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Research

Constitutive Expression of Cyclotide Kalata B1 Gene in Transgenic Rice Conferring Resistance to Golden Apple Snail (*Pomacea canaliculata*)

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ABSTRACT

The golden apple snail, also known as *Siput Gondang Emas* in Malaysia, is a serious pest of paddy fields and native aquatic plants throughout Southeast Asia. Agrobacterium-mediated transformation was used to transform a synthetic *Oak 1* gene encoding kalata B1 (kB1), which is toxic to golden apple snails, into Malaysian indica rice MR219. The synthetic *Oak 1* gene was placed under the control of a strong constitutive *Zea mays* ubiquitin promoter. Twelve transgenic lines containing the *Oak 1* gene were obtained from the regenerated calli selected on hygromycin. *Oak 1* gene expression was determined using quantitative reverse transcriptase- PCR (RT-qPCR). The resistance of the transgenic line to snail infestation was evaluated by feeding experiments. One dimensional 1H nuclear magnetic resonance (NMR) spectroscopy revealed that the kB1 produced in transgenic rice is in the form of an acyclic peptide. Phenotypic analysis of the transgenic plants revealed that they have fewer leaves and grains than wild-type MR219. In a molluscicidal activity bioassay, feeding juvenile snails with different concentrations of leaf extracts resulted in molluscicidal activity against snails that was comparable to the synthetic molluscicide metaldehyde, thus farmers can overcome the golden apple snail infestation problem by using genetically modified rice containing the kB1-encoding gene. This technology also has the potential to reduce the toxic effects of chemically synthesized molluscicides on the environment and ecosystem.

Key words: Cyclotide kalata B1, golden apple snail, rice transformation

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INTRODUCTION

Global rice production increased from 3.9 million tonnes to 751.9 million tonnes as a result of the Green Revolution in 2016 (FAO, 2017). Rice yields are predicted to record a decline of up to 5% by 2030 in Southeast Asia, Central America, and Brazil (Lobell *et al*., 2008). The impact of pests and diseases could result in decreased productivity of rice, which in turn affected rice production in the long term.

Golden apple snail (GAS) was first reported as a new rice pest in Malaysia in year 1987 (Mochida, 1991). *Pomacea canaliculata* (Gastropoda: Ampullariidae) is a major pest threat to rice farmers, resulting in a drastic decline in rice yields (Horgan, 2018). The GAS attack was a severe pest that destroyed 4,000 ha of rice fields (Department of Agriculture, 2021), so the government has implemented various measures to control GAS attacks on rice crops, including biological control. These biological methods include releasing ducks into rice fields before the start of the paddy planting season to eat snails. However, the release of duck into rice fields in the early stage of transplantation will damage the young rice seedling. Besides, the natural predators such as dogs were a great problem because the dogs would always attack the duck released to the field. Chemical pesticides specifically designed for snails have

also been used. Farmers frequently use excessive amounts of chemical pesticides to control insects and pests, especially GAS. Although metaldehyde is more specific in targeting slugs and snails compared to other mollucicides, but remains toxic to some other non-targeted species including mammals, and would destroy the ecosystem. Unfortunately, these chemicals have the potential to kill or harm beneficial living organisms as well as the environment. The increased use of chemical pesticides has harmed the farmers, who have reported having difficulty breathing during or after pesticide spraying (Fuad *et al*., 2012). Other side effects include poisoning, vomiting, and fainting. The most severe cases of poisoning were reported in 2009, resulting in two deaths (Fuad *et al*., 2012; Mazlan *et al*., 2018). Due to the unknown effects of the continued use of synthetic pesticides on the environment, much attention has been given to studying an environmentally friendly method in recent years in the control of GAS. These include the use of biopesticides or plant-based molluscicides because they are biodegradable and safe for mankind and the environment (Duke *et al*., 2010). However, the application of either synthetic or natural molluscicides in control of GAS will be futile if water is allowed to flow continuously into the field. Therefore, to reduce the losses caused by snails and the use of chemical pesticides, the development of new varieties resistant to GAS using transgenic technology may serve as an alternative approach to combat GAS attacks.

Cyclotides have been proven to have insecticidal and molluscicidal activities with backbone cyclized, cysteine-rich peptides (~30 amino acid residues) produced by plants in the Rubiaceae and Violaceae families (Craik *et al*., 1999; Grover *et al*., 2021). Cyclotides are the largest group of plant peptides rich in disulfide cyclic (Barbeta *et al*., 2008). Plants that have cyclotides are mostly found in dicotyledonous plants. Nevertheless, in monocotyledon plants, there is a nucleic acid sequence similar to the cyclotide sequence but in a linear structure (acyclotide) which has cysteine folding but no cyclic backbone (Weidmann & Craik, 2016; De Veer *et al*., 2019). The five major plant families that have cyclotides are Rubiaceae, Violaceae, Cucurbitaceae, Solanaceae, and Fabaceae (Weidmann & Craik, 2016). The small embedded ring is formed by two intra-cystine backbone segments connected by two disulfide bonds (CysI-CysIV, CysII-CysV), and this ring is penetrated by a third disulfide bond (CysIII-CysVI) to form a cystine knot (Gründemann *et al*., 2019). The combination of cyclic and disulfide bonds in the formation of cyclic cystine knot motifs (CCKs), contributes to the stability of cyclotide structures that are highly resistant to heat, chemical, or enzymatic degradation (Camarero, 2017). The discovery of cyclotides was based on Lorents Gran's research on *Oldenlandia affinis* (Gentianales: Rubiaceae) plants in 1973 (Gran, 1973; Chen *et al*., 2017). The kB1 cyclotide can protect plants from pest attacks, as cyclotides can inhibit the growth and development of insect species, *Helicoverpa punctigera* (Leptidoptera: Noctuidae) and *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Keov *et al*., 2018; Craik, 2012). The effect of kB1 cyclotide on epithelial cell midline morphology was investigated in *Helicoverpa armigera* (Leptidoptera: Noctuidae) larvae showing that midgut epithelium cell membranes were disrupted (Barbeta *et al*., 2008; Slazak *et al*., 2022), therefore, the kB1 cyclotide may be applied to other plant pests. The crudes extract from the *O. affinis* plant and the purified peptides kB1 have also been reported to exhibit molluscicidal activities against GAS (Plan *et al*., 2008). These recent discoveries of cyclotide protein in molluscicidal activities have prompted investigations into the development of transgenic rice resistant against golden apple snails.

Transformation of the *Oak 1* gene has been observed in other species such as tobacco, *Nicotiana tabacum* (Solanales: Solanaceae), and *Arabidopsis thaliana* (Brassicales: Brassicaceae). The introduction of the *Oak 1* gene encoding kB1 into a non-cyclotide-producing plant with an additional biosynthetic enzyme, asparaginyl endopeptidases (AEP), could form peptide backbone cyclization (Poon *et al*., 2018). Moreover, naturally occurring acyclic forms or linear cyclotides are as stable as the cyclotides because of the cysteine knot structure but are susceptible to exoprotease activity because of a lack of head-to-tail cyclization (Tammineni *et al*., 2020).

This study aims to broaden the molluscicidal spectrum and develop transgenic rice snail resistance by introducing the *Oak 1* gene into Malaysian indica rice MR219 without the presence of AEP. Evaluation of changes to MR219 phenotype comprised extracting expressed molluscicide from transgenic rice leaves and using GAS in feeding experiments.

MATERIALS AND METHODS

Regulatory permission was sought and received by the Malaysia Biosafety Act (2007). This project has been notified to the National Biosafety Board (NBB) of the Malaysian Ministry of Natural Resources and Environment (NRE). NBB approved the project with the reference number JBK (S) 602-1/2/21.

Plasmid construction

The full-length *Oak 1* gene (GenBank accession number: FJ211184.1) encoding kalata B1 was used as a reference in synthesizing the *Oak 1* gene using *gBlocks*™ Gene Fragments by Integrated DNA Technologies, USA. *Not*I and *Sal*I sites were introduced to the 5' and 3'-end of the *Oak 1* gene using PCR with two specific oligonucleotide primers, *Not*I*Oak* F and *OakSal*I R (Supplementary Table S1). The expression cassette of the *Oak 1* gene was generated by joining the maize ubiquitin *(UBI)*

promoter and nopaline synthase *(NOS)* terminator sequence upstream and downstream of the *Oak 1* gene, respectively. The *Oak 1* expression cassette was then ligated into the pCAMBIA1301 T-DNA vector via directional cloning. The resulting construct was designated as pCAMBIA1301-*Oak 1* and was transferred into *Agrobacterium tumefaciens* strain LBA4404 via electroporation according to the method described by Nagel *et al*. (1990) with modifications. The transformants were selected by colony PCR, followed by restriction digestion (*Sac*I and *Eco*RI) of the recombinant plasmid.

Rice transformation

Rice (*Oryza sativa* subsp. indica var. MR219) seeds were obtained from the Malaysia Agricultural Research and Development Institute (MARDI). Embryogenic calli of MR219 rice were generated according to the method described by Rahman *et al*. (2010) with modifications in concentration and types of PGRs. Mature seeds were de-husked and surface sterilized with 70% (v/v) ethanol. The seeds were then washed with 30% (v/v) commercial bleach for 20 min before being rinsed three times rinses with sterile distilled water. The seeds were then dried in a laminar flow cabinet on sterile tissue paper before being transferred aseptically to a callus induction medium (30 g/L sucrose, 4 g/L phytagel, 3 mg/L 2,4-D, and 0.3 mg/L kinetin). After three weeks in the dark at 28 °C, the proliferated calli derived from the scutella were separated and selected for transformation.

Agrobacterium-mediated transformation of rice

The protocols used for transformation and co-cultivation were modified from (Bajaj *et al*., 2006). The agrobacteria carrying pCAMBIA1301-*Oak 1* were grown on YM media (0.04% yeast extract, 1.0% mannitol, 1.7 mM NaCl, 0.8 mM $MgSO_4.7H_2O$, 2.2 mM $K_2HPO_4.3H_2O$) containing 50 µg/mL streptomycin and 50 µg/mL kanamycin at 28 °C and shaken at 250 r.p.m overnight. Bacterial cell cultures were examined until the absorption reached OD_{600nm} 0.7–0.8, then centrifuged at 6000 r.p.m. for 10 min and the pellet was dissolved with 20 mL of YM media containing 400 µM acetosyringone. The cells were shaken in an orbital incubation machine at 50 r.p.m for 30 min. The embryogenic calli were soaked in bacterial suspension for 30 min at room temperature and blotted dry with sterile tissue paper to remove excess bacteria before transfer to co-cultivation solid media (MS media containing 3 mg/L 2,4-D, 0.3 mg/L kinetin, 400 µM acetosyringone, and 4 g/L phytagel, pH5.7). Six weeks embryogenic callus derived from MR219 rice seeds were co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harboring pCAMBIA1301-*Oak 1* vector for 3 days in the dark at 28 °C.

Selection and regeneration

The infected calli were transferred to a selection medium containing 25 mg/L hygromycin and 200 mg/L cefotaxime and incubated for two weeks to remove excess agrobacteria. The transformed callus was selected using 25 mg/L and 50 mg/L hygromycin B (Roche, Germany) for two cycles of 2-weeks each, then transferred to a callus induction medium with 3 mg/L 2, 4-D, 0.5 mg/L kinetin, and 50 mg/L hygromycin. Transformed calli were incubated in the dark at 25 °C.

Transgenic putative calli were transferred to regeneration media after 12 weeks of incubation in selection media. The optimal regeneration media composition of MS contained 30 g/L sucrose, 6 g/L phytagel, 1 mg/L NAA, and 2 mg/L BAP. The calli were transferred into the regeneration media and incubated in the dark for 1 week, then incubated under light photoperiod of 16 h and 8 h dark at 25 °C. After shooting regeneration media for about 4 weeks, the calli were transferred to half-strength MS media for root regeneration. After 3 months the growing putative transgenic rice plants were transferred to the soil in a transgenic greenhouse. The calculation of the transformation frequency is based on Sahoo *et al*. (2011):

$$\text{Regeneration frequency (\%)} = \frac{\text{Number of calli producing shoot}}{\text{number of calli transformed}} \times 100$$

Molecular confirmation of transgenic plants

The rice genome DNA was extracted from young leaves using DNAeasy Kits (QIAGEN, USA) and the presence of the *Oak 1* gene in transgenic rice was determined via PCR using specific primers (Table 1). The PCR mixture for complete sequence amplification of the *UBI-Oak 1-NOS* promoter comprised: 1x GoTaq® Flexi buffer, 4.0 mM MgCl2, 0.2 mM dNTPs, 1.0 µM each primer forward and reverse, 0.2 µg/µL template DNA, 1.25 u GoTaq® DNA Polymerase, and PCR-grade water to make the volume up to 25 µL. The PCR cycling conditions were 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and extension 72 °C for 1 min.

Gene expression analysis by Quantitative Real-Time PCR

Total RNA was extracted from 100 mg of plant leaves with RNAeasy Kits (QIAGEN, USA), and quantified using a Nanodrop (Thermo Fisher Scientific, USA). Total RNA was converted to cDNA using random hexamers and oligo (dT)12 primers contained in Maxima H Minus First Strand cDNA Synthesis

kit RT-qPCR (Thermo Scientific, USA) as recommended by the manufacturer. The synthesized cDNA was diluted 1 in 10 with nuclease-free PCR water before qPCR analysis. Quantitative Real-Time PCR amplification was performed using IQ SYBR®Green in 25 µL reaction mixtures on a Biorad CFX system and analyzed using CFX Manager Software. Each RT-qPCR was repeated in three replications. Water added in the reaction mixtures was a blank control. The RT-qPCR reaction was performed in a 25 uL volume and included 1 µl (100 ng) of diluted cDNA, 12.5 µl of 2X IQ SYBR®Green master mix (Bio-Rad, USA), and primers to a final concentration of 100 nM. The following amplification conditions were used: initial denaturation and enzyme activation at 94 °C for 60 s, followed by 35 cycles at 94 °C for 60 s, and 58 °C for 60 s. Each assay included three technical replicates. The specificity of amplification was assessed by a single peak in the melting curve analysis. For melting curve analysis, a dissociation step cycle was performed at 65 °C for 0.5 s followed by a gradual increase of 0.05 °C for 0.5 s until 95 °C. The gene expression of the *Oak 1* transgene was normalized against the housekeeping gene(*GAPDH*) and estimated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Table 1. The gene transformation experiments

Experiment	Number of co-cultivation calli	Number of surviving calli after 50 mg/L hygromycin treatment	Number of regenerated plant
Experiment a	700	66	2
Experiment b	700	87	5
Experiment c	600	67	5
Total	2000	220	12

NMR spectroscopy analysis

Ground transgenic rice leaves (200 mg) were weighed into a 2 mL Eppendorf tube and the solvent (methanol 8:2 ratio with water) was added in a 1:10 sample-to-solvent ratio. The tube contents were sonicated using an ultrasonicator for 60 sec, centrifuged at 10 000 × *g* for 5 min and the pellet was oven dried at 40 °C. The sample was weighed and stored at RT for further use. The NMR spectra were obtained using a 600-MHz NMR spectrometer (Agilent, USA). The sample (10 mg) was mixed with 0.375 mL of tetradeuteromethanol (CD_3OD) and 0.375 mL buffer in D_2O (pH 6.0) containing TSP (0.1% (w/w)) in a 1.5-mL Eppendorf tube, vortexed for 60 s and sonicated at room temperature for 20 min before centrifuging at 10,000 × *g* for 10 min to obtain a clear supernatant. The supernatant (600 µL) was transferred to an NMR tube (5 mm, Norell, Sigma Aldrich, Canada) for NMR analysis. A pre-saturation pulse sequence (PRESAT) experiment was performed to remove the large signal for the HOD to determine the ^1H NMR spectra at 25 °C. The number of proton scans was 8 and the water peak was suppressed by PRESAT 64 scans. The spectra of chemical shifts are in parts per million (ppm) and the spectra were analyzed using 'Chenomx Profiler'.

Bioassay molluscicidal activity of crude extracts from transgenic rice

The toxicity of transgenic rice to GAS was assessed based on the method described in (Plan *et al*., 2008) with modifications to determine the performance of cyclotides that are expected to be expressed in the rice. GASs were bred in glass aquariums in the laboratory and fed aquatic plants such as *Pistia stratoites* (Alismatales: Araceae). Solutions of the ground transgenic leaf and metaldehyde pesticides (positive control) were prepared in sterile distilled water at 10 ppm and twenty snails ranging in sizes from 10 mm to 15 mm were placed in a 50 mL beaker containing 10 mL of each cyclotide solution. Non-transgenic rice leaves as the negative control. All beakers were incubated at room temperature. A mortality rate was recorded at 120 h by observing the heartbeat under a dissecting microscope to determine whether the snail was alive or dead. This experiment was repeated three times and the mortality percentage was calculated as follows:

$$\text{Mortality rate percentage (\%)} = \frac{\text{Number of dead snails}}{\text{Number of snails treated}} \times 100$$

Data from a randomized experiment were analyzed with MINITAB 20 software. A one-way analysis of variance (ANOVA) was employed to identify statistically significant deviations.

RESULTS AND DISCUSSION

Agrobacterium-mediated transformation and regeneration of rice

The pCAMBIA1301-*Oak 1* plasmid was successfully transformed into Agrobacterium cells via electroporation. Fifteen colonies were randomly selected and screened by PCR using specific primers

(Supplementary Table S1) to ensure that the *Oak 1* gene was integrated into the plasmid. As shown in Figure S1a (Supplementary Figure S1a), five colonies: pCAMBIA1301-*Oak 1-3*, pCAMBIA1301-*Oak 1-6*, pCAMBIA1301-*Oak 1-9*, pCAMBIA1301-*Oak 1-14*, and pCAMBIA1301-*Oak 1-15* contained PCR fragments of 380 bp. The plasmid integrity was confirmed using *SacI* and *EcoRI* restriction enzymes where the band at ~2kb (see supplementary data Figure S1(b)) corresponds to the *Oak 1* gene expression cassette (Maize *UBI-Oak 1-NOS* terminator, Figure 1). Fragments were sequenced to ensure that the recombinant construct encoded *Oak 1* in the correct reading frame before being used for transformation.

The rice callus was inoculated with *Agrobacterium tumefaciens* strain LBA4404 harboring the pCAMBIA1301-*Oak 1-14*. A total of 2000 embryogenic calli were transformed in Agrobacterium-mediated systems, grown in selective media, and subcultured four times in selective media (Figure 2). Only 220 calli survived after the selection process (Table 1) to obtain twelve transgenic plants with an approximately 0.6% from the initial callus transformed: a_6, a_11, b_4, b_1, b_3, b_11, b_12, c_6, c_21a, c_21b, c_39 and c_50. The identification of these transgenic rice is based on a series of gene transformation experiments performed in Table 1 and each transformation group involved a total of 2000 calli. The six-week-old embryogenic callus was deemed the most suitable callus for co-cultivation with the *A. tumefaciens* strain LBA4404 containing the pCAMBIA1301-*Oak 1* construct. Calli infected with Agrobacterium were allowed to grow on selection media containing 25 mg/L hygromycin for the first cycle of 2 weeks (Figure 2a), then the callus was transferred to selection media containing 50 mg/L hygromycin for two weeks and subcultured three times (Figure 2b). A lower concentration of hygromycin (25 mg/L) was used in the early stages to obtain more calli (Zhao *et al*., 2011). After 2 weeks the calli were subcultured into media containing 50 mg/L of hygromycin. At this stage, calli containing the plasmid pCAMBIA1301-*Oak 1* will survive and most calli will change color to brown and black and undergo necrosis (Figure 2c).

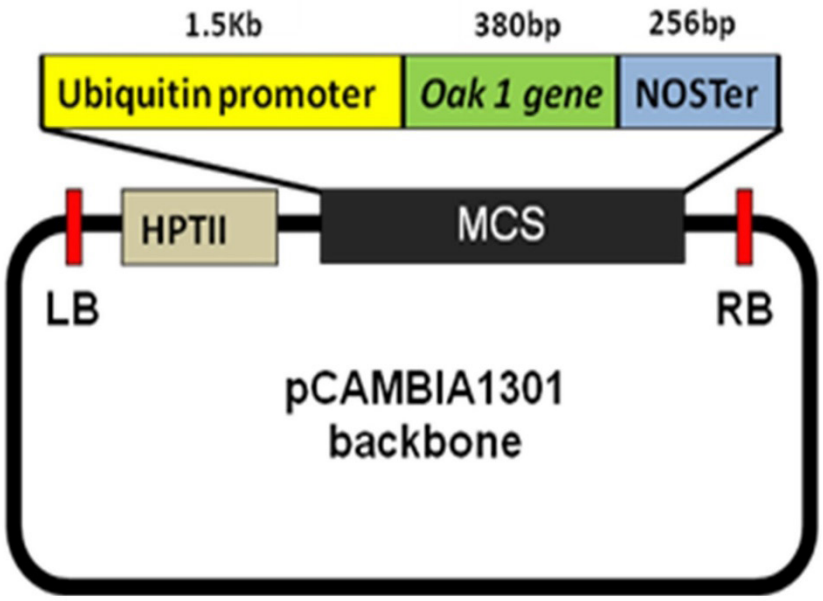


Fig. 1. Diagramatic representation of pCAMBIA1301-*Oak 1* T-DNA construct. Different color bars indicate the various components of the *Oak 1* gene expression cassette. The red bars represent the left and right borders of pCAMBIA1301, and the backbone of pCAMBIA1301 is represented by thick black lines. This expression has a *UBI* promoter and *NOS* terminator, as well as a hygromycin (*hptII*) resistance gene as a plant selection marker upstream of the transgene.

The embryogenic callus transformation efficiency was calculated by counting the number of hygromycin-resistant plantlets after three selection cycles (Figure 2d-f) and the transformation efficiency was 11%. The transformation efficiency of rice is genotype-dependent, as the transformation efficiency reported in Japonica rice was 93% (Hiei & Komari, 2006) and 22% Indica rice (Rashid *et al*., 1996), whereas Ratanasut *et al*. (2017) reported that the transformation efficiency of RD41 fragrant rice (Indica) was 1.4%. An extremely low transformation efficiency of 10% was reported in several genotypes (Azhakanandam *et al*., 2000; Yinxia & Te-Chato, 2015). According to Repalli *et al*. (2017), adding antioxidants (40 mg/L L-cystine, 5 mg/L argentum nitrate, and 15 mg/L ascorbic acids) to co-cultivation media increased the viability and regenerative ability of post-infection plants by 32-42% compared to control. Successful plant transformation depends on several critical factors, all of which must be meticulously evaluated and optimized throughout the genetic transformation process. A

significant factor is the dependence on genotype, which can influence transformation efficiency. Certain genotypes may exhibit efficient cell division and differentiation, leading to higher regeneration rates and better transformation efficiency, while others may have limited regenerative potential. Certain genotypes may support efficient cell division, thus promoting higher regeneration rates and improved transformation efficiency. While some genotypes are particularly susceptible to stress, the release of phenolic compounds, or other inhibitory factors during tissue culture, all of which can hinder the transformation process. Callus consists of a highly heterogeneous group of cells, many of which resemble organized structures similar to lateral root primordia (Atta *et al*., 2009; Fehér *et al*., 2019). However, most of these cells are regeneration-incompetent, with only a few capable of proliferating. This limited capability prevents, T-DNA from targeting the specific cells competent for dedifferentiation. Xing *et al*. (2009) noted that the meristematic cells in monocots can influence the pathogenicity of Agrobacterium, thereby reducing the function(s) of its *vir*-genes. Consequently, these cells might not release the inducer, leading to their inability to differentiate at an early stage of development.

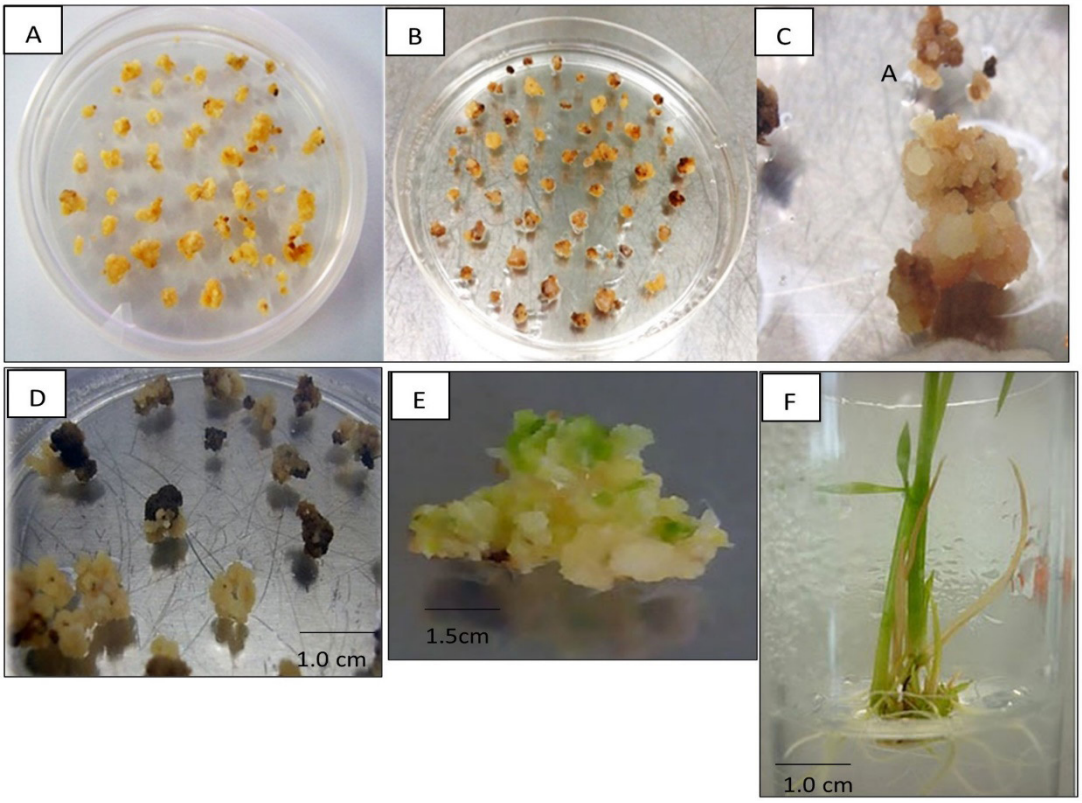


Fig. 2. The pCAMBIA-*Oak 1-14* plasmid was inserted by Agrobacterium cells into the callus grown in a selection medium containing a) 25 mg/L hygromycin, and b) 50 mg/L hygromycin. c) The transformed callus was resistant to hygromycin. *d-e*) Regeneration of the transformed rice shoots and roots. Many shoots grew from the callus clumps after the selection process using media containing 50 mg/L hygromycin. and the plant took 3 months to regenerate. f) Green shoots and roots plantlet of transgenic rice in regeneration media.

Several factors can contribute to this condition occurring during the transformation of foreign genes into the embryogenic callus, including the Agrobacterium culture concentration (OD), inoculation time and incubation time of Agrobacterium culture with the callus, and acetosyringone concentration (Cheng *et al*., 2004; Yaqoob *et al*., 2017). OD_{600} readings of Agrobacterium cultures containing plasmids must be between 0.5 to 0.8 (Maheshwari & Kovalchuk, 2016). During Agrobacterium and callus infection, OD_{600} was adjusted to 0.8 because high bacterial concentrations cause severe damage to the callus, lowering the percentage of regenerating plants. The co-culture period of Agrobacterium and the callus should be between 2 to 3 days, with 2 days resulting in better infection without excessive Agrobacterium growth. According to Saroj *et al*. (2015), acetosyringone concentrations must be between 100–150 µM to improve the efficiency of Agrobacterium-mediated genetic transformation. Poor transformation efficiency may contribute to the genotoxic effect of kB1 in transgenic rice. Qu *et al*. (2020) hypothesized that the kB1 molecule is toxic above a certain level, therefore limiting the kB1 yield in rice. Therefore, the low transformation efficiency in this study may be due to kB1 toxicity. A breakthrough report by Lowe *et al*. (2016) has revealed that the application of plant developmental regulatory genes during *in vitro* culture can contribute to the enhancement of plant transformation efficiency. The Agrobacterium nopaline

synthase promoter *(Nos:ZmWUS2*), which is known to be a weak promoter for monocots, was found to result in low expression of the maize *WUS2* gene. Conversely, the strong maize Ubiquitin promoter *(ZmUbi:ZmBBM)* led to high expression of the maize BBM gene. This differential gene expression pattern induced somatic embryogenesis and facilitated the regeneration of fertile transgenic plants in immature embryos and callus tissues of maize, sorghum, sugarcane, and other related species. This method significantly enhanced callus transformation efficiency accelerated regeneration and made various non-transformable genotypes transformable. For instance, a combination of *Nos:ZmWUS2* and *ZmUbi:ZmBBM* showed dramatically improved transformation efficiency from 0.0–2.0% to 25.3–51.7% in recalcitrant maize (Lowe *et al*., 2016; Maren *et al*., 2022).

Molecular analysis of *Oak 1* expression in transgenic lines

The presence of the *Oak 1* gene in the transgenic rice was confirmed by PCR. Of the 2000 calli transformed with the *Oak 1* gene, only twelve rice plants were regenerated (0.6%) (Figure 3). The number of transformed but not regenerable calli is most likely due to ineffective use of the selective systems, imperfect gene insertion, unstable transferred genes integrated into the rice genome, and missing during cell division (Saini *et* al., 2012*)*. Transgenic rice plants carrying the *Oak 1 gene* were grown in a transgenic greenhouse and gene segregation analysis was performed on selected seeds originating from the T_0 positive PCR test *Oak 1*. A total of 20 seeds were randomly selected and planted for T_1 for DNA isolation and DNA amplification was performed using specific primers for the *Oak 1* gene. The expression of the *Oak 1* gene in transgenic rice was determined by RT-qPCR (Figure 4). The relative gene expression of the transgenic lines was calculated based on Kumar *et al*. (2018). Based on the analysis of reference genes using NormFinder software, *GAPDH* gene stability was 0.685 whereas actin was less stable with a value of 4.563 (Kumar *et al*., 2018). Indicating that *GAPDH* is a more endogenous reference gene for transgenic rice. The a_11, b_12, and c_39 transgenic lines showed higher levels, with the lowest expression in the c_21_a transgenic line (Figure 4). Among the transgenic lines with high transgene expression, the a_11 line displayed the highest expression level. Although we are using the maize UBI constitutive promoter which generally produces high expression levels, the transgene expression level might be varied depending on the transgene insertion location in the genome. Different levels of transgene expression from independent transgenic lines have been reported by several groups. The factors contributed to this variability are differences in chromosome position where transgene is integrated, repeat sequences, and also transgene copy number (Matzke & Matzke 1998; Kooter *et al*. 1999; Selker, 1999; Nagaya *et al*., 2005).

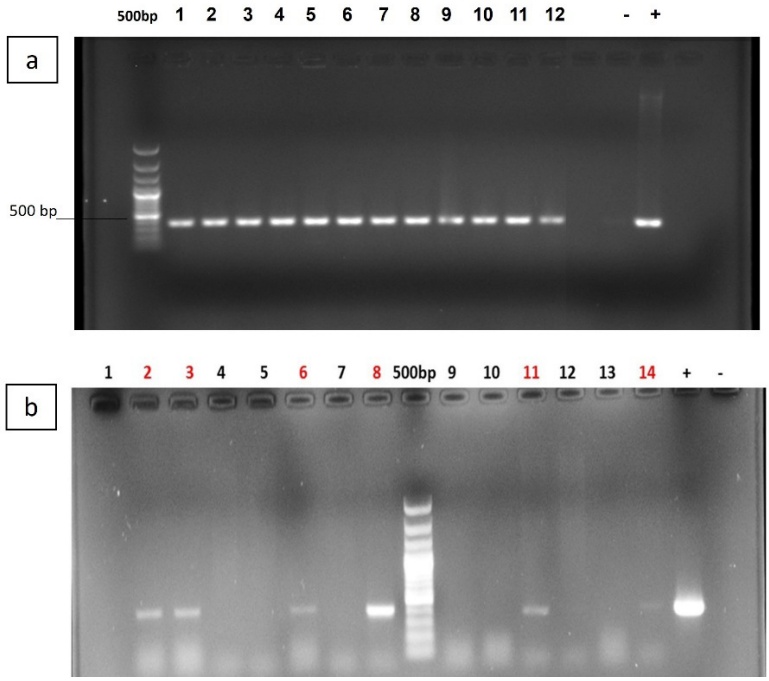


Fig. 3. a) PCR screening revealed that twelve transgenic (1-a_6, 2- a_11, 3- b_4,4- b_1, 5- b_3, 6- b_11, 7-b_12, 8- c_6, 9- c_21a, 10- c_21b, 11- c_39 and 12- c_50-12), rice plants showed the presence of the *Oak 1* transgene (380 bp) .b) Six transgenic from T_1 rice plants showed the presence of the *Oak 1*. Positive control (+) and negative control (-).

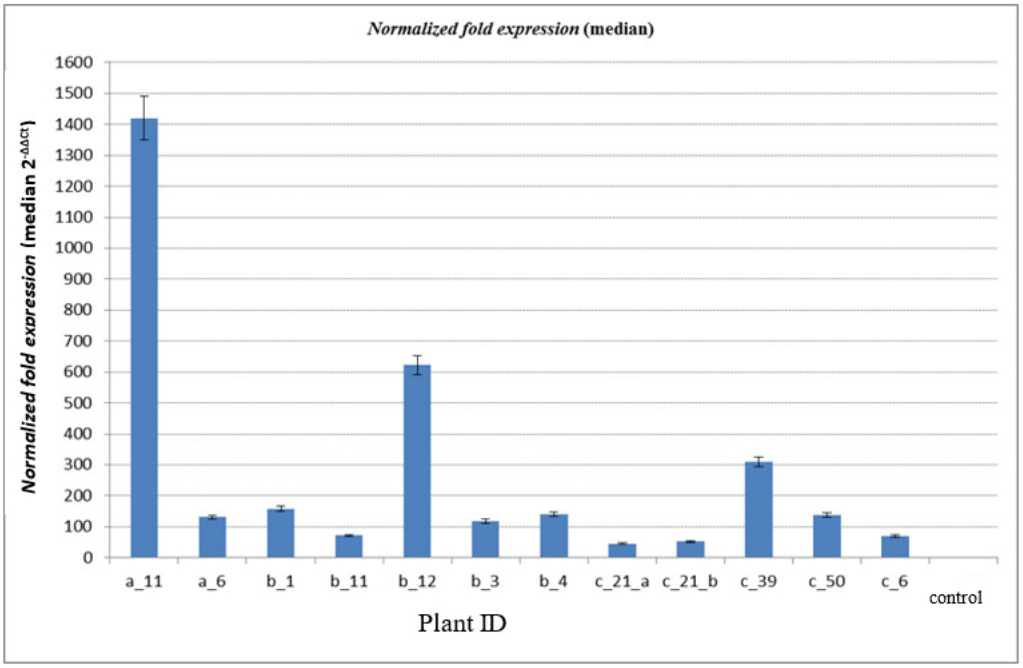


Fig. 4. Expression of the *Oak 1* gene in the leaves of transgenic rice normalized to GAPDH. All reactions were performed intechnical replicate with a null template. The vertical bars represent the standard deviations based on biological replicate experiments.

NMR analysis

When a protein folds correctly, the peaks in the 1D NMR spectrum are sharp and narrow, indicating distinct chemical environments. On the other hand, if the protein doesn't fold properly or only partially folds, the peaks in the spectrum become broader and less well-defined. An informative sign of a folded protein is a well-dispersed spectrum, where peaks span from 0 to 10 ppm. The focus of spectrum analysis is on the amide region, which spans from 7.0 to 9.0 ppm. This analysis is crucial because the hydrogen bonding patterns within proteins, as detected by NMR, rely on experiments involving deuterium exchange or temperature-induced shifts in amide proton chemical shifts (Handley et *al*. 2020).

As depicted in Figure 5, the 1H NMR spectroscopic analysis of the peptide extracted from the transgenic leaf reveals that they were in an unfolded state, with limited dispersion of the amide backbone chemical shifts within the range of 7.0 to 9.0 ppm. The spectrum of the peptide extracted from the transgenic leaf indicates the absence of proper folding, as evidenced by broader and less well-dispersed peaks within that range. The primary reason for broader peaks in an unfolded protein lies in the chemical shift's dependence on the local proton environment. In an unfolded protein, protons lack distinct environments, causing peaks to represent an average of all the different environments where those protons are located. Moreover, variations in proton environments aren't significantly pronounced in an unfolded protein, resulting in peaks that lack a broad chemical shift range. This is particularly evident in intensities near ~8.2 ppm, serving as a strong indicator of an acyclotide or linear peptide. Therefore, the nature of kB1 is more aligned with the acyclic peptide. Similarly, the obtained results were compared to the findings of Simonsen *et al*. (2004), who reported similar spectra from selected regions of the acyclic kB1 peptide.

Phenotypes of transgenic lines

Seven out of twelve transgenic lines had a lower number of rice seeds, which was around (461 to 700 seeds) compared to the control (Figure 6). The differences in the number of tilers, leaves, and panicles were not significant between the transgenic lines and control (Figure 6). The decrease in seed number might be due to the disruption of host biochemical pathways by foreign genes (Malone *et al*., 2001; Azizoglu *et al*., 2020) as reported in studies of transgenic trees for overexpression of the *OsGAD2* gene to produce aminobutyric acid (GABA) (Akama & Takaiwa, 2007; Akama *et al*., 2020). Acyclotides mainly arise because of mutations in their precursor proteins that do not allow cyclization to proceed, therefore, the roles of asparaginyl endoproteinase (AEP) enzymes in cyclotide biosynthesis were elucidated through the expression of cyclotide precursors in transgenic plants (Saska *et al*., 2007; Gillon *et al*., 2008). For instance, cyclotide biosynthesis pathways may affect the yield of kB1. A

requirement of AEP-mediated cyclization is the efficient proteolytic release of the emerging peptide at the N-terminus (a Gly residue in kB1) before cyclization (Rehm *et al*., 2019). It was demonstrated that the yield and formation of kB1 were seriously affected if N-terminal cleavage was disrupted (Rehm *et al*., 2019), which is reflected in the reduced vigor and lower seed number of the recovered transgenic lines. The endogenous AEPs play an important role in production and cyclization, thereby increasing the yield of cyclic kB1 (Qu *et al*., 2020). It is important to note that these findings do not represent the conclusive outcomes for commercial application. Due to low transformation efficiency also causes the selection of the finest phenotype transgenic plant was limited.

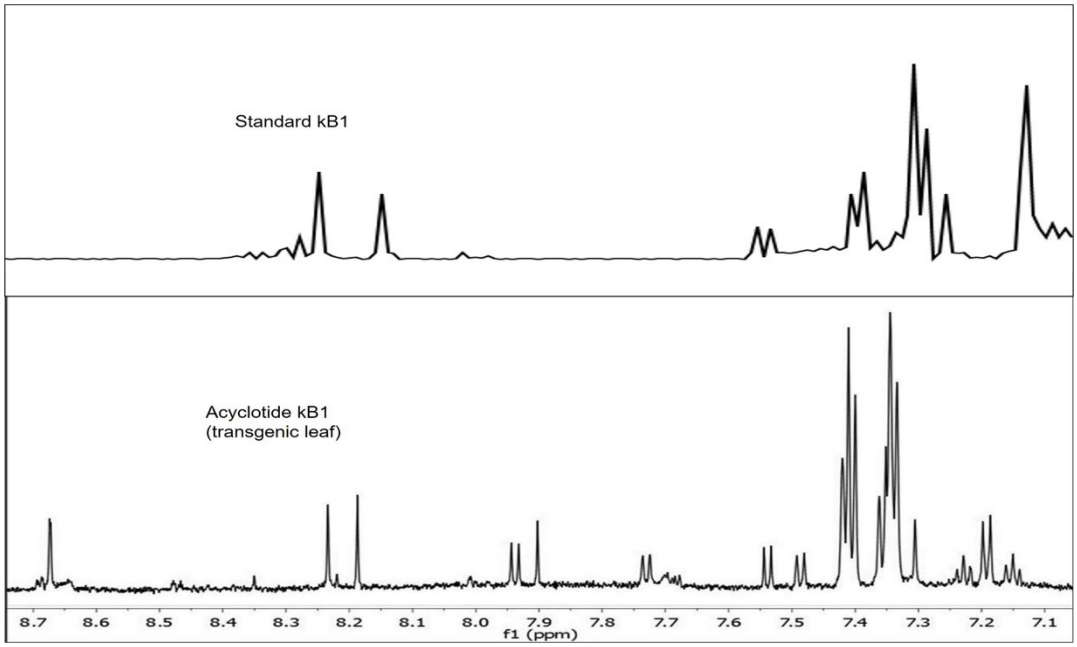
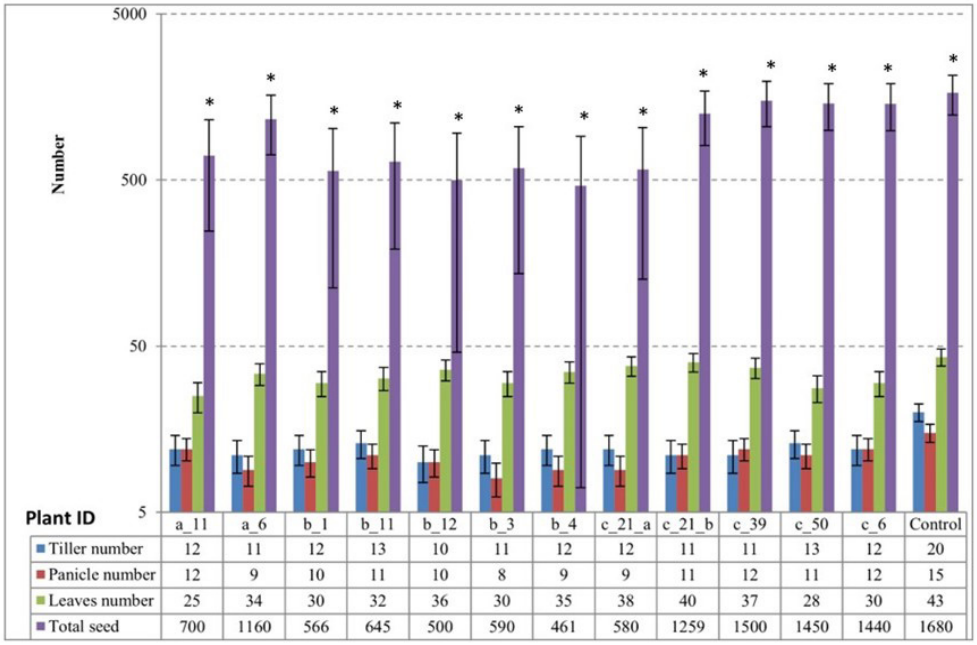


Fig. 5. One-dimensional ^{1}H NMR spectra analysis of the peptide extracted from transgenic leaf indicated that they were unfolded or acyclotide molecules at chemical shifts near ~8.2 ppm.



Plant ID	a_11	a_6	b_1	b_11	b_12	b_3	b_4	c_21_a	c_21_b	c_39	c_50	c_6	Control
Tiller number	12	11	12	13	10	11	12	12	11	11	13	12	20
Panicle number	12	9	10	11	10	8	9	9	11	12	11	12	15
Leaves number	25	34	30	32	36	30	35	38	40	37	28	30	43
Total seed	700	1160	566	645	500	590	461	580	1259	1500	1450	1440	1680

Fig. 6. Phenotypes of transgenic rice plants. The characterization of this phenotype is based on the number of tilers, panicles, leaves, and grains produced. The error bars indicate the standard deviations. The differences between the transgenic rice and control plants were compared using Tukey's HSD test at $p<0.05$. Asterisks indicate significant differences in the experiment.

Toxicity of leaves from transgenic lines against golden apple snail

The lethal effects of transgenic lines on GAS were evaluated *in vitro*. The transgenic leaf solution may cause disturbances to GAS, such as oxytocin effects. The mortality of GAS occurred on the fifth day (120 h) at a concentration of 10 ppm of transgenic rice leaf solution compared to the commercial molluscide, metaldehyde. Transgenic leaf solution (10 ppm) showed 76.65% mortality in golden apple snails compared to metaldehyde (10 ppm), which exhibited 60% mortality (Table 2). These promising outcomes encouraged us to further analyze the mechanism of action of the transgenic rice-derived acyclotide components against the golden apple snail. A highly visual response to the transgenic construct expressed in rice leaves was a dead snail operculum bursting out of the shell, as in Figure 7.

Table 2. Toxicity effect of the metaldehyde and transgenic leaf against *P.canaliculata* after 120 h treatment

Samples	Number of dead snails			mean±SD [a]	Mortality (%)
	I	II	III		
Metaldehyde solution	10/20	12/20	14/20	12.00±2.00 c	60%
Transgenic leaf solution	15/20	16/20	15/20	15.33± 0.57 b	76.65%
Control	0/20	0/20	0/20	0 d	0

[abc] *Means that do not share a letter are significantly different by Tukey pairwise comparison and 95% confidence level.*

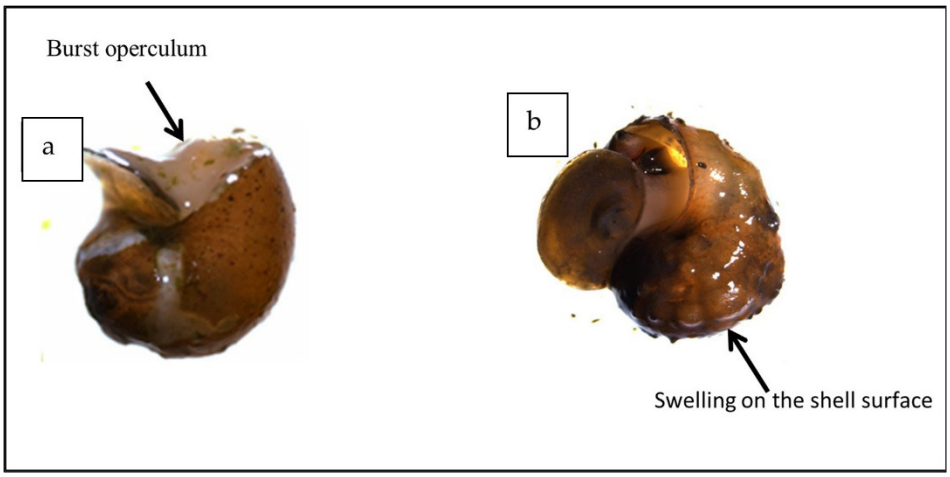


Fig. 7. The condition of the dead snails was observed under a surgical microscope: a) 10ppm of transgenic rice leaves solution. b) 10 ppm metaldehyde. GAS experiences swelling in the shell.

A comparison of the effects of transgenic rice leaf solution and metaldehyde chemical pesticides on GAS physical (Figure 7) showed that the GAS in the metaldehyde solution suffered severe damage to the inside of the operculum until it came out of its shell. The same situation applies to GAS in beakers containing transgenic rice leaves, but the snail shells do not swell. This was also evidenced by Plan *et al*. (2008) where the authors found crude extracts cyclotides such as cycloviolacin O1, kB1, and kB2 were more toxic to GASs than metaldehyde, while kB7 and kB8 did not cause significant mortality. Nevertheless, the mode of action of cyclotides on GAS is still unknown but mucus secretion and the shrinking operculum indicate toxic effects similar to those of metaldehyde. Cycloviolacin O1 was the most active peptide tested and contained the most hydrophobic residues (Narayani *et al*., 2020). Similarly, the molluscicide activity of kB1 peptides correlated with the hydrophobic properties of the peptides (B2> B1> B7> B8). Histological observations and immunochemical effects of metaldehyde on the mollusk *Deroceras reticulatum* showed that this cyclotide can damage the mucosal and skin digestive tract which initially has excessive mucosal secretion followed by changes in energy metabolism (Plan *et al*., 2008). However, it is still unclear how metaldehyde causes the proliferation of mucus cells and whether cyclotides also have a similar mechanism. Furthermore, the resulting application of metaldehyde not only affects the quantity but also the quality of mucus produced by slugs. Metaldehyde-induced effects in mucocytes could be linked to the molluscicide's effects on serotonin and energy metabolism (Triebskorn *et al*., 1998).

To date, there have been no reports of the efficacy of the molluscicide assay on GAS. Prabhakaran *et al*. (2017) used extracts from plants (*Nerium indicum*, *Azadirachta indica,* & *Nicotiana tabacum*) and showed that the combination of three plants caused high mortality (93.3%) of GAS. Nguyen *et al*. (2013) reported that although acyclotides lack peptide stability in terms of enzymatic degradation, they possess antimicrobial, hemolytic, and cytotoxic properties. Tammineni *et al*. (2020) suggest that the mechanism of action of acyclotides could be similar as they have the identical structural topology

as the cyclotides. Furthermore, cytotoxicity has been shown in twelve types of acyclotides (panitides L1-L8, Psyle C, chassatide C7, C8, C11) exhibiting varying degrees of cytotoxic activity against cancer cells. Therefore, as in the case of cyclotides, acyclotides may also be potential pesticides and can be applied to manage plant diseases and pests via the production of genetically modified plants expressing insecticidal acyclotide (Jackson & Gilding, 2015; Tammineni *et al*., 2020). Although kB1 expression in rice has been reported, kB1 expression was only detected by immunoprecipitation and the authors did not test the molluscicidal activity on GASs (Lim & Lai, 2017). The present study demonstrated that kB1 expression in transgenic rice leaves protects against GAS, thus it may be useful to protect plants from other pests. However, extensive research on the mode of action is required before it can become a reality and potentially reduce mollusk attacks in rice fields.

CONCLUSION

This study showed that the expression of the *Oak 1* gene in transgenic rice allows the plants to gain resistance against golden apple snail infestation, as the transgenic leaf caused significant snail mortality. The number of grains decreased, especially in the plant line with the highest expression of the *Oak 1* gene. Further studies will be conducted to ensure gene stability in the next generation of transgenic rice plants and investigate the mode of action of transgenic rice containing acyclotides on GAS.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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