzyme genes,[3,4] so we tested transcript abundance in guts of larvae fed Mcp4-expressing transgenic plants and their nontransgenic counterparts. As for the feeding trial, larvae were hatched on transgenic plants expressing Mcp4, which were then changed daily, with the intention of detecting macrocypin-responsive genes. In this way, larval adaptation to the heterologous protease inhibitor would not be masked by adaptation to potato defense compounds induced by larval feeding.

qPCR expression analysis revealed that CPB larvae feeding on transgenic plants expressing macrocypin are not affected by the reduced activities of digestive enzymes resulting from the presence of macrocypin in the larval diet, in the same way as has been shown for the presence of potato defense induced endogenous compounds.[3] None of the tested transcripts increased in abundance when larvae were fed a macrocypin-enriched diet (Figure 7), including genes coding for intestains

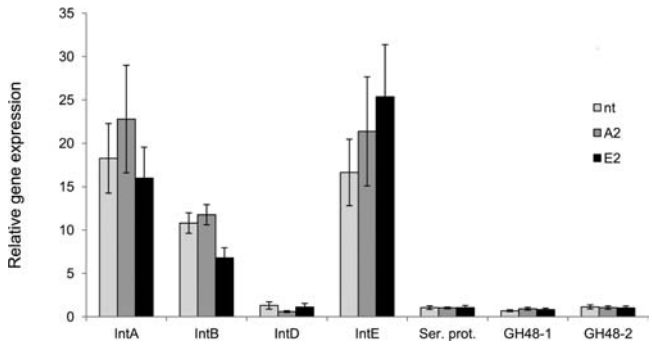

Figure 7. Expression of digestive enzyme genes in CPB larval guts fed with macrocypin-enriched diet. Relative expression of intestains A, B, D, and E (IntA, IntB, IntD, IntE), serine protease (Ser. prot.) and glycoside hydrolases GH48-1 and GH48-2 was measured in CPB larvae fed nontransgenic (nt) and Mcp4-expressing transgenic plants (independent transgenic potato lines A2 and E2).

A, B, D, and E, a serine protease, and two glycoside hydrolases. An interesting effect was, however, observed in the expression of *intC* gene. Expression was not detected in any of the six pools of larvae grown on nontransgenic plants or in 7 of 12 pools fed plants of both transgenic lines (A2 and E2). Transcript abundance in the five positive pools was comparable to that of other intestains and corresponds to a $>10^4$-fold difference in gene expression between the two sample types. As Mcp in diet delayed larval development (Figures 2 and 3) and such very large differences in gene expression were detected, we assume that *intC* genes are the first to shut down in the prepupal stage. Because all of our experiments were set to analyze larvae in the late fourth instar, we have additionally reviewed the raw data of our previous qPCR experiments.[3] These show that, in 5 of 32 pools, no *intC* transcript was detected in either control or adapted larvae, whereas, in all other samples, the expression was comparable to that of other intestains, additionally supporting our hypothesis of developmental regulation of *intC* genes.

It has been previously shown that in response to dietary protease inhibitors, CPB larvae (i) overexpress sensitive cysteine proteases, (ii) express insensitive cysteine proteases, (iii) express proteases of other catalytic classes, (iv) express proteases that degrade introduced protease inhibitors, and (v) readjust metabolic resources in compensation.[1,3−5,25] Differential digestive compensation was shown in CPB larvae fed potato plants in which different defense-related genes were induced, manifested as distinct changes of protease class ratios in insect midguts.[2] Dietary macrocypin, however, did not influence the expression of selected genes encoding digestive enzymes associated with adaptation in CPB larval guts, including proteases (intestains A, B, D, and E and a serine protease) and glycoside hydrolases (GH48-1 and GH48-2). Macrocypin targets in CPB larvae adapted to induced potato defense compounds include a group of proteins not directly involved in digestive processes, indicating a multilevel mechanism of action leading to the observed negative effects

on larval growth and development. The unaltered transcriptional response to dietary macrocypin at the level of proteases is exceptional, because regulation of protease-encoding genes was, as in CPB larvae, observed regularly in beetle larvae fed diets containing protease inhibitors: southern corn rootworm larvae (*D. undecimpunctata*) overexpressed cysteine and aspartic proteases,[9] and cowpea bruchid larvae (*Callosobruchus maculatus*) overexpressed cysteine proteases and carboxypeptidases in response to dietary soybean cysteine protease inhibitor.[6] In the red flour beetle larvae (*T. castaneum*) dietary cysteine or serine protease inhibitor led to regulation of differential expression of many protease-encoding genes, which were up- or down-regulated depending on the inhibitor treatment.[49]

In conclusion, this study showed a great potential of macrocypins for pest control. A gene coding for a cysteine protease inhibitor of fungal origin, macrocypin, has been successfully introduced into and expressed in potato, and the growth rate of CPB larvae was significantly decreased when feeding on macrocypin-containing diet. The observed reduction of larval growth and development is probably due to inhibition of a specific set of digestive enzymes but could be more complex. Macrocypins effectively inhibit the *p*Glu-Phe-Leu-*p*NA-hydrolyzing cysteine protease activity in adapted larval guts, show weak inhibition of Z-Arg-Arg-*p*NA-hydrolyzing activity, and have no inhibitory effect on other proteases. Their exceptional trait is lack of compensatory transcriptional response, as seen with protease inhibitors from other sources, to dietary macrocypins, and there is evidence suggesting that the negative effect on CPB larval growth and development is mediated through multiple levels. These mushroom-derived protease inhibitors have several other advantages, including their great resistance to exposure to high temperature and extremes of pH[32] as well as resistance to proteolytic degradation. Macrocypins could be applied in the form of transgenic potato as well as in the form of recombinant proteins produced in *E. coli*, where very high yields[32] provide another advantage. To enhance their effectiveness, macrocypins could be applied in combination with other protease inhibitors, for example, aprotinin that targets serine proteases. Furthermore, macrocypins can contribute to plant resistance to other biotic and abiotic stress conditions in which proteases play important roles,[50] and as cysteine protease inhibitors, they could be evaluated as control agents for other pests that depend predominantly on cysteine proteases in their digestion. On the basis of this study revealing several advantageous traits of mushroom-derived protease inhibitors in pest control, further research on the use of potential pesticidal proteins from mushrooms is of great interest.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the Research Agency of the Republic of Slovenia under Grants P4-0127 (J.K.), P4-0165 (M.R.), J4-2022 (J.Ž.,) and 1000-09-310204 (I.Š.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Notes

The authors declare the following competing financial interest(s): Parts of the results described in this publication including application of macrocypins have been included in a patent application "Use of macrocypins as pesticidal agents', PCT/EP2012/065373, which was filed at the European Patent Office on August 6, 2012. We declare that we have no financial, personal, or professional interests that could be construed to have influenced the manuscript.

■ ACKNOWLEDGMENTS

We are grateful to Dr. Roger Pain for critical review of the manuscript.

■ ABBREVIATIONS USED

CPB, Colorado potato beetle; Cq, quantitation cycle; DP1, diapause protein 1; ESI-MS/MS, electrospray mass spectrometry; Int, intestains; LOD, limit of detection; Mcp, macrocypin; nt, nontransgenic; qPCR, quantitative PCR; rMcp, recombinant macrocypin; RT-qPCR, reverse transcription qPCR

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