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GENE CLONING AND EXPRESSION OF A TRITERPENE SYNTHASE FROM *ALANGIUM LAMARCKII*

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INTRODUCTION

The triterpenoids are a large group of terpenoid compounds found widely as natural products. Many structures of tetracyclic and pentacyclic triterpenes have been discovered in higher plants and reported to have various biological and pharmacological activities [1, 2]. However, the physiological functions of this secondary product group of triterpenes in plants are still not clear. Triterpenes are biosynthesized by the group of enzymes, called either as oxidosqualene cyclases (OSC), named after their common substrate, or as triterpenoid synthases, named after their preferred products. Each of these enzymes can facilitate the mechanism of cyclisation which comprises protonation, cyclisation, rearrangement and deprotonaton. All of these steps occur within one enzyme to give the structure of tetracyclic or pentacyclic triterpene [3]. Due to the complex mechanism of the OSC enzymes and the diversity of the triterpene products, it is interesting to know how each triterpene synthase produces each product specifically. Several studies have been reported on cloning and characterization of OSCs from plant species (for review, see [4]). The data of triterpene synthase genes from many plant species can help predicting the formation of various triterpenes which are taken place in active sites of the enzymes.

Alangium lamarckii is a small to medium tree belonging to the family Alangiaceae. Previous study has shown that friedelin can be found in *A. lamarckii* leaves [5]. Therefore, this plant is an ideal source of this triterpene ketone. Here, we report the cDNA cloning of OSCs from *A. lamarckii* and heterologous expression in a mutant yeast lacking lanosterol synthase, which is a member of OSC family.

MATERIALS AND METHODS

Plant material Young leaves of *Alangium lamarckii* were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Cloning of OSC from A. lamarckii The leaves of *A. lamarckii* were ground by using liquid nitrogen in a mortar and pestle. Total RNA was extracted by using RNeasy mini kit (Qiagen) followed by cDNA synthesis using reverse transcriptase (Fermentas). The resulting cDNA was used as template for amplification by PCR in a total volume of 50 µl containing the first strand cDNA 2 µl, primers (5'-ATGTGGAGGCTGAAAGTAGCAGAAGG-3' and 5'-TCAGAGCCTCTGGGGAGGGAACTGAA-3'), dNTP mixture (0.2 mM), 10x buffer (5 µl) and *taq* DNA polymerase (Invitrogen). The PCR condition was set for 35 cycles, at 94°C for 30 seconds, 55°C 40 seconds 72°C for 3 min and final extension 72°C for 10 min. A 2.3-kb DNA band corresponding to the length of OSC genes was separated by agarose gel electrophoresis. After sequencing, 5 genes from the first PCR were used as templates for the second PCR under the same PCR condition in the solution. Primers containing yeast consensus sequence (5'-GTACTGAACGTTHAMAMAATGTCSAGGCTGAAAGTAGCAGAAG-3' and 5'-AAGTCAAGGGA GGGGTCTCCGAGAGATCTATCTAG-3') were used to get the suitable constructs for expression in yeast following the yeast expression vector pYES2 protocol (Invitrogen). The second PCR products were digested with *Not*I and *Xba*I, and ligated into multiple cloning site of a yeast expression vector pYES2 which was digested by the same restriction enzymes.

Functional expression in yeast and product detection The full length cDNAs were cloned into a yeast expression vector pYES2 (Invitrogen). Forty constructs were transformed into a *Saccharomyces cerevisiae* strain GIL77 [6] by using Forzen-EZ Yeast Transformation II™ kit (ZYMO RESERCH).

The transformed cells were picked and cultured in synthetic complete medium without uracil (SC-U) at 30°C with shaking 200 rpm, and supplemented with ergosterol, hemin, and Tween80. After 2 days, the cells were collected and re-suspended in SC-U without glucose, supplemented with ergosterol, hemin, Tween80 and 2% galactose, and incubated using the same condition for 1 day. Cells were collected and re-suspended in 0.1 M potassium phosphate pH 7.0, supplemented with 3% glucose and hemin, and incubated using the same condition for 1 day. The cells were then collected and refluxed in 20% KOH/50% EtOH, and extracted with hexane. The obtained 40 crude extracts were checked for the

presence of triterpene products using thin layer chromatography (TLC). The plate was double developed using hexane-acetone (19:1) as mobile phase with the presence of standards (β-amyrin and friedelin). The TLC plate was visualized by spraying anisaldehyde-sulphuric acid reagent (AS).
The sample with potential product forming was chosen to scale up for detecting the structure of the products. The obtained extract was then partial purified on a preparative TLC glass plate and double developed using hexane-acetone (19:1) as mobile phase. The corresponding positions to the standard triterpene mono-alcohols were scratched and eluted with acetone. The eluate was concentrated, separated and analysed by high pressure liquid chromatography (HPLC) using a SUPER-ODS column (4.6 x 250 mm. Tosoh) eluted with 95% CH_3CN as solvent (flow rate 1 ml/min, temperature 40 °C, detection UV 254 nm).

RESULTS AND DISCUSSION

Five targeted genes from *A. lamarckii* cDNA were amplified by PCR. The genes consist of 2292 bp nucleotides encoding 763 amino acids. The genes have conserved QW motif, which presumably has a role to stabilize the protein structure, and DCTAE motif, which is responsible for substrate protonation [7]. These motifs are conserved in the OSC family. Among the 5 genes, 4 of them showed 80% identity to the amyrin synthase from *Catharanthus roseus* [8], and the other one showed 79% identity to the mixed amyrin synthase from *Olea europaea* [9].
The results suggested that the genes from *A. larmarckii* leaves are OSC genes. Each gene was then constructed into yeast expression vector and expressed in a mutant yeast, GIL77, lacking lanosterol synthase. The yeast was extracted and screened on a TLC plate. Interesting samples showed a pink spot corresponding to the standard β-amyrin and positive control pOEA [9]. The sample no. 35, which is one of the 4 genes, showed 80% identity to amyrin synthase from *C. roseus*. It was scaled up to a large volume to increase the amount of product which has the same Rf to the standard. The extract was purified by TLC and analysed by HPLC. Only the obtained HPLC chromatogram (Fig.1) of the sample showed a peak at the retention time of 15 min compared to the standards and negative control (empty vector pYES2). Compared to the standards, this peak is presumably the triterpene mono-alcohol taraxasterol or β-amyrin. This is being confirmed by using the technique of liquid chromatography mass spectrometry (LC-MS).

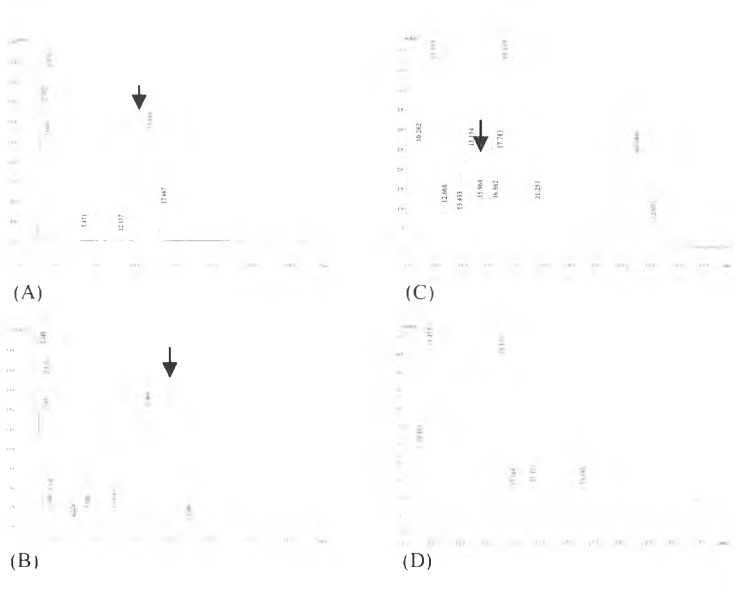

Figure 1 HPLC chromatograms of (A) standard taraxasterol (15.86 min), (B) standard β-amyrin (15.91 min), (C) sample no. 35 and (D) negative control pYES2.

CONCLUSION

It has been reported that friedelin is present in *A. larmarckii* leaves [5]. Therefore, we targeted the genes that are involved in the formation of fridelin. However, based on the results presented, we can conclude that the gene obtained would be involved in the formation of triterpene mono-alcohols. This suggested that this plant has other triterpenoid compounds which have not been reported, presumably due to their

presence as minor compounds. However, the finding of OSC genes in this study would contribute to the knowledge of the triterpenoid natural products in this Thai plant.

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REFERENCES

1. Dzubak, P., et al., Pharmacological activities of natural triterpenoids and their therapeutic implications. Natural Product Reports, 2006. 23(3): p. 394-411.
2. Salvador, J.A.R., et al., Ursane-type pentacyclic triterpenoids as useful platforms to discover anticancer drugs. Natural Product Reports, 2012. 29(12): p. 1463-1479.
3. K. U. Wendt, et al., Enzyme Mechanisms for Polycyclic Triterpene Formation. Angewandte Chemie International Edition, 2000. 39: p. 2812-2833.
4. Haralampidis, K., M. Trojanowska, and A. Osbourn, Biosynthesis of Triterpenoid Saponins in Plants, in History and Trends in Bioprocessing and Biotransformation, N.N. Dutta, et al., Editors. 2002, Springer Berlin Heidelberg. p. 31-49.
5. Gupta, N.C., B. Singh, and D.S. Bhakuni, Steroids and triterpenes from Alangium lamarckii, Allamanda cathartica, Abrus precatorius and Holoptelea integrifolia. Phytochemistry, 1969. 8(4): p. 791-792.
6. Kushiro, T., M. Shibuya, and Y. Ebizuka, β-Amyrin synthase. European Journal of Biochemistry, 1998. 256(1): p. 238-244. Thoma, R., et al., Insight into steroid scaffold formation from the structure of human oxidosqualene cyclase. Nature, 2004. 432(7013): p. 118-122.
7. Thoma, R., et al., Insight into steroid scaffold formation from the structure of human oxidosqualene cyclase. Nature, 2004. 432(7013): p. 118-122.
8. Yu, F., et al., Functional characterization of amyrin synthase involved in ursolic acid biosynthesis in Catharanthus roseus leaf epidermis. Phytochemistry, 2013. 91(0): p. 122-127.
9. Saimaru, H., et al., Production of triterpene acids by cell suspension cultures of Olea europaea. Chemical and Pharmaceutical Bulletin 2007. 55(5): p. 784-788.