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Original Article

Development of somatic embryos for genetic transformation in *Curcuma longa* L. and *Curcuma mangga* Valeton & Zijp

Vachiraporn Pikulthong,[a] Tharathorn Teerakathiti,[b] Arinthip Thamchaipenet,[c] Surin Peyachoknagul[c, *]

[a] *Bioscience Program, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[b] *National Center for Genetic Engineering and Biotechnology, Pathum Thani 12120, Thailand*
[c] *Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

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ABSTRACT

Buds from rhizomes of *Curcuma longa* L. variety 'Chumphon' and *Curcuma mangga* Valeton & Zijp variety 'Phetchaburi' were cultured on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L N^6-benzyladenine (BA) for multiple shoot induction. Their shoots were cultured on MS medium supplemented with various concentrations of one of two plant growth regulators or a combination of both—2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA). Interestingly, the medium containing both auxins (5 mg/L 2,4-D and 5 mg/L NAA) was best for somatic embryo induction after culturing for 4 weeks. Somatic embryo formation reached 87.50% for *Curcuma longa* and 95.83% for *Curcuma mangga* with a high quality of loose, friable and yellowish characters. The best conditions for the formation of shootlets occurred after transferring the somatic embryo to MS medium supplemented with 3.0 mg/L BA, 0.5 mg/L NAA and 3% maltose. The shootlets were rooted by transferring to MS medium containing 3.0 mg/L NAA. This is the first report of a complete *in vitro* regeneration system from somatic embryos of *C. longa* and *C. mangga* which was further used for gene manipulation in these plants. Diketide CoA synthase (*DCS*) and curcumin synthase (*CURS*) genes, which are the two genes involved in curcuminoid biosynthesis in turmeric, were cloned and transferred to these two species using *Agrobacterium*-mediated transformation. The presence of both target and marker genes, *hpt*, in the transformed somatic embryos was confirmed by polymerase chain reaction assay. After culturing, the transformed somatic embryos could survive for 4 weeks.

Introduction

Turmeric (*Curcuma longa* L.) is a tropical herb belonging to the Zingiberaceae family and its rhizome is an important source of natural products called curcuminoids which have been widely used in traditional medicines (Medicinal Plant Research Institute, 2001). *Curcuma longa* is also frequently used as a food additive in Indian and Thai food, in cosmetics and in the pharmaceutical industry (Medicinal Plant Research Institute, 2001). Its underground rhizome has a very long history of medicinal uses due to its anti-tumor, anti-inflammatory, antioxidant, cholesterol-level-lowering and wound-healing properties (Viu et al., 2009; Roopadarshini, 2010). Curcuminoids in *C. longa* rhizome have been synthesized by the coordination of two enzyme clusters, diketide CoA synthase (DCS) and curcumin synthase (CURS), which are members of a type III polyketide synthase (Katsuyama et al., 2009a, 2009b). White turmeric (*Curcuma mangga* Valeton & Zijp) is a related species with a very low curcuminoid content and has been used only as a vegetable crop (Thaikert and Paisooksantivatana, 2009). *C. longa* and *C. mangga* are normally propagated vegetatively during the rainy season (Medicinal Plant Research Institute, 2001); therefore, their products are available only during certain periods of the year. The horticultural production of turmeric is also affected by many factors such as the slow propagation rate, soil-borne disease infection, deterioration of rhizomes caused by bacteria, and fungal and insect attacks (Shirgurkar et al., 2001). Improvement of these crops through conventional breeding is therefore difficult (Shirgurkar et al., 2001). The annual productivity of turmeric is also

* Corresponding author.
E-mail address: fscisrp@ku.ac.th (S. Peyachoknagul).
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affected by rhizome dormancy and irregular flowering (Nayak, 2000).

In vitro culture in some *Curcuma* species has been previously reported, for example, in *C. longa* (Salvi et al., 2000; Shirgurkar et al., 2001; Prathanturarug et al., 2003; Tyagi et al., 2004), in *C. mangga* (Raihana et al., 2011), in *Curcuma aromatica* (Nayak, 2000), in *Curcuma alismatifolia* (Mahadtanapuk et al., 2006), and in *Curcuma zedoaria* (Loc et al., 2005). Callus induction by the *Curcuma* genus is restricted to *C. longa* (Salvi et al., 2000), *Curcuma amada* (Prakash et al., 2004) and *C. aromatica* (Mohanty et al., 2008). However, there is no report of callus induction and somatic embryogenesis in *C. mangga* Valeton & Zijp. Therefore, the aim of this study was to apply a callus-mediated plantlet regeneration protocol, which would provide a prerequisite to horticultural production and genetic transformation for *C. longa* and *C. mangga*.

Genetic engineering is an important tool for the production of plants with desirable traits and is helpful to improve species with little genetic variation (Shirgurkar et al., 2006). Induction of secondary metabolite production in an *in vitro* culture of normal or transformed tissues is another way to obtain a desired product from plants (El-Nabarawy et al., 2015). At present, there are only a few reports on gene transfer in turmeric (Shirgurkar et al., 2006; He and Gang, 2014). Therefore, an efficient plant transformation protocol is necessary for the successful genetic manipulation of turmeric (He and Gang, 2014).

Materials and methods

Plant materials and shoot cultures

Healthy rhizome buds of *C. longa* L. variety 'Chumphon' and *C. mangga* Valeton & Zijp variety 'Phetchaburi' were collected from southern Thailand and kept in the dark to allow sprouting. Clean rhizome pieces with sprouted buds (1–2 cm) were excised and surface sterilized by treating in liquid soap for 5 min before thoroughly rinsing several times with running tap water. Explants were then immersed in 70% (volume per volume; v/v) ethanol for 30 s, soaked in 20% (v/v) Clorox® (Clorox Co., Ltd.; Oakland, CA, USA) with 0.1% Tween 20 for 20 min and washed 3–4 times with sterilized distilled water. Clean sprouts were dissected to remove the outer few layers of leaf sheath under aseptic conditions. Excised buds (0.5 cm each) were transferred to solid MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg/L N^6-benzyladenine (BA) and 30 g/L sucrose, the pH of the medium was adjusted to 5.7 before adding 2.5 g/L Phytagel® (Sigma–Aldrich, Co.; St Louis, MO, USA). The explants were subjected to shoot induction under culturing conditions at 25 ± 2 °C, having a light/dark regime of 16:8 h for 2 weeks. The plantlets were then transferred to a new medium for shoot multiplication for 4 weeks.

Somatic embryo induction

Somatic embryos of *C. longa* and *C. mangga* were induced by cutting the shoots longitudinally and cultured on solid MS medium supplemented with one of two plant growth regulators (PGRs) or a combination of both—2,4-dichlorophenoxyacetic acid (2,4-D) at 1 mg/L, 3 mg/L, 5 mg/L or 8 mg/L or naphthaleneacetic acid (NAA) at 1 mg/L, 3 mg/L, 5 mg/L or 8 mg/L. The percentage of somatic embryo induction was calculated after 4 weeks of culturing. A suitable concentration for the induction of somatic embryogenesis was selected for further experiments. Every 2 weeks, the somatic embryos were subcultured on solid MS medium containing the relevant PGR concentration or combination. Cultures were maintained at 25 ± 2 °C under dark conditions.

Each experiment was carried out in four replicates and each replicate contained six explants. For statistical analysis, SPSS version 11 (SPSS Inc., Chicago, IL, USA) was used and all data were analyzed using one-way ANOVA and Duncan's multiple range test. Differences at $p < 0.05$ were considered significant.

Shoot regeneration

Somatic embryos were transferred to a solid MS medium supplemented with various concentrations of BA and NAA. A suitable condition for shoot regeneration was selected. Shoot regeneration ability was further improved using MS media supplemented with different sugars (3% sucrose, 3% maltose and 1.5% sucrose plus 1.5% maltose). The culture was maintained at 25 ± 2 °C under a light/dark regime of 16:8 h for 5 weeks. Well developed plantlets with leaves were selected and transferred to MS medium for leaf elongation.

RNA extraction and reverse transcription

Total RNA was extracted from young healthy rhizome buds of *C. longa* using the CTAB method (Yu and Goh, 2000) and lithium chloride precipitation as described by Stiekema et al. (1988). DNA was eliminated from the total RNA solution using the RQ1 RNase-Free DNase treatment (Promega; Madison, WI, USA). Five micrograms of DNase-treated RNA were reverse transcribed using a SuperScript™III First-Strand Synthesis System (Invitrogen; Waltham, MA, USA) following the manufacturer's instructions. The obtained cDNAs were used as templates for polymerase chain reaction assay (PCR).

Amplification of full-length cDNAs and gene cloning

The full-length cDNA of diketide CoA synthase (*DCS*) and curcumin synthase2 (*CURS2*) were amplified using designed primers based on the previously reported complete coding sequences of these genes (National Center for Biotechnology Information; NCBI accession number AB495006 and number AB506762, respectively) as follows: DCS-F 5′–ATGGAAGCGAACGGCTAC–3′, DCS-R 5′–CTAGTTCAGTCTGCAACTATGG–3′, and CURS2-F 5′–ATGGCGATGATCAGCTTGCA–3′, CURS2-R 5′–CTAAAGCGGCACGCTTTGG–3′. The amplified products were cloned in pGEM®-T Easy vector (Promega; Madison, WI, USA) and transformed into *Escherichia coli* DH5α. Plasmid DNA was purified using a FavorPrep™ Plasmid DNA Extraction Mini Kit (FAVORGEN®, Taiwan), sequenced by Macrogen (Macrogen Inc.; Seoul, South Korea) and analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Construction of pCXUN expression vector

A pCXUN vector is the ZeBaTA stable expression vector derived from the binary vector pCAMBIA1300 (www.cambia.org). Amplification of 1170 bp of the *DCS* gene and 1176 bp of the *CURS2* gene from *C. longa* was carried out using the DCS or CURS2 primers for the *DCS* and *CURS2* genes, respectively. PCR products were cloned into a *Xcm*I-cut linearized pCXUN expression vector. The expression cassette, driven by the ubiquitin-1 promoter and terminated by a nopaline synthase terminator (*NOS*), was then transformed into *Agrobacterium tumefaciens* EHA105 by electroporation.

Test for concentration of selective agent

Non-transformed somatic embryos were tested for survival on MS1 medium containing 0 mg/L, 200 mg/L, 300 mg/L, 400 mg/L,

500 mg/L and 700 mg/L of cefotaxime or 0 mg/L, 10 mg/L, 20 mg/L, 30 mg/L, 50 mg/L and 70 mg/L of hygromycin (Table 1). Each treatment used 24 explants (six somatic embryos/plate, four plates for replication). Cultures were grown and subcultured every 2 weeks. Four weeks later, the survival of somatic embryos was observed.

Agrobacterium mediated plant transformation

A. tumefaciens EHA105 samples harboring pCXUN-*DCS* and pCXUN-*CURS2* were cultured in 5 mL of liquid LB medium containing 50 mg/L kanamycin and 50 mg/L rifampicin at 28 °C with continuous shaking at 200 revolutions per minute for 2 d. The density of bacteria was adjusted to OD_{600} (the absorbance, or optical density, of a sample measured at a wavelength of 600 nm) at 0.8, and added with 200 μM acetosyringone in MS3 medium (Table 1) and used as *Agrobacterium* inoculum. Somatic embryos of *C. longa* and *C. mangga* were immersed in bacterial suspension for 5 min. Excess bacteria were removed by decanting the liquid. Subsequently, the inoculated somatic embryos were transferred onto sterile filtered paper (Kumar et al., 2005) for blot-drying and then placed on MS4 medium (Table 1) for co-cultivation. The cultures were kept in the dark at 25 °C for 2 d. After co-cultivation, the inoculated somatic embryos were washed gently 3–4 times with sterile distilled water containing 300 mg/L cefotaxime and blotted dry on sterile filtered paper. The inoculated somatic embryos were cultured on MS5 (Table 1) and then transferred to MS6 (Table 1) selective medium, cultivated at 25 °C in the dark for 2 weeks. The selected somatic embryos were then transferred to regeneration medium (MS2, 300 mg/L cefotaxime and 30 mg/L hygromycin) and cultivated under the same conditions for shoot regeneration.

polymerase chain reaction analysis of transformed somatic embryos

Genomic DNA was extracted from transformed and non-transformed (control) somatic embryos using the CTAB method (Doyle and Doyle, 1987). PCR analysis was carried out using DCS-F or CURS2-F as the forward primer and a *NOS* terminator primer on the vector (5′-CGAATCTCAAGCAATCAAGC-3′) as a reverse primer. PCR was also performed with primers within the hygromycin phosphotransferase, *hpt* gene on the pCXUN vector (HPT-F primer; 5′–GCCTCCAGAAGAAGATGTTG–3′ and HPT-R primer; 5′-ATGTCCTGCGGGTAAATAGC-3′).

Results and discussion

Multiple shoot formation

A suitable concentration for multiple shoot formation of *C. longa* and *C. mangga* was the MS medium supplemented with 2 mg/L BA. At this concentration, the explants showed shoot formation within 7 d. However, differences in the shoot multiplication were not observed when the concentration of BA was increased to 3 mg/L (data not shown). It was found that at 2 mg/L BA, the explants responded readily to the culture conditions. Expansion and swelling of explants at the cut end were obtained within 1 week and 2–3 tubercules had emerged in 2 weeks, and then stretched to young shoots. After 2 weeks, roots were generated from the young shoots. Three weeks later, healthy and robust multiple shoots were well-developed and young leaves had emerged from shoots. The explants grown in MS medium containing 2 mg/L BA gave rise to 4–6 shoots/explant when regularly subcultured every 2 weeks. It was found that *C. mangga* was better developed into multiple shoots than *C. longa*. Strong and disease-free plants were obtained which could be further used for somatic embryogenesis induction. However, more than 6 month of subculturing of explants in the same medium resulted in small, stretched and weak plantlets.

Establishment of contamination-free cultures of curcuma was difficult because the explants were taken from sprouting rhizomes. However, once a healthy culture was established, there was no further contamination. For the *in vitro* multiplication of shoots, the use of a relatively high concentration of cytokinin combined with a lower concentration of auxin in the medium has been recommended (Rahman et al., 2004). Both NAA and BA induced multiple shoots and roots in ginger vegetative bud explants; however, the culture responded differently in different treatments (Salvi et al., 2001; Nasirujjaman et al., 2005).

In the present study, shoot multiplication of both *Curcuma* species was induced using BA regardless of the concentration of auxin in the medium. It is believed that a small quantity of auxin may be synthesized by apical rhizome buds grown *in vitro;* therefore, exogenous auxin was unnecessary. BA was also one of the most potent and affordable cytokinins to induce multiple shoots (Balachandran et al., 1990; Naz et al., 2009). The use of BA in the development of a healthy plant system in *Curcuma* species is in agreement with earlier reports (Rahman et al., 2004; Naz et al., 2009). The shoots obtained were successfully rooted with high frequency regeneration and opened up the potential of using this explant in somatic embryogenesis induction for genetic manipulation of these two species.

Somatic embryo induction

For somatic embryo induction, *in vitro* shoot bud sections of about 0.5–1.0 cm in length were cultured horizontally on MS media supplemented with two different PGRs—NAA (1 mg/L, 3 mg/L, 5 mg/L and 8 mg/L) and 2,4-D (1 mg/L, 3 mg/L, 5 mg/L and 8 mg/L)—at various concentrations (Fig. 1). After 2 weeks, callus formation started at the cut end of the explants. The somatic embryos of *C. longa* and *C. mangga* were induced from vegetative bud explants using either NAA or 2,4-D alone, but the induction was not efficient. An increase or decrease in the NAA or 2,4-D concentrations resulted in the reduction of somatic embryo formation. At lower concentrations the somatic embryos were pale yellowish and friable in appearance, whereas at higher concentrations they became brownish and semi-compact.

In the solid MS media with NAA or 2,4-D alone, pale-yellowish granular and soft, friable callus was developed from the incision

Table 1
Composition of media used for *Agrobacterium*-mediated transformation.

Medium	Composition
MS1 (somatic embryo induction)	Murashige and Skoog (MS) medium, 5.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 5.0 mg/L naphthaleneacetic acid (NAA)
MS2 (regeneration)	MS medium, 3.0 mg/L N6-benzyladenine (BA), 0.5 mg/L NAA, 3% maltose
MS3 (*Agrobacterium* suspension)	Liquid MS medium, 5.0 mg/L 2,4-D, 5.0 mg/L NAA with 200 μM acetosyringone added just before use
MS4 (co-cultivation)	MS medium, 5.0 mg/L 2,4-D, 5.0 mg/L NAA with 200 μM acetosyringone
MS5 (resting)	MS medium, 5.0 mg/L 2,4-D, 5.0 mg/L NAA, 300 mg/L cefotaxime
MS6 (selection)	MS medium, 5.0 mg/L 2,4-D, 5.0 mg/L NAA, 300 mg/L cefotaxime, 30 mg/L hygromycin

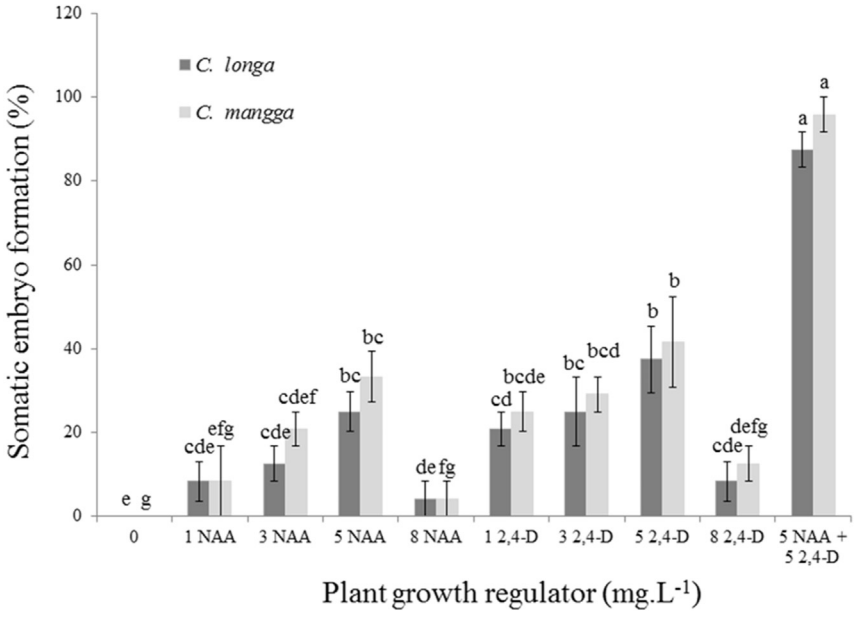

Fig. 1. Percentage of somatic embryos induction of *Curcuma longa* and *Curcuma mangga* after culturing for 5 weeks in different concentrations of the plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA). The same lowercase letters above bars indicate no significant difference using Duncan's multiple range test at $p < 0.05$.

surface of the explant within 2 weeks. It was clearly shown that NAA alone could induce callus production while 2,4-D alone produced a few somatic embryos. Although 3 mg/L and 5 mg/L NAA induced a few soft, somatic embryos in *C. longa* and *C. mangga* explants, the induced somatic embryos from both PGRs could not continuously proliferate and eventually turned brown when the culture was maintained for a long time. However, some explants could differentiate into green shoot primodia with root formation. When the concentration of NAA or 2,4-D was increased to 8 mg/L, somatic embryo formation was reduced to 4.2% and 8.3% in *C. longa*, and 4.2% and 12.5% in *C. mangga*, respectively.

The best conditions for somatic embryo induction were observed in the medium having 5 mg/L NAA together with 5 mg/L 2,4-D (Table 3). After 1 week culturing of explants in this medium, the wounded surface of the tips became swollen. Compact, soft, mucilaginous, yellowish callus and watery callus were observed within 5 weeks (Fig. 2B and F). A mass of callus was formed and multiplied. Then friable, yellowish, granular, somatic embryos were formed on the surface of the callus after further culturing. This type of callus is a somatic embryo which was chosen for multiplication and the somatic embryos were subcultured every 2 weeks. It was clearly shown that the combination of 2,4-D and NAA could induce a continuous growth of somatic embryos. *C. longa* and *C. mangga* had the highest production rate of somatic embryos on the MS medium containing 5 mg/L 2,4-D and 5 mg/L NAA, but there was no correlation observed between the time required and the rate of somatic embryo formation. However, *C. mangga* showed higher somatic embryogenesis compared to *C. longa*. After 36 d of culturing in the dark, the somatic embryos of *C. mangga* became yellowish, friable and granular callus, whereas *C. longa* needed 60 d to induce the somatic embryos with a mucilaginous, yellowish and friable appearance (Fig. 2). In contrast, the highest amount of shoot formation was obtained in the medium with no PGR and this medium was also good for root formation (Table 2).

Table 2
Effects of different plant growth regulators on shoot and root formation of *Curcuma longa* and *Curcuma mangga* on Murashige and Skoog medium containing naphthaleneacetic acid (NAA) alone, 2,4-dichlorophenoxyacetic acid (2,4-D) alone and a combination of NAA and 2,4-D after 4 weeks.

Plant growth regulator (mg/L)		Shoot formation* (%)		Root formation* (%)	
NAA	2,4-D	*C. longa*	*C. mangga*	*C. longa*	*C. mangga*
0	0	0.79 ± 0.10^{a}	0.92 ± 0.04^{a}	0.67 ± 0.06^{a}	0.75 ± 0.04^{a}
0	1	0^{d}	0^{d}	0^{d}	0^{d}
0	3	0^{d}	0^{d}	0^{d}	0^{d}
0	5	0^{d}	0^{d}	0^{d}	0^{d}
0	8	0^{d}	0^{d}	0^{d}	0^{d}
1	0	0.38 ± 0.04^{bc}	0.38 ± 0.04^{b}	0.38 ± 0.04^{b}	0.37 ± 0.04^{b}
3	0	0.45 ± 0.08^{b}	0.46 ± 0.07^{b}	0.75 ± 0.11^{a}	0.75 ± 0.04^{a}
5	0	0.21 ± 0.15^{cd}	0.25 ± 0.04^{c}	0.21 ± 0.10^{c}	0.25 ± 0.04^{c}
8	0	0.04 ± 0.08^{d}	0.08 ± 0.04^{d}	0.08 ± 0.04^{cd}	0.17 ± 0.00^{c}
5	5	0^{d}	0^{d}	0^{d}	0^{d}

*Mean values of 24 replications (±SE) followed by the same letter within a column are not significantly different by Duncan's multiple range test at $p \leq 0.05$.

Table 3
Effects of different plant growth regulators on somatic embryogenesis induction of *Curcuma longa* and *Curcuma mangga* on Murashige and Skoog medium containing naphthaleneacetic acid (NAA) alone, 2,4-dichlorophenoxyacetic acid (2,4-D) alone and a combination of NAA and 2,4-D after 4 weeks.

Plant growth regulator (mg/L)		Nature of somatic embryos*		Color of somatic embryos* (brown to yellowish = 0–4)	
NAA	2,4-D	*C. longa*	*C. mangga*	*C. longa*	*C. mangga*
0	0	0^{e}	0^{f}	0^{f}	0^{f}
1	0	0.79 ± 0.16^{cd}	0.86 ± 0.25^{cd}	1.75 ± 0.22^{bcd}	1.88 ± 0.28^{bcd}
3	0	1.25 ± 0.40^{bc}	2.00 ± 0.56^{b}	2.13 ± 0.25^{bc}	2.00 ± 0.56^{bcd}
5	0	1.50 ± 0.58^{b}	2.00 ± 0.30^{b}	2.67 ± 0.58^{ab}	2.71 ± 0.58^{ab}
8	0	0.58 ± 0.29^{d}	0.67 ± 0.30^{de}	1.04 ± 0.53^{de}	1.17 ± 0.47^{de}
0	1	1.13 ± 0.21^{bc}	1.17 ± 0.24^{c}	1.38 ± 0.83^{cde}	1.50 ± 0.86^{cd}
0	3	1.17 ± 0.43^{bc}	1.25 ± 0.32^{c}	1.92 ± 0.82^{bcd}	2.04 ± 0.77^{bc}
0	5	1.38 ± 0.50^{b}	1.38 ± 0.50^{c}	2.38 ± 1.05^{ab}	2.17 ± 0.41^{bc}
0	8	0.33 ± 0.24^{de}	0.33 ± 0.14^{ef}	0.50 ± 0.36^{ef}	0.54 ± 0.28^{ef}
5	5	2.25 ± 0.17^{a}	2.50 ± 0.24^{a}	3.08 ± 0.29^{a}	3.42 ± 0.62^{a}

*Mean values of 24 replications (±SE) followed by the same letter within a column are not significantly different by Duncan's multiple range test at $p \leq 0.05$.

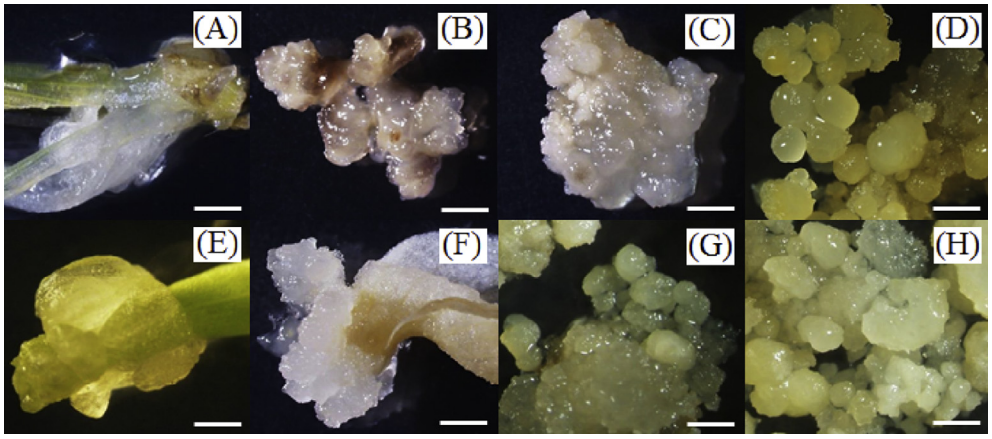

Fig. 2. Different stages of somatic embryo induction of *Curcuma longa* (A–D) and *Curcuma mangga* (E–H) on Murashige and Skoog (MS) medium supplemented with 5 mg/L 2,4-dichlorophenoxyacetic acid and 5 mg/L naphthaleneacetic acid for 1 week: (A, E) cultured explants; (B, F) mucilaginous callus formation at the cut end from the explants after 5 weeks; (C, G) mass of somatic embryos developed from shoot bud after 9 weeks; (D, H) cluster of somatic embryos. (scale bar = 100 μm).

The response of tissues to become somatic embryos depends on the chemical nature, the combination and the concentration of PGRs according to the plant species and the origin of the explant. It may be assumed that the response of the base of the stem of *C. longa* and *C. mangga* was due to the existence of a meristematic area observed in the basal disk, from which the leaves and the roots were produced (Zapata et al., 2003). Accordingly, the shoot base explants and the combination of 2,4-D and NAA were selected for somatic embryo induction.

Although there are some reports on callus induction under light and dark conditions (Mohanty et al., 2008; Zhang et al., 2011), it seems that the light conditions had no obvious influence on callus induction, but did have some effect on the state of the callus. The callus cultured under light conditions gave a tighter structure and more regular shape with a white color, while that under dark conditions was less compact and more irregular with a yellowish color. The current results clearly showed that large, loose, friable, somatic embryos with no brown coloration were induced at a significant level under dark conditions. The somatic embryos were separated and used for subsequent cultures on the same medium. The differentiation of somatic embryos from successively sub-cultured calli for shoot initiation was tested in the next steps.

Shoot regeneration from somatic embryos

Shoot initiation was induced by culturing 2 week-old somatic embryos on MS media supplemented with various concentrations of BA and NAA. Somatic embryos were maintained for 5 weeks under these conditions. After this period, the beginning of shoot primordia was observed in all treated somatic embryos (Fig. 3). The MS medium without growth regulator failed to induce shoot formation from somatic embryos, nor in the culture medium supplemented with 20% coconut water. The yellowish tissue was unable to develop on the surface of somatic embryos within 5 weeks. In contrast, MS medium with different combinations of BA and NAA showed better results in somatic embryo response to shoot initiation (Fig. 3).

The media with different concentrations of BA (0.5–2.0 mg/L) combined with NAA (0.5–1.0 mg/L) were unable to promote greenish meristematic tissue on the surface of the somatic embryo when maintained for a normal observation period of 5 weeks. The tested MS medium with 3.0 mg/L BA and 0.5 mg/L NAA showed the best response, with green spots and proliferated calli appearing on the surface of somatic embryos within 5 weeks. The shootlets were elongated after 5 weeks of culture. Although green meristematic tissue with green shootlets was obtained, it was unable to differentiate into plantlets after 5 weeks of culturing.

Higher concentrations of BA (4.0–5.0 mg/L) with 0.5 mg/L NAA showed lower callus formation which contained green spots, was white in color and had a dry texture on the surface of somatic embryos. When 1.0 mg/L thidiazuron (TDZ) was added in the MS medium with 5.0 mg/L benzyladenine (BA) and 0.5 mg/L NAA, the groups of yellow–green increased, and loose callus with a white color and dry texture was observed on the surface of the callus. However, these groups were unable to develop into shootlets when maintained on the same medium over a long period.

It has been reported that the addition of either BA or NAA in the culture medium improved the response of *C. longa* callus as well as of Zingiberaceae (Balachandran et al., 1990). The combination of BA and NAA was also needed to produce more multiple shoots of *C. longa* (Noguchi and Yamakawa, 1998). In the current report, the induction of shoots was achieved by the addition of auxin and cytokinin. The optimum response was observed in 3.0 mg/L BA combined with 0.5 mg/L NAA which was the same as the regeneration medium used for *C. aromatica* (Mohanty et al., 2008). In many *Curcuma* species, callus is known to be capable of plant regeneration (Salvi et al., 2001; Prakash et al., 2004; Mohanty et al., 2008; Zhang et al., 2011). Therefore, the results of the current study provide a good tool to study plant cell metabolism, beside providing the precursors needed for certain plant species to trigger cell differentiation.

Although sucrose is the most commonly used carbohydrate for shoot initiation (Vu et al., 1993), it promoted poor results for cell growth of both species studied. Two-week-old somatic embryos of the two plant species were further tested on the MS medium containing 3.0 mg/L BA and 0.5 mg/L NAA along with different types of sugar, (sucrose alone, maltose alone and sucrose combined with maltose). Shoots were observed after 5 weeks of culturing in the different media (Fig. 4). Among the different types of sugar, the medium containing sucrose as standard sugar produced shoots with short, thin, unfolded leaves, and the roots were relatively thin and short. The combination of sucrose and maltose produced green, spotted calli, with delicate and stunted shootlets with unfolded leaves. Having maltose in the medium produced the best results with healthy, green shootlets with one or two leaves longer and better than in the other treatments. From this investigation, the difference between sucrose and maltose offers the possibility of using alternative sugars as carbon sources for shoot initiation. In

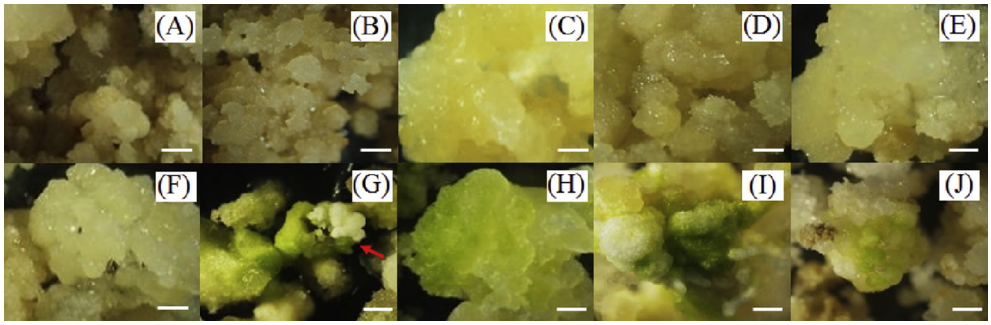

Fig. 3. Effect of Murashige and Skoog (MS) medium supplemented with various concentration of plant growth regulator to induce organogenesis in *Curcuma longa* after culture for 5 weeks: (A) MS medium; (B) MS + 20% coconut water; (C) MS + 0.5 mg/L N^6-benzyladenine (BA) + 0.5 mg/L kinetin + 1 mg/L naphthaleneacetic acid (NAA); (D) MS + 1 mg/L BA + 0.5 mg/L NAA; (E) MS + 1 mg/L BA + 1 mg/L NAA; (F) MS + 2 mg/L BA + 1 mg/L NAA; (G) MS + 3 mg/L BA+0.5 mg/L NAA, where arrow shows shoot formation; (H) MS + 4 mg/L BA + 0.5 mg/L NAA; (I) MS + 5 mg/L BA + 0.5 mg/L NAA; (J) MS + 1 mg/L BA + 1 mg/L NAA + 1 mg/L thidiazuron. (scale bar = 100 μm).

conclusion, maltose was the most suitable and effective carbon source for the cell growth of both the species studied. Finally, the shootlets were transferred to the MS medium supplemented with 3.0 mg/L NAA for root formation under a light/dark photoperiod (Fig. 5). The plantlets were regenerated and produced prolific roots within 5 weeks.

Cloning of cDNAs for DCS and CURS2 and plasmid construction

Total RNA was extracted from rhizomes of *C. longa* and cDNA encoding curcuminoid biosynthesis genes were synthesized using PCR. As a result, a 1170 bp product of the *DCS* gene and a 1176 bp product of the *CURS2* gene were successfully amplified. The cDNA complete coding sequences were submitted to the DNA Data Bank of Japan under the accession numbers LC064067 for *DCS* and LC064068 for *CURS2*. The sequence identity between the *DCS* gene and the sequence in the GenBank (accession number AB495006) was 99% and the sequence identity between the *CURS2* gene and the sequence in the GenBank (accession number AB506762) was also 99%. Pair-wise analyses of the translated amino acid sequences of the DCS and CURS2 proteins indicated that they have overall identities of 62.9–99.7% among themselves (Fig. 6). The amino acid identity between DCS and data from GenBank (accession number BAH56225) and amino acid identity between CURS2 and data from the GenBank (accession number BAH85780) was 98.7%. Because of the high sequence similarity of the *DCS* and *CURS2* genes and the deduced amino acid sequences with recently reported curcuminoid synthesizing genes of *C. longa* (Katsuyama et al., 2009a, 2009b), it was hypothesized that these genes might also have a significant role in curcuminoid biosynthesis. The putative DCS and CURS2 proteins possess a Cys-His-Asn catalytic triad which is conserved in all known type III polyketide synthase (Jiang et al., 2008), suggesting that these genes are functional and can catalyze the formation of curcuminoid in the *C. longa* rhizome. However, the tissue-specific expression patterns have not been investigated. In the current study, the two biosynthetic genes, *DCS* and *CURS2* were cloned from *C. longa* rhizome which could be used for genetic transformation for over expression and increase production of curcuminoid compositions in turmeric rhizome. The *DCS* and *CURS2* genes were inserted into pCXUN plasmid (Chen et al., 2009) (Fig. 7) to become pCXUN-*DCS* and pCXUN-*CURS2* for plant transformation.

Sensitivity of explants to hygromycin and cefotaxime

Prior to transformation, an optimal concentration of antibiotic for the selection of transformed tissue was determined by culturing somatic embryos of *C. longa* and *C. mangga* on MS medium containing various concentration of cefotaxime and hygromycin. Cefotaxime at the concentration of 300 mg/L was sufficient for somatic embryos of *C. longa* and *C. mangga* to survive. This concentration had no effect on the growth of somatic embryos, but could control *Agrobacterium* growth. Hygromycin at the concentration of 30 mg/L caused almost total inhibition of somatic embryos, so this concentration was used for selection of transformed somatic embryos of *C. longa* and *C. mangga*.

Gene transformation

Somatic embryos of *C. longa* and *C. mangga* were inoculated with *Agrobacterium* containing either pCXUN-*DCS* or pCXUN-*CURS2*, or both, for 5 min because any longer period of incubation caused problems in the elimination of bacteria and contamination in subsequent *in vitro* cultures of somatic embryos. After inoculation, the somatic embryos were incubated for co-cultivation for 2 d. *Agrobacterium* was eliminated from the somatic embryos by

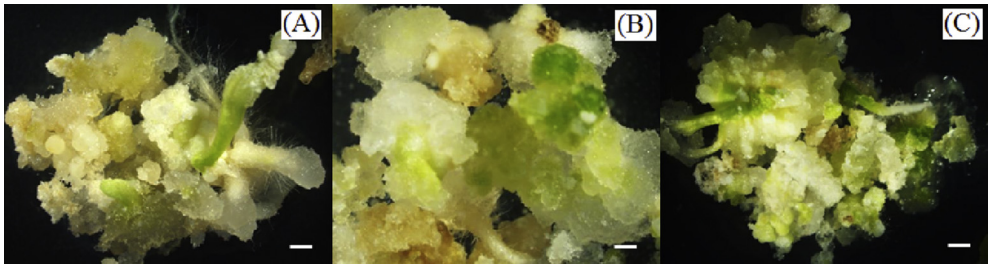

Fig. 4. Effect of different sugars in Murashige and Skoog medium with 3.0 mg/L N^6-benzyladenine and 0.5 mg/L naphthaleneacetic acid for shoot initiation of *Curcuma longa* for 5 weeks: (A) 3% sucrose; (B) 1.5% sucrose and 1.5% maltose; (C) 3% maltose. (scale bar = 100 μm).

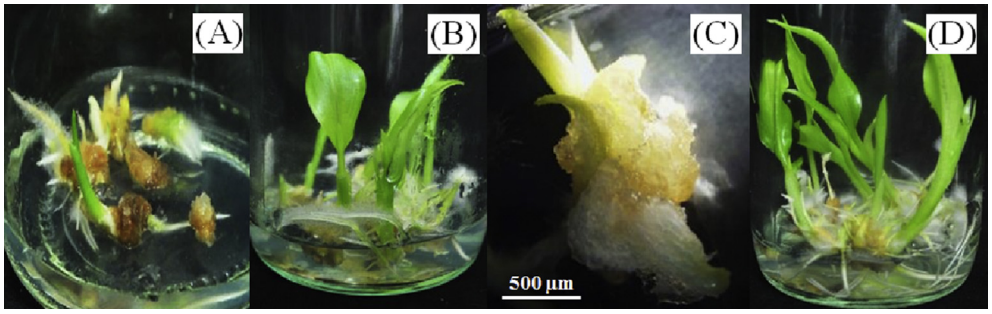

Fig. 5. Growth of *Curcuma longa* and *Curcuma mangga* somatic embryo after 5 weeks of culture on Murashige and Skoog medium containing 3.0 mg/L naphthaleneacetic acid for plant regeneration: (A) shoot initiation of *C. longa*; (B) plant regeneration of *C. longa*; (C) shoot initiation of *C. mangga*; (D) plant regeneration of *C. mangga*.

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DCS          ---MEANGYRITHSADGPATILAIGTANPTNVVDQNAYPDFYFRVTNSEHLQELKAKFRR 57
BAH56225     ---MEANGYRITHSADGPATILAIGTANPTNVVDQNAYPDFYFRVTNSEYLQELKAKFRR 57
CURS2        MAMISLQAMRKAQRAQGPATILAVGTANPPNLYEQDTYPDYYFRVTNSEHKQELKNKFRR 60
BAH85780     MAMISLQAMRKAQRAQGPATILAVGTANPPNLYEQDTYPDYYFRVTNSEHKQELKNKFRL 60
                :. :. * :: *:*******:*****.*: :*::***:******** **** ***

DCS          ICEKAAIRKRHLYLTEEILRENPSLLAPMAPSFDARQAIVVEAVPKLAKEAAEKAIKEWG 117
BAH56225     ICEKAAIRKRHLYLTEEILRENPSLLAPMAPSFDARQAIVVEAVPKLAKEAAEKAIKEWG 117
CURS2        MCEKTMVKRRYLYLTPEILKERPKLCSYMEPSFDDRQDIVVEEVPKLAAEAAEKAIKEWG 120
BAH85780     MCEKTMVKRRYLYLTPEILKERPKLCSYMEPSFDDRQDIVVEEVPKLAAEAAENAIKEWG 120
             :***: :::*:**** ***:*.*.* : * **** ** **** ***** ****:******

DCS          RPKSDITHLVFCSASGIDMPGSDLQLLKLLGLPPSVNRVMLYNVGCHAGGTALRVAKDLA 177
BAH56225     RPKSDITHLVFCSASGIDMPGSDLQLLKLLGLPPSVNRVMLYNVGCHAGGTALRVAKDLA 177
CURS2        GDKSAITHLVFCSISGIDMPGADYRLAKLLGLPLAVNHLMLYSQACHMGAAMLRIAKDIA 180
BAH85780     GDKSAITHLVFCSISGIDMPGADYRLAQLLGLPLAVNRLMLYSQACHMGAAMLRIAKDIA 180
               ** ******** *******:* :* :***** :**::***. .** *.: **:***:*

DCS          ENNRGARVLAVCSEVTVLSYRGPHPAHIESLFVQALFGDGAAALVVGSDPVDGVERPIFE 237
BAH56225     ENNRGARVLAVCSEVTVLSYRGPHPAHIESLFVQALFGDGAAALVVGSDPVDGVERPIFE 237
CURS2        ENNRSARVLVVACEITVLSFRGPDERDFQALAGQAGFGDGAGAMIAGADPVLGVERPLYH 240
BAH85780     ENNRSARVLVVACEITVLSFRGPDERDFQALAGQAGFGDGAGAMIVGADPVLGVERPLYH 240
             ****.****.*..*:****:***.  .:::*  ** *****.*::.*:*** *****::.

DCS          IASASQVMLPESAEAVGGHLREIGLTFHLKSQLPSIIASNIEQSLTTACSPLGLSDWNQL 297
BAH56225     IASASQVMLPESAEAVGGHLREIGLTFHLKSQLPSIIASNIEQSLTTACSPLGLSDWNQL 297
CURS2        IMSATQTTVPESEKAVGGHLREVGLTFHFFNQLPAIIADNVGNSLAEAFEPIGIKDWNNI 300
BAH85780     IMSATQTTVPESEKAVGGHLREVGLTFHFFNQLPAIIADNVGNSLAEAFEPIGIKDWNNI 300
             * **:*. :*** :********:*****: .***:***.*: :**: * .*:*:.***::

DCS          FWAVHPGGRAILDQVEARLGLEKDRLAATRHVLSEYGNMQSATVLFILDEMRNRSAAEGH 357
BAH56225     FWAVHPGGRAILDQVEARLGLEKDRLAATRHVLSEYGNMQSATVLFILDEMRNRSAAEGH 357
CURS2        FWVAHPGNWAIMDAIETKLGLEQSKLATARHVFSEFGNMQSATVYFVMDELRKRSAAENR 360
BAH85780     FWVAHPGNWAIMDAIETKLGLEQSKLATARHVFSEFGNMQSATVYFVMDELRKRSAAENR 360
             **..***. **:* :*::****:.:**::***:**:******** *::**:*:*****.:

DCS          ATTGEGLDWGVLLGFGPGLSIETVVLHSCRLN 389
BAH56225     ATTGEGLDWGVLLGFGPGLSIETVVLHSCRLN 389
CURS2        ATTGDGLRWGVLFGFGPGISIETVVLQSVPL- 391
BAH85780     ATTGDGLRWGVLFGFGPGISIETVVLQSVPL- 391
             ****:** ****:*****:*******:*  *
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Fig. 6. Alignment of type III PKS amino acid sequences of curcuminoid biosynthesis gene generated by CLUSTALW: *DCS* and BAH56225 (diketide CoA synthase); *CURS2* and BAH85780 (*curcuminoid synthase2*). The amino acids of the catalytic Cys-His-Asn triad are marked by rectangular boxes. The star below each line of alignment indicates identical sites.

Fig. 7. Structures of the pCXUN-*DCS*/*CURS2* overexpression cassette generated by *Xcm*I digestion. LB, T-DNA left border; RB, T-DNA right border.

culturing on MS5 medium for 2 weeks. Then, the fresh, surviving, somatic embryos were transferred to MS6 containing hygromycin for selection of the transformed tissue. A few somatic embryos of *C. longa* and *C. mangga* survived (Fig. 8) and formed new somatic embryos on MS6 medium after 4–5 weeks but non-transformed somatic embryos of *C. longa* and *C. mangga* could not survive (Fig. 8A and E).

There are few reports (discussed below) describing genetic transformation of *C. longa* and *C. mangga* using somatic embryos. Transformed somatic embryos of *C. longa* and *C. mangga* were established by *Agrobacterium*-mediated transformation using pCXUN plasmid containing the *DCS* and/or *CURS2* genes and the transformation efficiency was reported to vary depending on the bacterial strain, composition of the culture medium and concentration of *Agrobacterium* (Suma et al., 2008; He and Gang, 2014). In the current study, the optimum concentration of *Agrobacterium* EHA105 for co-cultivation of the somatic embryos of *C. longa* and *C. mangga* was 0.8–1.0 OD_{600}. The transformation efficiency was determined by observing the survival of somatic embryos on MS6 selection medium. However, the transformed somatic embryos were unable to grow and became necrotic after 4 weeks. *A. tumefaciens* EHA105 used in this study, was reported to be a highly virulent strain and so would also damage plant cells, which resulted in lower cell recovery and reduced the transformation efficiency (Ziemienowicz, 2014). However, the transformation of somatic embryos using EHA105 which resulted in survival at this stage was better than anticipated. Therefore, the concentration of *Agrobacterium* and the inoculation time should be adjusted for better results. However, the availability of an *in vitro* regeneration system is a prerequisite for effective genetic transformation. The current study clearly showed that *Agrobacterium* with a pCXUN expression vector was an efficient system for *Agrobacterium*-mediated transformation in *C. longa* and *C. mangga*.

Screening of transgene in transformed tissue by polymerase chain reaction

DNA extracts from transformed and non-transformed control somatic embryos were used for PCR analysis using *hpt* and gene-specific primers. The 573 bp amplified fragment from the *hpt* gene was detected in transformed somatic embryos of *C. longa* and *C. mangga* but not in the control (Fig. 9). Because there are endogenous *DCS* and *CURS* genes in their tissue, other PCR was performed with DCS-F and NOS-R primers or CURS2-F and NOS-R primers. The amplified product of the *DCS* transgene was found in transformed somatic embryos of *C. longa* and *C. mangga*, but not in the control sample (Fig. 10). The size of the band confirmed the presence of the *DCS* transgene in these somatic embryos. In contrast, the band of the *CURS2* gene was not found in the transformed somatic embryos of either *C. longa* or *C. mangga*.

The bud explants of *C. longa* var. 'Chumphon' and *C. mangga* var. 'Phetchaburi' cultured in MS medium supplemented with 2.0 mg/L BA for 4 weeks could regenerate shoots at 4–6 shoots/explant. The

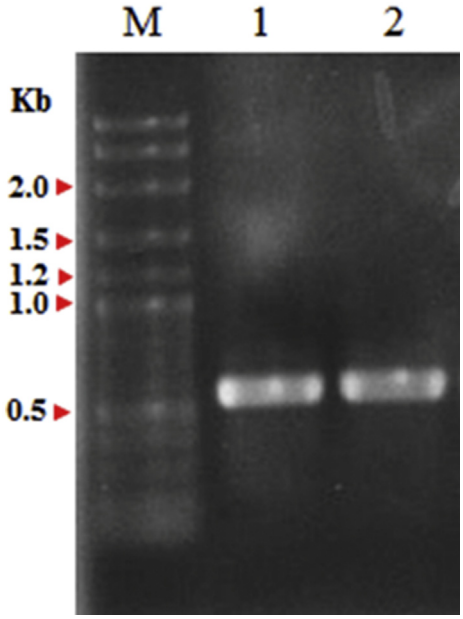

Fig. 9. Detection of 573 bp polymerase chain reaction products of *hpt* gene fragment in transformed somatic embryos of *Curcuma longa* (1) and *Curcuma manga* (2). Lane M is a DNA marker.

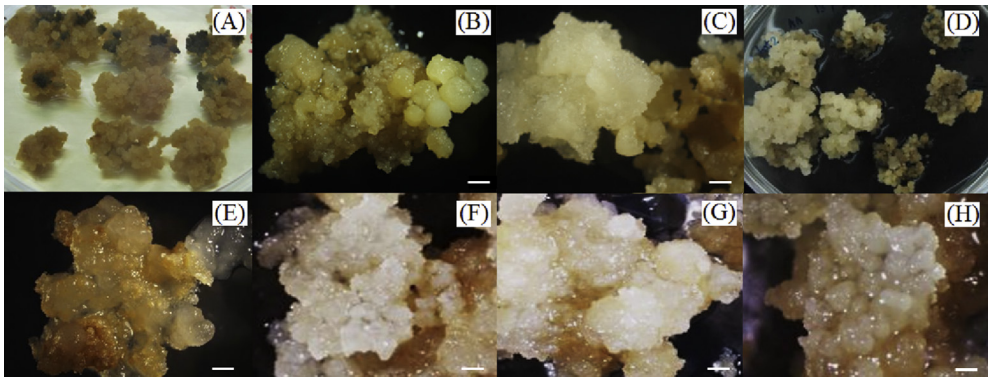

Fig. 8. *Agrobacterium*-mediated transformation of *Curcuma longa* and *Curcuma mangga* cultured on MS6 medium (see Table 1) for 4 weeks: (A, E) untransformed somatic embryos; (B, F) pCXUN-*DCS* transformed somatic embryos; (C, G) pCXUN-*CURS2* transformed somatic embryos (D, H) pCXUN-*DCS* and pCXUN-*CURS2* transformed somatic embryos (scale bar = 100 μm).

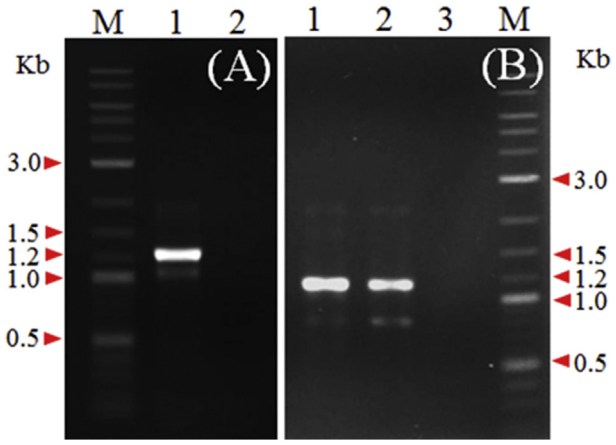

Fig. 10. Detection of about 1200 bp polymerase chain reaction assay products of *DCS* gene in transformed somatic embryos of *Curcuma longa* (A) and *Curcuma mangga* (B): M is the 2-Log DNA ladder markers; (A) transformed *C. longa* (lane 1) and non transformed tissue (lane 2); (B) transformed *C. mangga* (lanes 1, 2); and non transformed tissue (lane 3).

in vitro shoots were suitable for somatic embryo induction in which a modified MS medium supplemented with 5 mg/L 2,4-D and 5 mg/L NAA (MS1) was the optimal medium for somatic embryo induction under dark conditions. These conditions could increase growth resulting in a high quality, loose, friable, somatic embryo that was yellowish in color. The shoots were then re-induced in MS medium with 3.0 mg/L BA and 0.5 mg/L NAA and 3% maltose and the formation of roots occurred by transferring shoots to MS medium containing 3.0 mg/L NAA.

cDNAs of complete coding *DCS* and *CURS2* sequences were cloned from *C. longa* rhizomes and inserted into pCXUN plasmid. These plasmids were transformed to *Agrobacterium* for transformation of *C. longa* and *C. mangga*.

The availability of an *in vitro* regeneration system is a prerequisite for effective genetic transformation. The current work showed that the *Agrobacterium*-mediated transformation of somatic embryos of *C. longa* and *C. mangga* gave rise to transformed somatic embryos on selective medium. These results showed that somatic embryo culture is an efficient system for *Agrobacterium*-mediated transformation in these plants. Optimized transformation conditions were established using 2 week-old somatic embryos, co-cultivated with 0.8–1.0 OD_{600} *Agrobacterium* for 5 min with the culture being continued on MS1 medium without antibiotics for 2 d. The target *DCS* gene and marker gene, *hpt*, were detected in transformed somatic embryos.

Therefore, the protocol reported here is feasible for gene transfer in *C. longa* and *C. mangga* for which conventional breeding is impossible owing to its sterile nature. Further study on the function of *DCS* and *CURS2* in responding to curcuminoid biosynthesis will contribute to a better understanding of the curcuminoid biosynthetic pathway in *C. longa*.

Conflict of interest

There is no conflict of interest.

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