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Backbone Cyclised Peptides from Plants Show Molluscicidal Activity against the Rice Pest *Pomacea canaliculata* (Golden Apple Snail)

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Golden apple snails (*Pomacea canaliculata*) are serious pests of rice in South East Asia. Cyclotides are backbone cyclized peptides produced by plants from Rubiaceae and Violaceae. In this study, we investigated the molluscicidal activity of cyclotides against golden apple snails. Crude cyclotide extracts from both *Oldenlandia affinis* and *Viola odorata* plants showed molluscicidal activity comparable to the synthetic molluscicide metaldehyde. Individual cyclotides from each extract demonstrated a range of molluscicidal activities. The cyclotides cycloviolacin O1, kalata B1, and kalata B2 were more toxic to golden apple snails than metaldehyde, while kalata B7 and kalata B8 did not cause significant mortality. The toxicity of the cyclotide kalata B2 on a nontarget species, the Nile tilapia (*Oreochromis niloticus*), was three times lower than the common piscicide rotenone. Our findings suggest that the existing diversity of cyclotides in plants could be used to develop natural molluscicides.

KEYWORDS: Cyclotides; Rubiaceae; Violaceae; *Oldenlandia affinis*; *Viola odorata;* golden apple snail (*Pomacea canaliculata*); Nile tilapia (*Oreochromis niloticus*); metaldehyde; rotenone

INTRODUCTION

The golden apple snail (*Pomacea canaliculata*) is a serious pest on rice (*Oryza sativa*) and native aquatic plants throughout South East Asia. Originally imported into Taiwan from South America in the early 1980s (*1*), the freshwater mollusc (family Ampullariidae) has now spread to hundreds of thousands of hectares of agricultural wetlands in Japan, the Philippines, and Taiwan where it has caused billions of dollars worth of crop damage (*1, 2*). Field studies have also shown that the invasive snail disrupts the functioning of natural ecosystems by altering aquatic plant composition (*3, 4*).

Golden apple snails are freshwater herbivores that are able to respire in both air and water (*5*). They prefer submerged environments and succulent plants, and consequently, damage to rice crops is most severe in the weeks following transplanting of rice seedlings into paddies (*6*). Several strategies have been explored for the control of golden apple snails in rice crops. These include drainage of paddies after seeding (*5, 7*), mechanical methods such as physically removing the snails (*7*), biological control regimes, i.e., by fish and ducks (*8, 9*), and the use of chemical pesticides (*5, 10*). Metaldehyde (2,4,6,8-tetramethyl-1,3,5,7-tetraoxacyclooctane) is a commonly used molluscicide that targets slugs and snails. It is more specific than other commercially available molluscicides such as niclosamide (2′,5-dichloro-4′-nitrosalicylanilide) but remains toxic to some nontarget species, including mammals (*11, 12*). These factors, in addition to the unknown effects of the continued use of synthetic pesticides on the environment, have prompted investigations into effective molluscicides from natural sources that target the golden apple snail.

Cyclotides are a family of backbone cyclized, cysteine rich, peptides (~30 amino acid residues) produced by plants in the Rubiaceae and Violaceae (*13*) families. In combination, the cyclic backbone and cystine knot core, derived from six absolutely conserved cysteine residues, make the peptides extremely stable. **Figure 1** shows the amino acids sequences and representative three-dimensional (3D) structures of the cyclotides used in this study. Cyclotides are resistant to degradation by proteolytic enzymes and can tolerate extremes of pH and temperature (*14*). Plants typically produce a suite of cyclotides distributed throughout different tissues (leaves, roots, seeds and flowers (*15*)), and it has therefore been proposed that they function in plant defense. The cyclotides kalata B1 and kalata B2, isolated from the African plant *Oldenlandia affinis* (Rubiaceae), exhibit insecticidal activity against the cotton budworm *Helicoverpa punctigera* and the cotton bollworm *H. armigera* (*16, 17*).

The objective of the present study was to evaluate the efficacy of cyclotides as natural molluscicidal agents targeting the golden apple snail. Initially, leaf extracts from representative cyclotide-containing Rubiaceae and Violaceae plants were assayed for

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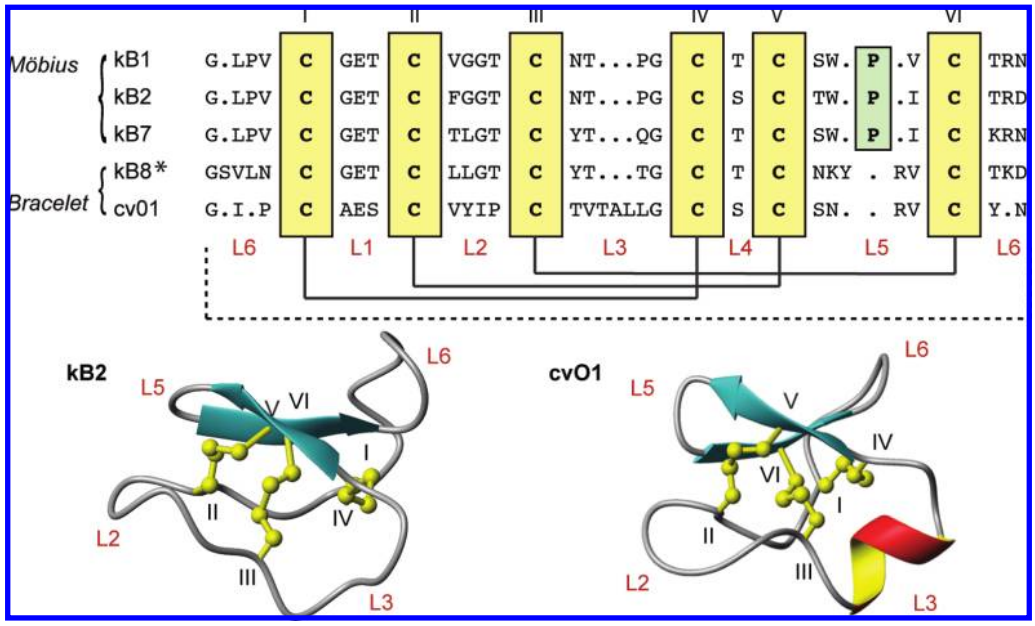

Figure 1. Cyclotide structures. Five cyclotides were used in this study; kalata B1 (kB1), kalata B2 (kB2), kalata B7 (kB7) and kalata B8 (kB8) from *O. affinis*, and cycloviolacin O1 (cvO1) from *V. odorata*. Amino acid sequences are represented in one-letter code, with the cysteine residues that make up the disulfide knot labeled in Roman numerals. Solid bars represent disulfide connectivity. The intervening regions between the cysteine residues, defined as loops 1 to 6, are labeled in red. The dashed line represents the protein backbone, which occurs between an N-terminal Gly and a C-terminal Asn or Asp residue in all of the peptides. Cyclotides have been classified into Möbius and bracelet subtypes on the basis of the presence of a Cis-Pro residue in loop 5 of the former (highlighted in green). Kalata B8 is marked with an asterisk to indicate that it is somewhat of a hybrid of the Möbius and bracelet subtypes. The 3D structures of kB2 (pdb 1pt4) and cvO1 (pdb 1nbj), representing the Möbius and bracelet subtypes, respectively, are shown in ribbon format.

molluscicidal activity. Once this was established, an LC_{50} value (median lethal concentration) was determined for the purified cyclotide kalata B2, which is the most highly produced cyclotide in *O. affinis* leaves. This value was used to guide tests on a range of purified cyclotides from both *O. affinis* and *Viola odorata* (Violaceae). Activity was compared to the synthetic molluscicide metaldehyde. To gauge the effect of cyclotides on nontarget organisms, the survival of Nile tilapia fish (*Oreochromis niloticus*) exposed to kalata B2 and the common piscicide rotenone (2*R*,6a*S*,12a*S*)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-*b*]furo(2,3-h)chromen-6-one) was compared. Several cyclotides exhibited more potent activity than metaldehyde against golden apple snails, indicating the potential for the development of the cyclic peptides as natural alternatives to the synthetic molluscicide.

MATERIALS AND METHODS

Cyclotide Extraction from Plants. *O. affinis* and *V. odorata* plants were grown in a glasshouse facility at the University of Queensland. The extraction of cyclotides from the aerial parts of the plants was conducted as previously described in refs *13 and 18*. Plant material was homogenized and extracted in dichloromethane/methanol (1:1) then filtered through cotton wool and partitioned by the addition of water. The aqueous water/methanol layer was collected, concentrated using a rotary evaporator, and loaded onto a reverse phase C18 flash column. Hydrophilic substances were removed from the extract by flushing the column with 25% (v/v) acetonitrile. Hydrophobic compounds, including cyclotides, were eluted from the column with 90% (v/v) acetonitrile, collected, and lyophilised. This lyophilised material is referred to as the crude cyclotide extract.

Purification and Quantification of Cyclotides. Purification of the cyclotides kalata B1, kalata B2, kalata B7, and kalata B8 from *O. affinis* and cycloviolacin O1 from *V. odorata* was carried out by reverse phase-high pressure liquid chromatography of the crude cyclotide extracts as previously described (*18*). Molar concentrations of the cyclotides were calculated using UV absorbance at 280 nm and extinction coefficients predicted by the ProtParam software program (http://kr.expasy.org/tools/protpar-ref.html). Stock solutions were prepared in water containing up to 20% (v/v) of ethanol, to increase peptide solubility. The final concentration of ethanol in the working solutions did not exceed 0.2% (v/v). Ethanol concentrations between 0−2% (v/v) did not affect golden apple snail mortality relative to a water control (data not shown).

Golden Apple Snails. Golden apple snails (*Pomacea canaliculata*) were collected from rice fields at the Central Luzon State University (CLSU), Muñoz, Nueva Ecija, Philippines. Juvenile snails 10 ± 1 mm in shell length (i.e., along the spine and body whorl) were selected for all experiments because they have been reported to have the greatest foraging capacity (per gram of snail) of all the life stages (*19*) and therefore potentially pose the biggest threat to rice crops. Prior to conducting the experiments, the snails were preconditioned in an aquarium containing water and rolled oats for two days. The water was changed twice a day.

Molluscicidal Assays with Cyclotide Extracts. Solutions of the crude cyclotide extracts from *O. affinis* and *V. odorata* were made up to 145, 290, 435, and 580 μg/mL in distilled water. Ten snails were placed in 200 mL glass beakers containing 50 mL each of the cyclotide solutions and incubated for 24 h at room temperature (28 ± 2 °C). During this period, a plastic mesh was fitted into the beakers to prevent the snails from moving above the level of the solution. The snails were not fed. After this time, the snails were removed from the solutions, washed 10 times in water, and then placed in 50 mL of water for a further 24 h to recover. During the recovery period, the snails were provided with rolled oats (~50 mg). Mortality rates were determined at the end of the recovery period by observing the heartbeat (which can be seen through the shell) and/or the retraction reflex of the foot of the snail after being pulled gently. The experiment was repeated four times at each concentration.

Molluscicidal Activity of Kalata B2. Molluscicidal activity assays were conducted as described above using solutions containing 0, 25, 75, and 100 μM of kalata B2 and 0, 25, 75, 100, 250, 500 and 1000 μM of metaldehyde. The peptide concentrations were determined by UV absorption spectroscopy. Each experiment was conducted six times.

Statistical Analysis. All data were analyzed using GraphPad Prism 4.0 software. Dose−response curves were fitted using a four parameter

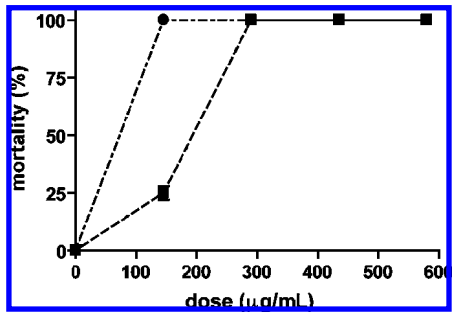

Figure 2. Molluscicidal activities of crude cyclotides extracts. The mortality of golden apple snails exposed to cyclotide extracts from *O. affinis* (■) and *V. odorata* (●). Data points represent the mean of four replicates ± SEM.

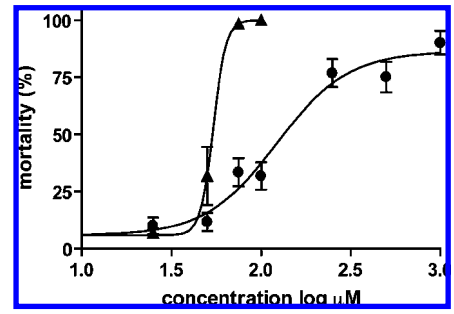

Figure 3. Molluscicidal activity of kalata B2. Golden apple snail mortality was recorded after incubation in solutions containing increasing concentrations of the cyclotide kalata B2 or metaldehyde. Data points represent the mean of six replicates ± SEM. LC_{50} values of 53 µM for kalata B2 (▲) and 133 µM for metaldehyde (●) were calculated from the fitted curves.

logistic function, which is equivalent to a sigmoidal dose−response curve with variable slope. The lethal concentration for half the treated population (LC_{50}) was calculated from the dose−response curve.

Relative Molluscicidal Activity of Cyclotides. Molluscicidal activity assays were conducted as described above. Snails were incubated in solutions containing 65 µM of the purified cyclotides kalata B1, kalata B2, kalata B7, and kalata B8, and cycloviolacin O1 and metaldehyde. Distilled water was used as a negative control. Each experiment was repeated six times except for the cycloviolacin O1 experiment, which was repeated four times.

Nile Tilapia Assay. Freshwater Nile tilapia (*Oreochromis niloticus*) fish fingerlings (155 ± 15 mg; 15 × 5 ± 1 mm) were purchased from the Freshwater Aquaculture Center at CLSU. Experiments were conducted in 450 mL plastic beakers containing 10 fish and 200 mL of a solution of kalata B2 or rotenone in aerated distilled water at room temperature (28 ± 2 °C). Kalata B2 was tested at 0, 3, 9, 15, 21, and 27 µM, and rotenone was tested at 0, 1, 3, 5, 10, 15, and 30 µM. Each assay was repeated four times. Fish mortality was observed at 1 h and at 1, 3, 6, 12 and 24 h for the 15 µM solutions.

RESULTS

Molluscicidal Activity of Extracts From Cyclotide-Containing Plants. Crude cyclotide extracts were prepared in dichloromethane/methanol (1:1) from *O. affinis* and *V. odorata* leaves using a well established protocol (*13, 18*). Lyophilised material from the aqueous water/methanol layer was resuspended in water and analyzed by liquid chromatography−mass spectrometry (LC-MS) for the presence of cyclotides. Cyclotides have a characteristically hydrophobic surface and are typically identified as late-eluting peaks on reverse phase-HPLC, with a mass of ∼3 kDa. According to these criteria, a suite of previously characterized cyclotides (*18, 20, 21*) was identified in both the *O. affinis* and *V. odorata* extracts. LC-MS analysis confirmed the presence of the cyclotides kalata B1 to kalata B17 (excluding kalata B5) in the *O. affinis* extract and cycloviolacin O1 to O12 in the *V. odorata* extract. Kalata B2 and cycloviolacin O1 were among the most abundant cyclotides in each extract.

To test for molluscicidal activity, golden apple snails collected from rice fields and preconditioned in the laboratory were incubated in solutions containing different concentrations (µg/mL) of the *O. affinis* and *V. odorata* extracts. After 24 h of incubation and a further 24 h of recovery, the mortality rates of the snails were recorded. **Figure 2** shows the snail mortality in plant extracts at 0, 145, 290, 435 and 580 µg/mL. These concentrations correspond to approximately 0, 50, 100, 150 and 200 µM of cyclotides (calculated using a molecular weight of 2900). This concentration is comparable to the total cyclotide concentration *in planta* of approximately 300 µM (*13*). As can be seen in **Figure 2**, both crude extracts from *O. affinis* and *V. odorata* had molluscicidal activities below this level and were therefore investigated further.

Molluscicidal Activity of Kalata B2. After the toxicity of the crude cyclotide extracts was confirmed, the toxicity of purified kalata B2 was examined. Kalata B2 was selected because it was the most abundant cyclotide in *O. affinis* leaves and has previously been well characterized (*16, 17*). Purification was carried out by repeated RP-HPLC of the crude cyclotide extract and confirmed by mass spectrometry. The toxicity of kalata B2 to the golden apple snail was compared with metaldehyde at 0, 25, 75, 100, 250, 500, and 1000 µM. **Figure 3** shows a plot of golden apple snail mortality after incubation for 24 h with increasing concentrations of kalata B2 and metaldehyde. Fitting the data gave an LC_{50} of 53 µM for kalata B2 and 133 µM for metaldehyde. Notably, at high concentrations of both test compounds, the snails retracted into their shells, closed their operculum and began to exude excess colorless mucus. This behavior has previously been described for metaldehyde induced toxicity in slugs (*22*). To exclude the possibility that changes in peptide concentration were affecting pH and thus mediating toxicity, snail mortality was measured in solutions ranging from pH 3−8. After 24 h, the pH of all of the solutions stabilized within a neutral pH range (6.75 ± 0.2), and snail mortality was equivalent to the neutral water control.

Relative Molluscicidal Potencies of Cyclotides. Guided by the LC_{50} value determined for kalata B2, bioassays were conducted to test the relative molluscicidal activity of other cyclotides that were less abundant in the *O. affinis* and *V. odorata* extracts. A single concentration of 65 µM was used to test the toxicity of five different cyclotides against the golden apple snail relative to metaldehyde; cycloviolacin O1, kalata B1, B2, B7, and B8. The amino acid sequences of the cyclotides tested is outlined in **Figure 1**. **Figure 4** is a plot of the mortality rates observed after the snails were incubated in solutions containing 65 µM pure peptides or metaldehyde. Kalata B1, kalata B2, and cycloviolacin O1 displayed an average mortality of 68%, 78%, and 100%, respectively, which was approximately equal to or higher than metaldehyde at 60%. In contrast, kalata B7 and kalata B8 had little effect on snail mortality.

Piscicidal Activity of Kalata B2. To ascertain the effect of cyclotides on nontarget aquatic organisms, the piscicidal activity of kalata B2 against Nile tilapia fish (*Oreochromis niloticus*) was compared to the positive control rotenone, a naturally occurring piscicidal agent from pea plants in the genus *Derris* (Fabaceae). The LC_{50} for kalata B2 was determined to be at least 3-fold weaker than rotenone. **Figure 5A** shows a plot of fish mortality after incubation in 0−30 µM solutions of the compounds for 1 h. By fitting these data, an LC_{50} value of 16.8 µM was calculated for kalata B2 and 5.0 µM for rotenone. The effect of the compounds was also investigated over 24 h to

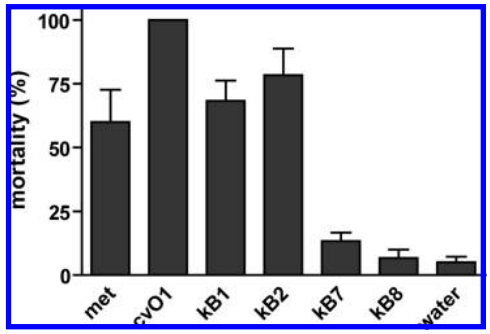

Figure 4. Relative molluscicidal activities of cyclotides. Mortality of golden apple snails exposed to solutions containing 65 μM of metaldehyde (met) or the cyclotides cycloviolacin O1 (cvO1), kalata B1 (kB1), kalata B2 (kB2), kalata B7 (kB7), and kalata B8 (kB8). Water was used as a control. Bars represent the mean of six replicates ± SEM except for cycloviolacin O1, which is the mean of four replicates.

determine the effect of the duration of exposure on toxicity, at a concentration approximating the LC_{50} of kalata B2 (15 μM). As is evident from **Figure 5B**, at this concentration the mortality of the Nile tilapia did not increase substantially over time as both kalata B2 and rotenone appear to illicit their maximum effects within 1 h of treatment.

DISCUSSION

The cyclotides are topologically complex backbone cyclized peptides derived from plants belonging to the Rubiaceae and Violaceae families. Many cyclotides were originally discovered as a part of bioactivity screens directed at identifying potential drug candidates from plant extracts. These include assays for anti-HIV activity (circulin A and B (*23*)) and the ability to inhibit neurotensin binding (cyclopsychotride A (*24*)). Subsequent studies found that some cyclotides also possess cytotoxic (*25*), antitumor (*26*), antifouling (*27*), antimicrobial (*25*), and insecticidal (*16, 17*) activity. These findings, particularly the insecticidal activity of cyclotides, suggest that they serve a defense function *in planta*, albeit with a broad spectrum of activity. In this study, we wanted to test whether cyclotides have the potential to protect plants from a molluscan pest, the golden apple snail.

Both the crude cyclotide extracts and the purified cyclotides were toxic to golden apple snails, and variations in the level of activity were seen with individual cyclotides. Kalata B1 and kalata B2 from *O. affinis* and cycloviolacin O1 from *V. odorata* all induced higher levels of snail mortality than metaldehyde when applied at the same concentration (**Figure 4**). This was reflected in the 2.5-fold lower LC_{50} of kalata B2 (~53 μM) compared to that of metaldehyde (~133 μM) (**Figure 3**). By contrast, kalata B7 and kalata B8 showed low toxicity against the snails.

The differences in molluscicidal activity of individual cyclotides indicate that changes in sequence modulate activity, although the mechanistic basis for this is not yet clear. From the amino acid sequences in **Figure 1**, it can be seen that the kalata peptides from *O. affinis* show greater similarity to each other than to cycloviolacin O1 from *V. odorata*. Despite this kalata B1, kalata B2, and cycloviolacin O1 show the highest molluscicidal activities. One possibility for explaining the trends in activities is based on trends in hydrophobicity. Cycloviolacin O1 is the most active of the peptides tested and contains the most hydrophobic residues. Similarly, the molluscicidal activity of the kalata peptides correlates with their hydrophobicity (B2 > B1 > B7 > B8).

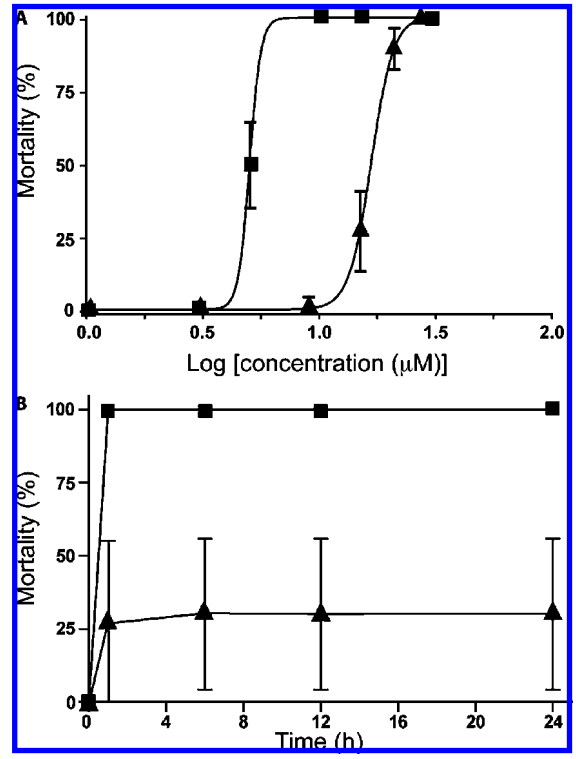

Figure 5. Piscicidal activity of kalata B2. (**A**) The effect of kalata B2 on the mortality of Nile tilapia fish compared to rotenone after 1 h. Fitting the data points yielded an LC_{50} value of 17 μM for kalata B2 (▲) and 5 μM for rotenone (■). (**B**) The effect of 15 μM of kalata B2 or rotenone on the survival of Nile tilapia fish over 24 h. Data points represent the mean of four replicates ± SEM.

The mode of action of cyclotides against golden apple snails is unknown, but the excessive secretion of mucus and the retraction of the snails into their shells in the high concentration treatments suggests a process of toxicity similar to that of metaldehyde. Histological and immunochemical examinations of the effect of metaldehyde on the slug *Deroceras reticulatum* have shown that it damages the mucocytes of the digestive tract and skin leading initially to excessive mucus secretion followed by changes to energy metabolism (*22*). It is not clear how metaldehyde causes the disintegration of the mucus cells and whether cyclotides adopt the same mechanism.

The effect of cyclotides on nontarget organisms was investigated by testing the effect of kalata B2 on Nile tilapia fish. This and other fish species have previously been investigated as biological control agents in rice fields as part of integrated pest management strategies (*8, 9*). The fish can have positive effects on pest control, and it is therefore of interest that they persist in the semiaquatic environment of the rice paddies. Nile tilapia fish exposed to powdered metaldehyde in field trials showed no mortality, although up to 13% mortality was observed for pelleted formulations presumably due to the ingestion of pellets (*10*). Kalata B2 had a moderate effect on fish mortality in laboratory experiments with an LC_{50} approximately 3-fold weaker than the piscicide rotenone. This effect appeared to be lower than the ability of the peptide to target the golden apple snail, but further investigations are required to establish whether the toxicity of the cyclotide toward the mollusc can be separated from its toxicity toward nontarget organisms. Determining whether the molluscicidal activity of the other cyclotides in this study correlates with their toxicity toward Nile tilapa may provide insight into this relationship.

The golden apple snail causes considerable damage to rice

paddies and other aquatic environments in South East Asia. As the effects of persistent synthetic pesticide use on the environment are not known, integrated approaches to controlling this pest are preferred and also better suited to the limited financial resources of many rice farmers in the region (*5*). Using the recently developed knowledge of the biosynthesis of cyclotides (*28, 29*), the potential exists for these gene-derived peptides to be used in transgenic crops that produce their own biopesticides, reducing economic and environmental costs. This approach to the delivery of biopesticides also has the potential to reduce toxic effects on nontarget organisms, compared to direct external delivery. In this study, we have shown that backbone cyclized peptides occurring endogenously in Rubiaceae and Violaceae plants have comparable molluscicidal activity to the commonly used synthetic molluscicidal agent metaldehyde. Both crude plant extracts and purified peptides possess molluscicidal activity, and our initial findings suggest that it may be possible to modulate the specificity of the peptides to target and nontarget organisms by exploiting their natural sequence diversity in plants. With further investigations into the mode of action of the cyclotides in golden apple snails, it may be possible to develop novel cyclotide-based molluscicidal agents to help defend rice plants against this devastating pest.

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