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Research article

Cloning and characterization of an elicitor-responsive gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase involved in 20-hydroxyecdysone production in cell cultures of *Cyanotis arachnoidea*

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ABSTRACT

Cyanotis arachnoidea contains a rich source of bioactive phytoecdysteroids (i.e. analogues of insect steroid hormones). 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) supplies mevalonate for the synthesis of many secondary metabolites including 20-hydroxyecdysone (20E), one of metabolism-enhancing phytoecdysteroids. In this study, in order to develop a sustainable source of 20E, cell suspension cultures were established from shoot cultures of *C. arachnoidea*, and a full length cDNA encoding HMGR (designated as *CaHMGR*) was cloned and characterized. The cDNA contained 2037 nucleotides with a complete open reading frame (ORF) of 1800 nucleotides, which was predicted to encode a peptide of 599 amino acids. Expression analysis by real-time PCR revealed that *CaHMGR* mRNA was abundant in *C. arachnoidea* stems, roots and leaves. When cultivated in Murashige & Skoog medium supplemented with 0.2 mg L^{-1} 1-naphthlcetic acid (NAA) and 3.0 mg L^{-1} 6-benzyladenine (6-BA), *C. arachnoidea* cells in suspension culture grew rapidly, yielding 20E (124.14 μg L^{-1}) after 12 days. The content of 20E in cell cultures elicited by 0.2 mM methyl jasmonate (MeJA), 100 mg L^{-1} yeast elicitor (YE) or 25 μM $AgNO_3$ was increased 8-, 2-, and 6-fold over the control, respectively. Quantitative real-time PCR analysis showed that *CaHMGR* was expressed at a higher level under the treatment of MeJA or Ag^+ elicitor. Our results suggested that 20E accumulation may be the result of the expression up-regulation of *CaHMGR* involved in the biosynthesis under the treatment of various elicitors.

1. Introduction

Cyanotis arachnoidea C. B. Clarke, a perennial grass from the family Commelinaceae, has been used in traditional Chinese medicine as a therapeutic for curing limb numbness, rheumatoid arthritis or as a tonic for promoting the circulation of blood, relaxing the muscles and joints (Tan et al., 2003). It produces a rich array of bioactive phytoecdysteroids including 20-hydroxyecdysone (20E, also reported as β-ecdysone or ecdysterone), dihydroxyrubrosterone, rubrosterone, poststerone and cyanosterone B (Tan et al., 2002, 2003). 20E was the most abundant among the major ecdysteroids, reaching 1.2% (w/w) in air-dried whole plants and 2.9% (w/w) in roots (Zhu et al., 2011). 20E has various agricultural applications such as enhancing synchronous development of the larvae of *Bombyx mori*, elevating significantly the yield of silk from the cocoon (Trivedy et al., 2003) and reducing the length of the moult cycle of shrimp *Alpheus heterochelis* (Mellon and Greer, 1987). 20E and its derivatives also displayed a wide variety of biomedical activities including growth-promoting, wound-healing, stimulating protein synthesis and beneficial pharmacological effects (e.g., antidepression, antioxidation, antidiabete and neuron protection) (Lafont and Dinan, 2003; Dinan and Lafont, 2006). The commercial anabolic preparations and 20E supply for agricultural and therapeutic uses are mainly based on extraction from collected wild *C. arachnoidea* plants. Hence, there is a need to develop more reliable and sustainable means for 20E production and other bioactive ecdysteroids. Plant tissue and cell cultures are alternative production systems for ecdysteroids (Lev et al., 1990; Wang et al., 2013). Although adventitious root growth and hairy root cultures transformed with *Agrobacterium rhizogenes* in *C. arachnoidea* have been reported (Zhou et al., 1996; Zhou and Yang, 1996), little information is available up to date about the production of pharmacologically significant 20E in the cultured *C. arachnoidea* cells.

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The biosynthesis of the ecdysteroid backbone proceeds through the classical mevalonate (MVA) pathway. Grebenok and Adler (1993) reported that spinach leaves synthesized ecdysteroids from MVA and the synthesis was developmentally regulated. Using labeling with [^{14}C]MVA, Bakrim et al. (2008) found that aged spinach leaves transformed 2–3.5% of the applied MVA into 20E after 24 h incubations. With labeled MVA, 20E reached 0.4–2.7% of the radioactivity recovered in hairy roots of *Serratula tinctoria* (Delbecque et al., 1995). The regulatory enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC 1.1.1.34), which catalyzes the irreversible conversion of HMG-CoA to MVA, is thought to be rate limiting in this pathway (Chappell, 1995). The recent characterization of a loss of HMGR function *hmg1 Arabidopsis* mutant with reduced sterol levels, demonstrated the regulatory role of HMGR in the sterol biosynthesis (Suzuki et al., 2004). The predominant effect of mevinolin, a highly specific competitive inhibitor of HMGR on free phytosterol accumulation in radish seedlings suggested a rate-limiting role of HMGR activity for phytosterol synthesis (Bach, 1986). In a tobacco mutant callus with higher HMGR activity (3- to 4-fold), phytosterol accumulation reached 10 times higher than that of the wild-type genotype. Simultaneously, HMG-CoA synthesis by the coupled enzyme acetoacetyl-CoA thiolase/HMG-CoA synthase and its conversion to acetyl-CoA plus acetoacetate by HMG-CoA lyase were not affected (Gondet et al., 1992). These results confirmed the key-regulating role of HMGR in phytosterol biosynthesis. Although much progress has been made in understanding 20E biosynthesis in insects (Huang et al., 2008) and some phytoecdysteroid biosynthesis (Dinan et al., 2009), studies on the 20E biosynthesis in plants are limited primarily to metabolic studies on the conversion pathway from some precursors such as cholesterol or lathosterol to 20E by labeling experiments (Grebenok and Adler, 1993; Festucci-Buselli et al., 2008). However, there is still no reported work on the cloned HMGR gene involved in phytoecdysteroid biosynthesis.

Thus, as a follow-up to our previous works in the biosynthesis regulation and biotechnological production of 20E (Zhao et al., 2010b; Wang et al., 2013), we tried to established *C. arachnoidea* cell suspension culture for 20E production. A full length cDNA encoding HMGR was cloned and characterized in *C. arachnoidea*, which was designated as *CaHMGR* (GenBank Accession No. KJ608269). The expression pattern of *CaHMGR* in various tissues including roots, stems and leaves of *C. arachnoidea* plants was also studied. As HMGR was found to be elicitor-responsive in plants (Stermer and Bostock, 1987; Liao et al., 2009), the expression level of *CaHMGR* was examined in response to elicitors including methyl jasmonate (MeJA), yeast elicitor (YE) and $AgNO_3$, and its relationship to elicitor-induced 20E biosynthesis in cell suspension cultures was elucidated. This is the first report about the isolation, characterization of a novel HMGR gene and the establishment of *C. arachnoidea* cell suspension cultures for 20E production.

2. Materials and methods

2.1. Plant material and callus induction

C. arachnoidea seeds, collected in September 2011 from the suburbs of Luquan County, Yunnan Province of China with its voucher specimen (SCU-110923) identified by Prof. C.Y. Liu deposited in the Herbarium of Soochow University, were sterilized with 0.1% (w/v) $HgCl_2$ for 60 s and washed three times with sterile distilled water. Seeds were germinated on hormone-free Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium containing 2% (w/v) sucrose at pH 5.8. Germination started within 6 days and plantlets were used for callus induction. Young leaves of 4-week old plantlets were cut into small pieces and callus was induced on MS medium containing 3.0 mg L^{-1} 6-benzyladenie (6-BA) and 0.5 mg L^{-1} 1-naphthylacetic acid (NAA) in continuous darkness at 25 ± 1 °C. Subcultures of callus were carried out in MS medium containing 6-BA 3.0 mg L^{-1}, NAA 0.2 mg L^{-1} in continous darkness every 20 days.

2.2. Cell suspension cultures of C. arachnoidea

For cell suspension cultures, 5 g fresh friable homogeneous callus was cultured in 150 mL erlenmeyer flasks containing 50 mL liquid MS medium supplemented with 6-BA 3.0 mg L^{-1} and NAA 0.2 mg L^{-1}. The flasks were incubated in a rotary shaker at 120 rpm in the dark at 25 ± 1 °C. During the 15 days culture period, cell growth was evaluated by determining biomass (dry weight, DW) every three days. Measurements were performed until there was no longer increase in cell growth (stationary phase). Each experiment was done in triplicate.

2.3. Elicitor preparation and treatment

MeJA (Sigma, USA) was dissolved at 1.0 M followed by filter sterilized. YE was the polysaccharide fraction of yeast extract (Sigma, USA) prepared by ethanol precipitation as described by Zhao et al. (2010a). The concentration of YE was represented by total carbohydrate content determined by the anthrone test using glucose as a reference. Silver nitrate (Sigma, USA) was dissolved to the desired concentrations and adjusted to pH 5.8. The concentration of MeJA, YE and $AgNO_3$ in the cell culture of *C. arachnoidea* was fixed at 0.2 mM, 100 mg L^{-1} and 25 μM respectively, as the most effective doses chosen from preliminary tests. MeJA, YE or $AgNO_3$ was applied separately with the cell cultures at the end of exponential growth phase, and the cells were harvested for analysis after 24, 48, 60, 72 and 96 h. All treatments were performed in triplicate, and the results were averaged.

2.4. Cloning of full-length cDNA of CaHMGR

Total RNA was isolated from *C. arachnoidea* plants by RNAprep pure plant kit (Tiangen, China) following the manufacture's procedure. cDNA was amplified from the total RNA by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the instruction. All PCR products were cloned into pCR2.1 (Invitrogen, Karlsruhe, Germany) vector and transformed into *Escherichia coli* Top 10 for sequencing. A pair of degenerated primers HMGRF (5′-ATGGGIATGAAYATGGTWSIAARGG-3′) and HMGRR (5′-TCIACRTTYTGIGCIGGRTCYTG-3′) were designed and synthesized according to the conserved regions of previously reported HMGRs of other plant species such as *Eucommia ulmoides* (Jiang et al., 2006), *Ginkgo biloba* (Shen et al., 2006), *Euphorbia pekinensis* (Cao et al., 2010). PCR was conducted in a total volume of 25 μL containing 10 ng μL^{-1} cDNA, 5 μmol HMGRF, 5 μmol HMGRR, 5 μmol dNTPs, 2.5 μL 10 × DreamTaq™ Green Buffer and 0.625 U DreamTaq™ Green DNA Polymerase (Fermentas, Canada), following the protocol: the cDNA was denatured at 94 °C for 3 min followed by 35 cycles of amplification (94 °C for 60 s, 55 °C for 60 s, 72 °C for 30 s) and by 10 min at 72 °C. To clone *CaHMGR* full length cDNA 5′RACE and 3′RACE were carried out using the SMARTer™ RACE Amplification Kit (Clontech) with 5′RACE gene specific primer CaHR1 (5′-TCTTGACCAGTAGCAATGTAGATA-3′) and 3′RACE gene specific primer CaHF2 (5′-GCTAGCAATATAGTCTCTGCTATCTA-3′) respectively. The PCR conditions were: the cDNA was denatured at 94 °C for 3 min followed by 35 cycles of amplification (94 °C for 60 s, 58 °C for 60 s, 72 °C for 90 s) and by 10 min at 72 °C.