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Agrobacterium-Mediated Transformation of *Cry8db* Gene in Vietnam Sweet Potato Cultivar

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Abstract: Sweet potato [*Ipomoea batatas* (L.) Lam.] is an important food crop in the world as well as in Vietnam. It is well known as a recalcitrant crop for gene transformation and tissue culture because of its genotype dependent in vitro responses. In present study, *Agrobacterium*-mediated transformation of *cry8Db* from *Bacillus thuringiensis* into KB1 sweet potato variety has been studied. The C58cv strain carrying a pBI121 backbone which contained *cry8Db* delta-endotoxin gene regulated under 35S CaMV promoter, and the selection marker gene, neomycin phosphotransferase (*nptII*) gene, was subjected for plant transformation. Callus induced from shoot tips and leaf explants were inoculated and cocultured with *A. tumefaciens*. The selection occurred during callus producing and plant regenerating steps. A total of 201 transgenic putative plant lines were produced, and 21 transgenic lines were positively confirmed by PCR and finalized by Southern blot. Four putative transgenic lines confirming a single copy of the *cry8Db* gene were transferred into soil pots in greenhouse. Biological activity evaluation for the insecticidal capacity of these transgenic lines under controlled conditions showed that the level of infestation by sweet potato weevils (*Cylas formicarius*) in untransformed plants was higher than that of transgenic lines.

Key words: Sweet potato, *Agrobacterium tumefaciens*, plant transformation, *cry8Db*, sweet potato weevil resistance, *cylas formicarius*.

1. Introduction

Ipomoea batatas (L.) Lam. (Convolvulaceae, Dicotyledons) produces storage roots rich in carbohydrates and β-carotene, a precursor of vitamin A, and its leaves are rich in proteins. The roots also contain vitamins C, vitamin B complex, and vitamin E as well as potassium, calcium, and iron [1]. In Vietnam, sweet potato ranks fourth in the top list of important crops after rice, corn and cassava. According to the world crop statistics, the production of sweet potato in Vietnam ranks sixth, after China, Nigeria, Uganda, Indonesia, and Tanzania with an annual production around 1.367 Mt. However, the average yield of sweet potato in Vietnam (9 tons per ha) is extremely lower than that in Japan (23 tons per ha) and China (21 tons per ha). Increasing the production of sweet potato is limited by the severe damage caused by pests and diseases [2].

Improvement of sweet potato production through conventional breeding is a complicated process and has not been very successful. This is due to the fact that sweet potato is a hexaploid crop and genes from the cultivated sweet potato gene pool are not easily accessible by direct sexual hybridization. A highly promising alternative to the conventional breeding is the introduction of foreign genes into plants through genetic transformation. Attempts over the last few years to produce transformed sweet potato plants utilized different gene transfer systems such as

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transformation by electroporation [3-5], particle gun bombardment [6-11] or *A. rhizogenes* [12]. Since the *Agrobacterium* mediated transformation system does not involve sophisticated equipment and frequently produces cleaner events (intact integrations and single copy) than other methods [13], it remains to be the more favorable approach. Previous studies on sweet potato transformation via *Agrobacterium tumefaciens* transformation have been reported including herbicide resistance [14, 15], viral resistance [16], insect resistances related to the CryIIIA delta-endotoxin, cowpea trypsin inhibitor and snowdrop lectin [17, 18] and enhancing starch accumulation in tubers [19, 20]. To date, effective transformation protocols have been developed for only few of sweet potato cultivars, indicating that it is a very recalcitrant species and transformation methods of sweet potato are still genotype dependent.

In Vietnam, as almost worldwide, sweet potato is the most susceptible to *C. formicarius*. More than 50% of sweet potato crops are lost due to this pest. The expression of insecticide genes into sweet potato plants to control pest resistance is an appropriate alternative to reduce crop loss. Recently, the activities of some insecticidal genes from *Bacillus* to control coleopteran species have been well characterized. Bt galleriae SDS-502 [21] and Bt BT185 [22] were isolated as strains possessing toxicity to larvae of the scarab beetles. Two *cry* genes, *cry8Ca* from Buibui and *cry8Da* from SDS-502 were cloned, and their protein showed toxicity against certain scarab beetle species [21, 23]. Another novel *cry* gene, *cry8Db*, highly toxic to scarab beetles such as the Japanese beetle, was cloned from an isolate of *Bacillus thuringiensis* (Bt), BBT2-5. The *cry8Db* gene has the length of 3525 bp and codes for a protein of 1,174 amino acid residues. Studies also showed that the deletions of domain 3 (517-686) of *cry8D* appeared to be not involved in the insect host specificity. The activity against insects is determined by Domain 2 (290-516) [24].

The objective of present study was to generate transgenic sweet potatoes resistant to sweet potato weevil. Particularly, the study is designed to 1) optimize codon usage of an active *cry8Db* gene region for expression in a plant; 2) construct transformation vector carrying the synthesis *cry8Db* gene; 3) transform *cry8Db* into sweet potato via *Agrobaterium tumefaciens*; and 4) analyze the transformants and biological tests.

2. Materials and Methods

2.1 Plant Materials and Culture Conditions

KB1 sweet potato variety (*Ipomoea batatas* [L.] Lam.) used in the experiments was kindly provided by Root Crop Research and Development Center, Vietnam Academy of Agricultural Sciences.

CP and EP basal medium [25] was used in the experiment. The pH of all media was adjusted to 5.8 before autoclaving at 118 °C for 20 min.

To establish *in vitro* cultures, shoots with the length of 10 cm were excised from greenhouse-grown plants and were rinsed under running tap water for 15 min. Then, the shoot tips were cut to 3 cm in length. The shoots were sterilized by briefly immersing in 70% (v/v) ethanol for 20 s, rinsing with sterile, distilled water, immersing into 0.1% mercuric chloride solution for 3 min, and then rinsing three more times with sterile, distilled water. The surface-sterilized shoot tips were blotted dry on sterile filter paper. All of the explants were cultured on the MS basal medium for propagation.

A two-stage protocol for plant regeneration was followed Vu Thi Lan et al. [26, 27]. The stage 1 culture consisted of basal medium supplemented with 0.5 mg/L picloram and 2,237 mg/L KCl for callus production (CP medium) for 3-4 weeks. In the stage 2, the EP basal medium was supplemented with 1.0 mg/L ABA and 1.0 mg/L GA_3 for for 2 weeks (IRM). Shoots were regenerated from callus in MS medium containing 0.5 mg/L kinetin and 1.0 mg/L BAP [18]. Shoots 2-3 cm in length were rooted on MS medium.

2.2 Agrobacterium and the Binary Vector

The roughly 2.1 kb of *cry8Db* gene (AB303980) was optimized codon usage for expression in plants and synthesized artificially. For easier purification and immunological detection, *cry8Db* gene was fused to a poly-histidine tag and a cMyc-tag at the C terminus. In order to clone *cry8Db* into pBI121 under the control of CaMV promoter, the nucleotide sequences of BamHI and SmaI recognition sites were added into the N-terminal and C-terminal of *cry8Db* gene, respectively. The generating binary vector pBI121/*Cry8Db* was transformed into *A. tumefaciens* strain C58cv. The *nptII* gene was designed for the selection of kanamycin resistance (Fig. 1)

Cry8Db is transcriptionally controlled by the P35S promoter and T35S terminator. In addition *cry8Db* gene was fused to a poly-histidine tag and a cmyc-tag at the C terminus. The *nptII* gene was designed for the selection of kanamycin resistance in plant.

2.3 Genetic Transformation

A colony of *A. tumefaciens* strain C58cv carrying a binary vector pBI121 containing *cry8Db* gene was cultured on 50 mL LB medium at 28 °C, 200 rpm on an orbital shaker for 14-16 h. Then, the bacterial cells were collected by centrifugation at 5,000 rpm for 10 min and resuspended in 25 mL ½ MS medium [28].

Shoot tips and leaf explants were cut in 0.3-0.4 cm in ½ MS liquid medium and placed on CP for induction. After three days, explants were transferred onto a new plate containing 25 mL of agrobacterial suspension and incubated for 30 min with gentle shaking. The bacteria were discarded and the explants were dried on sterile filter paper and placed on CP solid medium under low light intensity. After three days inoculated with bacteria, the explants were placed onto new CP plates containing 500 mg/L cefotaxime (Brithol Michcoma, Holland) and 100 mg/L kanamycin. Survival callus were subsequently transferred to the selective IRM after 3 weeks. Putative transgenic shoots regenerated on RM selective medium. Shoots with 2-3 cm in length were rooted on MS selective medium.

2.4 Detection of Transformed Plants by PCR Analysis

Genomic DNA from putative transformants was isolated according to protocol [29]. Rapid detection of transformants was done by PCR analysis. Primer pairs for amplification are forward primer Cry8F (5'-CTATTTACAATAAACAATGGCGGGA -3') and reverse primer Cry8R (5'-AGATGCGTCTCTAAGCAAAAGGAG -3'). PCR reaction was performed in 20 µL volume containing 0.5-1 µg of total plant DNA made as follows: 2 µL of 10X Buffer; 0.5 µL from each 10 µM primer solutions, 0.4 µL of 10 mM dNTP; 0.1 µL of Taq polymerase (1 unit; ThermoScientific), and adjusted to 20 µL with distilled water. The PCR amplification cycles were as follows: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. This cycle was repeated 35 times and ended up with an elongation step at 72 °C for 10 min. Visualization of PCR products of 700bp were done on standard 1% agarose gel electrophoresis using 20 µL of the PCR reaction.

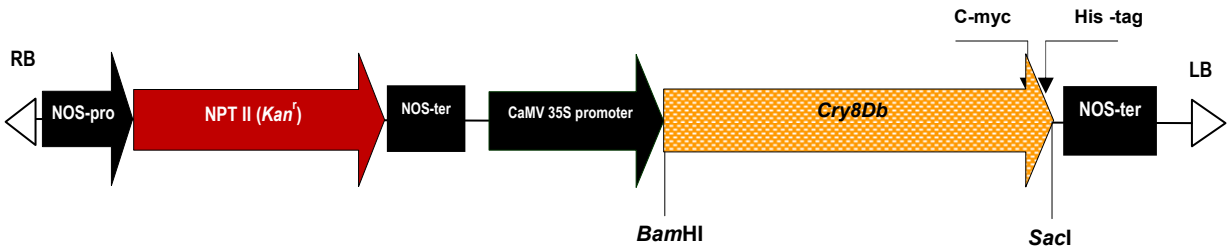


Fig. 1 NptII-*Cry8Db* cassettes of pBI121 binary vector.

2.5 Transgene Insertion Analyzed by Southern Blot Hybridization

About 40 µg of total DNA from transgenic plants were digested with *BamHI* (40 units) overnight at 37 °C. DNA fragments was electrophoresed in a 1.0% agarose gel and transferred onto nylon membrane Hybond-N^+ by alkaline transfer method. The probe was developed by PCR amplification of a fragment of the *Cry8Db* gene. This DNA was used as the probe in the Southern hybridization and was labeled with BIOTIN-11-dUTP. The blots were hybridized at 42°C and washed at high stringency (0.1 x SSC, 0.1% SDS at 65 °C). Finally, blot was detected by Biotin Chromogenic Detection Kit (Thermo scientific).

2.6 Biological Tests

Four transformants exerting *Cry8Db*-positive PCR and Southern blot were selected to test for weevil resistance. These plantlets were grown in pots and tubers from each transformants separately and harvested after 4 months of planting. The plastic box containing 1-2 pre-weight sweet potato tubers were used to evaluate damage by sweet potato weevil test. Approximately 10 adults per 50 g sweet potato tubers were released into each box, and the boxes were covered with cloth and kept in dark incubator at 28 °C. After about 35-40 days, the sweet potatoes tubers were cut, and the degree of damage caused by sweet potato weevil wad observed. Damage was evaluated following Moran's method (Fig. 2), considering the parameters: infestation percent (I), and PDI (pondered degree of infestation) [18].

3. Results and Discussion

3.1 Transformation of Cry8db into Sweet Potato via Agrobacterium tumefaciens

Genetic transformation of plants relies on two independent but concurrent processes: integration of foreign DNA into plant cells and regeneration of whole plants from these transformed cells. In previous experiments the regeneration and transformation protocol of sweet potato via *A. tumefaciens* infection has been demonstrated. Vu and colleagues established an efficient regeneration system for KB1 sweet potato variety with highest shoot regeneration efficiency (67.75%) [26, 27]. Furthermore, study on factors affecting *agrobacterium*-mediated transformation of KB1 sweet potato variety suggested that shoot tip or apical explants infected with *Agrobacterium tumefaciens* strain C58 at the concentration of OD_{600nm} 0.8, 150 µM acetosyringone supplied in bacterial suspension solution and 20-30 min infection time gave the highest percentage of GUS positive transformants (38%) [26, 27]. In the present study, genetic transformation of sweet potato via *A. tumefaciens* harboring the binary vector construct pBI121/*cry8Db* comprising the DNA coding insecticidal crystal protein *Cry8Db* has been carried out (Fig. 1).

Approximately 1000 pre-culture explants per experiment were infected with *A. tumefaciens* C58cv harboring pBI121/*cry8Db*. After transformation, these explants were cultured into the selective medium

Control Value 1 Value 2 Value 3 Value 4

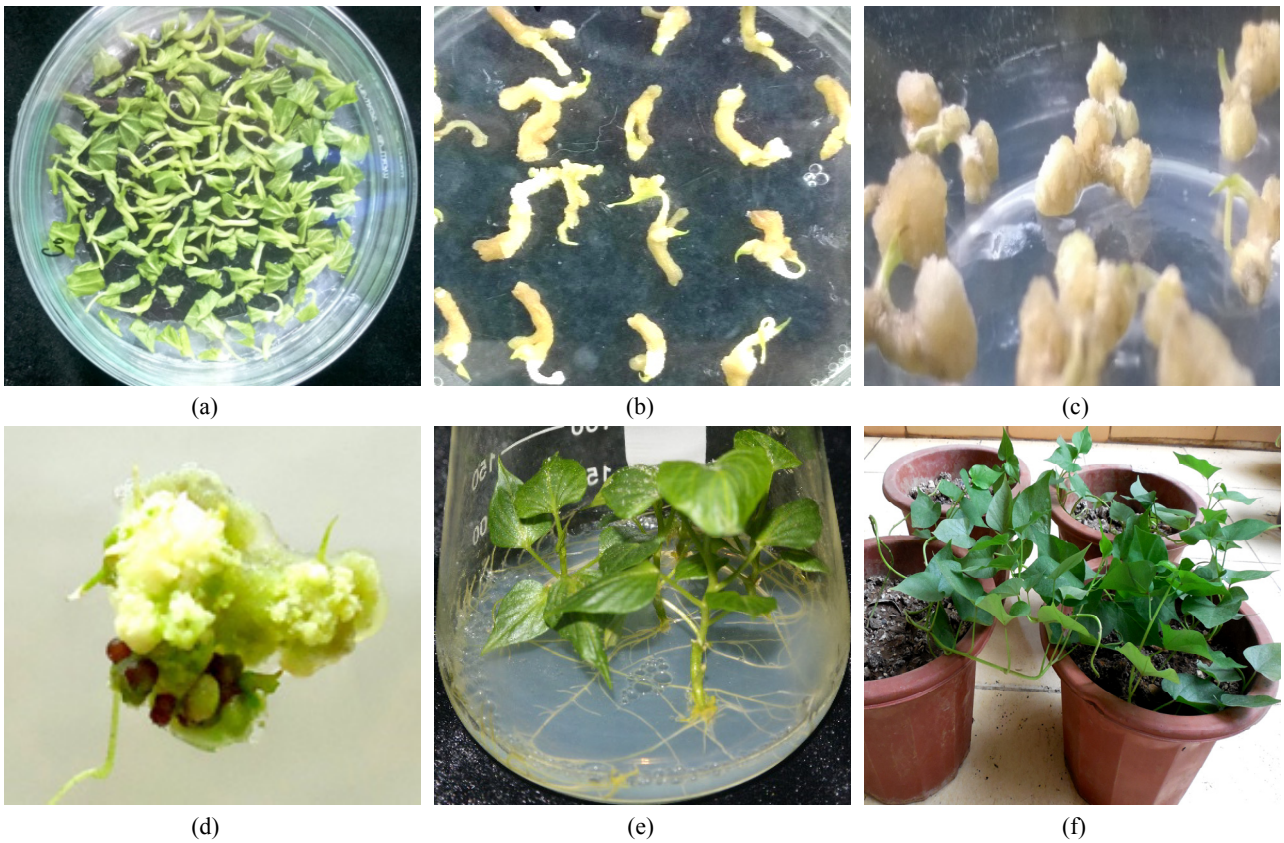
Fig. 2 Evaluation of transgenic sweet potato tubers for resistance to sweet potato weevil (*C. formicarius*) under controlled conditions.

Values of scale of damages (from 1 to 4) deal with the percent of the tuber that is damaged.

Table 1 Agrobaterium-mediated transformation efficiency of *cry8Db* gene into KB1 sweet potato cultivar.

Transformation No.	Explants infected	No. of survival calli on selection medium			No. regenerated calli	No. regenerated shoots	No. rooted shoots	PCR positive (%)	Transformation efficiency (%)
		CP	IRM	RM					
1	893	595	262	45	12	42	19	2	
2	1,210	785	355	53	10	57	21	2	
3	1,020	680	300	50	13	44	16	2	
4	780	666	352	63	15	57	12	2	
5	1,130	753	332	59	20	51	23	3	
6	800	533	533	42	6	38	20	1	
7	1,150	338	172	50	9	55	25	3	
8	1,120	880	382	56	15	52	23	2	
9	1,012	674	297	53	17	48	22	2	
10	1,048	698	308	65	29	55	20	2	
Sum	10,163	6,602	3,293	536	146	499	201	21	0.21
Wt	100	100	32	0	0	0	0	0	0

Wt: untransformed experiment; CP: callus production medium; IRM: induction regeneration medium; RM: regeneration medium.



(a) (b) (c)

(d) (e) (f)

Fig. 3 Transformation and regeneration sweetpotato KB1 plants.

(a) Explants infected with *A. tumefaciens C58* on co-cultivation medium for 2 days; (b) Callus induction from explants in selective medium; (c) Transgenic callus formation from shoot tip segments after 3-4 weeks in selective medium; (d) Shoot regeneration from survival embrygenic callus after 4 weeks on regeneration medium; (e) Transgenic plants in rooted medium; (f) Morphological characterization of transgenic lines in soil pots in the greenhouse.

according to two stage regeneration protocol. Shoots usually developed after 7-8 weeks from the wounded on the selective regeneration medium (Fig. 3). In present study, ten sets of transformation experiments were carried out with about 10,163 explants in total. After three weeks in CP selective medium, about 6,602 putative transformed calluses (65%) were obtained based on antibiotic resistance selection. These survival calluses were transferred continuously into the IRM and then RM selective medium. After three to five weeks under kanamycin selective pressure, 146 embryogenesis calluses produced 499 shoots (Table 1). These regenerated shoots with 2-3 cm in length were rooted on MS selective medium. From these shoots, 201 rooted plantlets were chosen for PCR and further analyses. Under kanamycin pressure, almost all of these shoots grew slowly and there are two kinds of root emergence from shoots. The thin and weak roots arising from above stem and the healthy and long roots arising from the end of cutting stem were observed (Fig. 3).

3.2 Molecular Analysis of Transgenic Plants

In order to confirm the presence of the *cry8*Db gene, the gel electrophoresis of PCR products from the putative transformants and the wild type as well as *A. tumefaciens* using *Cry8Db*-specific primer pairs showed that *cry8*Db fragment was present in 21 transgenic clones but not in the wild type (Fig. 3) whereas the virC PCR product was absent (Data not shown). These results proved that the transgenic plants were actually transformed with *Cry8Db* and were not contaminated with *Agrobacterium* [30]. This result also showed that the use of high concentration of kanamycin at 100 mg/L for selection of putative transformed clones still gave ambiguous results. Most of the root formation of 21 positive PCR plantlets arising from the bottom of the cutting stem was observed.

The copy number of integrated DNA was analyzed by Southern blot. DNA of transgenic plants was digested with *Bam*HI, at the *Bam*HI unique cleavage site in the T-DNA region in the vector. The fragments were hybridized with the *cry8Db* gene probe (Fig. 4). These transgenic plants showed different patterns, and the number of integrated copies varied from 1 to 4. Four transgenic plants carrying a single copy of the *cry8Db* gene were used for biotest.

3.3 Biological Test Results

Four transgenic sweet potato plants proved positively in Southern hybridization with a single copy of the

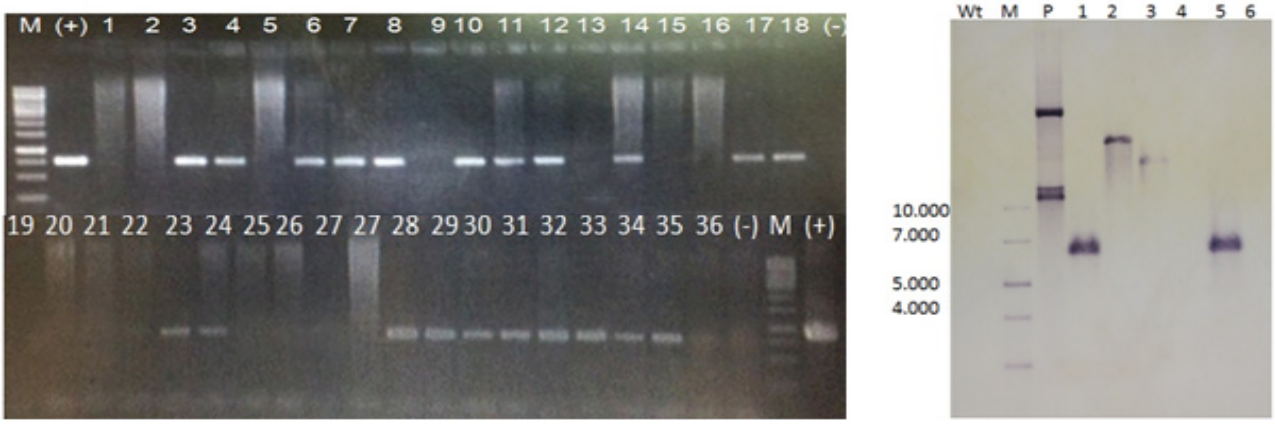


Fig. 4 Confirmation of *cry8Db* gene integration into the sweet potato genome by PCR (A) and Southern blot analysis (B).

A: (M) Molecular marker 1 kb (Fermentas); (+): positive control, plasmid DNA; (-): negative control, untransformed plant genomic DNA; Lane 1-36: putative transgenic sweetpotato lines;

B: 30-50 μg of total DNA from leaves of the transgenic clones and from the untransformed control were independently digested with BamHI; P: undigested plasmid DNA; Wt: untransformed plant genomic DNA; Lane1-6: genomic DNA of putative transgenic sweetpotato lines; M: Molecular marker.

*cry8*Db gene and the wild type were grown in pots in greenhouse and the roots were harvested after 4-5 months of planting. The size of sweet potato roots harvested in our study ranging from 70 g to 125 g (Data not shown). This could be due to the limited of the growing pots and farming methods. Damage was evaluated following Moran's method [18]. Scale of damages (ranging from 1 to 4) has been represented in Fig. 4. According to parameters including infestation percentage and pondered degree of infestation, biotests against sweet potato weevil of these transgenic lines showed that the level of infestation by weevils in untransformed control plants was higher than that of transgenic lines (Table 2). In the experiment, the pondered degree of infestation of transgenic lines was from 1.33 to 1.75 lower than untransformed plants (2.3).

The sweet potato weevil is the major sweet potato insect pest worldwide, however, only three reports on transformed plants with insecticidal genes cowpea trypsin inhibitor, snowdrop lectin genes and *cry*III gene have been published thus far [17, 18, 31]. Also, it is known that Bt is widely used in pest control agents, especially highly activate against lepidopteran pests. A few insecticidal proteins from Bt strains that are toxic for coleopteran insects such as *Cry8, Cry3, Cry7* have been reported [24, 32, 33]. These findings are in good agreement with the authors' previous studies that the activity of *cry8Db* gene in *E.coli* against sweetpotato weevil (*Cylas formicarius*) were confirmed (experimental unpublished data). In present study, for the first time, the novel of *cry8Db* gene from Bt (*Bacillus thuringiensis*) proved to be toxic to sweet potato weevil and was successfully used to transform into KB1 sweet potato variety.

From 201 putative transgenic shoots regenerated, 21 transgenic plants have been confirmed successfully by PCR and Southern blot. This result is in agreement with Luo's study [34] which showed that the untransformed escapes of all regeneration plantlets were observed. In the authors' experiments, the obtained transformation frequencies of about 0.21% indicates the difficulty of *Agrobacterium* mediated transformation of insecticidal genes into sweet potato. This result is also associated with our previous studies where insecticidal genes cry3ca1 and vip1-2 were used (experimental data unpublished) and other reports [17, 18]. To date, several reports on the *Agrobacterium*-mediated transformation of sweet potato have been published. However majority of the most efficient and robust *Agrobacterium* transformation systems succeed in transformation of marker genes such as *gfp, gus*A, *npt*II, *bar* [5, 10, 34-37]. Therefore, genetic transformation of sweet potato remains cumbersome and labor-intensive due to the difficulty in plant regeneration of several recalcitrant cultivars, and the reproducibility of the used protocol [15, 17, 34, 36-39] .

Four of transformants carrying a single copy of *Cry8Db* have been tested for biological activity against sweet potato weevil. Under the control conditions, levels of infestation by weevils in untransformed control plants were 58.3% and almost 1.7 times higher in comparison to the best transgenic clones (Table 2).

Table 2 Evaluation of transgenic sweet potato clones for resistance to sweet potato weevil (*C. formicarius*) under controlled conditions.

Clone	I	PDI
Wt	58.3	2.3
C8 (8.1)	37.5	1.5
C8 (8.2)	43.75	1.75
C8 (8.3)	40.0	1.6
C8 (8.4)	33.33	1.33

C8 (8.1-8.4): Four transgenic lines confirmed by PCR reaction and Southern blot; Wt: Untransformed plants; I: Infestation percent; PDI: Pondered degree of infestation.

For that reason, these transgenic plants were selected to be tested for cMyc using Western blot. Unfortunately, bands of transgenic clones were practically undetectable despite correct technical performance (detectable 25 ng positive SCFv_cmyc) (Data not shown). This result is coincident with a previous report which proposed that the CryIIIA toxin band in sweet potato transgenic plant is undetectably but the biological result still showed resistance against sweet potato weevil [18].

4. Conclusion

In conclusion, the sweet potato genetic transformation via *Agrobacterium* was performed successfully in our laboratory. With the importance of the sweet potato KB1 cultivar in Vietnam, the successful transformation of *Cry8Db* into KB1 cultivar and generation of weevil-resistant transgenic plants will contribute to increasing the yield and food security in Vietnam in the near future.

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