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SHORT COMMUNICATION

GENERATION OF TRANSGENIC RICE EXPRESSING CYCLOTIDE PRECURSOR *OLDENLANDIA AFFINIS* KALATA B1 PROTEIN

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ABSTRACT

Golden apple snail (*Pomacea canaliculata*) is a devastating pest on rice that causes heavy economic losses in South East Asia. In this study, we transformed mature seed-derived rice callus with plasmid containing *Oldenlandia affinis kalata B1* (*Oak1*) gene encoding precursor's protein for potent molluscicidal agent of cyclotidekalata B1 targeting the golden apple snails. A total of 11 independent T_0 transformants were recovered and 7 were positive *Oak1* transformants according to genomic PCR analysis. The *Oak1* mRNA transcript was successfully detected on all the tested T_0 transformants using real-time PCR. Further immunoprecipitation experiment using specific Oak1 antibodies confirmed the presence of Oak1 protein expression in the transformants. We report, for the first time, the generation of transgenic rice plants expressing Oak1 as a potential cropprotection strategy against the golden apple snail pest.

Keywords: Cyclotidekalata B1, Golden apple snail,Oak1,*Oryzasativa* ssp. Indica.

INTRODUCTION

Rice is an important food crop in the world, providing staple food for nearly one third of its population (Tang *et al.* 2001). However, rice yields can be severely compromised by diseases and pests (Khush and Brar 1991). The golden apple snail (*Pomacea canaliculata*) (GAS) is one of the most devastating pests on rice paddies and aquatic environment in South East Asia (Naylor 1996). GAS was first imported into Taiwan from South America in the early 1980s (Naylor 1996) but now,it has spread to hundreds of thousands of hectares agricultural wetlands across Asia that causes billions of dollars' worth of crop damage (Naylor 1996; Teo 2003). In addition, GAS also damages the natural ecosystem by altering aquatic plant composition (Carlsson*et al.* 2004; Carlsson*et al.* 2005).

GAS is a freshwater herbivore that feed on paddy leaves, and causes serious damage to rice crops (Manuel *et al.* 2008). Efforts to control the GAS include physically removing, biological control regime, i.e., ducks and the use of synthetic pesticide (Manuel *et al.* 2008). Synthetic pesticides like metaldehyde (2,4,6,8-tetramethyl-1,3,5,7-tetraoxacyclooctane) and niclosamide (2',5-dichloro-4'-nitrosalicyanilide) are commonly used molluscicides targeting the GAS. However, most of these synthetic pesticides are toxic to nontarget species, including mammals (Dolder 2003), led to contamination of water and food resources, and harmful to ecosystem. The adverse effect of synthetic pesticides on the environment has prompt public concern to seek for better and more sustainable method to control GAS. Recently, it has been reported that the natural cyclotide kalata B1 from African plant *Oldenlandia affinis* exhibits molluscicidal activity against the GAS (Manuel *et al.* 2008) and can be used as an effective biopesticide for the control of GAS. Cyclotides are family of backbone cyclized, cysteine rich peptides (~30 amino acid residues) produced by Rubiaceae and Violaceae plant families (Craik*et al.* 1999). Its cyclic backbone and cystine knot core, make the peptides extremely stable and resistant to degradation of proteolytic enzymes (Manuel *et al.* 2008). In plants, the cyclotide kalata B1 is synthesized from its precursor protein of Oak1 and function as a plant defensive protein. It has been shown that, the crude and purified cyclotide kalata B1 were toxic and lethal to GAS (Manuel *et al.* 2008).

To date, the development of plant genetic engineering has enabled various useful genes to be introduced into plant for pest control. For instance, plants expressing *Bacillus thuringiensis* (*Bt*) gene were successfully established in cotton, maize and potato for insect control (Chen*et al.* 2005).The development of Bt crops successfully reduced the used of synthetic insecticides and provide a more sustainable insect management system for better pest control. Besides, transgenic rice plants against *Xanthomonasoryzae* and lepidopteran were also generated through the expression of *ferredoxin-like amphipathic* (*ap1*) gene and *insecticidal crystal* (*cry2A**) gene respectively (Tang *et al.* 2001; Yang *et al.* 2011). Therefore, the aim of the present study was to develop transgenic rice plants

expressing Oak1 as a potential biological control strategy against the GAS pest. The developed transgenic rice plants that express its own natural pesticides will enhance the pest management system and reduce economic losses in rice industry.

MATERIALS AND METHODS

Plant material: Malaysia indica rice (*Oryzasativa* L. CV. MR 219) seeds were dehusked and surfaced sterilized using 70% ethanol for 1 min, 20% sodium hypochloride solution for 15 min and rinsed with sterile distilled water for 5 to 6 times. Sterilized seeds were cultured on solidified medium containing, MS basal medium (Murashige and Skoog, 1962), 3% sucrose, 0.3% gelrite agar, 5 mg/L NAA (α-naphthaleneacetic acid) and 1 mg/L 2, 4-D (2,4-dichlorophenoxyacetic acid) for callus induction. The callus was regenerated to a complete plantlet according to the protocol described by Zuraida *et al.*(2010). All growth conditions were set at 25°C with a photoperiod of 16 h light and 8 h darkness.

Expression Plasmid Construction: The full length 375 bp of Oak1 cDNA fragment (GenBank: AF393825) was synthesized by Integrated DNA Technologies, USA. PCR was performed using the synthesized Oak1 cDNA as a template and the resulting Oak1 fragment was cloned into pENTR^{TM}/D-Topo® plasmid (Invitrogen, USA) according to the manufacture's protocol. Using LR clonase (Invitrogen, USA), *Oak1* gene fragment was subcloned into the pBI121 plasmid producing 35S:Oak1/pBI121 expression plasmid (Fig. 1a). DNA sequencing was performed to confirm the presence of *Oak1* gene, its nucleotide sequences and orientation in the plasmid. Primers are listed in Table 1.

***Agrobacterium*-mediated Rice Transformation:** The 35S:Oak1/pBI121 expression plasmid was mobilized to the *Agrobacterium tumefaciens* strain LBA 4404 according to method described by Lai *et al.* (2012a). Two weeks old mature seed-derived calli (Fig-1b) were transformed using *A. tumefaciens* harbouring the 35S:Oak1/pBI121 expression plasmid following the protocol described by Khirod *et al.*(2011). The transformants were selected on 50 mg/L hygromycin selection media for 3 to 4 weeks until resistant calli were obtained (Fig. 1c).

Genomic PCR Analysis: Total genomic DNA was isolated from the hygromycin resistant and regenerated transformed seedlings using the NucleoSpin® Plant II kit (Macherey-Nagel, Germany) following the manufacturer's protocol.Genomic PCR was performed using the *Oak1* gene specific primers following the protocol described by Lai *et al.*(2012b)with slight modifications. A total of 25 µl reaction containing 2.5 µl 10X reaction buffer (Takara, japan), 0.5 µl Ex-taq polymerase (10u/µl), 2 µl 10 mM dNTP mixtures, 10 pmol gene specific forward and reverse primers, 1 µl genomic DNA (~30 ng/µl) and nuclease-free water were prepared. The PCR reaction conditions were 94°C for 5 min, 28 cycles of 94°C for 30s, 55°C for 30s, 72°C for 60 s and 72°C for 7 min. For control, PCR reactions were performed using the genomic DNA from wild type plant (negative control) and 35S:Oak1/pBI121 expression plasmid (positive control) as templates.

Real-time PCR Analysis: Total RNA from the genomic PCR positive transformants were extracted according to the method described previously (Lai *et al.* 2011; Lai *et al.*2013a) and treated using DNAse I (Invitrogen, USA). The purity and concentration of the RNA was measured using NanoDrop 2000c (Thermo Scientific, USA). A total of 50 ng per sample was used to performance the real-time PCR analysis according to the protocol described by Lai *et al.*(2013b). The 18S rRNA was used as an internal control for data normalization. Data were collected using the Roche Light Cycler 480 sequence detection system according to manufacturer's protocol. Primers are listed in Table 1.

Dot-blot Analysis: Total soluble protein of all selected transformnats was extracted according to the method described by Lai *et al.* (2011) with slight modifications. Approximately 2 g of leaves was ground to fine powder in a pre-cooled mortar using two-volume of cold PBS extraction buffer. The homogenate was centrifuged at 12,000 g for 20 min at 4℃ and the resulting supernatant was used for dot blot analysis. For Dot blot analysis, Oak1 antibodies were employed to detect the presence of Oak1 protein expression following the method described by Lai *et al.* (2012b). Total soluble protein from wild-type (WT) plant was used as negative control.

RESULTS AND DISCUSSION

The GAS is destructive to rice by feeding on the rice seedlings soon after sowing. Over the years, farmers have used various methods to reduce the economic losses caused by GAS through good culturing practices and the used of synthetic pesticides. With the advancement in plant genetic engineering, now it is possible to generate plants with enhance pest resistance. In the present study, we employed the used of full length cDNA sequences of Oak1, which serve as precursor's protein for potent molluscicidal agent of cyclotide kalata B1. The full length *Oak1* gene was synthesized, amplified and cloned into plant expression plasmid (Fig. 1a). Then, the exogenous *Oak1* gene was transformed into the rice calli. A total of 11 independent T_0 hygromycin resistant transformed calli were successfully recovered from the

selection media and regenerated as described previously (Fig. 1d) (Zuraida*et al.*2010). Further genomic PCR analysis using the *Oak1* gene specific primers confirmed that, 7 out of 11 tested T_0 transformants showed the presence of approximately 375 bp bands corresponding to the size of Oak1 cDNA (Fig. 2a). This result indicated the successful transformation and integration of *Oak1* gene fragment into the plant genome.As expected, no band was detected in WT plant (negative control).

In order to verify the presence of *Oak1* mRNA transcripts, quantitative real-time PCR analysis was performed on the transformants. Consistent with the genomic PCR result, all 7 T_0 transformants tested for quantitative real-time PCR analysis showed the presence of strong *Oak1* mRNA expression as compared to the WT plant (negative control) (Fig. 2b). The highest *Oak1* mRNA expression was detected on T_0 transformant line 4 (Fig. 2b). Hence, the used of strong constitutive promoters such as cauliflower mosaic virus 35S RNA promoter (CaMV 35S) is crucial to drive a high level of gene expression in plant. Moreover, the real-time PCR analysis infers that Oak1 cDNA was successfully and actively transcribed in all the selected transformants.

To further evaluate the presence of Oak1 protein expression, dot-blot analysis using specific Oak1 antibodies was performed. As shown in Fig. 2c positive signal was detected for all the tested transformants. No positive signal was detected in WT plant (negative control). The immunoprecipitation analysis confirmed that, Oak1 protein was produced in all the tested transformants. More importantly, the Oak1 protein was successfully expressed to the level detectable by the immunoblot analysis.

We have shown the successful generation of transgenic rice plants expressing th eOak1protein. To the best of our knowledge, this is the first report on development of transgenic rice plants expressing Oak1 as a biocontrol strategy against the GAS. Although the mechanism of how cyclotide kalata B1 is lethal to GAS remains unclear, the potential application of this natural molluscicidal agent in crop protection is promising. Further study will be carried out in the field trial to confirm the transgenic plants toxicity towards the GAS. Besides, the segregation analysis of the transgenic lines will also be monitored. Nevertheless, the successful development of transgenic rice plants expressing the Oak1 will serve as a good platform for better pest control management against the GAS. Concisely, the used of gene-derived peptides in transgenic rice that produce their own biopesticides represent one of the great and sustainable solutions to reduce economic damages on rice crop.

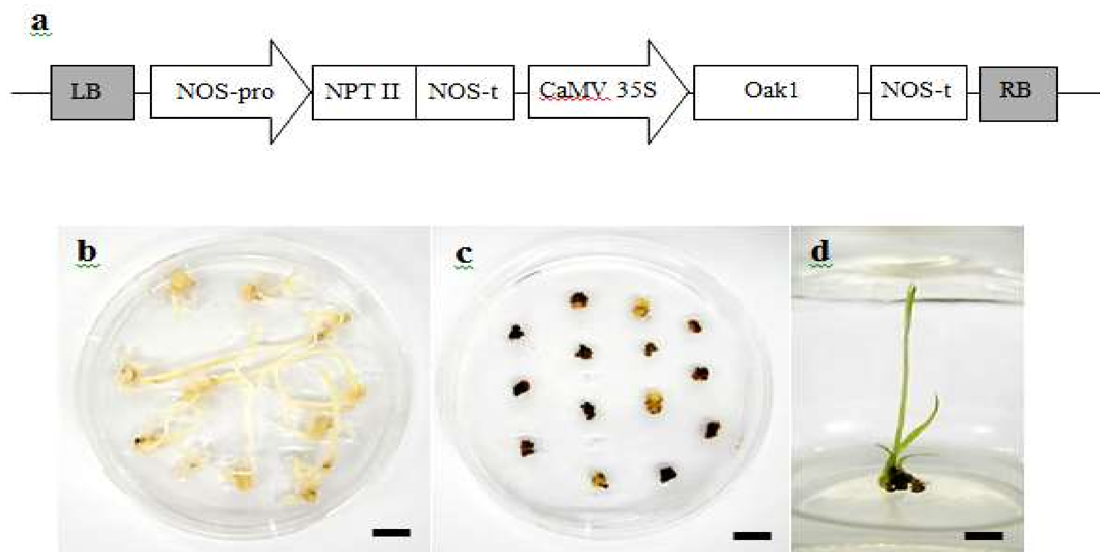

Fig. 1: *Agrobacterium*-mediated transformation and regeneration of Malaysia indica rice calli.(a) *Oak1* gene and schematic map of expression vector 35S:Oak1/pBI121 driven by the CaMV 35S promoter. NPT II: neomycin phosphotransferase II. NOS-pro: nopaline synthase promoter. NOS-t: nopaline synthase terminator. LB: left border. RB*:* right border. (b) Calli initiation from mature rice seeds. (c) Selection of transformed calli in presence of hygromycin (50 mg/L). (d) Regeneration of shoot from hygromycin resistant callus. Bars: 1 cm.

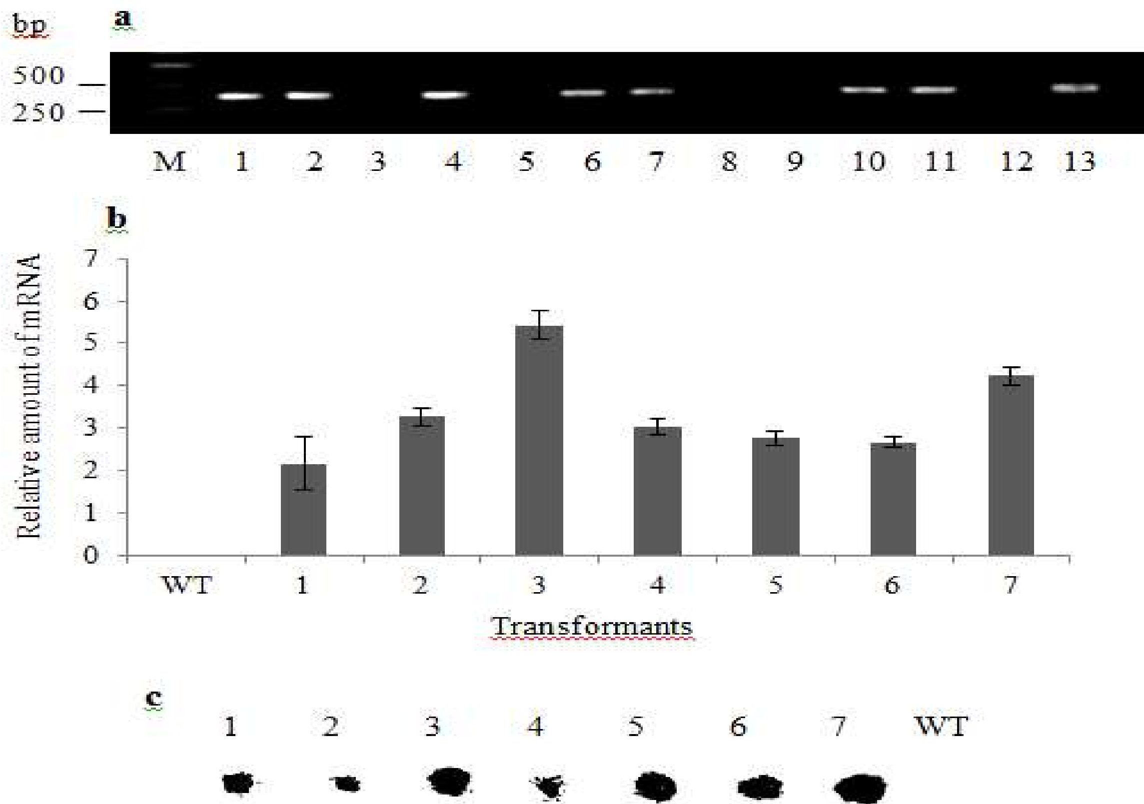

Fig. 2: Molecular characterization of rice T_0 transformants. (a) Genomic DNA PCR for detection of *Oak1* gene in T_0transformants. Lane M: 1kb DNA ladder (Fermentas, USA).Lanes 1-11: transformants line 1 to11 with 35S:Oak1/pBI121 vector. Lane 12: wild type plant used as negative control. Lane 13:*Oak1* gene PCR amplification from 35S:Oak1/pBI121 vector used as positive control. (b) Relative amount of *Oak1* mRNA transcripts in T_0 transformants measured by quantitative real-time PCR. The relative mRNA levels represent the amount of mRNA expression normalized with 18S rRNA. Lane WT: wild type plant. Lanes 1-7: transformants line 1, 2, 4, 6, 7, 10 and 11. The data are mean ± S.E. of three measurements per transformant. (c) Dot blot analysis of Oak1 protein expressed inT_0 transformants. Lanes 1-7: transformants line 1,2, 4, 6, 7, 10 and 11. Lane WT: wild type plant (negative control).

Table 1. Primers used in the study

Primer name	Sequences (5'-3')
F_Oak1	CACCATGCATCATCACCATCACCACGCTAAGTTCACCGTC
R_Oak1	TTATGCGGCCAAACTAGGAAGG
qPCR_F_Oak1	CAGCTCAAAGGACTTCCAGTATGC
qPCR_R_Oak1	TTATGCGGCCAAACTAGGAAGG
qPCR_F_18SrRNA	CTACGTCCCTGCCCTTTGTACA
qPCR_R_18SrRNA	ACACTTCACCGGACCATTCAA

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