ISOLATION OF GENE TRANSCRIPTS DURING AGARWOOD RESIN ACCUMULATING BY SUBTRACTIVE HYBRIDIZATION TECHNIQUE

Thitikorn Chomtong[1], Saowanee Suputtitada[1], Supachai Vuttipongchaikij[1,2] and Somsak Apisitwanich[1,2] *

[1] Department of Genetics Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand
[2] Center for Advanced Studies in tropical Natural Resource, National Research University, Kasetsart University, Chatuchak, Bangkok 10900, Thailand

*e-mail: fscissa@ku.ac.th

Abstract

Resin production of *Aquilaria* species, one of the plant defense mechanisms, is triggered by external stimuli such as chemical and biological treatment. However, mechanism of gene expression during resin accumulation has been fully understood. This study acquired subtractive hybridization technique (SH) to find genes involved in the mechanism. Based on sequence similarity, most genes were identified as fungal genes. Only one sequence was predicted to be a plant gene which was *tropinone reductase* (*TR*) gene containing two important motifs, TGxxxGxG motif and YxxxK motif. *A.crassna* TR could be categorized into short-chain dehydrogenases/reductases (SDRs) family. There is no evidence suggesting a relationship between TR and resin production in agarwood, which should be studied further. Moreover, the result of dot blot hybridization indicated that three unkown sequences gave positive dots, which were likely to be unidentified plant genes. Therefore, these three unknown sequences should be given gene annotation in the future.

Keywords: agarwood, *Aquilaria crassna*, resin, subtractive hybridization, tropinone reductase

Introduction

Agarwoods (*Aquilaria* species) are resinous heartwood plants producing resin to protect themselves from fungal infection. Resin production is considered as one of the plant defense mechanisms which retard the fungal growth and activate the healing process of plants. Agarwood resin is composed of volatile compounds, including terpenoids, aromatic and aliphatic constituents which are used in various applications such as antimicrobial agents, medical treatment and cosmetic additives (Naef 2011). Nowadays, the demand of agarwood resin is increasing, leading to illegally destroying agarwoods in forest. As a result, the population of agarwoods in nature decreases significantly and it affects genetic diversity of agarwoods. Therefore, considered as endangered species, agarwoods are in the list of Appendix II, according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in order to control cultivation as well as import and export of agarwood products (Oldfield et al. 1998). Because of their economic importance, a number of approaches have been developed to increase resin yield such as mechanical wounding and fungal infection. However, at present the mechanism of resin production remains unclear.

Therefore, the understanding of gene expression during resin production may lead to an effective way to increase resin accumulation in agarwoods. Because of an effective method, this study used subtractive hybridization (SH) technique to find genes expressed during resin production and accumulation.

Methodology

Plant material

Young leaves and xylem tissue of both resin-producing and nonresin-producing agarwoods (*Aquilaria crassna*) were collected from plantations in Trad, the eastern province of Thailand. Samples were frozen immediately in liquid N_2.

DNA and RNA extractions

Approximately 1 g of frozen agarwood leaf tissues was ground in liquid N_2. Genomic DNA was extracted according to Agarwal *et al*. (1992) with some modifications and then was kept at -20°C until use. For RNA extraction, approximately 100 mg of frozen stem tissues of *Aquilaria crassna* was ground in liquid N_2 and RNA was extracted using the Favor Prep™ Plant Total RNA Purification Mini Kit (Favorgen, TW). DNA was eliminated by DNase I digestion with RNase-free DNase set (Qiagen, US).

Subtractive cDNA and library constructions

Double-stranded cDNAs of nonresin-producing xylem tissues were synthesized using SMART™ PCR cDNA Synthesis Kit (Clontech,US), while single-stranded cDNA of resin-producing xylem tissues were synthesized by SMART™ cDNA Library Construction Kit (Clontech,US). Double-stranded cDNAs of nonresin-producing xylem tissues called tester were labeled with biotin -16- dUTP using nick translation method but single-stranded cDNAs of resin-producing xylem tissues called driver were not labeled. To hybridize the tester and the driver, both cDNAs were mixed with hybridization buffers and the mixture was incubated for denaturing at 95°C for 15-20 minutes and for annealing at 65°C overnight. To separate the hybridized cDNAs, the magnetic beads (Dynabeads®MyOne™Streptavidin C1, Invitrogen, US) were applied according to the manufacturer's protocol. The rest of cDNAs was precipitated by adding (2 v/v) 80% alcohol and (0.1v/v) 3 M NaOAc. Subsequently, double-stranded cDNAs were synthesized and cDNA library was generated using SMART™ cDNA Library Construction Kit according to the manufacturer's protocols. The library was analyzed by PCR and the cDNA fragments longer than 500 bp were sequenced.

Data analysis and Conformation

Nucleotide sequences were assembled by CAP3 (http://pbil.univ-lyon1.fr/cap3.php). Blastn and Blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to identify the identities and conserved domain. PCR products of inserted cDNA were dotted on positive charge nylon membrane and hybridized at 55°C overnight with biotin-labeled genomic DNA of leaf tissue which has been done using the random primed PCR labeling method. The result was detected after shaking the membrane in nitro-blue tetrazolium chloride (NBT, 18.75 mg/ml) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP, 9.4 mg/ml) (GIBCOBRL®, US) solutions.

Results

Sequence similarity in database

In subtractive hybridization technique, the hybridized cDNAs of the tester and the driver were removed and the rest of cDNAs were used to construct the cDNA library. After PCR screening, cDNAs which were longer than 500 bp in length, were selected for sequencing analysis. Results of CAP3 indicated that there were 17 contigs and 128 singletons. Besides, Blastx results revealed that 3 contigs and 49 singletons tended to be fungal genes while 5 singletons and 2 singletons were identified as slime mold genes and protobacterial genes respectively. Interestingly, only one singleton tended to be plant gene based on sequence similarity. However, 113 sequences could not be identified.

Analysis of putative plant gene fragment

Only one singleton showed sequence similarly to plant tropinone reductases (TRs). It encoded an enzyme in tropane alkaloid biosynthesis. TRs have two different stereospecific forms namely tropinone reductase I (TRI) and tropinone reductase II (TRII). These enzymes play an important role in reduction of a keto group in the tropane ring leading to the structural change of tropane alkaloid (Nakajima et al. 1993; Dräger 2006). Previous studies suggested that *TR* gene expressed when plants were treated with external stimuli such as ethylene treatment, mechanical wounding or fungal infection (González-Candelas et al. 2010). In this study, the full-length cDNA of *A. crassna* tropinone reductase (*AcTR*) was 1,013 bp in length containing an 819-bp open reading frame encoding a deduced protein of 272 amino acid residues. The deduced amino acid sequence had homology with TR sequences from other plant species (table 1). AcTR and those TR sequences were analyzed by sequence alignment using ClustalW (http://www.ebi.ac.uk/Tools /msa/clustalw2/) to identify two important motifs. The result suggested that AcTR sequence had two highly conserved motifs namely TGxxxGxG motif, coenzyme binding at position 23, and YxxxK motif, catalytic tyrosine at position 167 (Figure 1). These two motifs are commonly found in short-chain dehydrogenases/reductases (SDRs) family (Oppermann et al. 1997; Kavanagh et al. 2008). Therefore, AcTR could then be categorized into SDRs family. Moreover, although unidentified sequences were not found in GENBANK, they might be plant cDNAs which were not reported in the database. Therefore, dot-blot hybridization was used to detect whether unknown sequences were plant cDNAs. The result showed that three unknown sequences gave positive dots of which sequences have not yet been reported.

	Accession	Description	Max score	E-value	Max identity
PtTR	XP_002308174.1	Predicted protein [*Populus trichocarpa*]	352	1e-118	63%
VvTR	XP_002271837.1	Tropinone reductase homolog [*Vitis vinifera*]	333	3e-111	58%
GmTR	XP_003552193.1	Tropinone reductase homolog isoform1[*Glycine max*]	324	6e-108	59%
AlTR	XP_002879208.1	Hypothetical protein ARALYDRAFT_481843 [*Arabidopsis lyrata* subsp. lyrata]	312	3e-103	60%
RcTR	XP_002532998.1	Tropinone reductase, putative [*Ricinus communis*]	308	8e-102	57%
MtTR	AFK36946.1	Unknown [Medicgo truncatula]	305	2e-100	56%
SdTR*II*	CAQ19732.1	Tropinone reductase II [*Solanum dulcamara*]	290	1e-94	51%
DnTR*II*	AFD23289.1	Tropinone reductase II, partial [*Dendrobium nobile*]	290	1e-94	54%
AaTR	ABI_78943.1	Tropinone reductase [*Anisodus acutangulus*]	288	5e-94	52%

Table 1 Homology of AcTR amino acid sequence with other plant TR proteins

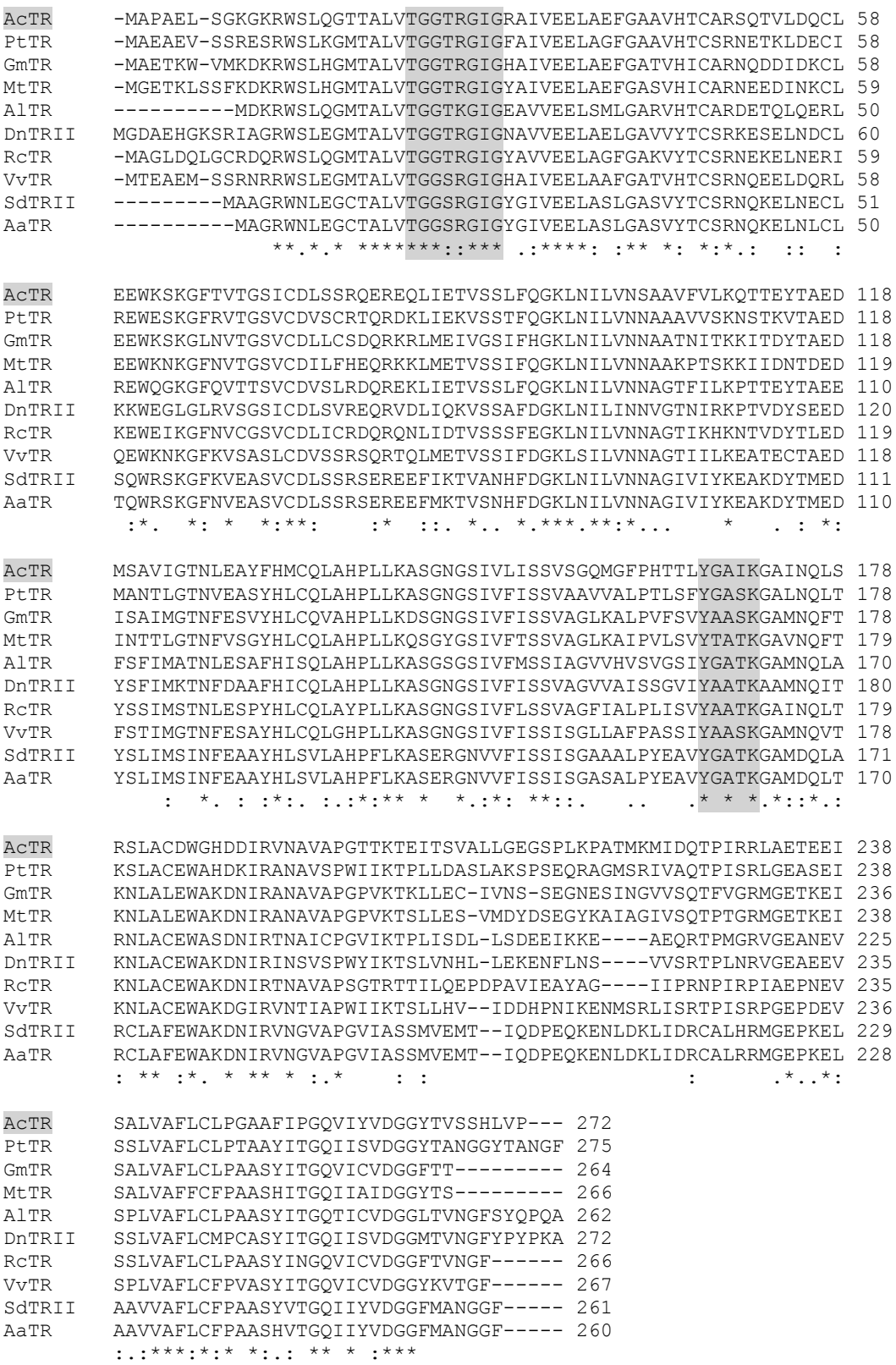

Figure 1 Mutiple sequence alignments of AcTR with other plant TR sequences

Discussion and Conclusion

Effect on subtraction in *Aquilaria crassna*

Aquilaria species could produce resin because insects or microbes-attacked them in order to protect themselves. The resin production is related to the plant stress and defensive responses. Therefore, it would be significant to investigate the molecular mechanism of these species in response to various stresses. Understanding of relationship between resin formation and plant stress could lead to develop the effective way to increase resin yield for commercial purposes. This study, subtractive hybridization (SH) technique is introduced to study differential gene expression between infected and uninfected xylem tissues of the same agarwood plant. Results indicated that many microorganisms lived in the area of resin production. This was proved by mRNA contamination. This research is corresponding with the previous study that resinous tissues of *Aquilaria* trees were naturally infected by various fungi (Mohamed et al. 2010; Cui et al. 2011) and the effectiveness of subtractive hybridization technique reported in several publications for studying the upregulate and down regulate gene-expression. Our studies, RNA from plant tissue were contaminated with RNAs of foreign species which made difficulty to identify only expressed genes of plant.

This study was the first report of *tropinone reductase* (*TR*) gene in *Aquilaria* species. TR is a key enzyme in tropane alkaloid biosynthesis. Tropinone reductases distribute in seven families of angiosperm, namely the Proteaceae, Convolvulaceae, Brassicaceae, Euphorbiaceae, Rhizophoraceae, Solanaceae, and Erythroxylaceae (Griffin and Lin 2000) but there was no evidence in Thymelaeaceae family. Therefore, this finding could lead to the further study of alkaloid biosynthesis of Thymelaeaceae, *Aquilaria* species. In *Hyoscyamus*, *Duboisia*, *Atropa* and *Scopolia* of Solanaceae plants, hyosyamine and scopolamine play important roles in protecting plants from pests and diseases. Hyosyamine isolated from *H. muticus* inhibits wide range of fungal species (Abdel-motall et al. 2009). Although alkaloid is highly toxic to many fungi, some fungal species have alkaloid detoxifying mechanism which helped them growing in the presence of alkaloid. For example, *Fusarium oxysporum* could detoxicate α-tomaine, an anti-fungal alkaloid (Ito et al. 2004).

Acknowledgments

This research is partially supported by Kasetsart University Research and Development Institute.

References

1. Abdel-motall FF, Nassar MSM, El-zayat SA, EL-SAYED MA, Ito S (2009) Responses of fungi to tropane alkaloids produced by a medicinal plant *Hyoscyamus muticus* (Egyptian Henbane). Folia Microbiol 54(3):207–212.
2. Agrawal GK, Pandey RN, Agrawal VP (1992) Isolation of DNA from *Choerospondias asillaris* leaves. Biotech Biodiv Lett 2:19-24.
3. Cui JL, Guo SX, Xiao PG (2011) Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*. J Zhejiang Univ Sci B 12(5):385–392
4. Dräger B (2006) Tropinone reductases, enzymes at the branch point of tropane alkaloid metabolism. Phytochemistry 67:327-337.

5. González-Candelas L, Alamar S, Sánchez-Torres P, Zacarias L and Marcos JF (2010) A transcriptomic approach highlights induction of secondary metabolism in citrus fruit in response to *Penicillium digitatum* infection. BMC Plant Biology 10:1-17.
6. Griffin WJ, Lin GD (2000) Chemotaxonomy and geographical distribution of tropane alkaloids. Phytochemistry 53:623-627.
7. Ito S, ETO T, Tanaka S, Yamauchi N, Takahara H, Ikeda T (2004) Tomatidine and lycotetraose, hydrolysis products of α-tomatine by *Fusarium oxysporum* tomatinase, suppress induced defense responses in tomato cells. FEBS Lett 571:31–34.
8. Kavanagh KL, Jörnvall H, Persson B, Opermann U (2008) Medium- and short-chain dehydrogenase/reductase gene and protein families: The SDR superfamily: Functional and structural diversity within a family of metabolic and regulatory enzymes. Cell Mol Life Sci 65:3895-3906.
9. Mohamed R, Jong P L, Zali MS (2010) Fungal diversity in wounded stems of *Aquilaria malaccensis*. Fungal Diversity 43:67–74.
10. Naef R (2011) The volatile and semi-volatile constituents of agarwood, the infected heartwood of *Aquilaria* species. Flavour Fragr J 26:73-89.
11. Nakajima K, Hashimoto T,Yamada Y (1993) Two tropinone reductases with different Stereospecificites are short-chain dehydrogenases evolved from a common ancestor. Proc Natl Acad Sci USA 90:9591-9595
12. Oldfield S, Lusty C, MacKinven A (1998) The world list of threatened trees. World conservation press, Cambridge
13. Opermann UC, Filling C, Berndt KD, Persson B, Benach J, Ladenstein R, Jörnvall H (1997) Active site directed mutagenesis of *3β/17β*-hydroxysteriod dehydrogenase establishes differential effects on short-chain dehydrogenase/reductase reactions. Biochemistry 36:34-40.