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Transformation *CodA* Gene to Lily Plants by *Agrobacterium tumefaciences* Mediated

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Abstract: Glycinebetaine (GB), an osmotic substance, could improve some stress tolerance in plants. *CodA* gene, originating from bacteria, could translate choline oxidase which stimulates the synthesis of GB in plants. To create lily lines resistant to heat, Belladonna lily and Yelloween lily had been transferred *CodA* gene through *Agrobacterium tumefaciens*. The bacteria harbored a binary vector carrying the hygromycin phosphotransferase, choline oxidase (*CodA*) and intron-containing β-glucuronidase (*Gus*) genes were co-cultivated with lily bulb scales slides. The result showed that most the bulb scales had developed into bulblets in a regulator-free growth medium, while some expressed the hygromycin-resistance, heat tolerance and *Gus* gene expression. Among them, one line demonstrated primarily the transcription level expression through reverse transcription polymerase chain reaction (RT-PCR). Moreover, they were tested with the accumulation of GB which was evident that the transferred line had four times of GB volumes higher than that of wild type. The original evidence could open a right approach to enhance stress tolerance in lily plants.

Key words: *Agrobacterium tumefaciens*, *CodA* gene, genetic transformation, glycinebetaine, lily, stress tolerance in plants.

1. Introduction

High temperature and some other abiotic stresses including cold, salinity and drought could harm plants at any time in their life. However, some can overcome these bad conditions with complicated mechanisms. One of the most important mechanisms is that plants could accumulate compatible solutes such as polyols, sugars, amino acids and betaines. Among them, glycinebetaine (GB), a compatible solute, was accumulated rapidly in many plants under stress conditions [1]. It also demonstrated that GB clearly enhanced the tolerance of plants to high temperature stress [2-4]. The GB, however, enhanced tolerance to abiotic stress that appeared to involve the GB-stimulated expression of stress tolerance-related genes [5]. Kathuria *et al.* [6] found that levels of expression of 165 genes were higher in *CodA*-transgenic rice than in wild-type (WT) rice. These genes have been shown to be involved in stress responses, regulations of gene expression, signal transduction, transport across membranes, cellular metabolism and the general growth and development of plants.

Lilium, a genus of *Lilium* spp. which belongs to the Liliaceae family, now is becoming one of the most favorite flowers with dramatically ornamental and high economical qualities because of the colorful flowers, plant forms and herbicide resistance [7]. However, there are few species which can have abiotic tolerance. That narrowed the range of lilies distributed worldwide. The lilies naturally grow in temperate zones. Therefore, it is necessary to establish an efficient genetic approach applying to lilies to improve the stress tolerance. In the *Lilium* genus, there were few publics reporting some transgenic interspecific hybrid lilies [8] or *L. formosanum* [9], which was only transferred indicator genes by *Agrobacterium*. This report had reported the investigative objective to transfer *CodA* gene to lily through lily bulb scales mediated by *Agrobacterium*. These plantlets were verified to be transgenic lines by

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Gus histo-chemical assay, reverse transcription polymerase chain reaction (RT-PCR) and GB detection analysis.

2. Materials and Methods

2.1 Materials

In vitro lily bulb scales of oriental trumpet (OT) hybrid Belladonna lily (Bel) and OT hybrid Yelloween lily (Yel) were used for transformation with the target gene, artificial *CodA*, choline oxidase gene. The gene was roughly transcoded from *Arthrobacter globiformis*' *CodA* gene to suitable genetic code in plants. It was also added with a nucleotide segment encoding a peptide with 30 nucleotides in length, namely *Cmyc* gene, which could help to check the transgene expression. Moreover, another version of the artificial *CodA* gene, namely *TP-CodA* gene, was created by being added at the 5' end of *CodA* gene a 216 nucleotide segment. The "TP" means transited peptide, leading transportation of the choline oxidase into the chloroplast created. The two target genes, with and without TP segment had been ordered to artificially be synthesized by the Fermentas Company, USA. The gene had been located in an *Agrobacterium tumefaciens* carrying a binary vector pCAMBIA1301 which included the hygromycin phospho transferase, choline oxidase (*CodA*) and intron-containing β-glucuronidase (*Gus*) [10].

2.2 Inoculation and Co-cultivation

The bacterial strain carrying target gene was inoculated into liquid LB medium [11] containing 20 mg/L acetosyringone (AS, Sigma-Aldrich), 50 mg/L kanamycin (Kanamycin Monosulfate; Wako) and was incubated for 24 h at 28 °C with reciprocal shaking (200 cycles/min), then it was centrifuged at 4,500 rmp for 15 min at 4 °C for collecting cells. These bacteria cells were suspended to a final inoculation medium with OD_{600} of 0.5, following with immersing bulb scale slides of *in vitro* lily bulb scales, 4-5 mm in length in 30 min. After that, the samples were co-cultivated at 25 °C in the dark for 3 d on co-cultivation media, which consisted of Murashige and Skoog (MS) medium [12], 30 g/L sucrose, 3 g/L gellan or 7 g/L agarose and 100 mg/L AS [13].

2.3 Selection and Regeneration of Transformed Plants

After co-cultivation, the bulb scale slides were cultured at 25 °C in the dark, on MS medium added 3 g/L gellan gum, 90 g/L sucrose and 250 mg/L cefotaxime, forming new small bulbs namely bulblets. The slides carrying bulblets with 10-15 mm in diameter, white and occasionally containing leaf-shoots were transferred to a selection medium, which consisted of MS medium, 75 mg/L hygromycin, 90 g/L sucrose and 3 g/L gellan gum (slightly followed by Han *et al.*, 1997 [14]). Survival bulblets called hygromycin-resistant (Hygr) bulblets were cultured on the MS medium added neither antibiotic substance nor plant-grow regulators in order to induce rooting. All plant tissue culture medium used in the study were adjusted to pH 5.8 and were autoclaved at 120 °C for 15 min.

2.4 High-Temperature Treatment during Growth of Bulblets

Bulblets, approximately 1 cm^2 in width, were cultured on MS medium and incubated at 37 °C for two weeks in growth chambers. The response of bulblets to high temperature was quantified in terms of the survived race in the recovery stage.

2.5 Gus Histochemical Assay

The heat-tolerant bulblets were tested by the technique which was performed similarly as the method of Jefferson (1987) [15]. Specifically, tissues were incubated for 24 h at 37 °C in 100 mM sodium phosphate buffer (pH 7.0) containing 2 mM 5-bromo-4-chloro-3-indoyl glucuronide (Wako).

2.6 Extraction and Quantification of GB

The bulblets of all transferred lines and the control,

untransformed one were cultured in every 8 h per day in light condition aiming to generate the *in vitro* leaves. The leaves were extracted and measured GB amount through the method of Grieve and Grattan [16].

2.7 Inverse PCR Analysis

RNA was extracted and purified by the protocol of Sigma-GenElute^{TM} Total RNA Miniprep. Then, RNA samples were measured by a NanoDrop Lite (Thermo Scientific) and used to synthesize cDNA which was followed by protocol of First Strand cDNA Synthesis (Fermentas). In the next step, the RT-PCR amplification mixture was mixed, containing 100 mg of each sample as a template, 200 mM dNTP mixture, 2.5 mM $MgCl_2$, PCR 10× buffer, 5 U DreamTaq (Fermentas), 0.2 mM primer *CodA* 5'GCTCTAGAATGCACATCGATAATATTGA3' (or *TP-CodA* 5'GCTCTAGAATGGCACAAATTAACA3') and 0.2 mM primer *Cmyc* 5'CGAGCTCTCAATTCAGATCCTCTTC3'. It was to amplify the fragments of the total cDNA with 35 cycles performed by a temperature control system in Veriti^{TM} 96-well Thermal Cycler (Applied Biosystems, USA) programmed under the following conditions: 1 min at 94 °C, 50 s at 58 °C and 90 min at 72 °C. The amplified products were analyzed by electrophoresis on a 1% (w/v) agarose gel.

3. Results and Discussion

3.1 Regeneration and Selection of Hygromycin Culture Lines

Table 1 shows that, being cultured from around four to six weeks in medium without hygromycin, most of the transferred bulb scales slides generated new small bulbs, namely bulblets. Specifically, over 82% was the rate of transferred slides of both varieties Yel and Bel forming bulblets. Moreover, the coefficients of them were notable, with each slide regenerating nearly one bulblet whenever it was co-cultured with the bacteria carrying *CodA* or *TP-CodA* gene.

Lily bulb scales carrying bulblets had been selected in an MS medium containing 75 mg/L hygromycin which was previous confirmed for inhibiting growth of the control as sufficiently as stimulating the successful lines [13]. The data (Table 2) illustrates that while some bulblets grew normally, named the Hygr lines, others occasionally developed at the early period, but subsequently turned brown and died later. As a result, after one, two or three months culturing, Yel

Table 1 The bulblet formation from transferred bulb scale slides.

Target gene	Variety	Number of experiment	Number of transferred slides	Number of slide forming new bulblet	Rate of bulblet forming (%)	Number of new bulblets	Total number of new bulblets	Coefficient (bulblet/slide)
CodA	Yel[1]	1	60	50	85.4	53	159	0.893
		2	60	52		54		
		3	58	50		52		
	Bel[2]	1	155	135	87.3	132	398	0.871
		2	152	133		132		
		3	150	131		134		
TP-CodA	Yel	1	58	50	86.6	50	147	0.890
		2	50	43		44		
		3	57	50		53		
	Bel	1	130	105	82.2	100	303	0.780
		2	130	108		102		
		3	128	106		101		

[1] Oriental trumpet Yelloween lily; [2] Oriental trumpet Belladonna lily.

Table 2 The survival ability of transferred bulblets in the selection medium (75 mg/L hygromycin).

Transferred gene	Variety	Numberof tested bulblets (line)	The percentage of survival bulblets in the selective medium (%)			Numberof survival bulblets (line)
			One month after	Two months after	Three months after	
CodA	Yel[1]	159	65.8	41.1	7.5	12
	Bel[2]	398	48.9	19.1	11.0	44
TP-CodA	Yel	147	87.1	38.8	6.1	9
	Bel	303	33.0	26.4	17.2	52

[1] Oriental trumpet Yelloween lily; [2] Oriental trumpet Belladonna lily.

variety bulb scales transferred *CodA* gene and *TP-CodA* genemade up 65.8%, 41.1%, 7.5% survival and 87.1%, 38.8%, 6.1% survival, respectively. In contrast, Bel variety samples transferred the target genes experienced the lower percentages in the first two months but the highest rates after three months with 11% of *CodA* lines and 17.2% of *TP-CodA* lines. In three months after selecting, there were only 12 *CodA* and nine *TP-CodA* bulblets of Yel variety survival; whereas the figures of *CodA* and *TP-CodA* transgenic Bel lines were 44 and 55, respectively.

3.2 Select the Heat Tolerance of Transferred Lines

All Hygr bulblets, continuously cultured in an MS medium without plant growth regulator substance and hygromycin, were tested on the heat tolerance in the heat stress condition (at 37 °C, in two weeks). After the heat treatment, they were completely transferred to the recovery phase in the normal conditional culture on the same medium. The results had shown a decrease in the number of bulblets during the recovery phase. It is evident that Bel variety had better heat resistance than the Yel sample, with the former receiving *CodA* gene and *TP-CodA* gene witnessing 40.9% and 56.2% survival after 30 d recovered phase, while only *CodA* transgenic lines of the latter get 40.9% survival. Besides, all non-transformation lines died in the 30th day in the recovery phase (Table 3).

3.3 Gus Histochemical Assay

To temporarily check the expression of the *Gus* gene, pieces or total plantlets were dyed with Xgluc. The result in Table 4 demonstrates that the number of lines which were Hygr and the heat tolerance and expressed the *Gus* gene in root, leaf, or in bulblet scales (Fig. 1) was modest, only around 0.5%-1% plantlet segments of lines after three to six months of transformation. Besides, it was evident that no endogenous *Gus* activity was detected in the control plants. It was similar to a preliminary research [13].

3.4 Inverse PCR Analysis

Inverse transcriptional analysis (RT-PCR), which amplified the fragments of inserted DNA, was carried out on the four plantlets. The result (Fig. 2) shows that there was only a transferred line called YelCodAT1.4, having a DNA fragment marked by a band in the lane 4, whereas other lines did not express the *CodA* gene.

3.5 GB Detection

Roots and leaves of non-transgenic and transgenic plantlets cultured on the MS medium at 25 °C for several days had been used to extract GB. The result shown in Table 5 illustrates that all samples had stimulated GB. The control also had an amount GB, however, the transgenic lines got higher amount of it. Specially, YelCodAT1.4, heat tolerant and positive expression *Gus* and *CodA* gene would accumulate the highest amount of GB, 3.7 mg/g.

Although there were some publications about/on transgenic lily plants by particle bombardment [17], or electrophoresed force through pollen protoplasts [18], no or few plants were obtained though callus mediated by *Agrobacterium* [7, 19-21]. The present study was the first to demonstrate *Agrobacterium*-mediated production of *CodA* transgenic lily plants by the thin layer of bulb scales slides. It demonstrated the effectiveness of using the

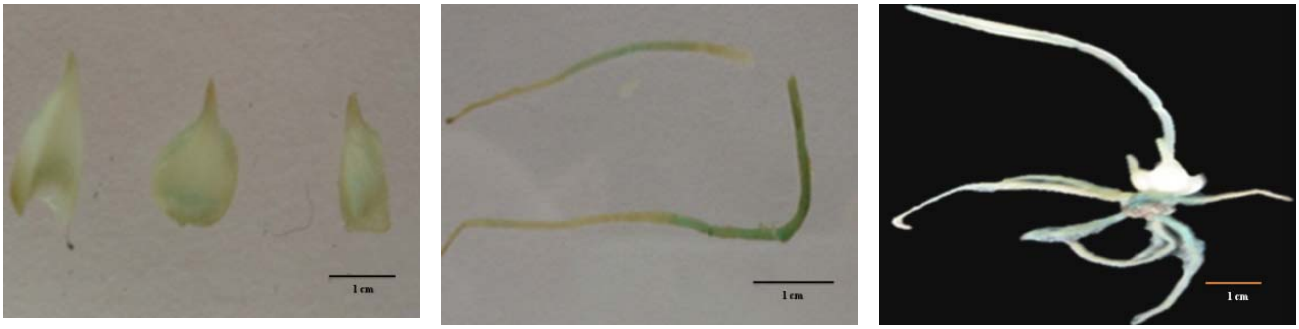
Table 3 The survival ability of transferred bulblets in and after heat stress.

Transferred gene	Variety	Numberof bulblets in heat stress	The percentage of survival bulblets (%) in recover phase				Numberof survival bulblets
			0 d	10 d	20 d	30 d	
CodA	Yel[1]	12	66.7	58.3	58.3	41.7	5
	Bel[2]	44	93.2	79.5	79.5	40.9	18
TP-CodA	Yel	9	33.3	0.0	0.0	0.0	0
	Bel	52	80.8	65.4	65.4	46.2	24
Non transformation	Yel	30	30.0	13.3	0.0	0.0	0
	Bel	30	33.33	26.7	3.33	0.0	0

[1] Oriental trumpet Yelloween lily; [2] Oriental trumpet Belladonna lily.

Table 4 The expression of the *Gus* gene of transgenic lily.

Transferred gene	Variety	Number of bulblets formed (line)	*Gus* assay after three months transformation			*Gus* assay after six months transformation		
			Number of positive lines	The rate of *Gus* expression (%)	Name of positive lines with Xgluc	Number of positive lines	The rate of *Gus* expression (%)	Name of positive lines with Xgluc
CodA/*Gus*	Yel	159	3	1.89	VCodAT1.4; VCodAT1.1; VcodAT4.1	2	1.26	VCodAT1.4; VCodAT1.1
	Bel	398	2	0.5	BelCodAT2.1; BelCodAT7.3	1	0.25	BelCodAT2.1
TP-CodA/*Gus*	Yel	147	0	0	-	0	0	-
	Bel	303	5	1.65	BelSpT9.1; BelSpT9.2 BelSpT8.1; BelSpT8.2; BelSpT8.3	2	0.66	BelSpT9.1; BelSpT9.2;



(a) (b) (c)

Fig. 1 Lily bulblets showed the *Gus* expression as being treated with Xgluc (scale bar: 1 cm).

a: bulb scales; b: roots; c: plantlet.

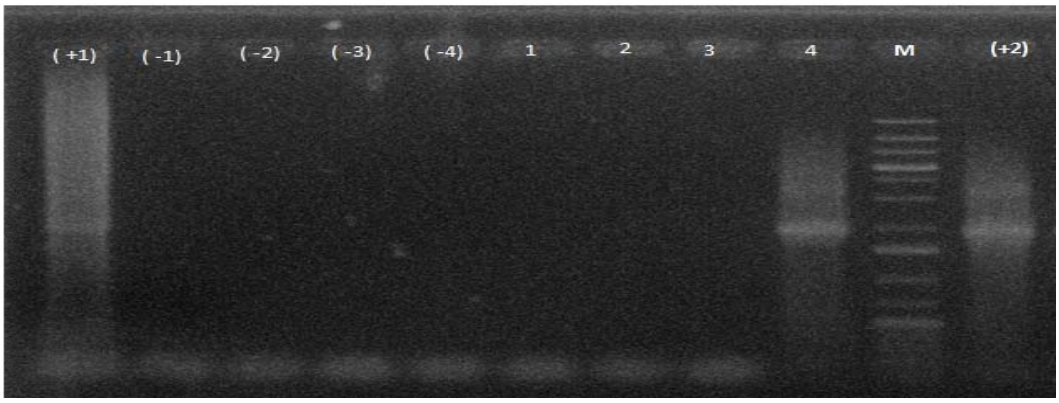


Fig. 2 Reverse transcription polymerase chain reaction (RT-PCR) of transferred line samples.

Lane (+1): positive control (*TP-CodA* gene); lane (+2): positive control (*CodA* gene); lane (-1), (-2), (-3), (-4): negative control (PCR with total RNA sample of transferred lines); Lane 1, 2, 3, 4: transgenic lines; M: standard 1 kb DNA ladder.

Table 5 Levels of glycinebetaine (GB) in wild-type (WT) and four *CodA*-transformed lines.

No	Line	Plasmid	Heat tolerance	*Gus* expression	*CodA* gene expression at transcriptional level	Glycine betaine (mg/g)
1	Bel(WT)	No	-	-	No test	1.04
2	BelSpT8.1	*1	+	-	-	1.19
3	BelSpT9.1	*2	+	+	-	1.09
4	BelCodAT8	*2	+	-	-	0.95
5	BelCodAT2.1	*2	+	+	-	2.04
6	YelWT1	No	-	-	No test	1.48
7	YelCodA4	*2	+	-	No test	1.37
8	YelCodAT1.4	*1	+	+	+	3.70

(+): positive expression; (-): negative expression

right material (which was bulb scale slides because of their high effectiveness). RT-PCR had been used to characterize the junctions of integrated DNA and host genomic DNA [22-25]. RT-PCR analysis was substituted and succeeded in obtaining the sufficiently clear signals with a *CodA* band, at a length of about 1.9 kb.

4. Conclusions

More than 80% lily bulb scales slices, being co-cultivated with *A. tumefaciens,* were successfully formed the bulblets with coefficient around 0.8. These bulblets had been selected by culturing in medium added 75 mg/L hygromycin that made up 6% to 17% samples to Hygr after three months. Some Hygr lines expressed heat tolerance *in vitro* when culturing in high temperate (37 °C). Especially, one of heat tolerant line initially showed the *CodA* gene expression at the transcriptional level (RT-PCR reaction) and accumulated nearly four times amount of GB higher than that of non-transgenic lines (WT).

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