Agriculture and Natural Resources 50 (2016) 276–285

Contents lists available at ScienceDirect

Agriculture and Natural Resources

journal homepage: http://www.journals.elsevier.com/agriculture-and-natural-resources/

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*

ARTICLE INFO

Article history:
Received 23 June 2015
Accepted 6 August 2015
Available online 8 October 2016

Keywords:
Agrobacterium-mediated transformation
Curcuma mangga Valeton & Zijp
Curcuminoid
Somatic embryogenesis
Turmeric (*Curcuma longa* L.)

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).
Peer review under responsibility of Kasetsart University.

http://dx.doi.org/10.1016/j.anres.2015.08.004

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,