Studies on Terpenoid Biosynthesis of Mangrove Tree Species

(マングローブにおけるテルペノイド生合成に関する研究)

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Certification

We, the undersigned, herewith certify that this dissertation, entitled “Studies on Terpenoid Biosynthesis of Mangrove Tree Species” not previously presented for application of a degree, and the researches contained herein were independently conducted by Mohammad Basyuni under our supervision, and which is hereby submitted to the United Graduate School of Agricultural Sciences, Kagoshima University, Japan, in partial fulfillment for the degree of Doctor of Agriculture is accepted for presentation.

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List of Abbreviations

AMV	:	Avian myeloblastosis virus
ANOVA	:	Analysis of variance
βAS	:	β-amyrin synthase
BgAct	:	*Bruguiera gymnorrhiza* actin
BgbAS	:	*Bruguiera gymnorrhiza* β-amyrin synthase
BgLUS	:	*Bruguiera gymnorrhiza* lupeol synthase
CAS	:	Cyloartenol synthase
cDNA	:	Complementary DNA
CDS	:	Cucurbitadienol synthase.
CM	:	Chloroform-methanol
CTAB	:	Cetyltrimethylammonium bromide
DCTAE	:	Aspartic Acid (Asp) Cysteine (Cys) Threonine (Thr) Alanine (Ala) Glutamin Acid (Glu)
DNA	:	Deoxyribonucleic acid or deoxyribonucleate
EDTA	:	Ethylene diamine tetraacetic acid
EI	:	Electron impact
FID	:	Flame ionization detection
GC-MS	:	Gas chromatography mass spectrometry
KcAct	:	*Kandelia candel* actin
KcCAS	:	*Kandelia candel* cyloartenol synthase
KcMS	:	*Kandelia candel* multifunctional triterpene synthase
LAS	:	Lanosterol synthase
LUS	:	Lupeol synthase
MFS	:	Multifunctional triterpene synthase

MLCYCR : Methionine (Met) Leucine (Leu) Cysteine (Cys) Tyrosine (Tyr) Cysteine (Cys) Arginine (Arg)

mRNA : Messenger ribonucleic acid

MRT : Multiple range test

MS : Mass spectrum

MWCYCR : Methionine (Met) Tryptophan (Trp) Cysteine (Cys) Tyrosine (Tyr) Cysteine (Cys) Arginine (Arg)

NMR : Nuclear magnetic resonance

NSL : Nonsaponifiable lipid

ORF : Open reading frame

OSC : Oxidosqualene cyclase

PCR : Polymerase chain reaction

QW : Glutamine (Gln) Tryptophan (Trp)

RACE : Rapid amplification of cDNA ends

RNA : Ribonucleic acid or ribonucleate

RsCAS : *Rhizophora stylosa* cyloartenol synthase

RsM : *Rhizophora stylosa* multifunctional triterpene synthase

RT-PCR : Reverse transcription polymerase chain reaction

SC-Ura : Synthetic complete medium without uracil

TIC : Total ion chromatogram

TLC : Thin layer chromatography

TMS : Trimethylsilyl

YPD : Yeast peptone dextrose

Acknowledgements

"By the name of Allah, the Lord the Most Beneficent, the Most Merciful"

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Abstract

Mangroves plants are distributed in the inter-tidal zone of tropical and sub-tropical areas, and prosperous sources of triterpenoids alcohols are mostly derived from oleanane, lupane, and ursane type of terpenoids. Despite the ubiquitous distribution of terpenoids in mangrove trees, their physiological functions are not well understood. The present study thus sheds the light on the biosynthesis of terpenoid with the special emphasis on its relevance to salt tolerance.

First of all, nonsaponifiable lipid composition (NSL) of Okinawan mangroves were analyzed to characterize their terpenoid profile. Triterpenoids and phytosterols comprised the major proportion of NSL. The terpenoids and phytosterols mainly consisted of 11 and 6 compounds, respectively. The major components were lupeol, β-amyrin and taraxerol for terpenoids, and were β-sitosterol and stigmasterol for phytosterols. Terpenoid compositions of the root are not always similar to that of leaf, suggesting that terpenoids in the root are produced by biosynthesis in situ, not a translocation of the synthate from the leaf. Terpenoids existed in greater proportion in the outer parts of the root, suggesting the protective roles of terpenoids in mangrove.

The diversity in the NSL composition has been noted with mangrove species for both leaves and roots, implying the occurrence of divergent enzyme systems for biosynthesis. Despite diversity in the carbon skeleton, all triterpenes and phytosterols are biosynthesized from a common precursor substrate 2,3-oxidosqualene via oxidosqualene cyclases (OSCs). To get more insight into the physiological significance of terpenoid, gene cloning of terpenoids synthase was attempted. Five oxidosqualene cylase (OSC) cDNAs were cloned from young roots of *Kandelia candel* and the leaves of *Bruguiera gymnorrhiza* and *Rhizopora stylosa* by a homology-based PCR method. The Open reading frames (ORFs) of full-length clones termed *KcMS*, *BgbAS*, *BgLUS*, *RsM1* and *RsM2* were ligated into yeast expression plasmid pYES2 under the control of the *GAL1* promoter. Expression of *BgbAS* and *BgLUS* in GIL77 resulted in the production of β-amyrin and lupeol. This showed that the gene encoded β-amyrin and lupeol synthase, respectively. Furthermore, *RsM1* produced germanicol, β-amyrin, and lupeol in the ratio of 63:33:4, whereas *RsM2* produced taraxerol,

β-amyrin, and lupeol in the proportions 70:17:13. The *KcMS* transformant accumulated a mixture of lupeol, β-amyrin and α-amyrin in 2:1:1 ratio. These results indicated that these were multifunctional triterpene synthases. Phylogenetic analysis and sequence comparisons revealed that *BgbAS* and *RsM1* showed high similarities to β-amyrin synthases, and were located in the branch of β-amyrin synthase. *BgLUS* and *KcMS* formed a new branch for lupeol synthase that was closely related to the β-amyrin synthase cluster, while *RsM2* was found in the first branch of the multifunctional triterpene synthase evolved from lupeol to β-amyrin synthase.

Furthermore, cDNAs expected to encode the OSCs for phytosterol biosynthesis were cloned from roots of *K. candel* and leaves of *R. stylosa*: *KcCAS* and *RsCAS*. Functional expression of these genes found that both *KcCAS* and *RsCAS* encoded cycloartenol synthase.

Finally, correlation between mRNA expression of these genes and salt concentration was examined. mRNA level of *KcMS* was increased with salt concentration in both roots and leaves of *K. candel*. Similarly, salt stress increased the mRNA levels of *BgLUS* and *BgbAS* in the root of *B. gymnorrhiza*. In contrast to these observations, the mRNA level of *KcCAS* was not modulated by salt stress in the roots, and decreased in the leaves. These results therefore suggest that the terpenoid but not phytosterols are playing important role to cope with the salt stress in mangrove roots. The concentration and proportion of terpenoids increased with salinity in the seedlings of *K. candel* and *B. gymnorrhiza*, providing additional evidence for the protective role of terpenoids against salt stress.

Chapter I

General Introduction

Mangroves in Japan

Mangrove plants are halophytes that are distributed in the intertidal zone of tropical and sub tropical regions where they exist in conditions of high salinity, extreme tides, strong winds, high temperature and muddy, anaerobic soils (Tomlinson, 1986; Hogarth, 1999). In Japan, natural mangrove forests occupy an area of about 75 km^2, mainly exist throughout the Ryukyu Archipelago and they reach to Satuma Peninsular of Kyushu (Spalding et al., 1997; Kathiresan and Baba, 1999). The mangroves concentrated largely on the Iriomote and Ishigaki islands in the Okinawa prefecture and are the northernmost mangroves in Asia at latitude 31°22′ N. Ten Okinawan mangrove species naturally distributed: *Bruguiera gymnorrhiza* (L.) Lamk. (Rhizophoraceae), *Rhizophora stylosa* Griff. (Rhizophoraceae), *Kandelia candel* (L.) Druce (Rhizophoraceae), *Lumnitzera racemosa* Wild. (Combretaceae), *Avicennia marina* (Forsk.) Vierh (Avicenniaceae), *Pemphis acidula* Forst. (Lythraceae), *Sonneratia alba* J. Smith (Sonneratiaceae), *Heritiera littoralis* Dryand. (Sterculiaceae), *Nypa fruticans* Wurmb (Arecaceae), and *Excoecaria agallocha* L. (Euphorbiaceae), of which three Rhizophoracea tribe namely *B. gymnorrhiza, K. candel,* and *R. stylosa* are relatively dominant in Okinawa region (Kathiresan and Baba, 1999; Takeuchi et al. 2001).

Biological significance of terpenoids in mangrove tree

Mangrove plants have long been well known a source of phytochemical compounds or biologically active compounds (Bandaranayake, 2002). The plants produce various secondary metabolites, which is useful in its interaction with the environment, various environmental stresses as well as in the development of the resistance against external attack. The biotic and abiotic elicitors enhance the production of the secondary metabolite (Sudha and Ravishankar, 2002). By definition, secondary metabolites are regarded as chemical compounds that are not required or directly involved for growth, development or reproduction of the plants (Buckingham, 2001). Plant secondary metabolites also represent a vast resource of complex molecules valued and exploited for their pharmacological and other properties.

Pentacyclic triterpenoids and phytosterols are widely distributed in mangrove plants (Wannigama et al., 1981; Hogg and Gillan, 1984; Ghosh et al., 1985; Koch et al., 2003; Basyuni et al., 2007b). Because of their wide range of biological activities, isoprenoids are regarded as important as potential natural sources for medicinal compounds (Sparg et al., 2004) and mangrove plants have long been used in traditional

medicine to treat disease (Bandaranayake, 1998).

Several biological activities have been demonstrated for terpenoids: anti-inflammatory activity for taraxerol, α-amyrin, β-amyrin, lupeol and germanicol (Akihisa et al. 1996; Singh et al. 2002; Kim et al. 2005); anti-carcinogenic activity for taraxerol and germanicol (Takasaki et al. 1999; Jang et al. 2004); insecticidal activity for taraxerol (William, 1999), cardioprotective effect in hypercholesterolemic condition for lupeol (Sudhahar et al., 2007), hepatoprotection against acetaminophen-induced hepatotoxicity for α- and β-amyrin (Olievera et al., 2005), antileukemic activity of terpenoids from *Acanthus illicifolius* (Kokpol et al., 1986). For this reason, these tree species are potential medicinal plants, which may open up another possibility of mangrove utilization.

Saponins are important secondary metabolites in the Plant Kindom (Buckingham, 2001). Chemically, the term saponins has been accepted to define a group of structurally diverse molecules that consists of glycosylated steroids, steroidal alkaloids and triterpenoids (Haralampidis et al., 2002; Osbourn et al., 2003). Triterpenes are generally stored in plants as their glycosides and saponins that are recognized as secondary metabolites due to their apparent lack of physiological functions in the producing plants (Nes and Heftmann, 1981; Chappell, 1995). Whereas, sterols serve an essential membrane constituents, growth regulating substances, and precursors of various hormones in mammal, plants, fungi and yeasts, (Hodges et al., 1972; Chappell, 1995; Hartmann, 1998). However, their physiological function, if any, in mangrove plants has been rarely reported.

Mangroves are among the most productive ecosystems on earth, and the mangrove litter fall is the most important source of organic carbon in biogeochemical cycles in the mangrove ecosystem (Wafar et al., 1997; Clough et al., 2000) and a valuable indicator of mangrove productivity (Clough, 1998). Due to this high productivity, turnover rates of organic matter and the exchange with terrestrial and marine ecosystems, mangroves are of particular importance for the biogeochemical recycling of carbon and associated elements along the tropical coastal region.

Because lipid comprises a significant proportion of carbon output from mangroves (Wannigama et al., 1981; Hogg and Gillan, 1984), knowledge of the lipid composition of mangrove promisingly contributes to estimating the sources and accumulation rates of sedimentary organic matter. Nonsaponifiable lipids (NSLs) basically denote simple lipid fractions except for fatty acids (saponifiable lipids) after alkaline hydrolysis of the total lipids, and contain sterols, long-chain alcohols and alkanes. In

general, NSL represents a more stable lipid fraction than the saponifiable lipid fraction, and their resistance to microbial degradation has been considered to be a relatively important factor in controlling the diagenetic pathways (Killops and Frewin, 1994; Koch et al., 2005). Triterpenoids are common chemical constituents of higher plants, comprising the major proportions of NSLs, and have been identified in cuticular waxes of mangrove and other plant species (Beaton et al., 1955; Wannigama et al., 1981; Ghosh et al., 1985; Koch et al., 2003). Several lines of study have employed pentacyclic terpenoids as well-suited tracers for the primary source of organic matter from mangrove due to their stability during sedimentation and diagenesis (Killops and Frewin, 1994; Versteegh et al., 2004; Koch et al., 2005). Thus, analysis of terpenoids is a prerequisite for the interpretation of biomarker signals in sediment cores of mangroves.

Mangroves are unique in that these halophyte plants have cellular mechanisms to be tolerant with the high salinity, and have mechanism to take up water despite strong osmotic potentials (Tomlinson, 1986). Halophyte is a plant adapted to living in a saline environment, which include salt marshes, estuarine environments, and the lower parts of sea cliffs (Alberth, 1975; Flowers et al, 1977). A halophyte is a plant that naturally grows where it is affected by salinity in the root area or by salt spray, such as in saline semi-deserts, mangrove swamps, marshes and sloughs, and seashores (Scholander et al., 1962; Glen et al., 1999)

Mangrove plants tolerate salinity of the soil and water by the following ways (Scholander et al., 1962; Tomlinson, 1986):

1. Salt excretion: Some mangrove plants take saline water as such through roots. But in the tissues only water molecules and essential salts are retained. Excess salts are excreted through salt glands that are present in the leaves. Salt is partially excluded by the roots and the salt is excreted by the salt glands by the plant expending energy. The concentrated salt solution evaporates near the gland, becomes crystals which are removed by wind or rain. Examples species of salt excretion are *Avicennia* spp (family Avicenniaceae), *Aegialitis annulata* (family Plumbaginaceae), *Aegiceras* spp (family Myrsinaceae), and *Acanthus ilicifolius* (family Acanthaceae).
2. Salt exclusion: In some of the mangrove plants the roots possess an ultra filtration mechanism called reverse osmosis by which water and salts in the seawater are separated in the root zone itself and only water is taken inside and the salts are rejected such as *Bruguiera* (family Rhizophoraceae), *Lumnitzera* (family Combretaceae), *Rhizophora* (family Rhizoporceaea), or *Sonneratia* (family Sonneratiaceae)

species are non-secretors. They can selectively absorb only certain ions (electrically charged atom(s) andlor group of atom(s) which a salt becomes on going into solution) from the solutions they come into contact with by a process called ultra filtration.

Growth of the plants are impeded by the salt stress, due to water deficit, ion imbalance and ion toxicity. Although the stress-tolerance mechanisms of the plants appeared to be complex and divergent, the halophytic plants thus overcomes the salt stress by combination of several mechanisms: adjustment of the osmotic pressure by accumulation of small molecules of osmolytes such as glycine-betaine or sugar alcohols (Popp 1984; Fan et al. 1993; Bohnert et al. 1995; Sakamoto & Murata 2000); salt extrusion across the plasma membrane using ion transporters (Dupont, 1992; Allen et al. 1995; Shi et al. 2000; Kura-Hotta et al. 2001); accumulation of salt in vacuole using tonoplast transporter (Blumwald & Poole 1988; Matoh et al. 1989; Gaxiola et al. 1999; Mimura et al. 2003); induction of stress-related proteins (Sugihara et al. 2000; Yamada et al. 2002a; Banzai et al. 2002; Yamada et al. 2002b; Ueda et al. 2002; Ashihara et al. 2003). However, information about salt stress regulation of isoprenoids metabolites and the molecular mechanisms regulating these genes are poorly understood.

In addition to these mechanisms, our previous study showed that the salt stress induced a change in the concentration of terpenoids of roots and leaves in *Brugueira gymnorrhiza* and *Kandelia candel* suggesting another physiological significance of terpenoids in mangrove tree species (Oku et al. 2003). These results suggested that terpenoids function as self-protecting barrier against the external salt stress. Therefore studies on gene expression of mangrove oxidosqualene cyclases (OSCs) under different salt concentrations are required to obtain more insight into the physiological roles of phytosterols and terpenoids in mangrove plant species.

Biosynthesis of terpenoids

A variety of triterpenes and phytosterols widely distributed in plants are biosynthesized from a common precursor 2,3-oxidosqualene by the enzyme oxidosqualene cyclases (OSCs). 2,3-Oxidosqualene therefore locates at the branching point of isoprenoid pathway toward phytosterols or terpenes biosynthesis (Abe et al., 1993). The cyclization of oxidosqualene into terpenoids and phytsoterols are one of the most fascinating reactions found in nature and their biosynthetic pathway has been shown to be complicated and divergent (Fig. 1.1). The enzymes, which perform these reactions, belong to OSCs family. In higher plants,

OSC family member cycloartenol synthase and lanosterol synthase are responsible for sterol biosynthesis, and other OSCs are involved for triterpenes synthesis.

The cyclization, rearrangement and deprotonation reaction leading to the different product of triterpenoids. Enzymatic cylization of 2,3-oxidosqualene into sterols proceeds *via* the so-called "chair-boat-chair" conformation to yield the C-20 protosteryl cation (Fig. 1.2), which is then converted to cyloartenol or lanosterol. Triterpenoid synthesis, on the other hand, involves cylization of the corresponding "chair-chair-chair" conformation of the substrate to give the tetracylic dammarenyl cation. This cation may then either be converted to dammarene-like triterpenoids by the OSC dammarenediol synthase (Tansakul et al., 2006) or undergo expansion during the cyclization and rearrangement of 2,3-oxidosqualene to promote ring expansion and annulation to form lupenyl cation, after D-ring expansion *via* C-16 migration followed 18β E-ring cylization from dammarenyl cation to form lupeol with direct deprotonation without rearrangement. Additionally, β-amyrin, also originate from dammarenyl cation intermediates, and allow further E-ring expansion *via* C-21 migration and some rearrangement before deprotonation or by the addition of a hydroxyl group, resulting in a multi products of oleanane class of terpenoids, such as germanicol or taraxerol (Abe et al., 1993; Haralampidis et al., 2002; Xu et al., 2003).

Molecular biologists have made considerable use of tetracylic triterpenoids (e.g. sterols such as cyloartenol) and pentacylic triterpenoids with a five-membered E-ring (lupeol and compounds based on the lupane skeleton) or a six-membered E-ring (e.g. compounds based on oleanane or ursane skeletons) to synthesize sources of OSC genes in higher plants.

The biosynthesis of phytosterol and pentacyclic terpenoids shares the common pathway up to the branching point of 2,3-oxidesqualene. A number of studies have postulated that distinct OSC exists for each type of terpenoids (Kushiro et al. 1998; Zhang et al., 2003; Kajikawa et al., 2006; Sawai et al., 2006a) or a common enzyme can produce multiple terpenoids (Shibuya et al. 1999; Segura et al. 2000). These studies revealed the presence of two types of OSCs in higher plants. One is monofunctional synthases yielding one specific product, as exemplified by β-amyrin synthases or lupeol synthase. The other is multifunctional synthases producing more than one product.

Several lines of studies have been suggested that OSCs are regulatory steps in the isoprenoids pathway orienting the biosynthetic flux towards either pentacyclic triterpenes or phytosterols, which are the precursors of large variety physiologically active compounds (Henry et al., 1992; Taton et al., 1996).

The cyclisation of OSCs to triterpenes and sterols not only represents a branchpoint between primary and secondary metabolites, but plant OSCs also attractive tools for investigating the regulation of synthesis and the physiological role of triterpenoids, and particularly for manipulation of phytosterol and terpene content (Baisted, 1971; van der Heijden et al., 1989; Henry et al., 1992).

An important question in understanding triterpene biosynthesis has centered on whether triterpene synthases yielding different products are distinct proteins or they are generated post translationally from one gene product, since activities of OSCs are susceptible to the change of pH, detergents and electrolyte concentrations (Baisted, 1971). Additionally, there still remains a question as to whether one triterpene synthase might change its product specificity to synthesize another type of triterpene depending on different physiological conditions (Goad, 1983). A number of successful cloning of triterpene synthases has proved the presence of multiple OSCs as distinct proteins even in one plant species. Taken together, this hypothesis suggested that the cyclization reactions were carried out by different enzymes. However, in the absence of amino acid sequence information the possibility remained that the enzymes represented different forms of a single gene product. Therefore the cloning and characterization of genes encoding OSCs from mangrove plants were required to resolve this issue and to obtain more insight into its regulation mechanism and physiological significance.

In view of the diversity of the triterpene product formed in mangrove plants, it became interesting to determine how each triterpene synthase controls the product specificity. Moreover, triterpene synthase have also attracted our attention from the view of plant metabolism and physiology. The previous report in our laboratory showed that triterpenoids may play an important role in the adaptation of mangroves to salt stress (Oku et al., 2003). Therefore, OSC attracted our attention to understand the regulation and physiological role of mangrove terpenoids.

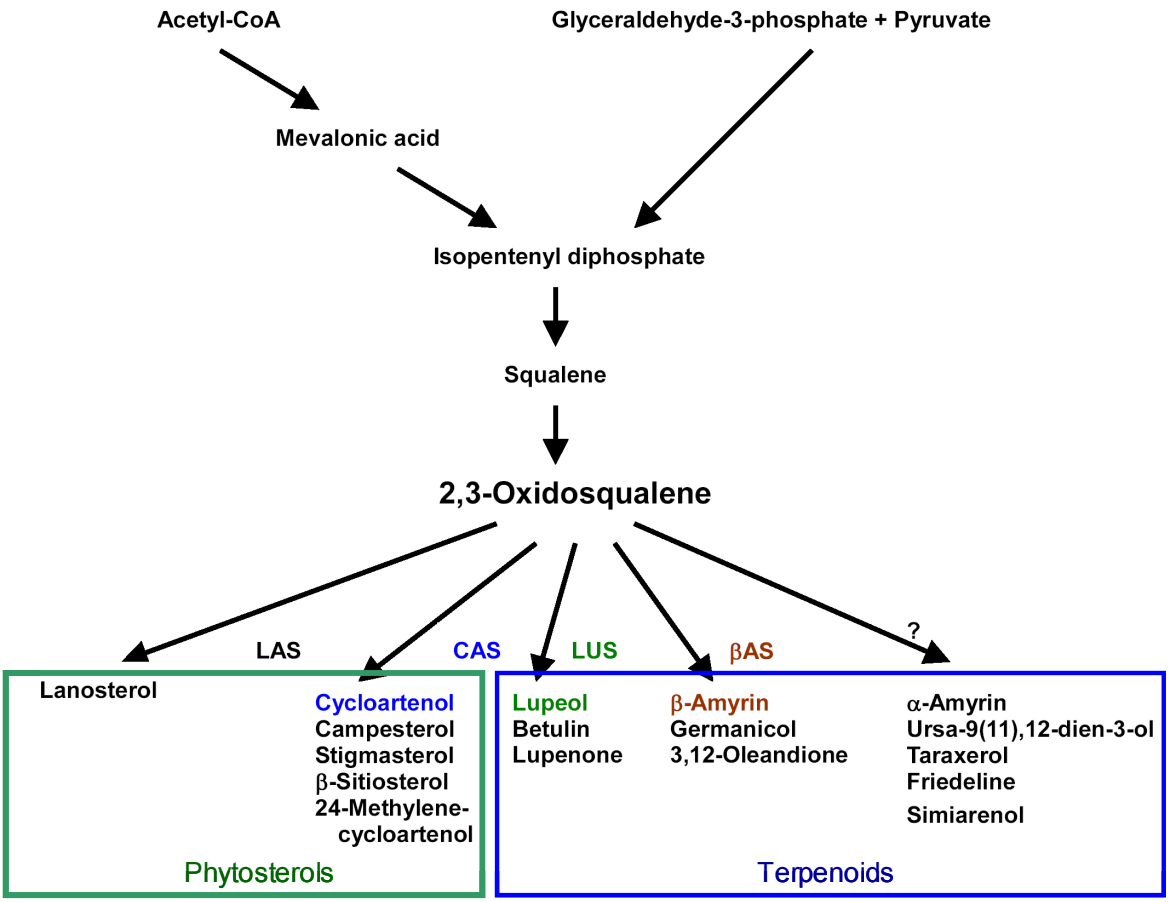


Fig. 1.1. A working-hypothesis for triterpenoid biosynthesis in mangrove plants. LAS, lanosterol synthase; CAS, cycloartenol synthase; LUS, lupeol synthase; βAS, β-amyrin synthase.

Thus in the present thesis, chapter II described terpenoid composition in the nonsaponifiable lipids (NSLs) fraction of Okinawan mangroves with special reference to lipid input to estuarine ecosystems and utilization as a source of medicinal compounds. Characterization of terpene synthases from the Okinawan mangrove tribe, Rhizophoraceae were studied in chapter III. Chapter IV delineated the cloning and functional expression of two cyloartenol synthases from mangrove species *Rhizophora stylosa* Griff. and *Kandelia candel* (L.) Druce. In chapter V, effect of salt stress on gene expression of mangrove OSCs and isoprenoids composition were studied.

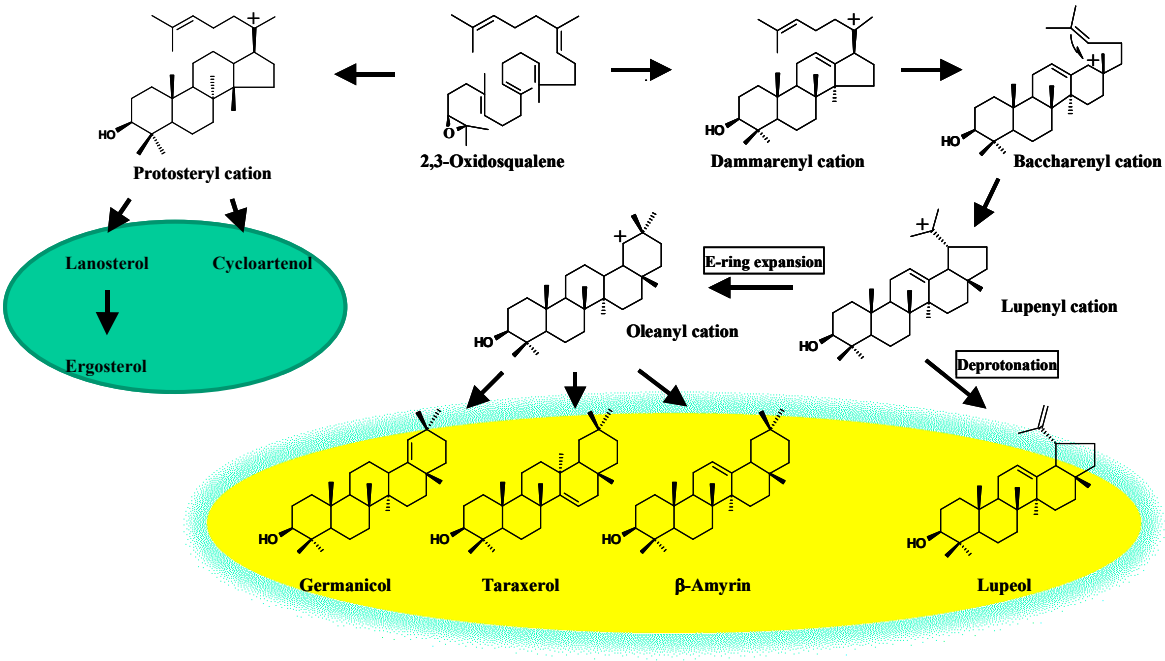


Fig. 1.2. Cyclization of 2,3-oxidosqualene to produce terpenoids and phytosterols.

Fig.1.3. Mangrove forest where dominated tree species is *Bruguiera gymnorrhiza* stands at Okukubi river, Okinawa prefecture.

Fig. 1.4. Mangrove forest where dominated tree species is *Kandelia candel* at Okukubi river, Okinawa prefecture.

Fig.1.5. Nakama river, Iriomote island with dominated tree species of *Rhizophora stylosa*.

Chapter II

Nonsaponifiable Lipid Composition of Seven Okinawan Mangroves

Part 1: Isoprenoids of Okinawan mangroves as lipid input into estuarine ecosystems

Introduction

Mangrove forests are widespread in the inter-tidal zone of tropical and subtropical regions, and form an important ecosystem for fish recruitment while protecting against coastal erosion (Tomlinson, 1986; Alongi, 2002). Because of its position along the land-sea interface, mangrove is highly vulnerable to abrupt changes in sea level and river sediment load (Nicholls et al., 1999). Thus, the present-day, human induced, rapid environmental changes demand a better understanding of the dynamics of this system.

Mangroves are among the most productive ecosystems on earth, and the mangrove litter fall is the most important source of organic carbon in biogeochemical cycles in the mangrove ecosystem (Wafar et al., 1997; Clough et al., 2000) and a valuable indicator of mangrove productivity (Clough, 1998). Due to this high productivity, turnover rates of organic matter and the exchange with terrestrial and marine ecosystems, mangroves are of particular importance for the biogeochemical recycling of carbon and associated elements along the tropical coastal region.

Because lipid comprises a significant proportion of carbon output from mangroves (Wannigama et al., 1981; Hogg and Gillan, 1984), knowledge of the lipid composition of mangrove promisingly contributes to estimating the sources and accumulation rates of sedimentary organic matter. In general, NSL represents a more stable lipid fraction than the saponifiable lipid fraction (fatty acids), and their resistance to microbial degradation has been considered to be a relatively important factor in controlling the diagenetic pathways (Killops and Frewin, 1994; Koch et al., 2005). Triterpenoids are common chemical constituents of higher plants, comprising the major proportions of NSLs, and have been identified in cuticular waxes of mangrove and other plant species (Beaton et al., 1955; Wannigama et al., 1981; Ghosh et al., 1985; Koch et al., 2003). Several lines of study have employed pentacyclic terpenoids as well-suited tracers for the primary source of organic matter from mangrove due to their stability during sedimentation and diagenesis (Killops and Frewin, 1994; Versteegh et al., 2004; Koch et al., 2005). Thus, analysis of terpenoids is a prerequisite for the interpretation of biomarker signals in sediment cores of mangroves. Furthermore,

it has been reported that mangrove leaves are a significant source of fatty acid (saponifiable lipid) and other lipids to surrounding ecosystems (Mfilinge et al., 2005). In spite of the importance of terpenoids as lipid input from mangrove into the environment or as biomarkers for organic matter, and as potential sources of medicines no information on the terpenoids composition of Okinawan mangrove has previously been available.

Furthermore, a number of biological activities from different mangrove plants have been reported for terpenoids (Kokpol et al., 1990; Miles et al., 1998; Williams, 1999). A triterpenoid saponin isolated from root *Acanthus illicifolius* had revealed antileukemic activity (Kokpol et al., 1986). Boiling extract of *Rhizophora apiculata* in Thailand has been used as folkloric medicine, and the biologically active compound has been identified as pentacyclic triterpenoids (Kokpol et al., 1990). These studies suggest that the mangrove tree species may become of a potential source of natural medicinal compounds, which may open up another possibility of mangrove utilization.

Mangroves are also unique in that these plants have cellular mechanisms to be tolerant with the high salinity (Tomlinson, 1986). Our previous study showed that the salt stress induced a change in the concentration of terpenoids of roots and leaves in *Brugueira gymnorrhiza* and *Kandelia candel* suggesting another physiological significance of terpenoids in mangrove trees (Oku et al., 2003). Thus, these observations in concert prompted us to analyze the nonsaponifiable lipid compositions of Okinawan mangroves with the special reference to isoprenoids.

Experimental procedures

Sample collection

Mangroves found in the southern part of Japan are largely concentrated on Iriomote Island, and are the northernmost mangroves in Asia at latitude 31°22′ N. Mangrove leaves and roots of mature trees were obtained from Iriomote Island, Okinawa, Japan, and were stored at –30 ˚C for lipid analysis. Seven species of mangroves thriving in the island were collected: *Bruguiera gymnorrhiza* (L.) Lamk. (Rhizophoraceae), *Rhizophora stylosa* Griff. (Rhizophoraceae), *Kandelia candel* (L.) Druce (Rhizophoraceae), *Lumnitzera racemosa* Wild. (Combretaceae), *Avicennia marina* (Forsk.) Vierh (Avicenniaceae), *Pemphis acidula* Forst. (Lythraceae), and *Sonneratia alba* J. Smith (Sonneratiaceae).

Lipid extraction

Main roots of mature trees were separated into epidermis, cortex and vascular bundle, as illustrated in Fig. 2.1. These parts are here named the outermost-, middle- and innermost-part, respectively. The outermost epidermis was peeled off with a razor blade, followed by separation at the junction of cortex and vascular bundle by hand. The leaves (5-6 leaves) or roots (5 to 10g wet weight, respectively) were first ground in liquid nitrogen, and extracted with 25 volumes of chloroform-methanol (2:1 by volume) (CM21). The cell wall debris insoluble in CM21 was removed by filtration through No. 2 filter paper (Advantec, Tokyo, Japan), and the extract was partially purified for lipid analysis as described previously (Folch et al., 1957). The extract was concentrated to dryness, and the lipid weight was measured gravimetrically. Total lipid content was expressed in relation to tissue weight (mg/g tissue).

Analysis of nonsaponifiable lipid (NSL)

The lipid extract containing 2 mg of total lipid was concentrated to dryness in a nitrogen stream, then saponified at 60 ℃ overnight with 3% KOH in 94% ethanol. The nonsaponifiable lipids (NSL) partitioned into hexane by vigorous mixing were analyzed by gas chromatography (GC

2010, Shimadzu, Kyoto, Japan) or gas chromatography-mass spectrometry (GC-MS QP 2010, Shimadzu).

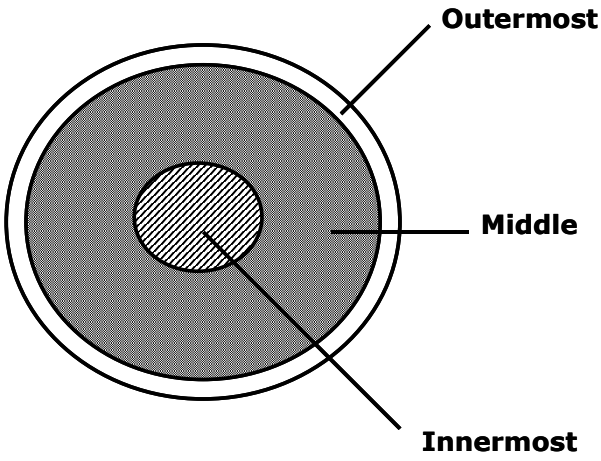


Fig. 2.1. Schematic representation of cross-section of mangrove root. The outermost, middle and innermost part correspond to epidermis, cortex and vascular bundle (central cylinder), respectively. The root of *B*. *gymnorrizha*, *R*. *stylosa*, and *K*. *candel* showed well-developed aerenchyma structure in cortex with wide medullated stele (central cylinder). Other species had rather broad lacunose or cancellous cortex and narrow medullated stele.

The column used was CBP1-M50-025 (Shimadzu), and the column temperature was programmed from 1 min hold at 50 ℃ to final temperature of 300 ℃ at a rate of 10 ℃/min. The carrier gas was helium with a flow rate of 0.70 ml/min (20 cm/s). To measure NSL content, approximately 30 mg of total lipid was saponified in 2 ml of 20% KOH (in 50% ethanol) at 90 ℃ for 10 min with reflux. NSL was extracted into 2 ml of hexane by vigorous shaking, and its weight was measured gravimetrically. NSL content was also expressed on the basis of fresh tissue weight (mg/g tissue) or total lipid weight (mg/mg total lipid).

Identification of chemical structure

The chemical structures of NSL were mainly identified by comparison of their retention time on the GC column with those of authentic standards, and by interpretation of the mass spectrum. Columns used and temperature programs were the same as described above for GC. The carrier gas was helium with a flow rate of 1.69 ml/min (47 cm/s). Samples were ionized by electron impact (EI) at 70 eV for estimation of chemical structure, or by chemical ionization with methane as reaction gas for the determination of molecular weight. The spectrum similarity search was carried out with the mass-spectrum library (NIST 147 and 27, Shimadzu). In case authentic standards were commercially unavailable, identifications were essentially based on similarity comparison of the mass spectrum with that in the spectrum database.

In the case of *R. stylosa*, the chemical structure of the major terpenoid component was unknown. To identify the chemical structure, the unknown component was purified for ^{13}C-nuclear magnetic resonance (NMR) spectrometry as follows. The unknown NSL component was first separated by silica gel column (1 cm ID x 50 cm length) with an eluent system of hexane/diethyl ether/acetic acid (70:30:1 by vol.) or hexane/ethylacetate/acetic acid (90:10:1 by vol.). Sample fractions containing unknown terpenoids were further purified by ODS column (1.1 cm ID x 30 cm length, Ultra Pack, Yamazen Co., Osaka, Japan) using 95% ethanol or 95% acetonitrile as an eluent. Purity of the specimen was checked by GC as described above. The chemical structure of the unknown lipid was analyzed by combination of ^{13}C-NMR and mass spectrometry. ^{13}C-NMR spectra were obtained at 125 MHz for ^{13}C by a Jeol α-500 spectrometer. The ^{13}C signals of the solvent (CD_3OD) were used as secondary references (49.0 from TMS, respectively). The ^{13}C-NMR and GC-MS spectral data identified the unknown terpene as taraxerol (Sakurai et al., 1987; Goad and Akihisa, 1992). ^{13}C-NMR ($CDCl_3$) δ : 158.1(C-14), 116.9(C-15), 79.1(C-3), 55.5(C-5), 49.3(C-18), 48.7(C-9), 41.3(C-19), 39.0(C-4), 38.8(C-8), 38.0(C-1, 17), 37.7(C-10, 13), 36.7(C-16), 35.8(C-12), 35.1(C-7), 33.7(C-21), 33.4(C-29), 33.1(C-22), 29.9(C-28), 29.8(C-26), 29.7(C-20), 28.0(C-23), 27.1(C-2), 25.9(C-27), 21.3(C-30), 18.8(C-6), 17.5(C-11), 15.5(C-24), 15.4(C-25). EI-MS *m/z*: 426[M^+] (17), 411 (12), 393 (3), 302 (39), 287 (37), 269 (17), 257 (13), 218 (31), 204 (100) (the

numbers in parentheses are the relative intensities of the fragment against the peak height of m/z=204).

Results and Discussion

The present study has extended our previous data on the lipid profile of mangrove, and described the NSL composition of Okinawan mangrove leaves and roots for the first time. Special emphasis was on the terpenoid compositions because these compounds are useful as biomarkers of organic matter from mangrove, as biologically active medicinal components.

The chemical structures of terpenoids identified in the mangrove leaves and roots are presented in Fig. 2.2. These terpenoids largely fall into three types of carbon skeleton: lupane (lupeol, lupenone, betulin); oleanane (β-amyrin, germanicol, taraxerol); ursane (α-amyrin, ursa-9(11),12-dien-3-ol). Among the terpenoids, taraxerol and lupeol were purified and identified by interpretation of ^{13}C-NMR and mass spectra as described respectively in our previous paper (Oku et al., 2003) and in the method section of this study.

α-Amyrin, β-amyrin, lupenone and betulin were identified by comparison of their retention time on a GC column and mass spectra with those of authentic standards. Identifications of others (germanicol, 3,12-oleandione, ursa-9(11),12-dien-3-ol, friedeline and simiarenol) were based only on the similarities of their mass spectra to those in the mass spectra library (NIST 147 and 27). The similarity indices were greater than 0.8 for these compounds.

Fig. 2.3 illustrates the chemical structures of phytosterol present in the mangroves. Campesterol, stigmasterol, β-sitosterol and lanosterol were identified by use of authentic references as described above, comparison of retention time on GC analysis, and mass spectra. Cycloartenol and 24-methylenecycloartenol were identified on the basis of the similarity search of the mass spectra.

Table 2.1 shows the total and NSL content of Okinawan mangrove leaves. Total lipid content of the mangrove leaves ranged from 3.2 to 10.6 mg/g tissues with an average of 6.0 mg/g. The average was almost one-half of that reported for mangroves of India: 11.9 mg/g (Ghosh et al., 1985). Lipid contents for *L. racemosa* and *P. acidula* were slightly lower than the other five species.

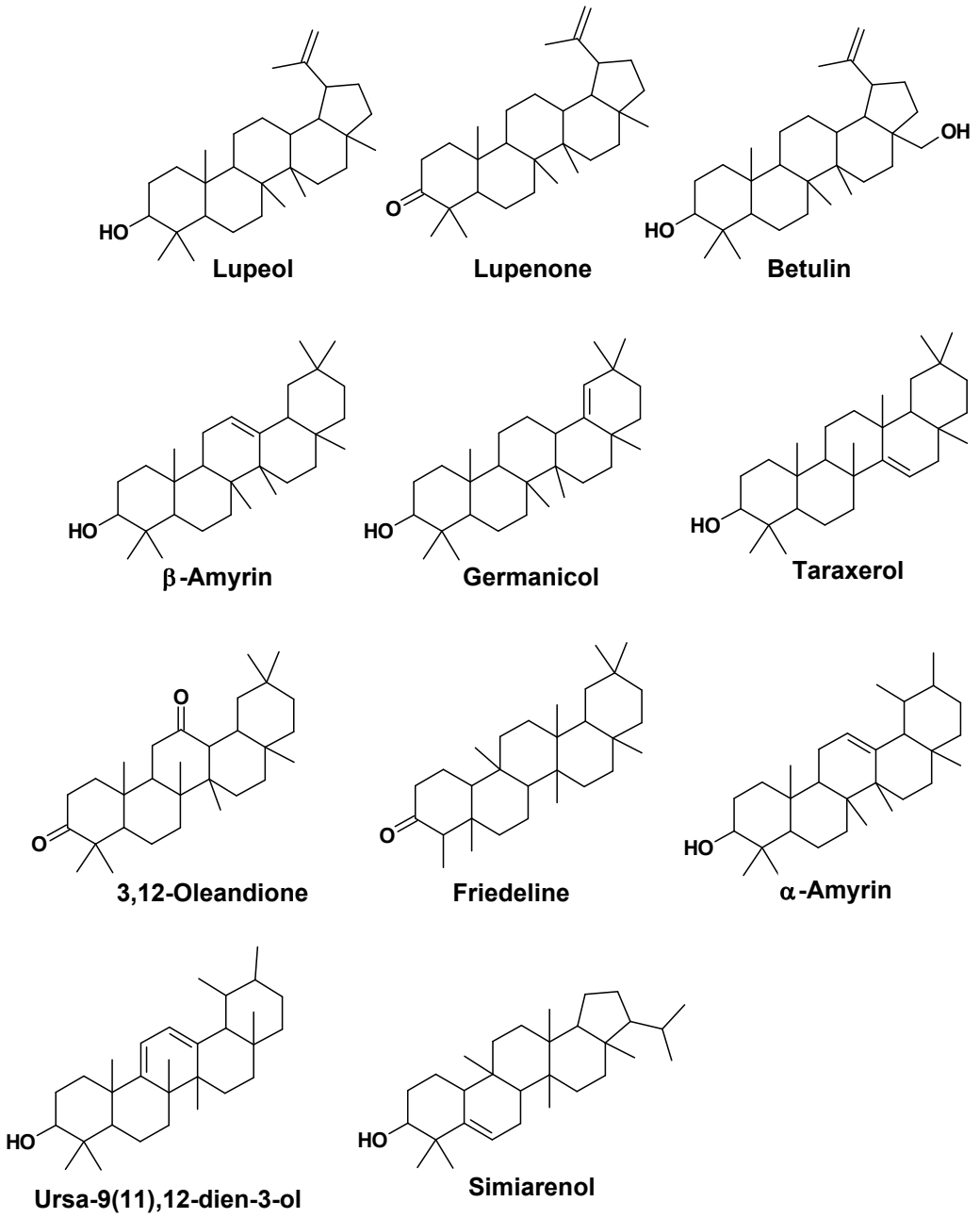


Fig. 2.2. Chemical structure of triterpenoids present in mangrove leaves and roots. Large-scale purification and identification by ^{13}C-NMR and GC-MS analyses were conducted for lupeol and taraxerol. Identifications of other terpenoids were based on GC and GC-MS analysis as described in the Experimental procedures section.

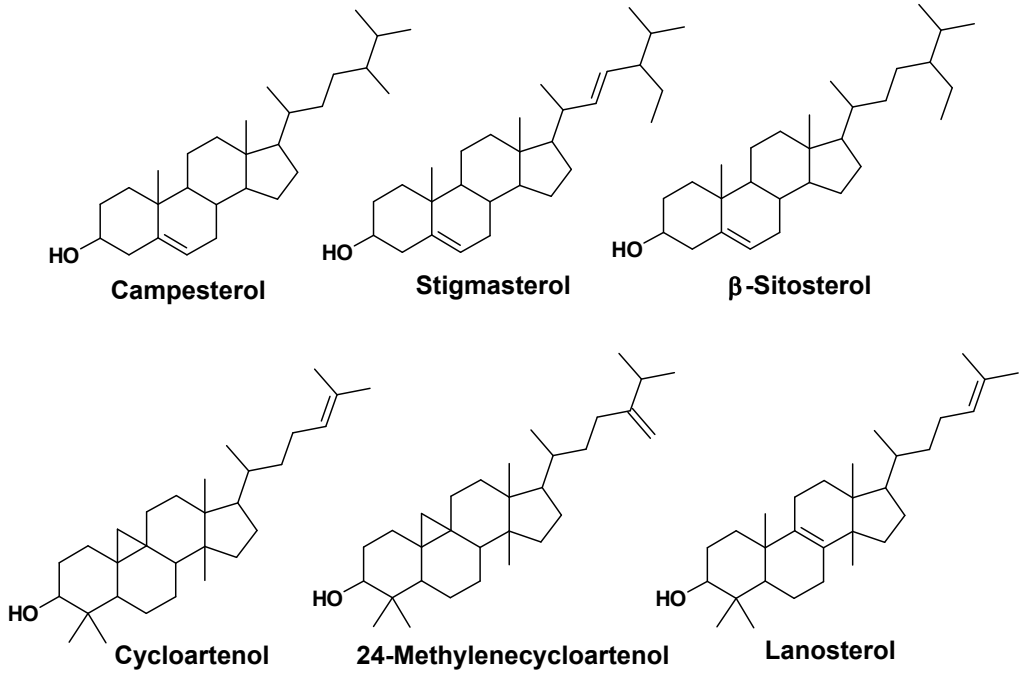


Fig. 2.3. Chemical structure of phytosterols present in mangrove leaves and roots. Identifications of the chemical structures were based on GC and GC-MS analysis as described in the Experimental procedures section.

Table 2.1. Total lipid and NSL contents of seven species of mangrove leaves

Species	Total lipid/Tissue (mg/g)	NSL/Tissue (mg/g)	NSL/Total lipid (mg/mg)
B. gymnorrhiza	5.34	0.91	0.17
R. stylosa	7.49	0.90	0.12
K. candel	6.36	1.48	0.23
L. racemosa	3.18	0.47	0.15
A. marina	5.47	0.99	0.18
P. acidula	3.29	0.63	0.19
S. alba	10.56	0.80	0.08

NSL content was the lowest for *L. racemosa*, and highest for *K. candel*. NSL comprised 8 to 23% of total lipids, and the average for seven species was 16%. Total and NSL content of root were not determined in the current study as the sample size was too limited for gravimetric measurement.

Table 2.2. Nonsaponifiable lipid composition (%) of mangrove leaves

Component	Species						
	B. gymnorrhiza	*R. stylosa*	*K. candel*	*L. racemosa*	*A. marina*	*P. acidula*	*S. alba*
Alkane (C25-C33)	2.9	0.6	11.1	16.5	11.9	9.2	48.9
Phytol	12.9	0.6	2.0	22.7	11.6	18.8	9.9
Squalene	3.2	3.3	6.9		6.0	5.7	7.5
Campesterol	2.0					1.7	1.2
Stigmasterol			0.6	2.3	1.5	4.3	1.9
β-Sitosterol	7.6	2.6	6.0	16.3	4.2	22.7	12.2
Cycloartenol			1.0				
Lanosterol							2.4
Lupeol	42.0	2.9		14.8	52.4		
Lupenone	4.7						
β-Amyrin	13.9	28.9	45.2		5.5	2.3	
Germanicol						13.1	
α-Amyrin			18.0	1.9		13.1	
Ursa-9(11),12-dien-3-ol			1.0				
Taraxerol		60.3					
Simiarenol				4.6			
Others	10.7	1.0	8.2	20.9	7.0	9.1	16.1

Data are mean of triplicate analyses

Table 2.2 summarizes the NSL composition of mangrove leaves. Diversity was notable in the composition of nonsaponifiable lipid. Terpenoids were the largest constituent of *B. gymnorrhiza*, *R. stylosa, K. candel* and *A. marina*. In contrast to these terpenoids-rich species, phytosterols were relatively dominant in the species of *L. racemosa*, *P. acidula* and *S. alba*, the largest concentration being β-sitosterol. The species of *S. alba* was distinguished from the others in that this species contained a larger quantity of alkanes. Phytol, probably derived from chlorophyll, was present in NSL fractions of all species. Squalene, an intermediate in isoprenoid biosynthesis, was also detectable at appreciable quantities in all species, except for the case of *L. racemosa*.

The NSL compositions of mangrove roots are listed in Table 2.3. Diversity within the species was also notable in the composition of root NSL. As was the case for the leaves, phytosterol and terpenoids comprised the largest proportion of NSL. The species of *B. gymnorrhiza*, *L. racemosa*, *A. marina* and *P. acidula* were abundant in lupeol. The root of *R. stylosa* and *K. candel* showed a higher concentration of fatty alcohols and phytosterol than other species. It was also noteworthy that the β-amyrin abundant in leaves was a minor component in the root.

The terpenoid composition showed variation with the mangrove species (Tables 2.2 and 2.3). This observation is in agreement with the previous result that the leaves of mangroves are

chemotaxonomically distinguishable on the basis of their marker component (Hogg and Gillan, 1984; Ghosh et al., 1985; ten Haven et al., 1992). Among the terpenoids detected, taraxerol was found to be more resistant to microbial degradation compared to other oleanane type terpenoids such as β-amyrin or germanicol, which makes this compound more ideal as a tracer of mangrove-derived organic matter (Killop and Frewin, 1994; Versteegh et al. 2004; Koch et al., 2005). The difference in the resistance to bacterial degradation can be explained by the stereoconfiguration of methyl group at the 13-carbon atom. The 13α-methyl group of taraxerol may hinder microbial enzyme processes (Koch et al., 2005). This, however, is only significant for *R. stylosa* because only this species contains a large amount of taraxerol in leaves, as was the case for *Rhizophora mangle* and *Rhizophora racemosa* (Killops and Frewin, 1994; Koch et al., 2003; Versteegh et al., 2004). Taraxerol was also reported as a minor common lipid in leaves and fruits of *Rhizophora mucronata* (Kokpol et al., 1990; Laphookhieo et al., 2004a), fruits of *Bruguiera cylindrica* (Laphookhieo et al., 2004b).

It is also noteworthy that the β-amyrin abundant in leaves was scarcely detected in the root. This was true for almost all mangrove species, and demonstrated that the type of terpenoid in leaf differed from that in the root, even in the same species. It has been reported that the leaf litter is the major contributor to the organic matter pool of an adjacent mangrove ecosystem, compared to root biomass (Fiala and Hernandez, 1993). However, the above observation should be taken into consideration in a particular case where the detrital flux from root biomass contributes to the surroundings to a greater extent. Furthermore this finding suggested that the triterpenoids in the root was biosynthesized in situ, not a translocation of the synthate from the leaves. The occurrence of squalene, the intermediate of isoprenoids biosynthesis, in the root may also support this view.

A number of studies have shown that phytosterols could be used as tracers of various inputs and transformation processes to environments due to their structural diversity, biosynthesis and stability (Volkman, 1986; Currie and Johns, 1989; Mudge and Norris, 1997). However, the phytosterol content of several Okinawan mangroves was low, as exemplified by *R. stylosa* leaves (Table 2.2). It has also been shown that β-sitosterol was degraded more efficiently in the sediments than other sterols such as cholesterol (Koch et al., 2005). Furthermore, some sterols are widely

distributed in the biological system, which makes them of limited value for assessing sources of organic matter in sediments. Taking these facts into consideration, the phytosterol output from Okinawan mangrove may have limited importance as a source of lipid input into the surrounding ecosystem.

Mflinge et al. (2005) studied the changes in the total lipid and fatty acid concentrations in mangrove leaves during microbial decay of two common mangrove species in Okinawa region: *Kandelia candel* and *Bruguiera gymnorrhiza*. They reported that the mangrove leaves are a significant source of fatty acids (saponifiable lipids) and probably other lipids to the adjacent environment. In this experiment, total lipid in the leaves of *K. candel* decayed faster than that of *B. gymnorrhiza* in both winter and summer. Although their study made no reference to NSL or terpenoid constituents, this may be explained in part by the difference in the terpenoid compositions. Our present data show that the major terpenoid was β-amyrin for *K. candel*, and lupeol for *B. gymnorrhiza*. Both components comprised the major proportion of NSL, which was rather more resistant to microbial degradation than fatty acids (Hogg and Gillan, 1984). The susceptibility to microbial degradation also showed variation between molecular species of terpenoids (Koch et al., 2005). Thus, it may be possible that the observed differences in the microbial decay of mangrove leave reflect the susceptibility of the major NSL component to microbial degradation. Therefore, our current results are likely to contribute to a better insight into lipid input from mangrove to estuarine sediments and the ocean.

Several biological activities have been demonstrated for terpenoids: anti-inflammatory activity for taraxerol, α-amyrin, β-amyrin, lupeol and germanicol (Akihisa et al. 1996; Singh et al. 2002; Kim et al. 2005); anti-carcinogenic activity for taraxerol and germanicol (Takasaki et al. 1999; Jang et al. 2004); insecticidal activity for taraxerol (William, 1999). In this respect, all of Okinawan mangrove species can be exploited as a source of medicinal compounds. It is however should be mentioned that *R. stylosa* may be particularly valuable because of its abundance in taraxerol. As stated above, taraxerol has several biological activities, and its abundance may be beneficial for the purification of this compound from the raw material. Exploration of other biological activities for terpenoids promisingly potentiates the usefulness of mangrove trees and the importance of mangrove

conservation.

Our previous study analyzed the lipid compositions of root by thin layer chromatography (TLC), and found histological uniqueness in the proportion of terpenoids (Oku et al., 2003). As summarized in Table 2.4, a site-dependent abundance of terpenoids or phytosterols was noted. The concentration of terpenoids showed inner to outer gradient, with the opposite gradient for phytosterols. Consequently, the outer and innermost parts of the root were respectively rich in terpenoids and phytosterols. These results agree essentially with our previous TLC data on lipid composition (Oku et al., 2003).

The concentration of terpenoids varied with the tissues of the root, inner or outer parts of the root. Sum concentration of terpenoids showed inner to outer gradient with a mirror images of descending gradient for the sum of phytosterol (Table 2.4). The phytosterols have been considered to be the membrane components (Bean, 1973) while the physiological significance for terpenoids has been an open question. The higher concentration in the outer tissue of the root may in part explain their protective role against external stresses including salinity, dryness, bacterial attack or herbivores. The cellular localization of the terpenoids may give an answer to this interesting question.

Table 2.3. Nonsapnifiable lipid composition (%) of mangrove roots

Component	Species																				
	B. gymnorrhiza			*R. stylosa*			*K. candel*			*L. racemosa*			*A. marina*			*P. acidula*			*S. alba*		
	O	M	I	O	M	I	O	M	I	O	M	I	O	M	I	O	M	I	O	M	I
Alkane (C24-C32)			8.8							6.7			6.8								
Fatty alcohol (C26-32)		2.9	17.0	51.0	0.6	0.8	48.2	9.7	13.5	5.6						6.1			30.0	3.9	
Squalene		0.3	1.0	1.9	2.4	2.0		1.6	1.3		0.6		0.6	1.2	6.0		9.7	9.1			2.5
Campesterol	4.9	5.8	5.2	1.1	5.3	8.5	5.8	14.5	14.1	3.1	1.2	1.9					1.7	2.0	1.9	4.2	9.8
Stigmasterol	22.7	19.0	12.5	3.6	4.0	9.4	13.0	30.2	24.0	5.5	9.1	10.6	9.2	7.4	20.2	2.2	1.9	1.5	34.3	4.3	
β-Sitosterol	27.2	21.4	19.8	21.1	50.5	63.1	17.6	36.6	36.1	7.5	51.3	73.1	12.4	13.7	46.5	3.6	42.2	46.8	10.8	21.5	68.2
Cycloartenol				0.7	2.5	1.2															
24-Methylenecycloartenol																		17.2			
Lanosterol													3.5	4.5	2.9		1.7	13.2			
Lupeol	7.5	45.5	21.6				0.8			37.3	25.4	1.5	42.2	37.0	7.3	57.3	29.8		11.7	46.6	3.8
Lupenone																6.1					
Betulin										5.8	3.0			3.9		7.8	1.1		2.2	0.6	
β-Amyrin		2.9	3.7	1.8	8.7	4.3							3.2	3.0	1.0						
3,12-Oleandione													18.5	27.3	6.7						
α-Amyrin				3.3	14.8	3.7															
Friedeline										19.4											
Taraxerol			1.1							3.7											
Unknown (M. W. 440)										3.5	2.4								2.6	11.3	
Others	37.6	2.3	9.3	15.5	11.3	7.0	14.6	7.4	10.9	1.8	7.0	12.9	3.6	2.1	9.4	16.9	11.8	10.1	6.5	7.6	15.7

Data are mean of triplicate analyses

O, outermost; M, middle; I, innermost

Table 2.4. Relative proportion of terpenoids and phytosterols in mangrove roots

Species		Outermost	Middle	Innermost
B. gymnorrhiza	Terpenoids	7.5 ± 0.1	48.3 ± 0.5	25.4 ± 3.1
	Phytosterol	54.9 ± 1.8	46.1 ± 0.6	37.4 ± 5.3
R. stylosa	Terpenoids	5.1 ± 0.1	23.5 ± 0.3	8.0 ± 0.6
	Phytosterol	26.5 ± 0.4	62.3 ± 0.8	82.1 ± 1.2
K. candel	Terpenoids	0.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
	Phytosterol	36.4 ± 1.3	81.3 ± 1.7	74.2 ± .3
L. racemosa	Terpenoids	62.5 ± 1.8	28.4 ± 0.3	1.5 ± 0.0
	Phytosterol	16.1 ± 0.8	61.6 ± 1.1	85.6 ± 0.5
A. marina	Terpenoids	63.9 ± 0.3	71.1 ± 0.1	15.0 ± 0.3
	Phytosterol	25.1 ± 0.6	25.6 ± 0.5	69.7 ± 0.2
P. acidula	Terpenoids	71.2 ± 0.1	30.9 ± 0.1	0.0 ± 0.0
	Phytosterol	5.8 ± 0.1	47.6 ± 0.3	80.8 ± 2.2
S. alba	Terpenoids	13.9 ± 0.2	47.2 ± 0.1	3.8 ± 0.0
	Phytosterol	47.0 ± 0.4	30.0 ± 0.5	78.0 ± 0.4

Data are mean ± SE of triplicate analyses

Chapter III

Characterization of Triterpene Synthases from the Okinawan Mangroves Tribe, Rhizophoraceae

Part 1: Molecular cloning and functional expression of a multifunctional triterpene synthase cDNA from a mangrove species *Kandelia candel* (L.) Druce

Introduction

Mangrove plants are distributed in intertidal tropical and subtropical regions and are rich sources of pentacyclic triterpenoids (Wannigama et al., 1981; Hogg and Gillan, 1984; Ghosh et al., 1985; Koch et al., 2003). Several biological activities of mangrove triterpenoids have been reported. For example, extract of *Rhizophora apiculata* has been used as a folklore medicine in Thailand and its biologically active compound has been identified as a pentacyclic triterpenoid (Kokpol et al., 1990). Triterpenoids from *Acanthus illicifolius* have been reported to show antileukemic activity (Kokpol et al., 1986). However, their physiological function, if any, in mangrove plants has been rarely reported.

Biosynthesis of triterpenes branches from phytosterols at the cyclization step of 2,3-oxidosqualene, which is catalyzed by oxidosqualene cyclases (OSCs) (Abe et al., 1993). Diverse skeletons of triterpenes such as lupane (lupeol), oleanane (β-amyrin) and ursane (α-amyrin) are constructed by OSCs (Fig. 3.1). A number of OSCs have been cloned and their enzyme functions identified by expression in lanosterol synthase deficient yeast (Xiang et al., 2006, and cited therein). These studies revealed the presence of two types of OSCs in higher plants. One is monofunctional synthases yielding one specific product, as exemplified by β-amyrin synthases (Hayashi et al., 2004, Iturbe-Ormaetxe et.al., 2003, Kajikawa et al., 2005, Kushiro et al., 1998, Morita et al., 2000, Sawai et al., 2006, Zhang et al., 2003), lupeol synthases (Guhling et. al., 2006, Hayashi et al., 2004, Shibuya et al., 1999, Zhang et al., 2003), isomultiflorenol synthase (Hayashi et al., 2001.), cucurbitadienol synthase (Shibuya et al., 2004), thalianol synthase (Fazio et al., 2004), marneral synthase (Xiong et al., 2006) and arabidiol synthase (Xiang et al., 2006). The other is multifunctional synthases producing more than one product, that include a multifunctional lupeol synthase (LUP1) (Segura et al., 2000) and another multifunctional OSC yielding more than nine products (Kushiro et al., 2000) from *Arabidopsis thaliana*, a mixed amyrin synthase from *Pisum sativum* (Morita et al., 2000), etc. It is noteworthy that more than one hundred triterpene skeletons are elaborated at

cyclization step of 2,3-oxidosqualene by contribution of both mono- and multi-functional triterpene synthases.

Despite the ubiquitous distribution of triterpenes in the plant kingdom, their physiological functions in the producing plants are not well understood, especially for those from mangrove species. Triterpenoids from *R. mangle* may be functioning as chemical defense substances as they show insecticidal activity (William, 1999), and our recent study provided a view that mangrove triterpenes may play a role in the defense system against salt stress (Oku et al., 2003). In this context, OSCs are likely to take part in the defense mechanism, as they are the first committed enzymes for triterpene biosynthesis. So far, no information on OSCs from mangrove species has been available. In order to obtain more insights into the physiological function of triterpenes in mangrove plants, present study therefore has been undertaken to clone OSC cDNAs from *Kandelia candel* (Rhizophoraceae), which is widely distributed in the Okinawan islands, Japan and produces a large amount of triterpenes (Majumdar and Patra, 1976; Oku et al., 2003). This section shows the first example of a multifunctional OSC from mangrove tree species.

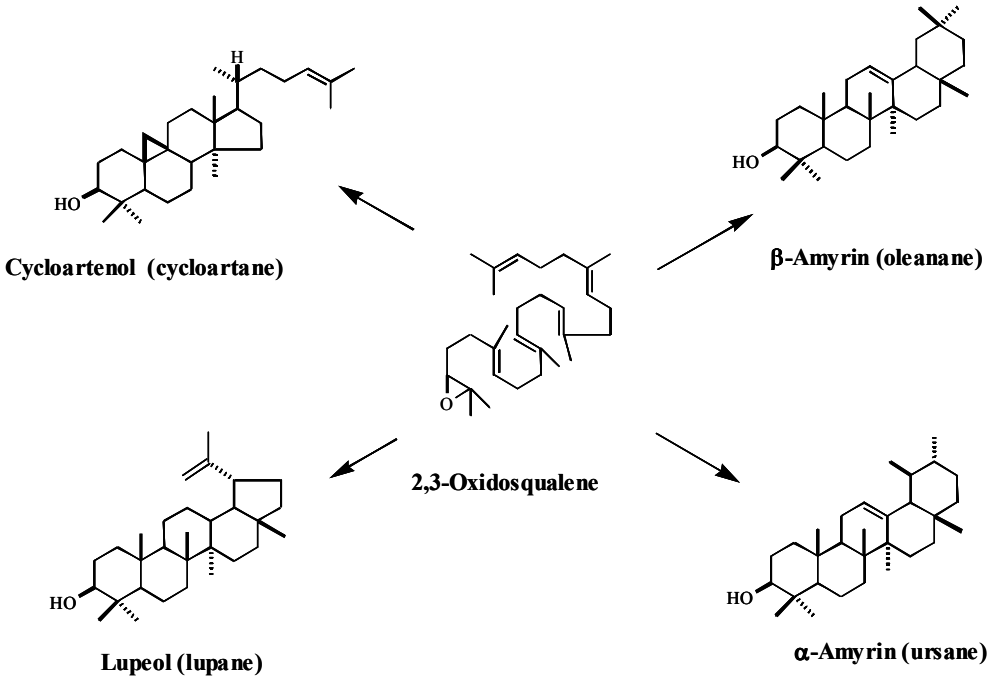


Fig. 3.1. Cyclization of 2,3-oxidosqualene to lupeol, β-amyrin, α-amyrin and cycloartenol.

Experimental procedures

Plant and culture conditions

Fresh young roots of *K. candel* were collected at Okukubi river, Okinawa, Japan. These materials were taken directly into liquid N_2 and stored at –80 ºC for RNA preparation. Yeast strain GIL77 (*gal2 hem3-6 erg7 ura3-167*) was used as the host, and maintained on a YPD medium (1.0 % yeast extract, 2.0% peptone, 2.0% dextrose) supplemented with hemin (13 μg/ml), ergosterol (20 μg/ml) and Tween 80 (5 mg/ml). Transformation of the yeast mutant was carried out by the Frozen-EZ Yeast Transformation II^{TM} Kit (Zymo Research, Orange, CA, USA). The transformant was cultured in synthetic complete medium without uracil (SC-Ura) supplemented with hemin and ergosterol (the same concentration as described above) at 30 ºC with shaking (220 rpm).

cDNA preparation

Total RNA was extracted from young root of *K. candel* using CTAB method (Simamoto and Sasaki, 1997) with a few minor modifications to improve the yield. Total RNA (2.42 μg) was reverse-transcribed with 0.5 μg oligo dT primer (RACE 32, 5'-GACTCGAGTCGACATCGA TTTTTTTTTTTTTT-3') to produce a cDNA in total volume of 20 μl for 5 min at 65 ºC, 1 h at 50 ºC, and 5 min at 85 ºC, and using cloned AMV First-Strand cDNA synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. The resulting cDNA mixture was diluted with 50 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and directly used as template for the following PCRs.

Cloning of Core Fragment

Four degenerate oligonucleotide primers (161S, 463S, 603A and 701A) corresponding to the highly conserved regions of known oxidosqualene cylases (OSCs) enzymes were synthesized. The nucleotide sequences of these primers are as follows: 161S = 5'-GAYGGIGGITGGGIYTICA-3' (DGGWGLH), 463S= 5'-MGICAYATHWSIAARGG-3' (RHISKGSW), 603A= 5'-CCCCARTTI CCRTACCAISWICCRTC-3' (DGSWYGNWG), 701A= 5'-CKRTAYTCIGCIARIGCCCA-3'

(FPMWALAEY). First PCR with 161S and 711A primers (10 ng) was performed by Ex Taq DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) with dNTP (0.2 mM) in the final volume of 100 µl according to the manufacturer's protocol. PCR amplification scheme was for 2 min at 94 ºC, followed by 30 cycles of 30 sec at 94 ºC, 30 sec at 50 ºC and 3 min 72 ºC, with final extension of 10 min at 72 ºC. The first PCR product was applied on Centricon centrifugal filter devices YM-30 (Millipore Co., Bedford, MA, USA) to remove the primers, and the volume was adjusted to 100 µl. The second PCR was carried out with 463S and 603A primers (10 ng) and 3 µl of the first PCR product as template under the same condition as the first PCR. The PCR product (≈445bp) was separated using 1 % agarose gel GTG and purified by Suprec^{TM}-01 (Takara Bio Inc.). The purified fragment was ligated to a plasmid vector of TOPO 10 (Invitrogen) and propagated in *Escherichia coli*, and sequenced by ABI PRISM^{TM} 3100-Avant Genetic Analyzer (Applied Biosystems, Tokyo, Japan) with Bigdye^{R} Terminator ver. 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). All the PCR products were subcloned and sequenced by the same procedure.

Cloning of 3′- and 5′- end amplification of cDNA

The "rapid amplification of cDNA ends" (RACE) method (Frohman et al., 1988) was applied for the 3'-end and 5'-end amplification using GeneRacer^{TM} Kit (Invitrogen). Based on the sequence of the core fragment, for 3'-RACE amplification, two specific oligonucleotide primers: S1 (5'-TGATGCCCCCTGAACTTG-3') and S2 (5'-CCTGAGCACAGGAGGAAAG-3') were synthesized. First PCR was carried out with S1 and GeneRacer 3' primer (5'-GCTGTCAACGATA CGCTACGTAACG-3') using *K. candel* cDNA as template, and the nested PCR with S2 and GeneRacer 3' nested (5'-CGCTACGTAACGGCATGACAGTG-3') primer was performed with the first PCR product as template, as described for the core fragment amplification except for that annealing temperature was 55 ºC.

For 5'-RACE amplification, two specific primers were synthesized i.e. A1 (5'-GAAACT CCACTGGATTAAGCCA-3') and A2 (5'-GAGGTTCCATCTTCCTACCA-3'). 5'-RACE was carried out as described above using A1 and GeneRacer 5' primer (5'-CGACTGGAGCACGAGGACA CTGA-3') for first PCR with cDNA template prepared by superscript III RT GeneRacer (Invitrogen).

The second PCR was conducted with A2 and GeneRace 5' nested primer (5'-GGACACTGACATG GACTGAAGGAGTA-3') using the first PCR product as template DNA and with annealing temperature of 57 ºC.

Cloning of the full-length cDNA

The full-length cDNA for *KcMS* was obtained using N-terminal and C-terminal primers, with introducing a KpnI site and XhoI site immediately upstream of the ATG codon and downstream of the TGA codon. The sequences were as follows: Kpn-KC-N1 (5'-GACT**GGTACC**ATGTGGAGGC TTAAGATTGCA-3'; KpnI site in bold face), Xho-KC-C2 (5'-GGC**CTCGAG**ATTTTTGGCC TATTTCAATCA-3'; XhoI site in bold face). PCR was performed with each set of primers with *K. candel* cDNA template and annealing temperature of 55 ºC. The obtained full-length cDNA was sequenced in both strands.

Expression in yeast

The 2.3-kb PCR product was digested with *Kpn*I and *Xho*I and ligated into *Kpn*I and *Xho*I sites of pYES2 (Invitrogen) to construct plasmid *KcMS*. The identity of the inserted DNA was verified by sequencing. The mutant GIL77 lacking lanosterol synthase activity was transformed with the plasmid using Frozen-EZ Yeast Transformation II^{TM} Kit (Zymo Research). The transformant of OSC-*KcMS* was inoculated in 25 ml synthetic complete medium without uracil (SC-Ura), containing hemin (13 μg/ml), ergosterol (20 μg/ml) and tween-80 (5 mg/ml), and incubated at 30 ºC for 2 days. Then, medium was changed to SC-Ura with the supplement of 2 % galactose for glucose. Cells were incubated at 30 ºC for 10 h, harvested by centrifugation at 3000 rpm for 5 min, resuspended in 20 ml of 0.1 M potassium phosphate buffer pH 7.0 and 3 % glucose and hemin, and incubated another 72 h. Cell pellet were collected and refluxed with 2 ml 20% KOH/50% ethanol at 90 ºC for 10 min. After extraction with the same volume of hexane, the extract was concentrated and applied to a TLC plate (Merck, Darmstadt, Germany), which was developed with benzene/acetone (19:1, v/v). The fraction corresponding to triterpene mono-alcohol was scraped, extracted with chloroform-methanol (2:1)

and served as the sample for GC or GC-MS analysis.

Gas chromatography (GC) and Gas chromatography-mass spectrometry (GC-MS) of triterpenoids

The reaction products of OSC in the extract were directly analyzed by gas chromatograph equipped with a flame ionization detector (Shimadzu, Kyoto, Japan). The column used was CBPI-M50-025 (0.25 mm ID x 50 m, Shimadzu). Column temperature program was at 50 ºC for 1 min, raised to 300 ºC with a rate of 10 ºC/min, and held at 300 ºC for 26 min. The carrier gas was helium with a flow rate of 20 cm/s, and the temperatures for injector and detector were 250 ºC and 300 ºC, respectively. The mass spectrometer used was GC-MS QP-2010 (Shimadzu). Column and GC conditions were described above. Ionization of sample was by electron impact (EI) at 70 eV to estimate the chemical structure, or by chemical ionization with methane as a reaction gas to determine the molecular weight. A similarity search of the spectrum was done with the mass-spectrum library (Nist 107, Shumadzu). Authentic standards of lupeol, β-amyrin, and α-amyrin were purchased from Extrasynthese, Genay, France.

Phylogenetic analysis of amino acid sequence

Sequence alignment of deduced amino acid *KcMS* was performed using the CLUSTAL W program (Thompson et al., 1994). Phylogenetic analysis of deduced amino acid alignment was conducted with CLUSTAL W program followed by drawing with TreeView software (Page, 1996) based on a neighbor-joining method. Bootstrap analysis with 1000 replications was used to assess the strength of the nodes in the tree (Felsenstein, 1985). The DDBJ/GenBank/EMBL accession numbers of the sequence used this analysis are as follows: AB025344 (*OEX*), AY520819 (*OSCCCS*), AB009029 (*PNX*), AB055509 (*BPX*), AB055510 (*BPX2*), U02555 (*CAS1*), AB025968 (*GgCAS1*), D89619 (*PSX*), AB181246 (*OSC5*), AB058507 (*CSOSC1*), AB025353 (*ALLOSC1*), AB033334 (*LcCAS1*), AB116238 (*CPQ*), AB116239 (*CPR*), AB033335 (*LcOSC2*), AB025346 (*TRV*), AB009031 (*PNZ*), AB025345 (*TRW*), AB025343 (*OEW*), AB055511 (*BPW*), AB116228 (*GgLUS1*), AB181245 (*OSC3*), AC007260 (*At1g78500*(*T30F21.16*)), U49919 (*At1g78970*(*LUP1*)), AC007152 (*At1g66960*(*F1019.4*)), AC002986 (*At1g78960*(*YUP8H12R.43*)), AB058643 (*LcIMS1*), DQ268869

(*RcLUS*), AB257507 (*KcMS*), AB206469 (*EtAS*), AB009030 (*PNY*), AB014057 (*PNY2*), AB055512 (*BPY*), AB034803 (*PSM*), AF478455 (*LjAMY2*), AB034802 (*PSY*), AF478453 (*MtAMY1*), AB181244 (*OSC1*), AB037203 (GgbAS1).

Recently, the enzyme function of *PNZ* was identified as lanosterol synthase (Suzuki et al., 2006).

Results and discussion

Cloning of OSC cDNA from K. candel

In order to clone a *K. candel* triterpene synthase, PCRs were performed using degenerate primers designed from the highly conserved regions of the known OSCs as described previously (Kushiro et al., 1998). The amplified DNA fragment (445 bp in length) was cloned into TOPO 10 vector (Invitrogen). More than three clones were sequenced and all of them showed an identical sequence. 3'-RACE and 5'-RACE (Frohman et al., 1988) were performed to clone 3'- and 5'-ends of the obtained clone with GeneRacer^{TM} Kit (Invitrogen) and yielded a full-length sequence of the cDNA, which was named *KcMS*.

The Open reading frame (ORF) of *KcMS* consists of 2286 bp that encodes a 761 amino acid polypeptide, which included five QW motifs: [(K/R)(G/A)X_{2-3}(F/Y/W)(L/I/V)X_3QX_{2-5}GXW] (Poralla et al., 1994; Abe and Prestwich, 1995a), which occurs repeatedly in the sequences of all known OSCs, are present in the *KcMS* sequence (Fig. 3.2). The consensus sequence is referred to as the QW motif as a consequence of the absolute conservation of Gln (Q) at position 10 and the carboxy-terminal Trp (W) at position 16 (Poralla et al., 1994) and by analogy, the sequence data revealed the presence of a DCTAE (Asp-Cys-Thr-Ala-Glu) motif that required for substrate binding (Abe and Prestwich, 1994; Feil et al., 1997), which has been claimed to be in the active site in the generation of the protesteryl cation for lanosterol synthase (Abe and Prestwich, 1994, 1995b). In addition to the aromatic amino acids (the QW motif), these repeats are likely to be important for protein structure and catalytic activity (Poralla et al., 1994; Wendt et al., 1997), although the function of the conserved amino acid residues inside the QW motif is still unknown (Haralampidis et al., 2002).

The deduced amino acid sequence of *KcMS* showed significant homology to the known triterpene synthases. Among them, the highest homology of 79% was shown with a *Ricinus communis* lupeol synthase (Guhling et. al., 2006). These results suggested that *KcMS* encodes triterpene synthase, most probably lupeol synthase.

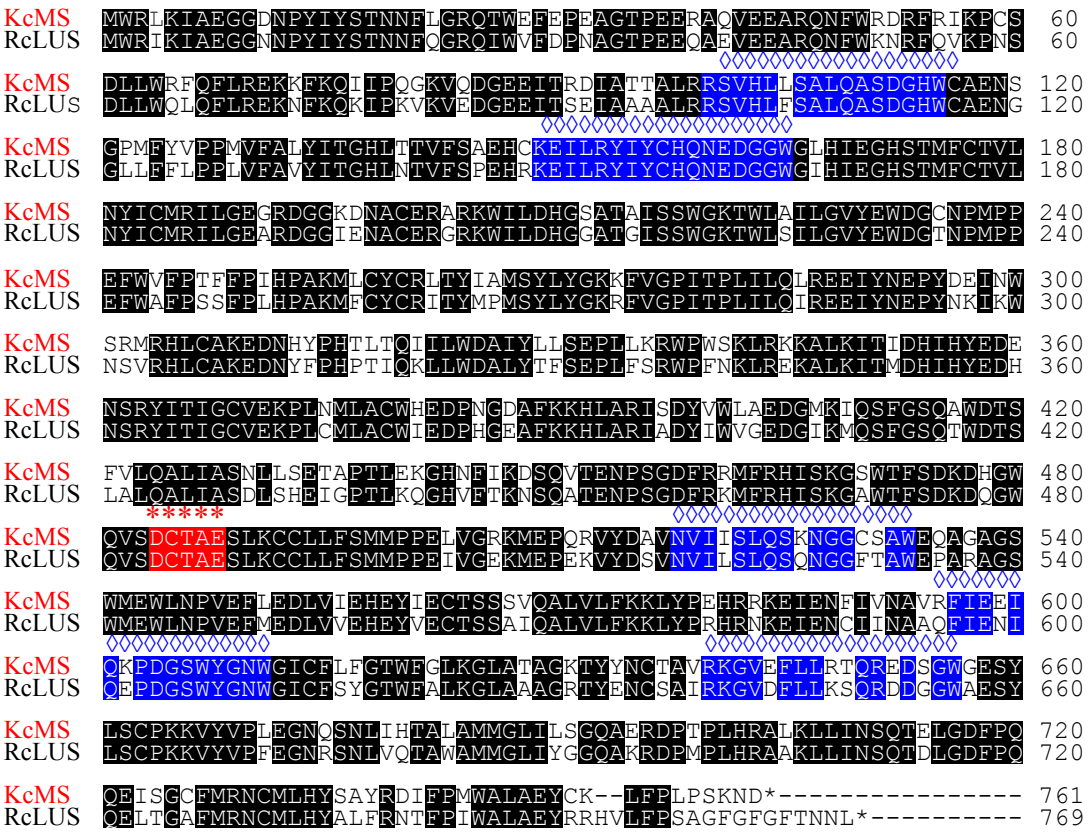


Fig. 3.2. Sequence alignment of the deduced amino acid from *K. candel* (*KcMS*) with lupeol synthase of *R. comunis* (*RcLUS*). DCTAE and QW motifs are indicated with # and ◊, respectively. Identical amino acid residues of two proteins are shaded and dashes indicate alignment gaps. The DDBJ/GenBank/EMBL accession numbers: *KcMS* (AB257507) and *RcLUS* (DQ268869).

Functional expression of KcMS in yeast

In order to investigate the function of the *KcMS* clone, functional expression in yeast was undertaken. *KcMS* cDNA was ligated to a yeast expression vector pYES2 (Invitrogen), and expressed under the control of GAL1 promoter in a lanosterol synthase deficient yeast mutant GIL77 that accumulates oxidosqualene inside cells (Kushiro et al., 1998). Introduction of *KcMS* gene into GIL77 resulted in the production of new compounds with the same mobility as β-amyrin on thin layer chromatography (TLC) (Fig. 3.3). However, its amount was smaller than that of the known OSCs such as β-amyrin synthase from *Panax ginseng* (Kushiro et al., 1998) (data not shown). Prolonged culturing slightly increased the band intensity but not to a great extent.

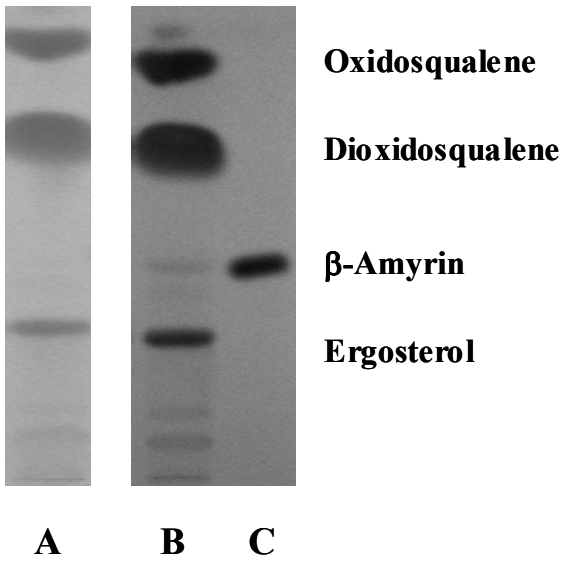


Fig. 3.3. TLC analysis of hexane extracts from transformed GIL77.

TLC was developed with benzene/acetone (19:1). To visualize the chromatograms, the plates were immersed in phosphoric acid/33% acetic acid/sulfuric acid/0.5% copper sulfate (5:5:0.5:90, by vol.) for 10 seconds, and heated at 140 ºC for 15 min (Brod et al., 1991). A, empty vector (pYES2) as control; B, lipid profile of extract from transformants (pYES2-*KcMS*) incubated 72 h after induction; C, β-amyrin standard.

In order to identify the chemical structures, products were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS) as described in the experimental section.

As shown in Fig. 3.4, three product peaks 1, 2 and 3 in 1:2:1 intensity are detected in GC and identified as β-amyrin, lupeol and α-amyrin, respectively, by comparing their Rts and mass fragmentation patterns on GC-MS with those of authentic standards. These results established that *KcMS* encodes a multifunctional triterpene synthase. Although *KcMS* shares high sequence homology (79%) with *R. communis* lupeol synthase, its product specificity is leaky as it produces significant amount of β-amyrin and α-amyrin in addition to the major product lupeol. So far, six multifunctional triterpene synthases have been identified (*LUP1*(At1g78970), Herrera et al., 1998; *PSM*, Morita et al., 2000; At1g78960, Kushiro et al., 2000; *LjAMY2*, Iturbe-Ormaetxe et al., 2003; At1g66960, Ebizuka et al., 2003; At1g78500, Ebizuka et al., 2003) as mentioned above and some of them produce lupeol as the major product. Their product patterns, however, are not identical and differ each other. *KcMS* is thus a new type of multifunctional OSCs with unique product specificity.

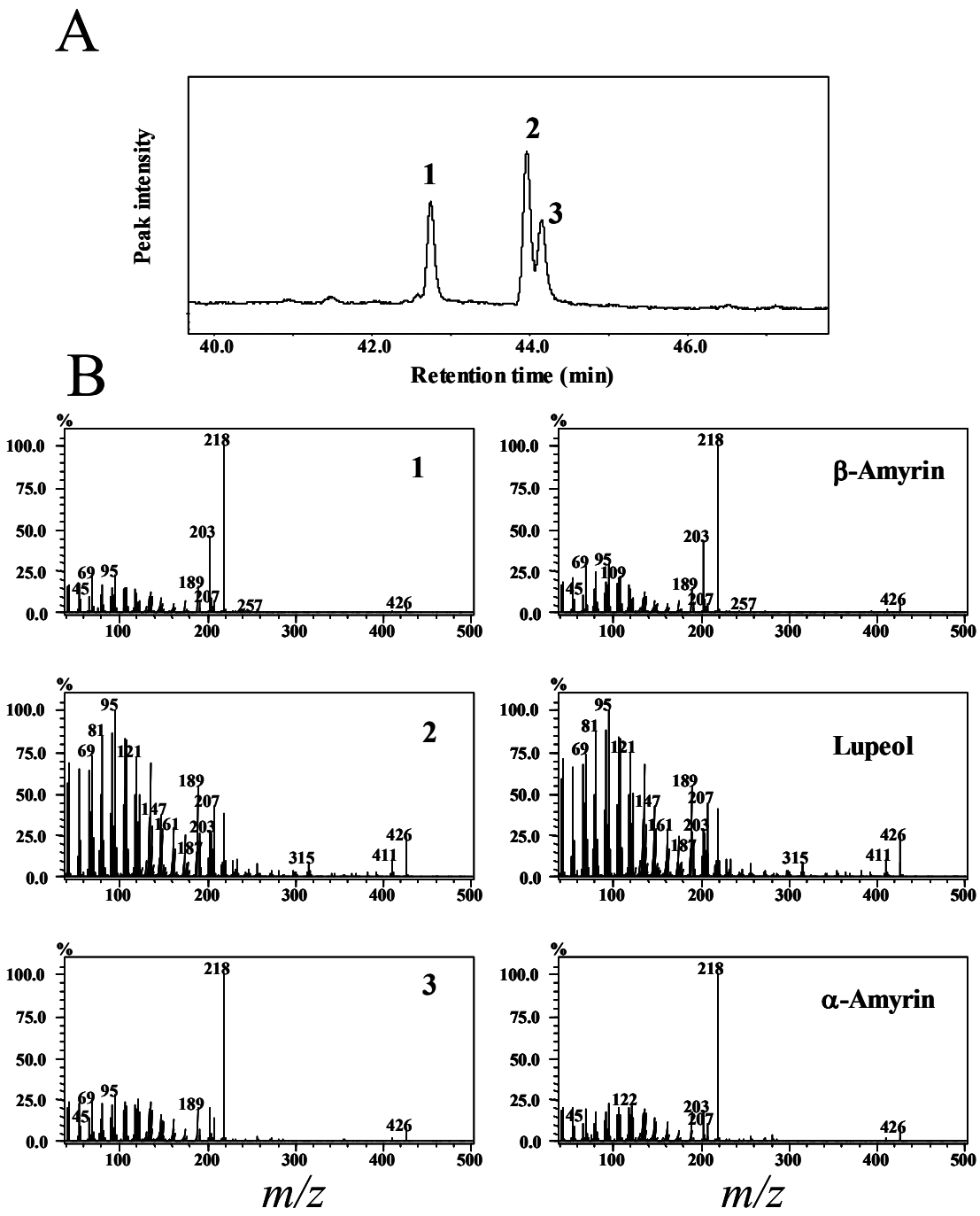


Fig. 3.4. GC-MS analysis of extracts from transformed GIL77.

Gas-chromatogram was monitored by FID (A). Left column of B (1-3) indicates EI-mass spectra of the products corresponding to the peak 1, 2 and 3. EI-Mass spectra of the authentic standards were shown in the right column of B.

2.3. Phylogenetic analysis of KcMS

To analyze the phylogenetic relationship of *KcMS* among plant OSCs, phylogenetic tree was constructed (Fig. 3.5). Cycloartenol synthase (CAS), lupeol synthase (LUS) and β-amyrin synthase (βAS) clones form each cluster in a tree. Based on the relationship between the distance in the tree and the reaction mechanism, sequential evolution of lupeol synthase and β-amyrin synthase from ancestral cycloartenol has been proposed (Shibuya et al., 1999; Wu and Griffin, 2002; Zhang et al., 2003). Multifunctional synthases do not form a branch but scatter in the tree and are proposed to be at an evolutionally transient state from one product specific OSC to another OSC. Recently, new product specific lupeol synthase, which does not join lupeol synthase branch and locates close to β-amyrin branch has been cloned from *R. communis*. *KcMS* shares highest homology with this clone, and thus might be an evolving clone from this new lupeol synthase to β-amyrin synthase.

Mangrove triterpenes

Our previous study demonstrated the presence of triterpenes lupeol, β-amyrin, and α-amyrin as fatty acid esters in the roots and leaves of *K. candel* (Oku et al., 2003). Product pattern of *KcMS* in transformed yeast is almost identical to that of triterpenes isolated from this plant. However, we can not exclude the presence of product specific β-amyrin synthase and lupeol synthase in this plant, since they are widely distributed in higher plants.

Our previous study also showed that the free triterpene concentration was higher in the outer part of the roots than inner part, and the proportion of free triterpenes increased with salinity in both leaves and roots of *K. candel* (Oku et al., 2003). These results might suggest that mangrove triterpenes play a role in the protection from external invasion by microbes, and/or in maintaining the physiological condition with change in salt concentration, which is often caused by exposure to marine water. The obtained cDNA will be useful and essential to clarify possible roles of triterpenes in mangroves.

It has been argued for a long time whether triterpene synthases yielding different products are distinct proteins or if they are generated post translationally from one gene product. This is because

activities of OSCs are susceptible to changes of pH, detergents and electrolyte concentrations (Baisted, 1971). A number of successful cloning of triterpene synthases has proved the presence of multiple OSCs as distinct proteins even in one plant species. However, this does not exclude the possibility of functional changes by post-translational modifications. Especially in mangrove trees, such a possibility cannot completely be eliminated, since the amounts and compositions of triterpenoids in the roots of *Bruguiera gymnorrhiza* and *K. candel* vary with the salt concentrations (Oku et al., 2003). These changes may be ascribed to changes of catalytic efficiency and/or specificity of *KcMS* by the alteration in tertiary protein structure, or in changes of expression of other OSCs with different functions by transcriptional regulation. A detailed biochemical analysis of *KcMS in vitro* and further cloning of other OSCs from mangrove species should provide an answer to these interesting and important questions.

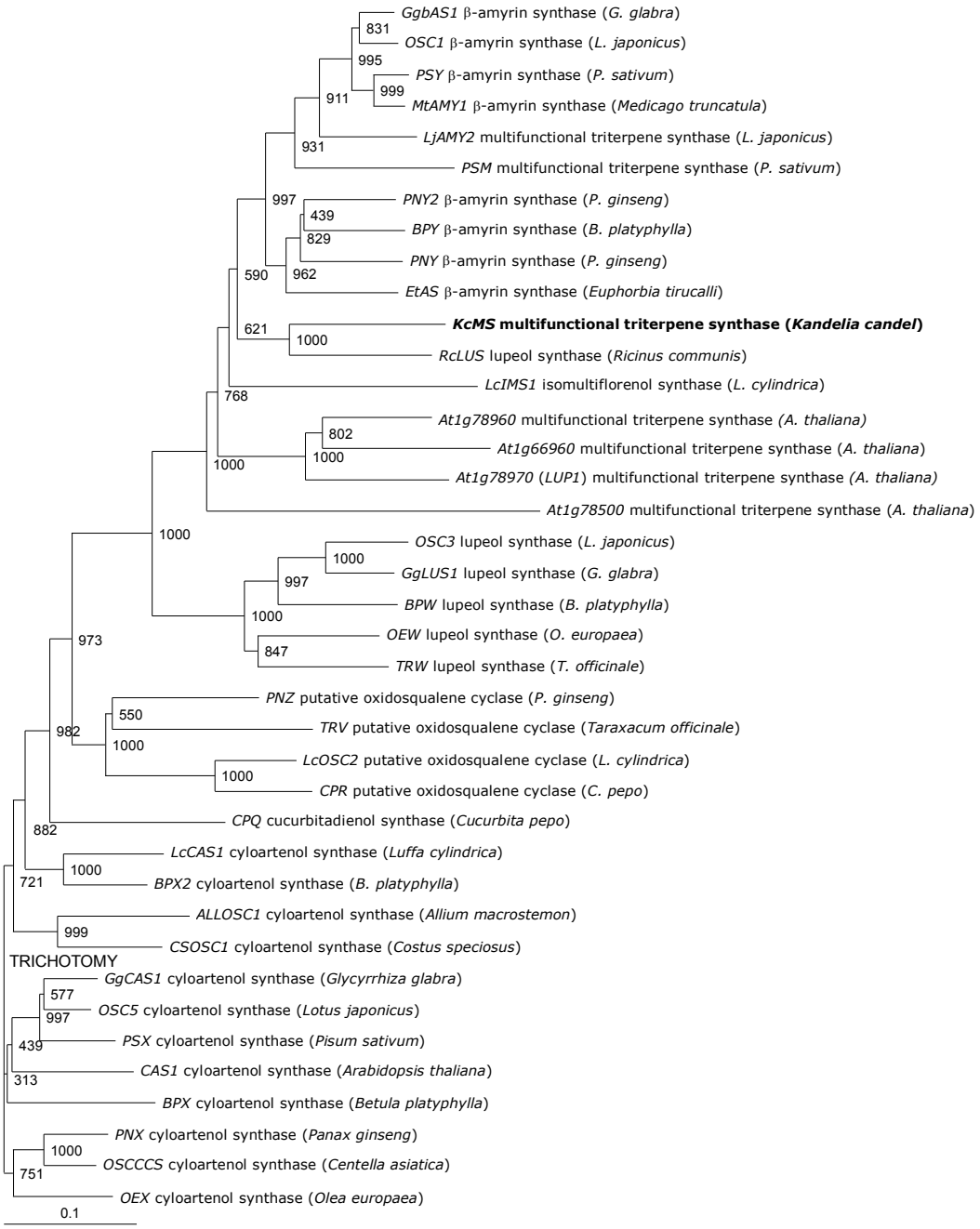


Fig. 3.5. Phylogenetic tree of plant OSCs including *KcMS* from *K. candel*. The phylogenetic tree was constructed using CLUSTAL W based on a neighbor-joining method. The indicated scale represents 0.1 amino acid substitutions per site. Numbers indicate bootstrap values from 1000 replicates. The DDBJ/GenBank/EMBL accession numbers of the sequence used this analysis are indicated in Experimental procedures.

Part 2: Triterpene synthases from *Bruguiera gymnorrhiza* (L.) Lamk. and *Rhizophora stylosa* Griff.

Introduction

More than 100 different triterpenoid carbon skeletons from the plant kingdom have been described (Xu et al., 2004). Despite a diversity in the carbon skeleton, all triterpenes and phytosterols are biosynthesized from a common precursor substrate, 2,3-oxidosqualene, with the participation of oxidosqualene cyclases (OSCs) (Abe et al., 1993). The diverse skeletons of triterpenoids, such as germanicol (oleanane), taraxerol (oleanane), β-amyrin (oleanane) and lupeol (lupane) are biosynthesized by various OSCs (Fig. 3.6) and these enzymes regulate the isoprenoids pathway controlling the biosynthetic flux towards either triterpenoids or phytosterols (Henry et al., 1992). Of the members of the OSC family, cycloartenol synthase (CAS) and lanosterol synthase are responsible for sterol biosynthesis in higher plants, and other OSCs for triterpene synthesis. Recently, a number of genes that are responsible for encoding plant OSCs, which include monofunctional and multifunctional triterpene synthases, have been cloned and their functions have been identified by heterologous expression in yeast (Phillips et al., 2006).

In spite of the ubiquitous distribution of triterpenes in the plant kingdom, their physiological functions in plants are poorly understood, and especially for those found in mangrove plant species. Taraxerol, identified in *Rhizophora mangle*, may function as a chemical defence molecule because it exhibits insecticidal activity (William, 1999). In addition, we recently proposed that triterpenes in mangrove plants may participate protection against salt stress (Oku et al., 2003). Furthermore, OSCs have been attracted the attention of many investigators because of their potential ability to modify the chemical structures of terpenoids, as well as, their importance as the first committed enzymes in triterpene biosynthesis. However, information on the OSCs from mangrove species is scarce. In order to gain information on the molecular structure of OSCs from mangrove plants, it is important to understand the biosynthetic pathway of terpenoids in these plant species, and to further our knowledge on the physiological significance of these compounds. Very recently, we were the first group to identify the *KcMS* gene that encodes a multifunctional triterpene synthase from a mangrove

tree species, *Kandelia candel* (Basyuni et al., 2006). The present section extends our previous result, and we now describe molecular cloning from other members of the Okinawan mangrove tribe, namely the Rhizophoraceae (*Bruguiera gymnorrhiza* (L.) Lamk. and *Rhizophora stylosa* Griff.).

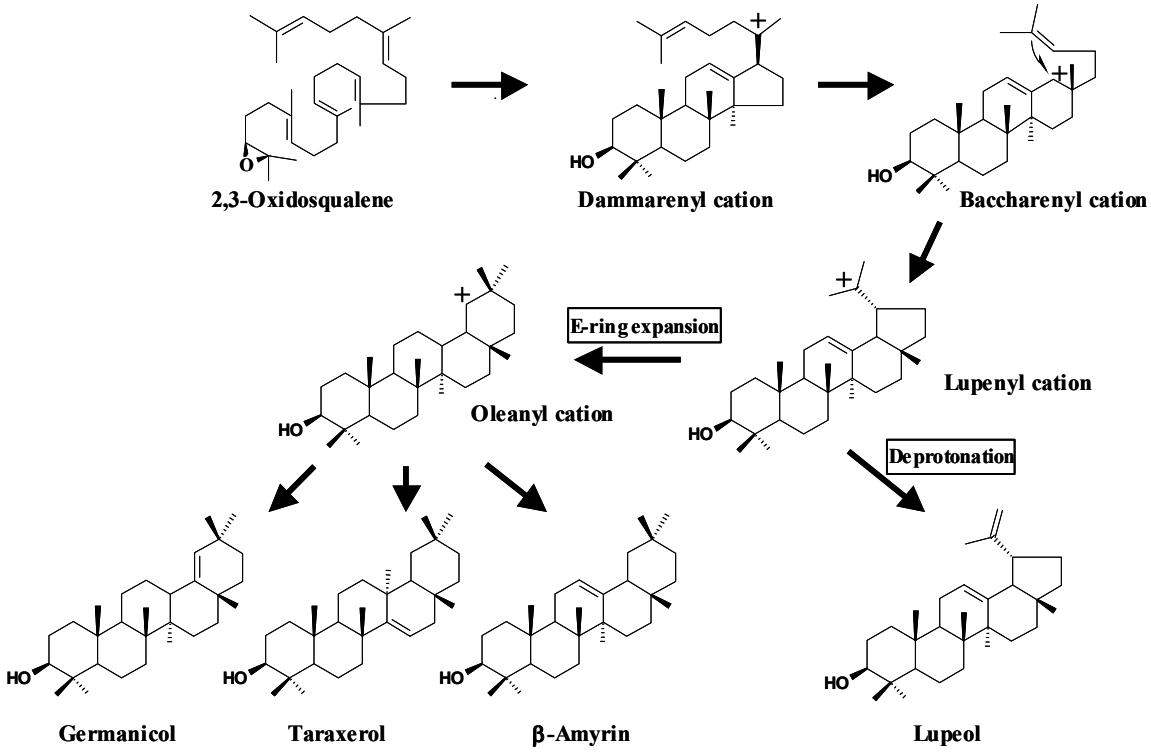


Fig. 3.6. Cyclization of 2,3-oxidosqualene to germanicol, taraxerol, β-amyrin and lupeol.

Experimental procedures

Chemicals

Authentic standards of β-amyrin, lupeol, α-amyrin and lupenone were purchased from Extrasynthese (Genay, France). Customized oligonucleotide primers were synthesized by Hokkaido System Science (Hokkaido, Japan).

PCR and sequence analysis

PCR was performed with a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA). The PCR reaction products were separated by SeaKem^{R} GTG^{R} agarose (BMA, Rockland, ME, USA), purified by Suprec^{TM}-01 (Takara Bio Inc., Otsu, Shiga, Japan), ligated to TOPO 10 (Invitrogen, Carlsbad, CA, USA), and introduced into electrocompetent *Escherichia coli* (Invitrogen) by Gene Pulser Xcell^{TM} (Bio-Rad, Tokyo, Japan). Plasmid DNA was extracted by LaboPass^{TM} plasmid mini purification Kit (Cosmo Genetech, Seoul, Korea). Sequencing was performed by ABI PRISM^{TM} 3100-Avant Genetic Analyzer (Applied Biosystems, Tokyo, Japan) using Bigdye^{R} Terminator ver. 1.1/3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Plant materials and culture conditions

Fresh leaves of *B*. *gymnorrhiza* and *R*. *stylosa* were collected from Okukubi river, Okinawa, Japan. These materials were snap frozen in liquid nitrogen immediately after collection and stored at –80 ºC for RNA extraction. For lipid analysis, the leaves of *B*. *gymnorrhiza*, *K*. *candel* and *R*. *stylosa* were sampled at the same place, and were stored at –30 ºC. The yeast strain GIL77 (*gal2 hem3-6 erg7 ura3-167*), was used for transformation and maintained on YPD medium (1.0 % yeast extract, 2.0% peptone, 2.0% dextrose) supplemented with 13 μg/ml hemin, 20 μg/ml ergosterol and 5 mg/ml Tween 80. Transformation of the yeast mutant was done using the Frozen-EZ Yeast Transformation II^{TM} Kit (Zymo Research). The transformant was cultured in complete medium (SC-Ura supplemented with 13 μg/ml hemin and 20 μg/ml ergosterol) at 30 ºC with shaking (220 rpm) for

functional gene expression.

Preparation of RNA

Total RNA was extracted from the leaves of *B*. *gymnorrhiza* and *R*. *stylosa* using the CTAB method [15]. Total RNA (2.42 µg/µl) was reverse transcribed with 0.5 µg oligonucleotide (dt) primer (RACE 32, 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTT-3') to produce a cDNA using a cloned AMV First-Strand cDNA synthesis Kit (Invitrogen) with 10 mM dNTP in a total volume of 20 µl for 5 min at 65 ºC, 1 h at 50 ºC, and 5 min at 85 ºC in accordance with the manufacturer's protocol. The resultant cDNA mixture was diluted with 50 µl Tris/EDTA (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and used as a template for PCR (described later).

Cloning of core fragment of triterpene synthase cDNA

The first PCR to amplify the core fragment was performed with degenerate primers, 161S (5'-GAYGGIGGITGGGIYTICA-3') and 701A (5'-CKRTAYTCIGCIARIGCCCA-3') (1 µg each) using Ex Taq^{TM} HS DNA polymerase (Takara Bio Inc.) and 0.2 mM dNTP in a final volume of 50 µl, according to the manufacturer's protocol. PCR amplification was carried out for 1 min at 94 ºC, followed by 30 cycles of 1 min at 94 ºC, 2 min at 50 ºC and 3 min at 72 ºC, with a final extension of 10 min at 72 ºC. The first PCR product was applied onto Microcon^{R} centrifugal filter devices YM-30 (Millipore Co., Bedford, MA, USA) and the volume was adjusted to 50 µl with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). The second PCR was carried out with 463S (5'-MGICAYATHWSIAARGG-3') and 603A (5'-CCCCARTTICCRTACCAISWICCRTC-3') using 1 µl of the first PCR product as the template and performed under the same conditions as described for the first PCR. The PCR product was cloned into the plasmid vector of TOPO 10 and propagated in *E*. *coli* TOPO 10. The number of clones that were sequenced for *BgLUS*, *BgbAS*, *RsM1* and *RsM2* gene was 10, 6, 2 and 6, respectively.

Cloning of the 3'- and 5'-ends

Based on the sequences of above four types of core fragment, specific primers to each gene

were designed to amplify the 3'- and 5'-ends of cDNAs by RACE (Basyuni et al., 2006). The primers used for 3'-RACE were as follows: BgbAS-S1 (5'-TGATGCCTCCAGAAATTG-3'), BgbAS-S2 (5'-CAGG GCACAGAAAGAAGGA-3'); BgLUS-S1 (5'-TGATGCCCCCTGAACTTG-3'), BgLUS-S2 (5'-CC TGAGCACAGGAGGAAAG-3'); RsM1-S1 (5'-TGATGCCTCCAGAAATTG-3'), RsM1-S2 (5'-CA GGGCACAGAAAGAAGGA-3'); and RsM2-S1 (5'-CAATGGCCCATGGTA TGG-3'), RsM2-S2 (5'-TACTGAAAACACAGTGTCAA-3').

The primers used for 5'-RACE were: BgbAS-A1 (5'-AAAACTCTGTAGGATTTAGTAG- 3'), BgbAS-A2 (5'-CAGGGACAGAAGGACATTGA-3'); BgLUS-A1 (5'-GAAATTCCACAGGGTT AAGCCA-3'), BgLUS-A2 (5'-GAGGTCCCATCTTCTCACCC-3'); RsM1-A1 (5'-AAAACTCTG TAGGATTTAGTAG-3'), RsM1- A2 (5'-CAGGGACAGAAGGACATTGA-3'); and RsM2-A1 (5'-GAAAGGTAGCTCTCTCCCCA-3'), RsM2-A2 (5'-CAACCCCTTGAGAGCAAAAT-3').

The PCR products of the 3'- and 5'-ends of each gene were cloned into a TOPO 10 plasmid vector, in the identical manner described for the core fragment, and propagated in *E. coli* for sequencing. In the case of 5'-RACE, three clones of each gene were sequenced in both strands. Similarly, two to four clones of each gene were sequenced for 3'-RACE.

Cloning of full-length cDNA

Finally, the full-length cDNAs for *BgbAS*, *BgLUS*, *RsM1* and *RsM2* were obtained using the following N-terminal and C-terminal primers with specific restriction enzyme sites at each end: Kpn-BgbAS-N1 (5'-AAGAGGTACCATGTGGAGAATAAAGATTGC-3'; KpnI site underlined), Xho-BgbAS-C1 (5'-CTTGCTCGAGTCAGGAAGGCAATGGAACGC-3'; XhoI site underlined); Kpn-BgLUS-N1 (5'-GATTGGTACCATGTGGAGGCTTAAGATTGC-3'; KpnI site underlined), Xho-BgLUS-C1 (5'-CCTGCTCGAGTCATTTTTGGAAGGCAATGG-3'; XhoI site underlined); Kpn-RsM1-N1 (5'-AACAGGTACCATGTGAGGCTAAAGATTGC-3'; KpnI site underlined), Xho-RsM1-C1 (5'-GCCACTCGAGTCAAATGCTTCAGGAAGGCA-3'; XhoI site underlined); Bam-RsM2-N1 (5'-GAGCGGATCCATGGGAGTGTGGAGGCTTAA-3'; BamHI site underlined), and Xho-RsM2-C1 (5'-TTGCCTCGAGTACTCCATCACCGAGGAGTG-3'; XhoI site underlined). PCRs were performed with each set of the above-described primers. The annealing temperatures for

BgbAS, *BgLUS*, *RsM1* and *RsM2* were 55 ºC, 57 ºC, 65 ºC and 58 ºC respectively. The full-length cDNAs were cloned into TOPO 10 plasmid vector, and propagated in *E. coli* for sequencing. The obtained full-length cDNA clones, *BgbAS* (10 colonies), *BgLUS* (7 colonies), *RsM1* (12 colonies) and *RsM2* (12 colonies), were sequenced in both strands. Of the clones sequenced, three to five clones for each gene were subsequently shown to be completely identical.

Expression in erg7 Saccharomyces cerevisiae strain GIL77

The 2.3-kb PCR product was digested with the restriction enzyme and ligated into the cloning sites of pYES2 (Invitrogen) to construct the plasmids OSC-pYES2-*BgbAS*, OSC-pYES2-*BgLUS*, OSC-pYES2-*RsM1* and OSC-pYES2-*RsM2*. The identity of the inserted DNA was confirmed by sequencing. The plasmid was then transferred to mutant GIL77, which lacks lanosterol synthase activity, using the Frozen-EZ Yeast Transformation II^{TM} Kit (Zymo Research). The transformant was then inoculated into 25 ml synthetic complete medium without uracil (SC-Ura), that contained 13 μg/ml hemin, 20 μg/ml ergosterol and 5 mg/ml Tween 80, and incubated at 30 ºC for 2 days. The medium was then replaced with fresh SC-Ura containing the same supplementation with the addition of 2% galactose as a replacement for glucose. The cells were incubated at 30 ºC for 12 h, harvested by centrifugation at 2000 rpm for 5 min, resuspended in 20 ml of 0.1 M potassium phosphate buffer, pH 7.0 containing 3 % glucose and 13 μg/ml hemin, and then incubated at 30 ºC for 48 h. The cell pellets were then collected and refluxed with 2 ml of 20% KOH/50% ethanol for 10 min. After extraction with 2 ml of hexane, the extract was concentrated and applied to a TLC plate (Merck) that was developed with benzene/acetone (19:1, v/v). The fraction that corresponded to the 4,4-dimethylsterol band was scraped off the plate, extracted with chloroform and served as the sample preparation for GC-FID and GC-MS analysis.

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS) of triterpenoids

The reaction products of OSC in the extract were analyzed by a gas chromatograph that was equipped a flame ionization detector (GC-2010 Shimadzu, Kyoto, Japan). The column used for gas chromatography was a CBPI-M50-025 (0.25 mm ID x 50 m, Shimadzu). The column temperature

program was initially 50 ºC for 1 min, then raised to 300 ºC at a rate of 10 ºC/min, and then held at 300 ºC for 26 min. The carrier gas was helium and was delivered at a flow rate of 20 cm/s. The temperatures for the injector and detector were 250 ºC and 300 ºC, respectively. The mass spectrometer used for gas chromatography/mass spectrometry was a GC-MS QP-2010 (Shimadzu). The column used and the GC conditions were identical to those already described. Ionization of sample was done by electron impact (EI) at 70 eV to estimate the chemical structure, or by chemical ionization using methane as the reaction gas to determine the molecular weight. A similarity search of the spectrum was done using the mass-spectrum library (Nist 147 and 27, Shimadzu).

NMR analysis

For NMR analysis of reaction products, a preparative-scale culture of the yeast transformant (500 ml) was performed. Induction was carried out using the previously described method. After refluxing the cell pellet with 50 ml of 20% KOH/50% ethanol for 10 min, the mixture was extracted with 50 ml of hexane, concentrated, and then applied to a 20 x 20 cm TLC plate (Merck). The terpenoids extracted were first separated using a silica gel column (1 cm internal diameter (ID) x 50 cm length) with an eluent system of hexane/diethyl ether/acetic acid (70:30:1 by vol.) or hexane/ethylacetate/acetic acid (90:10:1 by vol.). Further purification to homogeneity was done using 95% ethanol or 95% acetonitrile as an eluent in 1.1 cm ID x 30 cm long ODS column (Ultra Pack, Yamazen Co., Osaka, Japan). ^{13}C-NMR spectra were obtained at 125 MHz for ^{13}C by a Jeol α-500 spectrometer (JEOL Ltd., Tokyo, Japan). Shifts are expressed in parts per million downfield from tetramethylsilane. The following ^{13}C NMR data in $CDCl_3$ were obtained.

Germanicol: ^{13}C-NMR (125 MHz, $CDCl_3$) δ : 142.76 (C-18), 129.72 (C-19), 79.05 (C-3), 55.49 (C-5), 51.22 (C-9), 43.34 (C-14), 40.75 (C-8), 38.95 (C-13), 38.90 (C-4), 38.42 (C-1), 37.71 (C-16), 37.37 (C-22), 37.15 (C-10), 34.60 (C-7), 34.36 (C-17), 33.34 (C-21), 32.36 (C-20), 31.35 (C-29), 29.20 (C-30), 27.97 (C-23), 27.53 (C-15), 27.41 (C-2), 26.21 (C-12), 25.27 (C-28), 21.11 (C-11), 18.27 (C-6), 16.71 (C-26), 16.10 (C-25), 15.41 (C-24), 14.59 (C-27). The ^{13}C-NMR data of the isolated compound were identical with those reported previously (González et al., 1981).

Taraxerol: ^{13}C-NMR (125 MHz, $CDCl_3$) δ : 158.1(C-14), 116.9(C-15), 79.1(C-3), 55.5(C-5),

49.3(C-18), 48.7(C-9), 41.3(C-19), 39.0(C-4), 38.8(C-8), 38.0(C-1, 17), 37.7(C-10, 13), 36.7(C-16), 35.8(C-12), 35.1(C-7), 33.7(C-21), 33.4(C-29), 33.1(C-22), 29.9(C-28), 29.8(C-26), 29.7(C-20), 28.0(C-23), 27.1(C-2), 25.9(C-27), 21.3(C-30), 18.8(C-6), 17.5(C-11), 15.5(C-24), 15.4(C-25). The ^{13}C-NMR data were identical with those reported previously (Sakurai et al., 1987; Goad and Akihisa, 1997).

Similarity scores and phylogenetic analysis of amino acid sequence

The amino acid sequences were aligned and similarity scores were obtained using the CLUSTAL W ver. 1.83) program (Thompson et al., 1994) of the DNA Data Bank of Japan (Mishima, Shizuoka, Japan). The results are displyed in Table 4.1. The phylogenetic tree was constructed with the neighbor-joining method of the PHYLIP package ver. 3.66) (Felsentein, 1996). Amino acid distances were calculated using the Dayhoff PAM matrix method of the Protdist program of PHYLIP. The numbers indicate bootstrap values from 1000 replicates. The phylogenic tree was drawn using TreeView ver. 1.6.6 (Page, 1996). The DDBJ/GenBank/EMBL accession numbers of the sequences that were used in this analysis are as follows: AB055509 (*BPX Betula platyphylla*), AB055510 (*BPX2 B. Platyphylla*), U02555 (*CAS1 Arabidopsis thaliana*), D89619 (*PSX Pisum sativum*), AB181246 (*OSC5 Lotus japonicus*), AB025968 (*GgCAS1 Glycyrrhiza glabra*), AB025344 (*OEX Olea europaea*), AY520819 (*OSCCCS Centella asiatica*), AB009029 (*PNX Panax ginseng*), AB058507 (*CSOSC1 Costus speciosus*), AB025353 (*ALLOSC1 Allium macrostemon*), AB033334 (*LcCAS1 Luffa cylindrica*), AB116238 (*CPQ Cucurbita pepo*), AB244671 (*OSC7 L. Japonicus*), AB247155 (*LAS1 A. thaliana*), AB116239 (*CPR C. pepo*), AB033335 (*LcOSC2 L. cylindrica*), AB025346 (*TRV Taraxacum officinale*), AB009031 (*PNZ P. ginseng*), AB025345 (*TRW T. officinale*), AB025343 (*OEW O. europaea*), AB055511 (*BPW B. platyphylla*), AB116228 (*GgLUS1 G. glabra*), AB181245 (*OSC3 L. japonicus*), AC007260 (*At1g78500/T30F21.16 A. thaliana*), AB263204 (*RsM2 Rhizophora stylosa*), U49919 (*At1g78970/LUP1 A. thaliana*), AC007152 (*At1g66960/F1019.4 A. thaliana*), AC002986 (*At1g78960/YUP8H12R.43 A. thaliana*), AB058643 (*LcIMS1 L. cylindrica*), DQ268869 (*RcLUS Ricinus communis*), AB257507 (*KcMS Kandelia candel*), AB289586 (*BgLUS Bruguiera gymnorrhiza*), AB034803 (*PSM P. sativum*), AF478455 (*LjAMY2 L.*

japonicus), AF478453 (*MtAMY1 Medicago truncatula*), AB034802 (*PSY P. sativum*), AB181244 (*OSC1 L. japonicus*), AB037203 (*GgbAS1 G. glabra*)., AB206469 (*EtAS Euphorbia tirucalli*), AB263203 (*RsM1 R. stylosa*), AB289585 (*BgbAS B. gymnorrhiza*), AB009030 (*PNY P. ginseng*), AB055512 (*BPY B. platyphylla*), AB014057 (*PNY2 P. ginseng*).

Isolation of terpenoids from mangrove leaves

The mangrove leaves (5-6 leaves per species or 8-10 g wet weight, respectively) of *B. gymnorrhiza*, *R. stylosa* and *K. candel* were ground in liquid N_2, and then extracted with 25 volumes of chloroform-methanol (2:1, v/v) (CM21). The cell wall debris, which is insoluble in CM21, was removed by filtration through No. 2 filter paper (Advantec, Tokyo, Japan). The resultant extract was partially purified for lipid analysis using a previously-described method [47]. The purified extract was concentrated to dryness, and the lipid weight was measured gravimetrically.

The lipid extract, which contained 2 mg of total lipid, was concentrated to dryness using nitrogen gas stream, saponified with 3% KOH in 94% ethanol at 60 ºC overnight. The non-saponifiable lipids (NSL) were partitioned into hexane by vigorous mixing, and this mixture contained sterols, alcohols and alkanes. The NSL were analyzed by gas chromatography (GC 2010, Shimadzu) or gas chromatography-mass spectrometry (GC-MS QP 2010, Shimadzu). The columns used and the GC conditions were identical to those previously described.

Results and Discussion

Cloning of triterpene synthase cDNAs from B. gymnorrhiza and R. stylosa

In order to clone *BgbAS, BgLUS, RsM1* and *RsM2* triterpene synthases, PCRs were performed using degenerate primers whose designs were based on the highly conserved regions of known OSCs, as described previously (Basyuni et al., 2006). The amplified core DNA fragment of *BgbAS, BgLUS, RsM1* and *RsM2* (446, 446, 446 and 177 bp in length, respectively) were cloned into a TOPO 10 vector (Invitrogen). Ten clones for *BgBAS*, six clones for *BgLUS*, two clones for *RsM1* and six clones for *RsM2* were sequenced. 3'-RACE and 5'-RACE (Frohman et al., 1988) were employed to clone 3'- and 5' -ends of the desired clone using the GeneRacer^{TM} Kit (Invitrogen) and full-length sequences of the genes, which we named *BgbAS, BgLUS, RsM1* and *RsM2*, respectively, were produced.

The open reading frames (ORFs) of *BgbAS, BgLUS, RsM1* and *RsM2* consisted of DNA sequences whose lengths were 2280, 2286, 2280 and 2316 bp, respectively. These DNA sequences encoded proteins, which consisted of 759, 761, 759 and 771 amino acids residues, respectively. Each protein contained five QW motifs (Poralla et al., 1994) and a DCTAE motif (Abe and Prestwich, 1994) (Fig. 3.7). *RsM1* and *RsM2* shared 66% identities in their amino acid sequence and 71% in their DNA sequence.

The deduced amino acid sequence of *BgbAS*, *BgLUS*, *RsM1* and *RsM2* showed significant sequence similarity to known triterpene synthases (Table 3.1). Interestingly, *BgbAS* showed high similarity (93%) to the *RsM1* clone and 85 % similarity to *EtAS* β-amyrin synthase of *Euphorbia tirucalli* (Kajikawa et al., 2005). *BgLUS* exhibited 90% similarity to *KcMS* of *Kandelia candel* and 78 % similarity to RcLUS lupeol synthase of *Ricinus communis* (Guhling et al., 2006) (Table 3.1). The extent of similarity of *RsM1* with EtAS β-amyrin synthase of *E. tirucalli* was 84%, whereas *RsM2* also had relatively high similarities of (66%) to both β-amyrin synthases from *E. tirucalli* and *Panax ginseng* (*PNY2*) (Table 3.1). These results suggested that *BgbAS*, *BgLUS*, *RsM1* and *RsM2* encoded triterpene synthases.

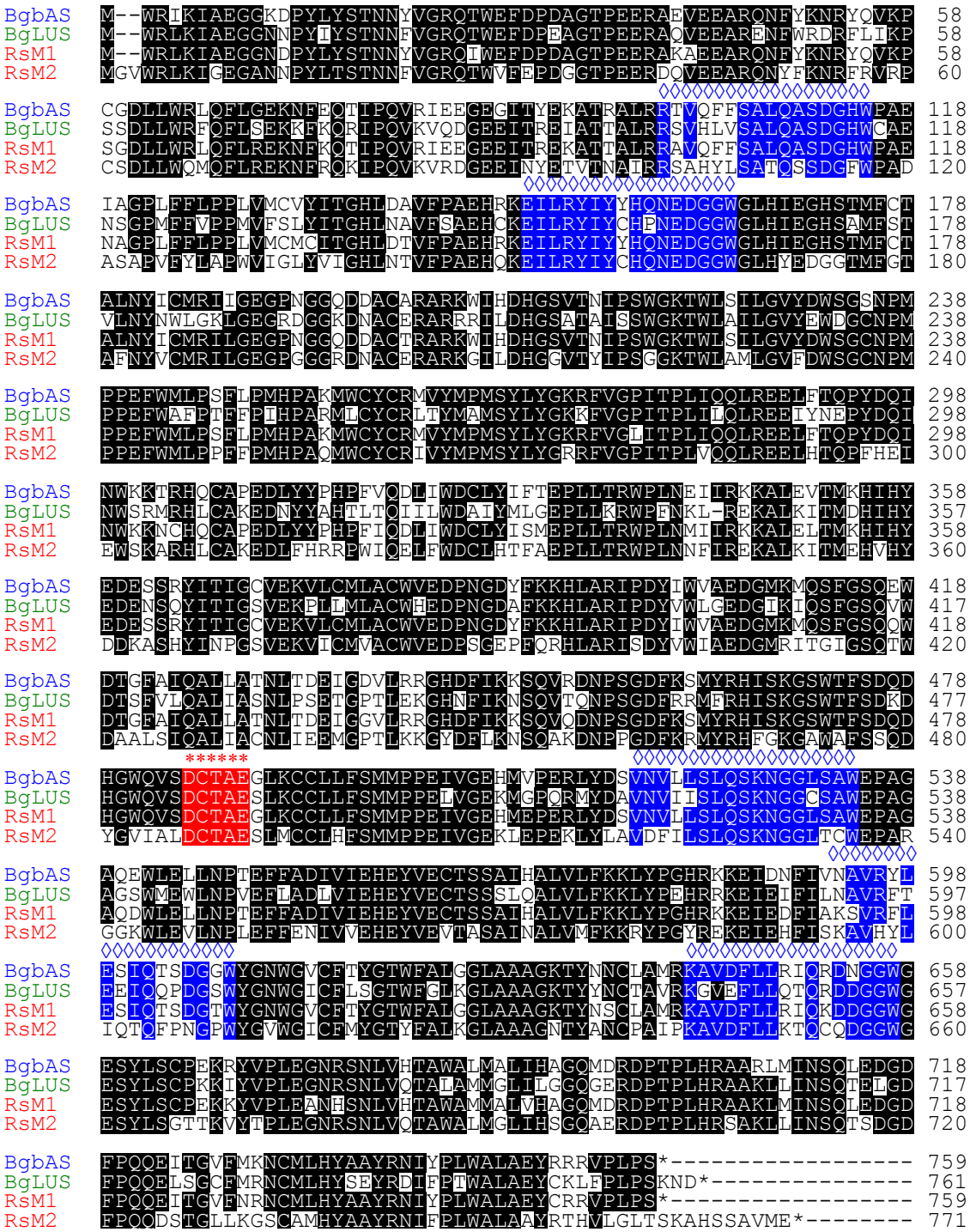


Fig. 3.7. Sequence alignment of the deduced amino acids from *B. gymnorrhiza* (*BgbAS* and *BgLUS*) and *R. stylosa* (*RsM1* and *RsM2*). DCTAE and QW motifs are indicated as * and ◊, respectively. Identical amino acid residues in three out of four proteins sequences are shaded and dashes indicate the alignment gaps.

Expression of BgbAS, BgLUS, RsM1 and RsM2 in erg7 deficient mutant GIL77

In order to confirm the identities of the *BgbAS*, *BgLUS*, *RsM1* and *RsM2* clones, functional expressions of these genes in yeast were undertaken. *BgbAS*, *BgLUS*, *RsM1* and *RsM2* cDNAs were ligated to a yeast expression vector pYES2 (Invitrogen), and expressed under the control of the *GAL1* promoter in an *erg7*-deficient yeast mutant GIL77, which accumulates oxidosqualene within cells (Kushiro et al., 1998).

Introduction of the *BgbAS*, *BgLUS*, *RsM1* and *RsM2* genes into GIL77 resulted in the production of dimethylsterols with the same mobility as β-amyrin on thin layer chromatography (TLC) plates (Kushiro et al., 1998). These products were then analyzed by gas chromatography/mass spectrometry (GC-MS) and ^{13}C-NMR spectroscopy in order to identify their chemical structures.

The gas-chromatogram profile demonstrated that the pYES2-*BgbAS* transformant accumulated β-amyrin as the sole product whereas the pYES2-*BgLUS* transformant produced lupeol only (Fig. 3.8.A). Identification of the chemical structures for β-amyrin and lupeol were accomplished by comparing their retention times and MS-spectra with those of authentic standards (Fig. 3.8.B).

In contrast, the reaction products of pYES2-*RsM1* consisted of three peaks whose relative proportions were 63:33:4 using GC-FID analysis (Fig. 3.9A). Using the database library, the MS spectrum of the largest peak (c) was similar to that for germanicol. This identification was verified by interpreting its ^{13}C-NMR spectrum. For the other two peaks, namely, β-amyrin (a) and lupeol (b), their identifications were verified by comparing their retention times and MS spectra with those of authentic standards.

The three products peaks (d, a and b) were detected in the lipid extract of the pYES-*RsM2* transformant, and were identified as taraxerol, β-amyrin and lupeol, respectively (Fig. 3.9A). The relative peak intensities for taraxerol, β-amyrin and lupeol were 70:17:13. The chemical structure of taraxerol was identified by interpretation of its ^{13}C-NMR spectrum.

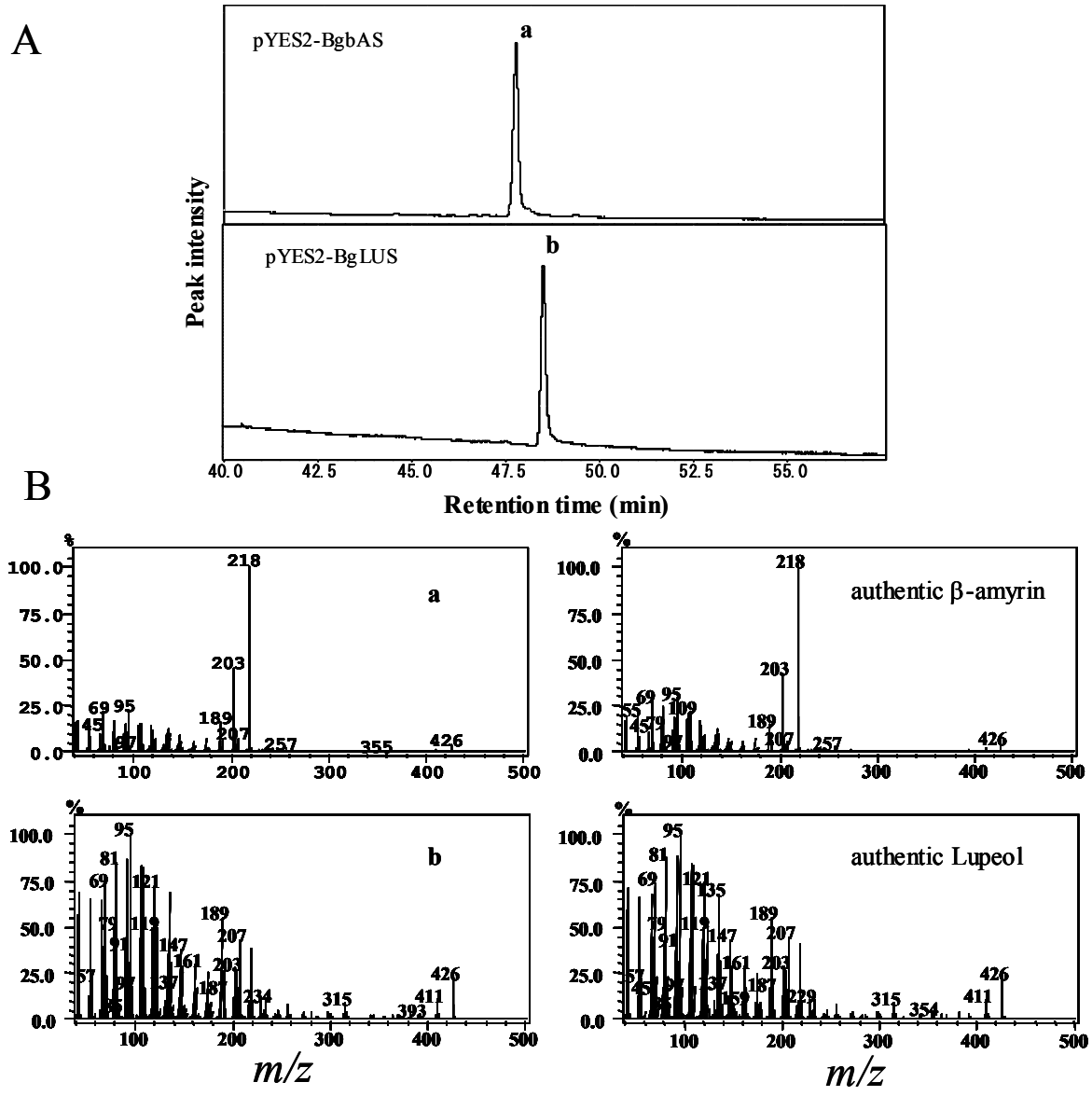


Fig. 3.8. GC-MS analysis of extracts from pYES2-*BgbAS* and pYES2-*BgLUS* transformants. Gas-chromatogram of the products of pYES2-*BgbAS* and pYES2-*BgLUS* were monitored by FID (A). Mass spectra of authentic β-amyrin and lupeol are shown (B).

These results clearly established that *BgbAS* and *BgLUS* respectively, encoded β-amyrin synthase and lupeol synthase, whereas both *RsM1* and *RsM2* encoded multifunctional triterpene synthases. Although *RsM1* and *RsM2* displayed high similarity with β-amyrin synthases from *E. tirucalli* in their amino acid sequence (84% and 66%, respectively), these biosynthesized germanicol and taraxerol as the major products, and also significant amounts of β-amyrin and lupeol as shown in Fig. 3.9.A. It is important to note that both *RsM1* and *RsM2* produced three distinct triterpenoids (Fig. 3.9.A). Until now, seven multifunctional triterpene synthases have been reported from only four plant species including the species that we described in our previous report: *Arabidopsis thaliana* (*LUP1*/At1g78970, Herrera et al., 1998; At1g78960, Kushiro et al., 2000a; At1g66960, Ebizuka et al., 2003; At1g78500, Ebizuka et al., 2003); *Pisum sativum PSM*, Morita et al., 2000; *Lotus japonicus LjAMY2*, Iturbe-Ormaetxe et al., 2003; and *Kandelia candel KcMS*, Basyuni et al., 2006. Nevertheless, none of these species synthesized germanicol and taraxerol as major products. Therefore, the results of this study suggest that *RsM1* and *RsM2* are new of multifunctional OSCs with distinctive product specificity.

Successful cloning of two cDNAs of multifunctional triterpene synthases (*RsM1* and *RsM2*) in the present study prove clearly the presence and expression of more two multifunctional triterpenes synthase genes in one plant species, like in *A. thaliana* which all triterpene synthases are multifunctional, producing more than one syclization product (Ebizuka et al., 2003). Our previous study from mangrove species *K. candel* (*KcMS*) was also identified as multifunctional triterpene synthase (Basyuni et al., 2006). Thus, such multi-product enzymes commonly occur in the plant kingdom, the number of enzymes to generate various triterpene skeletons would be greatly reduced.

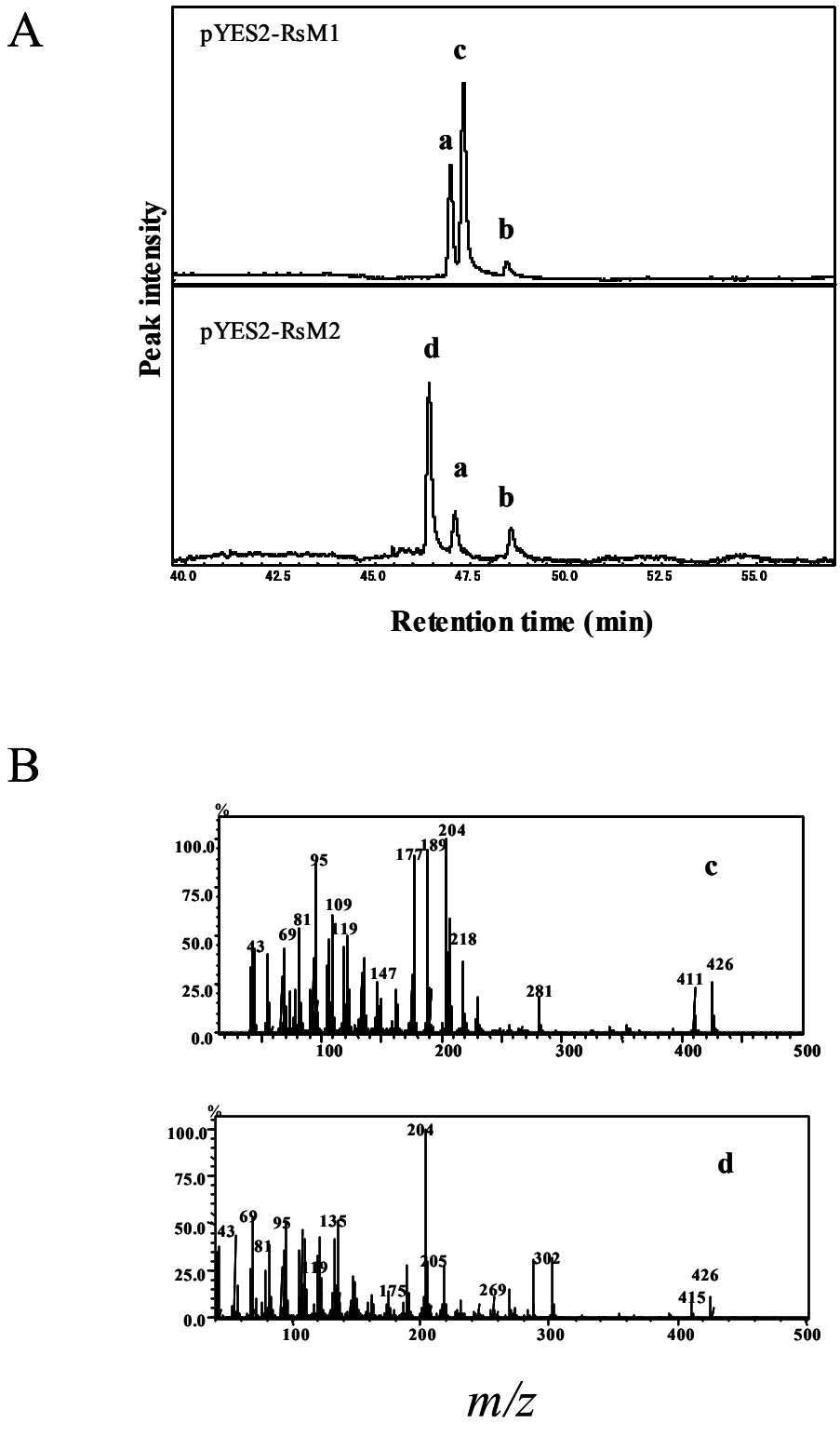


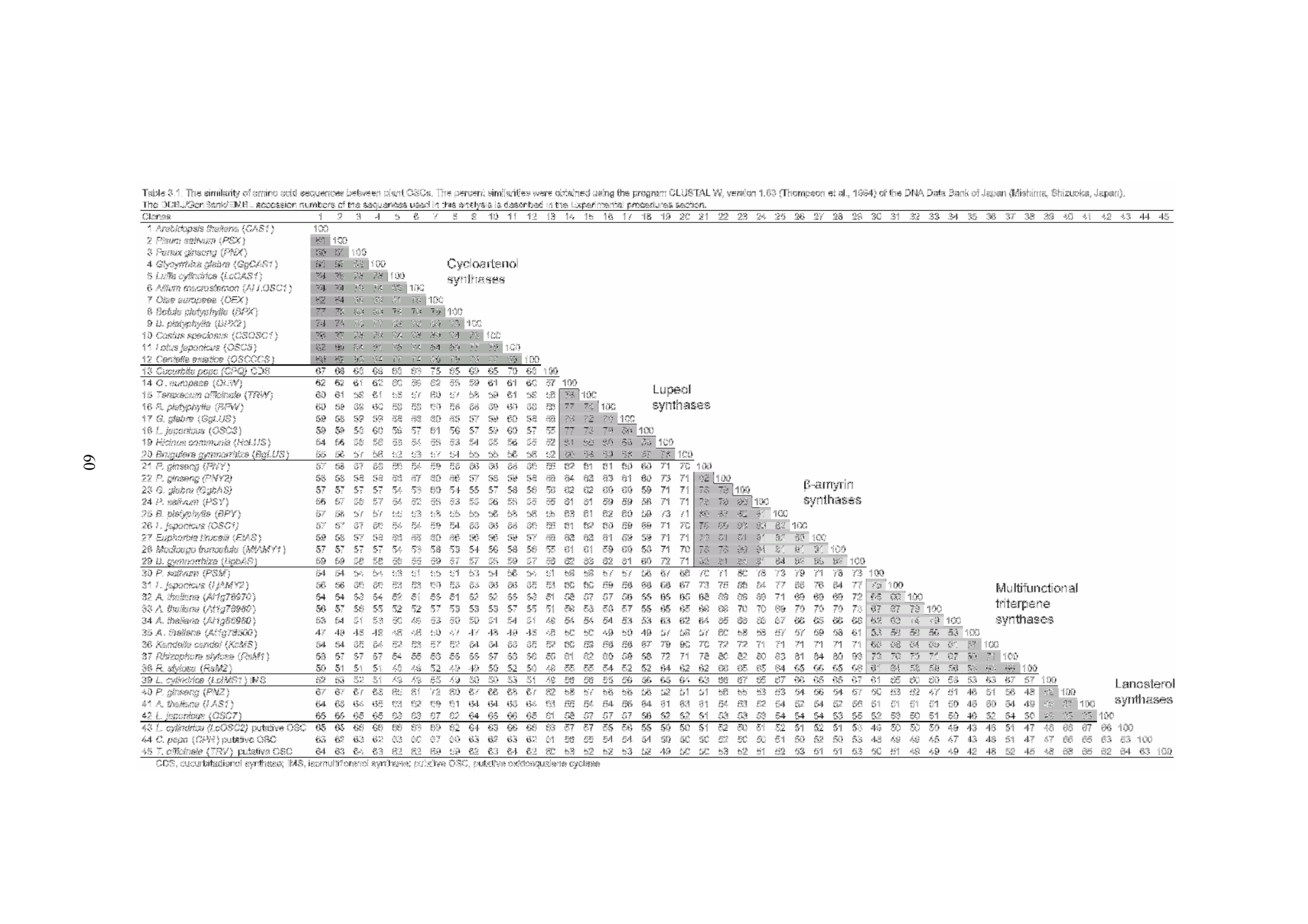
Fig. 3.9. GC-MS analysis of extracts from pYES2-*RsM1* and pYES2-*RsM2* transformants. Gas-chromatogram of the products of was monitored by FID (A). EI-Mass spectra of the major peaks are shown in the bottom panel (B).

Molecular evolution of the tribe Rhizophoraceae gene in the plant OSCs

In order to clarify the evolutionary relationships among plant OSCs, a phylogenetic tree was constructed on the basis of their amino acid sequences (Fig. 3.10). Ten dicotyledonous cyloartenol synthase (CAS) clones showed high similarities (70-91%) to each other and displayed slightly lower, but still high, similarities (69-80%) to the two clones that were isolated from the monocotyledonous plants, *Allium macrostemon* and *Costus speciosus* (Table 3.1). The cycloartenol synthase genes of plants form one large cluster in the tree and this finding, demonstrates that plant OSCs are evolutionary descendants from cycloartenol synthase (Fig. 3.10). The results of the present study are in almost full agreements with those described in previous reports in which plant cycloartenol synthase (CAS), lupeol synthase (LUS) and β-amyrin synthase (βAS) clones form distinct clusters in the tree (Shibuya et al., 1999; Zhang et al., 2003).

The LUS clones showed high identities (72-86%) to each other, except for the new branch of lupeol synthase that consisted of *BgLUS* and *RcLUS* lupeol synthase from *R. communis* (Guhling et al., 2006). This new LUS clone have evolved to the β-amyrin synthase branch because the two clones (a) display high similarity (70-73%) between them and (b) exhibit high similarity with *KcMS* from *K. candel* that also biosynthesizes lupeol as the major product (Basyuni et al., 2006). Our results are essentially consistent with those described in the previous report on the evolutionary generation of the two branches of lupeol synthase (Shibuya et al., 1999). However, the clone, *A. thaliana LUP1* that was classified as a new lupeol branch in these two studies (Herrera et al., 1999; Segura et al., 2000) is now located between the two branches of lupeol synthases in our study, together with the other members of multifunctional triterpene synthases. This location may be due to difference in the number of genes that were analyzed to construct the phylogenetic tree. Increasing the numbers of sequence data for the terpenoid synthases, including data from current study, allowed us to construct a more elaborated phylogenetic tree.

Table 3.1. The similarity of amino acid sequences between plant OSCs. The percent similarities were obtained using the program CLUSTAL W, version 1.83 (Thompson et al., 1994) of the DNA Data Bank of Japan (Mishima, Shizuoka, Japan). The DDBJ/GenBank/EMBL accession numbers of the sequences used in this analysis is described in the Experimental procedures section.



Clones	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
1 *Arabidopsis thaliana* (*CAS1*)	100																																												
2 *Pisum sativum* (*PSX*)	80	100																																												
3 *Panax ginseng* (*PNX*)	80	97	100																																										
4 *Glycyrrhiza glabra* (*GgCAS1*)	81	97	98	100																																									
5 *Luffa cylindrica* (*LcCAS1*)	79	96	98	98	100																																								
6 *Allium macrostemon* (*AmOSC1*)	78	94	96	96	96	100																																							
7 *Olea europaea* (*OEX*)	82	84	85	82	87	86	100																																						
8 *Betula platyphylla* (*BPX*)	77	78	80	80	79	79	79	100																																					
9 *B. platyphylla* (*BPX2*)	84	75	76	77	83	82	80	80	100																																				
10 *Costus speciosus* (*CSOSC1*)	78	77	78	78	74	78	80	84	78	100																																			
11 *Lotus japonicus* (*OSC5*)	82	80	82	81	79	82	84	80	83	89	100																																		
12 *Centella asiatica* (*OSCCC5*)	83	82	80	82	77	84	86	79	83	87	89	100																																	
13 *Cucurbita pepo* (*CPQ*) CDS	67	68	69	68	69	69	75	65	69	65	70	69	100																																
14 *C. sativus* (*CSW*)	62	62	61	62	60	66	62	65	59	61	61	60	67	100																															
15 *Taraxacum officinale* (*TRW*)	60	61	58	61	58	57	60	57	58	59	61	58	68	78	100																														
16 *K. platyphylla* (*KPW*)	60	59	60	60	59	58	60	56	60	59	60	58	69	77	76	100																													
17 *G. glabra* (*GgLUS*)	59	58	59	59	58	60	60	65	57	59	60	58	69	78	78	79	100																												
18 *L. japonicus* (*OSC3*)	59	59	58	60	58	57	61	58	57	59	60	57	68	77	78	79	94	100																											
19 *Ricinus communis* (*RcLUS*)	64	66	66	66	66	66	66	63	64	65	66	66	82	81	82	80	83	83	100																										
20 *Bruguiera gymnorrhiza* (*BgLUS*)	66	66	67	66	62	63	67	64	65	66	66	66	82	80	78	80	86	85	78	100																									
21 *P. ginseng* (*PNY*)	67	68	67	68	66	64	69	66	68	68	66	66	88	82	81	81	80	80	71	76	100																									
22 *P. ginseng* (*PNY2*)	66	66	66	66	66	67	68	66	67	66	66	66	88	84	83	83	81	80	73	71	92	100																							
23 *G. glabra* (*GgbAS*)	67	67	67	67	64	69	69	64	65	67	68	66	80	82	82	80	80	79	71	71	78	79	100																						
24 *P. sativum* (*PSY*)	66	67	66	67	64	69	68	63	65	66	66	65	80	81	81	80	80	80	71	71	78	79	99	100																					
25 *B. platyphylla* (*BPY*)	67	66	67	67	66	69	68	66	66	66	66	66	80	81	82	80	80	79	71	71	80	82	82	81	100																				
26 *I. japonicus* (*OSC1*)	67	67	67	66	64	64	69	64	66	66	66	66	80	81	82	80	80	80	71	70	78	80	80	80	83	81	100																		
27 *Euphorbia tirucalli* (*EtAS*)	69	68	67	68	68	66	69	66	68	68	68	67	80	82	82	81	82	82	73	71	81	81	81	81	82	80	82	100																	
28 *Medicago truncatula* (*MtAMY1*)	67	67	67	67	64	69	68	63	64	66	66	66	80	81	81	79	80	80	71	70	78	78	80	80	81	80	81	82	100																
29 *B. gymnorrhiza* (*BgbAS*)	69	69	68	68	68	66	69	67	67	66	69	67	80	82	80	82	81	80	72	71	80	81	80	81	84	82	85	86	100																
30 *P. sativum* (*PSM*)	64	64	64	64	63	61	65	61	63	64	66	64	81	80	80	80	80	80	72	70	78	79	79	79	80	78	80	80	80	100															
31 *I. japonicus* (*IjAMY2*)	66	66	66	66	63	63	69	63	63	66	66	65	81	80	80	80	80	80	69	67	78	78	80	80	81	80	81	82	82	82	100														
32 *A. thaliana* (*At1g78970*)	64	64	63	64	62	61	65	61	62	62	65	63	81	80	80	80	80	80	69	68	80	80	80	80	81	80	81	82	82	82	84	100													
33 *A. thaliana* (*At1g78960*)	66	67	66	65	62	62	67	63	63	63	67	65	81	80	80	80	80	80	69	68	80	80	80	80	81	80	81	82	82	82	84	98	100												
34 *A. thaliana* (*At1g80950*)	63	64	63	63	60	60	63	60	60	61	64	61	80	80	80	80	80	80	69	68	80	80	80	80	81	80	81	82	82	82	84	98	98	100											
35 *A. thaliana* (*At1g78500*)	67	68	68	68	68	68	68	67	67	68	68	68	82	82	82	80	80	80	67	68	80	80	80	80	81	80	81	82	82	82	84	98	98	98	100										
36 *Kandelia candel* (*KcMS*)	64	64	66	64	63	63	67	63	64	64	66	66	82	80	80	80	80	80	70	69	80	80	80	80	81	80	81	82	82	82	84	98	98	98	98	100									
37 *Rhizophora stylosa* (*RsM1*)	63	67	67	67	64	66	66	66	67	66	66	66	81	82	80	80	80	80	72	71	78	80	80	80	83	81	84	80	80	78	80	82	82	82	82	82	100								
38 *R. stylosa* (*RsM2*)	60	61	61	61	60	60	63	60	60	60	63	60	80	80	80	80	80	80	69	68	80	80	80	80	81	80	81	82	82	82	84	98	98	98	98	98	98	100							
39 *L. cylindrica* (*LcMS1*) IMS	69	69	69	69	69	69	69	69	69	69	69	69	84	84	84	84	84	84	73	73	84	84	84	84	85	84	85	86	86	84	86	86	86	86	86	86	86	86	100						
40 *P. ginseng* (*PNZ*)	67	67	67	68	66	67	72	69	67	68	69	67	82	80	80	80	80	80	72	71	81	81	83	83	84	82	84	84	84	82	84	84	84	84	84	84	84	84	98	100					
41 *A. thaliana* (*LAS1*)	64	66	66	66	63	63	69	63	64	64	66	66	83	82	82	82	82	82	71	69	82	82	82	82	83	82	83	84	84	82	84	84	84	84	84	84	84	84	98	98	100				
42 *L. japonicus* (*OSC7*)	66	66	66	66	63	63	67	63	64	66	66	66	81	80	80	80	80	80	69	68	80	80	80	80	81	80	81	82	82	80	82	82	82	82	82	82	82	82	98	98	98	100			
43 *L. cylindrica* (*LcOSC2*) putative OSC	65	65	66	66	66	66	69	62	64	63	66	66	83	81	81	80	80	80	69	68	81	82	80	81	82	81	82	81	82	80	82	82	82	82	82	82	82	82	98	98	98	98	100		
44 *C. pepo* (*CPH*) putative OSC	63	62	63	62	63	60	67	60	63	62	63	63	81	80	80	80	80	80	69	68	80	80	80	80	81	80	81	82	82	80	82	82	82	82	82	82	82	82	98	98	98	98	98	100	
45 *T. officinale* (*TRV*) putative OSC	64	63	64	63	62	62	69	59	62	63	64	62	82	82	82	82	82	82	71	70	82	82	82	82	83	82	83	84	84	82	84	84	84	84	84	84	84	84	98	98	98	98	98	98	100

CDS, cucurbitadienol synthase; IMS, isomultiflorenol synthase; putative OSC, putative oxidosqualene cyclase

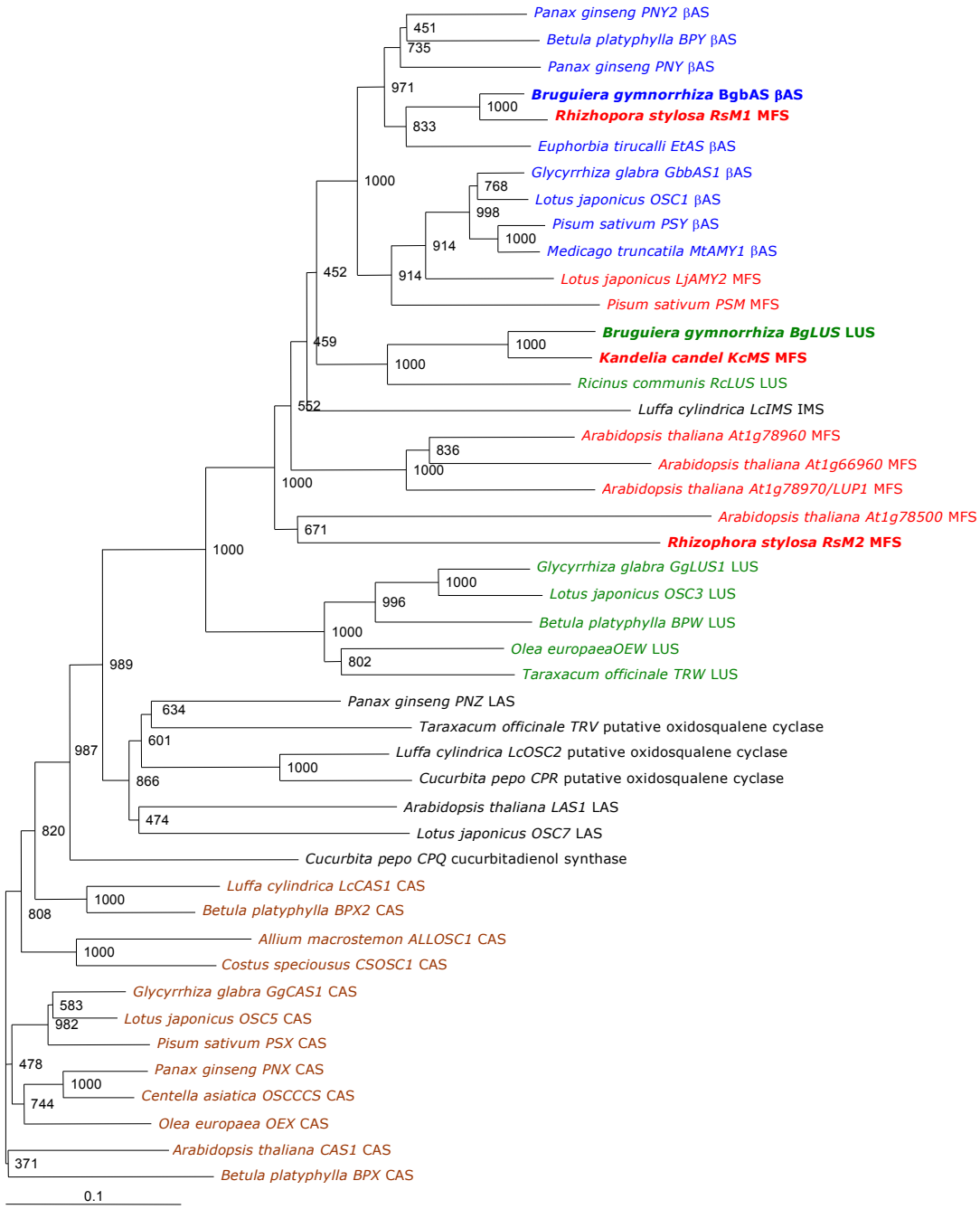


Fig. 3.10. Phylogenetic tree of plant OSCs that includes *BgbAS*, *BgLUS*, *RsM1*, *RsM2* and *KcMS*.

The deduced amino acid sequences were aligned by the CLUSTAL W program. The phylogenetic tree was constructed using the neighbor-joining method of PHYLIP package Version 3.66. Amino acid distances were calculated using the Dayhoff PAM matrix method of the Protdist program of PHYLIP. The indicated scale represents 0.1 amino acid substitutions per site. Numbers indicate bootstrap values from 1000 replicates. CAS, cycloartenol synthase; LAS, lanosterol synthase; βAS, β-amyrin synthase; LUS, lupeol synthase; MFS, multifunctional triterpene synthase. The DDBJ/GenBank/EMBL accession numbers of the sequence used in this analysis are described in the Experimental procedures section.

We considered the possibility that the presence of a new branch of lupeol synthase was due to evolutionary event or genetic noise. In this study, the presence of a new branch that consists of two monofunctional (*BgLUS* and *RcLUS*) and one multifunctional (*KcMS*) lupeol synthase favors the notion that the generation of the two branches of lupeol synthase gene occurred in the course of evolution. The reasons for the generation of a new branch during the course of evolution are not yet known. Successful cloning of triterpene synthases from a variety of plant species may reveal the reasons.

Ten β-amyrin synthase (βAS) genes including *BgbAS* exhibited high similarities (78-94%) between themselves (Table 3.1). In contrast to the high sequence similarity among those CAS, LUS and βAS, nine multifunctional triterpene synthases with different product patterns showed low identity (53-79%) to each other. As a result, they did not form one cluster in the tree but are distributed between the monofunctional β-amyrin synthase and lupeol synthase clusters.

The phylogenetic tree shows that *RsM1*, together with *BgbAS*, forms one branch of the β-amyrin synthase cluster with β-amyrin synthase from *E. tirucalli* (*EtAS*). Furthermore, *RsM1* close to the β-amyrin synthase cluster and shows high similarity (78-84%) to the known β-amyrin synthases (Table 3.1). This result, suggests that the *RsM1* is more evolved clone in the multifunctional synthase genes. Multifunctional synthases may represent evolutionally transient states between one product-specific OSC to another OSC. Of the OSCs, *RsM2* displays the highest similarity with At1g78500/T30F21.16 (Ebizuka et al., 2003) and forms the first branch of the multifunctional triterpene synthase that evolved from lupeol synthase to β-amyrin synthase. The enzymatic reaction products of *RsM1* and *RsM2* differed from those of their neighboring clones in the tree. This suggests that the relationships in the phylogenetic tree have limited significance in predicting the product profile of terpenoid synthases.

A number of studies have focused on identifying the active catalytic site of triterpene synthases, and have identified the MLCYCR or MWCYCR motifs for the product specificities of lupeol synthse and β-amyrin synthase, respectively (Kushiro et al., 1999; Kushiro et al., 2000b).

Thus, Leu of the MLCYCR motif and the Trp of the MWCYCR motif have been shown to play critical roles in product differentiation during lupeol and β-amyrin formation (Kushiro et al., 2000b).

		Motifs		
		MW(L)CYCR	MQSFGSQ	FIKKSQ
		*	●●	♦
Enzyme	Plant genes			
βAS	*Betula* BPY	256 MWCYCR 261	410 MQSFGSQ 416	446 FIKKSQ 451
	Pisum PSY	256 MWCYCR 261	410 MQSFGSQ 416	446 FIKKSQ 451
	Glycyrrhiza GgbAS1	256 MWCYCR 261	410 MQSFGSQ 416	446 FIKKSQ 451
	Panax PNY	258 MWCYCR 263	411 MQSFGSQ 417	447 FIKKSQ 452
	Euphorbia EtAS	256 MWCYCR 261	410 MQSFGSQ 416	446 FIKKSQ 451
	***Bruguiera* BgbAS**	256 MWCYCR 261	410 MQSFGSQ 416	446 FIKKSQ 451
LUS	*Betula* BPW	255 MLCYCR 260	407 IQSFGCQ 413	443 FVKASQ 448
	Olea OEW	255 MLCYCR 260	408 MQSFGCQ 414	444 FVKASQ 449
	Taraxacum TRW	257 MLCYCR 262	410 MQSFGCQ 416	446 FVKASQ 451
	Glycyrrhiza GgLUS	255 MLCYCR 260	407 IQSFGCQ 413	443 FVKASQ 448
	Lotus OSC3	255 MLCYCR 260	407 IQSFGCQ 413	443 FVKASQ 448
	Ricinus RcLUS	256 MFCYCR 261	409 MQSFGSQ 415	445 FTKNSQ 450
	***Bruguiera* BgLUS**	256 MLCYCR 261	409 IQSFGSQ 415	445 FIKNSQ 450
MFS	*Arabidopsis* At1g78970/LUP1	254 ILCYSR 259	408 MQSFGCQ 414	444 YIKASQ 449
	Arabidopsis At1g78500	259 LWIYFR 264	413 IQSFGSQ 419	453 FLNKSQ 458
	Arabidopsis At1g78960	257 TLCYTR 262	411 MQSFGSQ 417	447 FIKKSQ 452
	Arabidopsis At1g66960	257 AFSYTR 262	411 MQCFGSQ 417	447 YIKKSQ 452
	Lotus LjAMY2	256 MWCYCR 261	410 MQSFGSQ 416	446 FIKNSQ 451
	Pisum PSM	256 MLCYCR 261	410 LHSFGSQ 416	446 FIKKSQ 451
	***Kandelia* KcMS**	256 MLCYCR 261	409 IQSFGSQ 415	445 FIKDSQ 450
	***Rhizophora* RsM1**	256 MWCYCR 261	410 MQSFGSQ 416	446 FIKKSQ 451
	***Rhizophora* RsM2**	258 MWCYCR 263	412 ITGIGSQ 418	448 FLKNSQ 453

Fig. 3.11. Comparison of amino acid sequence alignment around the critical residues of plant OSCs.

Identical amino acid residues of all plant OSCs are shaded. The positions corresponding to the catalytically essential residues for β-amyrin synthase (Trp257) and lupeol synthase (Leu256) are marked with ∗. Lupeol and β-amyrin synthase also have two important residues: SerPhe (●) corresponds to the position to regulate the catalytic difference between cycloartenol synthase and lanosterol synthase. Another candidate amino acid residue to control terpenoid synthase product, Lys449 (♦) is also shown. βAS, β-amyrin synthase; LUS, lupeol synthase; MFS, multifunctional triterpene synthase. The DDBJ/GenBank/EMBL accession numbers of the multiple sequences used in this analysis is described in the Experimental procedures section.

Fig. 3.11 shows the alignment of the amino acid sequences around the MW(L)CYCR motif of multifunctional triterpene synthases. The motif was fairly well conserved throughout the plant species. However, the rationale for the importance of Trp or Leu in the motif may have limited significance in the cases of *A. thaliana* At1g78960 and *P. sativum PSM* because the Leu of MLCYCR motif in lupeol synthase was conserved in both clones, yet their main product was β-amyrin (Kushiro et al., 2000a; Morita et al., 2000). Likewise, the motif of MWCYCR for β-amyrin synthase was conserved in the clones of *RsM1* and *RsM2*, and their main enzymatic reaction products were germanicol and taraxerol, respectively. Therefore, these observations suggest that the presence of an additional protein domain acts to control the reaction product of terpene synthases.

The results of several mutagenesis studies have identified the catalytically important residues for cycloartenol synthase (Hart et al., 1999; Herrera et al., 2000; Meyer et al., 2002). The Tyr410 residue in *A. thaliana* cycloartenol synthase (*CAS1*) has been proven to be crucial in the catalytic sites of cycloartenol synthase and lanosterol synthase (Herrera et al., 2000; Meyer et al., 2002). The position that corresponds to Tyr410 is also well conserved in terpenoid synthases (Fig. 3.11). Monofunctional lupeol synthase and β-amyrin synthase have two residues: SerPhe instead of Tyr410 and a single amino acid deletion at this position. This is also applicable for multifunctional terpenoid synthases with one exception: SerPhe has been substituted by GlyIle in our clone *RsM2*. This position has been postulated to be located near the B/C ring, and has been implicated in facilitating the formation of the dammarenyl cation or in playing some other role specific to non-steroidal triterpenoid synthesis (Herrera et al., 2000).

An alternative strategy to the random mutagenesis studies for identifying catalytically important residues is to search for similar conservation patterns between the known terpenoid synthases. In the absence any proven data for identifying catalytically important residues using site-directed mutagenesis, we propose another candidate amino acid residues to control product formation of terpenoid synthase: Lys449 (Fig. 3.11). Lys449, which corresponds to *BgbAS* was strictly conserved in monofunctional β-amyrin synthase, while Ala or Asn has been substituted for Lys in monofunctional lupeol synthase. With respect to multifunctional terpenoid synthases, the

clones, in which Lys is located at this position, produced β-amyrin as the major product, and clones, in which Ala or Asn are located at this position, synthesized lupeol as the major product. The lupenyl cation represents the branch point from which numerous mechanistic pathways of oleanane or lupane type triterpene synthesis diverge (Fig. 3.6). Thus, the presence of basic amino acid residue of Lys at this position may favour E-ring expansion to produce oleanane or ursane type terpenoids, rather than lupane type terpenoids by deprotonation. We envisage that our results will trigger further studies using site-directed mutagenesis to shed light on the significance of Lys449.

Contribution of terpenoid synthase genes to the terpenoid composition

In order to extend our knowledge on the contribution of terpenoids synthase genes to the terpenoids of mangrove leaves, we analyzed the terpenoid composition of three major mangrove species in Okinawa (Table 3.2). *R. stylosa* leaves contained abundant quantities of taraxerol, β-amyrin and lupeol. This finding is in agreement with the results of other investigators who found that this species contains these three terpenoids as well as taraxerone, careaborin, and cis-careaborin (Basyuni et al., 2007b; Zhao et al., 2004). The product pattern of *RsM2* in the transformed yeast was almost identical to that of the triterpene profile in the leaves of *R. stylosa*. This result suggests that this gene is mainly responsible for terpenoid biosynthesis in this plant. However, this does not necessary negate the presence of product-specific β-amyrin synthase and lupeol synthase in this plant because these enzymes are widely distributed in higher plants.

Table 3.2. Terpenoids composition (%) of mangrove leaves and product profile of triterpene synthases (%)

Component	*Rhizophora stylosa*			*Bruguiera gymnorrhiza*			*Kandelia candel*	
	Leaves	*RsM1*	*RsM2*	Leaves	*BgbAS*	*BgLUS*	Leaves	*KcMS**
α-Amyrin							25	25
β-Amyrin	17	33	17	30	100		38	25
Germanicol		63						
Lupeol	10	4	13	59		100	36	50
Lupenone				11				
Taraxerol	73		70				1	

Terpenoids in the lipid extracts were analysed by GC-FID as described in the Experimental procedures section. Data on the terpenoids and the reaction products are expressed as the mean of quintuplicate and triplicate analyses, respectively. *KcMS from our previous paper (Basyuni et al., 2006)

The amounts of the main product of *RsM1* (germanicol) were negligible and almost below detectable levels in the leaves of *R. stylosa*. This observation suggests that this gene is not usually

expressed in the leaves of this plant. It is possible that there are a number of erratic terpene synthase genes that are not linked with molecular evolution, but can cause genetic noise. *RSM1* could be an example of such a gene. Mutagenesis studies have established that even a single mutation can dramatically alter the product specificity of OSC. By analogy, the product profile of *RsM1* completely differed from that of *RsM2*. This catalytic plasticity may potentially contribute to the diversity of terpenoids in this plant species.

β-amyrin and lupeol are the main triterpene components of *B. gymnorrhiza*. In contrast to *R. stylosa*, the triterpene synthase genes, *BgbAS* and *BgLUS*, which were cloned from this species, were found to be monofunctional and produced either only β-amyrin or lupeol. It is therefore very plausible that distinct enzymes are responsible for the formation of each terpenoid in this species. As a result, the composition of triterpenes may be a reflection of the distribution or expression of each monofunctional triterpene synthase in the cells of this plant.

In this regard, the circumstance for *K. candel* is more similar to that of *R. stylosa* than to that of *B. gymnorrhiza*. The triterpene composition of this species consists of almost equal amounts of lupeol, β-amyrin and α-amyrin and a small amount of taraxerol (Table 3.2). The triterpene composition in the leaves is almost comparable to the product profile of a multifunctional terpene synthase that was isolated from the roots of this species (*KcMS*). This finding also appears to support the view that multifunctional triterpene synthase is responsible for the formation of several terpenoids in the leaves of this species. The observed minor difference between the terpenoid composition of the leaf and the product profile of *KcMS* may be due, in part, to the differences in the tissues of this plant.

It should be noted that the OSCs of *B. gymnorrhiza* may differ evolutionally from those for *K. candel* and *R. stylosa*. *B. gymnorrhiza* expresses the monofunctional triterpene synthases, *BgbAS* and *BgLUS*, whereas *K. candel* and *R. stylosa* express the multifunctional triterpene synthases, *KcMS*, *RsM1* and *RsM2*, even though they originated from the same tribe of, Rhizophoracece. By association, a number of phylogenetic studies have been conducted in tribe of Rhizophoraceae based on molecular markers and morphological characters (Setoguchi et al., 1999; Schwarzbach and Ricklefs, 2000; Lakshmi et al., 2002). The mangrove tribe in Rhizophoraceae can be divided into

four genera, namely *Rhizophora*, *Bruguiera*, *Kandelia* and *Ceriops*. Members of the genus *Ceriops* are absent in the Okinawan mangrove habitat. Because of high similarity between *Rhizophora*, *Kandelia* and *Ceriops,* these genera form one cluster and the genus *Bruguiera* is loacted in another cluster (Setoguchi et al., 1999; Schwarzbach and Ricklefs, 2000; Lakshmi et al., 2002). Thus, the evolution of OSCs in mangrove plant species appeared to be at least partially associated with the lineage relationship.

Mangrove plants comprise a heterogeneous group of independently derived lineages that are defined ecologically by their location in tidal zones and physiologically by their ability to withstand high salt concentrations or low soil aeration. With respect to the Okinawan mangrove species, the distribution of *B*. *gymnorrhiza* is more inland than the coastal distribution of *R*. *stylosa* and *K*. *candel* (Baba, 1998). This distribution suggests that *B*. *gymnorrhiza* is less tolerant to salt stress than the other two species. Based on the evolutionary scheme of OSCs, which are descendants from cycloartenol synthase, multifunctional OSCs have been considered to represent a transitional state before their evolution to product-specific OSC. Therefore, the terpene synthase of *B*. *gymnorrhiza* may be considered to be a more highly evolved form when compared to the terpene synthase in the other two Okinawan mangrove species. The results of our previous study suggest that terpenoids plays a role in the protection of mangrove plants from salt stress (Oku et al., 2003). Furthermore, a large proportion of triterpenoids are found in the outer parts of the root and, this location may provide additional evidence for the protective roles of triterpenoids in mangroves species (Basyuni et al., 2007b).

In this context, it should be noted that the composition of terpenoids appears to be regulated by the product specificity of *RsM2* in *R*. *stylosa*. In contrast to the fixed ratio of terpenoids in *R*. *stylosa*, it is possible to alter the profile of terpenoids by regulating the gene expression of each monofunctional OSC in *B*. *gymnorrhiza*. The production of several terpenoids, whose molar ratio is defined by multifunctional OSCs may be beneficial to the plant by rendering it more tolerant to the environmental stress, such as osmotic pressure. Accordingly, it would be very interesting to further address the physiological significance of terpenoids in mangrove species. Such studies, may provide an explanation for the presence of divergent enzyme systems in mangrove species.

Chapter IV

Characterization of Cycloartenol Synthases from Mangrove Species *Rhizophora stylosa* Griff. and *Kandelia candel* (L.) Druce

Part 1: Cloning and functional expression of cycloartenol synthases from mangrove species *Rhizophora stylosa* Griff. and *Kandelia candel* (L.) Druce

Introduction

Phytosterol and terpenoids are common chemical constituents in mangroves and higher plants (Ghosh et al., 1985) and their biological activities have attracted the attention of many investigators. For example, stigmasterol, abundantly present in *Acanthus illicifolius*, has been found to have hypercholesterolemic effects (Kokpol et al., 1986). *Avicennia officinalis* produced pharmacologically important steroidal saponin and sapogenins (Bandaranayake, 1998). Mangrove triterpenoids and phytosterols as chemotaxonomical biomarkers also have been demonstrated (Koch et al., 2003) However, biosynthetic and physiological process in mangrove plants, have been rarely reported.

More than 100 different triterpene skeletons have been described from higher plants. They are elaborated from a common precursor 2,3-oxidosqualene *via* oxidosqualene cyclases (OSCs), which diverge in the cyclization of this substrate into either steroid cycloartenol and lanosterol or a variety of triterpenoids (Fig. 4.1). In higher plants, OSC family member cycloartenol synthase (CAS) and lanosterol synthase (LAS) are responsible for sterol biosynthesis, and other OSCs are involved for triterpenes synthesis.

A number of plant OSCs responsible for plant sterol have been cloned and their functions have been confirmed by expression in yeast, including CAS from dicotyledenous (dicot) plants, *Arabidopsis thaliana* (*CAS1*) (Corey et al., 1993), *Panax ginseng* (*PNX*) (Kushiro et al., 1998), [10] *Lotus japonicus* (*OSC5*) (Sawai et al., 2006a), *Ricinus communis* (*RcCAS*) (Guhling et al., 2006), etc; CAS monocotyledous (monocot) plants, *Avena strigosa* (*AsCS1*) (Haralampidis et al., 2001), *Costus speciosus* (*CSOSC1*) (Kawano et al., 2002); plant LAS, *A. thaliana* (*LAS1*) (Suzuki et al., 2006), *L. japonicus* (*OSC7*) (Sawai et al., 2006b) and cucurbitadienol synthase (*Cucurbita pepo*) (Shibuya et al., 2004)

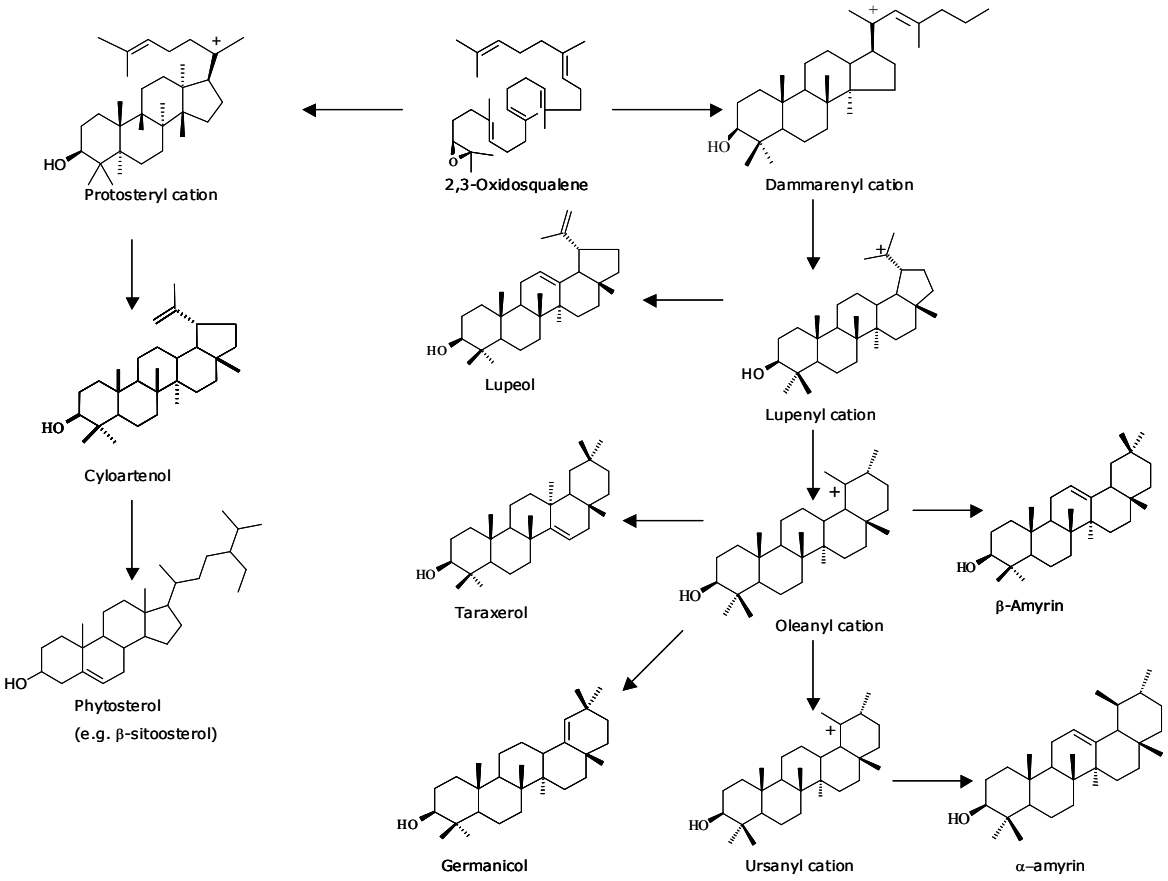


Fig. 4.1. Cyclization of 2,3-oxidosqualene cyclases in *K. candel* and *R. stylosa*.

Cyloartenol and lanosterol are important membrane constituents and can serve as precursors to steroid hormones (Nes and Heftmann, 1981). Despite phytosterols are ubiquitous and indispensable component of cell membrane, their physiological functions in mangrove plants are still unclear. Our previous report showed that membrane lipid (terpenoids and phytosterols) appeared to play a vital role in adaptation of mangrove to salt tolerance (Oku et al., 2003). It therefore became necessary to know the DNA sequence of OSC from mangrove to investigate its expression and the regulation mechanism. Furthermore, the genes encoding OSC responsible for phytosterol biosynthesis have not cloned from mangrove species so far, although OSC yielding multifunctional triterpene synthases have been successfully cloned from *Kandelia candel* (Basyuni et al., 2006) and *Rhizophora stylosa* (Basyuni et al., 2007c). Therefore in order to gain more insights into the

biosynthetic and physiological function of phytosterols in mangrove plants, present study therefore has attempted to clone OSC cDNAs from *R*. *stylosa* and *K*. *candel* (Rhizophoraceae), which are commonly distributed in Okinawa Island, Japan.

Experimental procedures

Chemicals

Authentic standard of cycloartenol was purchased from Extrasynthese, Genay, France, while β-sitosterol, stigmasterol and campesterol were purchased from Tama Biochemical (Tokyo, Japan), and lanosterol from Sigma-Aldrich Co. St. Louis, MO, USA. Customized oligonucleotide primers were synthesized by Hokkaido System Science (Hokkaido, Japan).

PCR and sequence analysis

PCR was performed with a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA). The PCR reaction products were separated by SeaKem^{R} GTG agarose (BMA, Rockland, ME, USA), purified by Suprec^{TM}-01 (Takara Bio Inc., Otsu, Shiga, Japan), ligated to TOPO 10 (Invitrogen, Carlsbad, CA, USA), and introduced into electrocompetent *Escherichia coli* (Invitrogen) by Gene Pulser Xcell^{TM} (Bio-Rad, Tokyo, Japan). Plasmid DNA was extracted by GenElute^{TM} Plasmid Miniprep Kit (Sigma-Aldrich Co. St. Louis, MO, USA). Sequencing was carried out by ABI PRISM^{TM} 3100-Avant Genetic Analyzer (Applied Biosystems, Tokyo, Japan) using Bigdye^{R} Terminator ver. 1.1/3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Plant materials and culture conditions

Young roots of *Kandelia candel* and fresh leaves of *Rhizophora stylosa* were collected from Okukubi river, Okinawa, Japan. These materials were taken directly into liquid nitrogen and stored at –80 ºC for RNA extraction and were stored at –30 ºC for phytosterol analysis. Lanosterol synthase deficient (ERG7) strain GIL77 (*gal2 hem3-6 erg7 ura3-167*) was used for transformation, and was maintained on a YPD medium (1.0 % yeast extract, 2.0% peptone, 2.0% dextrose) supplemented with hemin (13 μg/ml), ergosterol (20 μg/ml) and Tween 80 (5 mg/ml). Transformation of the yeast mutant was by the Frozen-EZ Yeast Transformation II^{TM} Kit (Zymo Research, Orange, CA, USA). The transformant was cultured in complete medium (SC-Ura supplemented with hemin and ergosterol as the case of YPD medium) at 30 ^{0}C with shaking (220 rpm) for functional gene

expression.

cDNA cloning

Total RNA was extracted from root of *K. candel* and leaf of *R. stylosa* using CTAB method. Total RNA (2.4 µg/µl) each was reverse transcribed with 0.5 µg oligo (dt) primer (RACE 32, 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTT-3') to produce a cDNA using cloned AMV First-Strand cDNA synthesis Kit (Invitrogen) with 10mM dNTP in total volume of 20 µl for 5 min at 65 ºC, 1 h at 50 ºC, and 5 min at 85 ºC according to manufacturer's protocol. The resultant cDNA mixture was diluted with 50 µl Tris/EDTA (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and directly used as template for the following PCRs.

First PCR was performed with degenerated primers, 161S (5'-GAYGGIGGITGGGIYTIC A-3') and 701A (5'-CKRTAYTCIGCIARIGCCCA-3') (1 µg each) using Ex Taq^{TM} HS DNA polymerase (Takara Bio Inc.) with dNTP (0.2 mM) in the final volume 50 µl following the manufacturer's protocol. PCR amplification was carried out for 30 sec at 94 ºC, followed by 30 cycles of 30 sec at 94 ºC, 30 sec at 50 ºC and 2 min 72 ºC, with final extension of 10 min at 72 ºC. The first PCR product was applied on Microcon centrifugal filter devices YM-30 (Millipore Co., Bedford, MA, USA) and the volume was adjusted to 50 µl with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). The second PCR was carried out with 463S (5'-MGICAYATHWSIAARGG-3') and 603A (5'-CCCCARTTICCRTACCAISWICCRTC-3') using 1 µl of the first PCR product as template under the same condition as the first PCR. The PCR products (453 bp and 440 bp) were subcloned to the plasmid vector of TOPO 10. Two new genes (2 clones each), respectively were cloned and named as *KcCAS* and *RsCAS*.

Based on the sequence of *RsCAS* and *KcCAS*, specific primers for each clone were designed to sequence the 3'- and 5'-ends. The rapid amplification of cDNA ends (RACE) PCRs methods were applied as described previously (Basyuni et al., 2006) with some minor modifications. Primers used for 3'-RACE were as follows: KcCAS-S1 (5'-TGGGGAACCGTTAAGTGAGA-3'), KcCAS-S2 (5'-GA AATAGAACGCTGTATCAG-3') and RsCAS-S1 (5'-TTCAGTGATTGTTGGGGAAC-3'), RsCAS- S2 (5'-CATACCCTGGGCATAGGCGAGA -3').

For 5'-RACE: KcCAS-A1 (5'-GCTGCCTTTCTGATACAGCG-3'), KcCAS-A2 (5'-TAATT GGTTCTCACTTAACG-3') and RsCAS-A1 (5'-ATGTCACCAAAGGTTTCAGCAG-3'), RsCAS-A2 (5'-AGCAGCTTTAAGTCCTTCAG-3').

The full-length cDNA for *KcCAS* and *RsCAS* were obtained using N-terminal and C-terminal primers with specific restriction enzyme sites at each end as follows: Kpn-KcCAS-N1 (5'-AGAAGGTACCATGTGGCGGCTCAAGATTGC-3'; KpnI site underlined), Xho-KcCAS-C1 (5'-CCAACTCGAGTCAAGAAGCCTGCAACACCC-3'; XhoI site underlined) and Kpn-RsCAS-N1 (5'-GAGAGGTACCATGTGGAGGCTCAAGATTGC-3'; KpnI site underlined), Xho-RsCAS-C1 (5'-CCAACTCGAGTCAAGAAGCCTGCAACACCC-3'; XhoI site underlined). PCRs were performed with each set of primers with *K. candel* and *R. stylosa* and cDNAs as template and annealing temperature of 64 ºC and 55 ºC and respectively. The obtained full-length cDNA clones, *KcCAS* (7 colonies) and *RsCAS* (11 colonies) were sequenced in both strands.

Expression in (Erg7) Saccharomyces cerevisiae strain GIL77

The 2.3-kb PCR product was digested with the restriction enzyme sites and ligated into cloning site of pYES2 (Invitrogen) to construct plasmid OSC-pYES2-KcCAS and OSC-pYES2-RcCAS. The identity of the inserted DNA was confirmed by sequencing. The plasmid was introduced into *Saccharomyces cerevisiae* strain GIL77 lacking lanosterol synthase activity, using Frozen-EZ Yeast Transformation II™ Kit (Zymo Research). The transformant was inoculated in 25 ml synthetic complete medium without uracil (SC-Ura), containing hemin (13 μg/ml), ergosterol (20 μg/ml) and Tween-80 (5 mg/ml), and incubated at 30 ºC for 2 days. Then, the medium was changed to SC-Ura with the same supplements but 2% galactose for glucose. Cells were incubated at the same condition for 24 h, harvested by centrifugation at 2000 rpm for 5 min, resuspended in 20 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 3 % glucose and hemin (13 μg/ml), and incubated at the same condition for 48 h. Cell pellets were collected and refluxed with 2 ml 20% KOH/50% ethanol for 10 min. After extraction with the same volume of hexane, the extract was concentrated and applied to a TLC plate (Merck, Darmstadt, Germany), and was developed with benzene/acetone (19:1, v/v). The fraction corresponding to 4,4-dimethylsterol

band was scraped off from the plate, extracted with chloroform and served as the sample preparation for GC and GC-MS analysis.

Gas chromatography (GC) and Gas chromatography/mass spectrometry (GC-MS) of Phytosterols

The method for GC and GC-MS has been described in the foregoing chapter (please refer to the experimental section of Chapter III, page 34).

Phylogenetic analysis of amino acid sequence

Multiple sequence alignments were performed using the CLUSTAL W program ver. 1.83 (Thompson et al., 1994) of the DNA Data Bank of Japan (Mishima, Shizuoka, Japan). Phylogenetic analysis of deduced amino acid alignment was conducted with CLUSTAL W program followed by drawing with TreeView, ver. 1.6.6 (Page, 1996) based on a neighbor-joining method. Bootstrap analysis with 1000 replications was used to assess the strength of the nodes in the tree (Felsentein, 1985). The DDBJ/GenBank/EMBL accession numbers of the sequence used this analysis are as follows: AB055509 and AB055510 *BPX* and *BPX2* cycloartenol synthase (*Betula platyphylla*), U02555 *CAS1* cycloartenol synthase (*Arabidopsis thaliana*), AB025968 *GgCAS1* cycloartenol synthase (*Glycyrrhiza glabra*), D89619 *PSX* cycloartenol synthase (*Pisum sativum*), AB025344 *OEX* cycloartenol synthase (*Olea europea*), AY520819 *OSCCCS* cycloartenol synthase (*Centella asiatica*), AB009029 *PNX* cycloartenol synthase (*Panax ginseng*), AB033334 *LcCAS1* cycloartenol synthase (*Luffa cylindrica*), AB181246 *OSC5* cycloartenol synthase (*Lotus japonicus*), AB292608 *RsCAS* cyloartenol synthase (*Rhizophora stylosa*), AB2922609 *KcCAS* cyloartenol synthase (*Kandelia candel*), AB058507 *CSOSC1* cycloartenol synthase (*Costus speciosus*), AB025353 *ALLOSC1* cycloartenol synthase (*Allium macrostemon*), AF169966 CAS1 cycloartenol synthase (*Oryza sativa*), AJ311790 CS1 cyloartenol synthase (*Avena strigosa*), AY618694 CS1 cyloartenol synthase (*A. ventricosa*), AF216755 CAS1 cyloartenol synthase (*Abies magnifica*), AB116238 *CPQ* cucurbitadienol synthase (*Cucurbita pepo*), AB009031 *PNZ* lanosterol synthase (*P. ginseng*), AB244671 *OSC7* lanosterol synthase (*L. Japonicus*), AB247155 *LAS1* lanosterol synthase (*A. thaliana*), AB116239 putative oxidosqualene (*C. pepo*), AB033335 *LcOSC2* putative oxidosqualene

cyclase (*L. cylindrica*), AB025346 *TRV* putative oxidosqualene cyclase (*Taraxacum officinale*).

Isolation of phytosterol composition of K. candel and *R. stylosa*

Identification and analysis of phytosterols have been described in the foregoing chapter (please refer to page 15).

Results and Discussion

Cloning of cyloartenol synthase cDNAs

In order to clone *K. candel* and *R. stylosa* cyloartenol synthase gene, PCR reactions were performed for cloning using degenerate primers designed from highly conserved regions of known OSCs enzymes as described previously (Basyuni et al., 2006). The amplification products as core sequences were 440 and 453 bp, respectively in length. The DNA fragments were cloned into vector TOPO 10 (invitrogen). 3'-RACE and 5'-RACE methods (Frohman et al., 1988) were used to clone each of the 3'- and 5'-end of cDNAs, using the template cDNA prepared by GeneRacer^{TM} Kit (Invitrogen). Cloning and sequencing of these fragments showed that each product contained an overlapping sequence with the core fragments, which enabled us to identify the open reading frame (ORF) of OSC. The full-length sequences of the cDNAs clone were obtained by sequencing in both strands.

The ORF of both *KcCAS* and *RsCAS* consisted of 2,277 bp, encoding 758 amino acids polypeptides. The deduced amino acid sequence of the both clones showed the highest homology of 82% to the *Lotus japonicus* cycloartenol synthase (OSC5) (Sawai et al., 2006a) and *Ricinus communis* cyloartenol synthase (Guhling et al., 2006), suggesting that *KcCAS* and *RsCAS* cDNAs encode cycloartenol synthase. As shown Fig. 5.2, there are five repeats of consensus structure of the QW motif (Poralla et al., 1994), which occurs repeatedly in the sequences of all known OSCs, are present in the *KcCAS* and *RsCAS* sequences. The amino sequence data also revealed the presence of a substrate binding DCTAE motif (Abe and Prestwich, 1994) (Fig.4.2), which has been claimed to be in the active site to generate the protesteryl cation (Abe and Prestwich, 1994). Thus, the high levels of sequence homology with cycloartenol synthases suggested that *KcCAS* and *RsCAS* encode cyloartenol synthases.

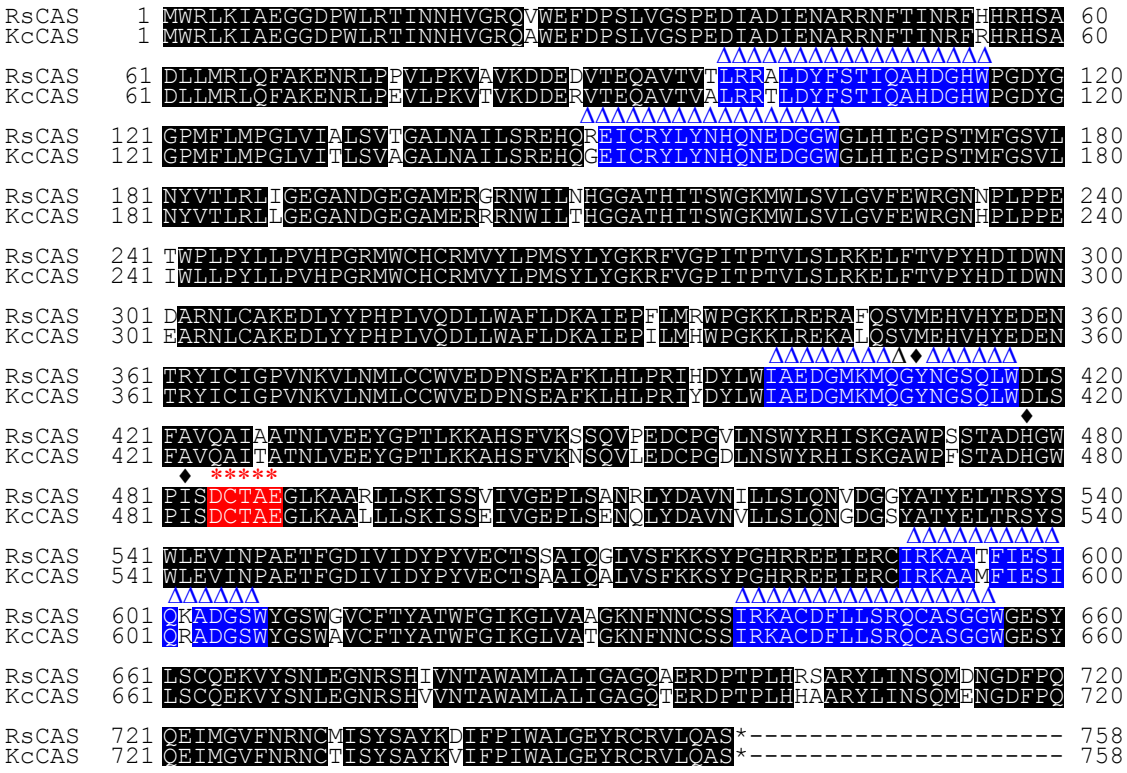


Fig. 4.2. Alignment of the deduced amino acid of cycloartenol synthases from *R. stylosa* (*RsCAS*) and *K. candel* (*KcCAS*). DCTAE motif, QW motifs, and important amino acid residues of Tyr411, His478, and Ile482 are indicated by ∗, Δ, and ♦, respectively. Identical amino acid residues in the two proteins are shaded.

Functional expression of KcCAS and *RsCAS in Erg7 deficient mutant GIL77*

In order to verify the enzymatic identities of *KcCAS* and *RsCAS* clones, functional expression in yeast of these genes were conducted. *KcCAS* and *RsCAS* cDNAs were ligated to yeast expression vector pYES2 (Invitrogen), and expressed under the control of GAL1 promoter in a lanosterol synthase deficient yeast (*Erg7*) mutant strain GIL77 that accumulates oxidosqualene inside cells (Kushiro et al., 1998). The cycloartenols produced in the mutant yeast were isolated by thin layer chromatography (TLC) (Fig. 4.3) as described in the Experimental procedures, and were analyzed by GC or GC-MS. Fig. 4.3 shows the thin layer chromatogram of the lipid extract from transformed GIL77. Introduction of pYES2-*RsCAS* gene into GIL77 cells resulted in the production

of a distinct sterols with the same mobility (R_f value) of cycloartenol standard, along with several spots that did not occur in the control transformant of the vehicle vector. This observation agrees with a previous report, in that cycloartenol was metabolized into ergosterol in a transformant yeast strain (Venkatramesh and Nes, 1995) with some cycloartenol metabolites on the way to ergosterol (Kushiro et al., 1998; Haralampidis et al., 2001; Guhling et al., 2006)

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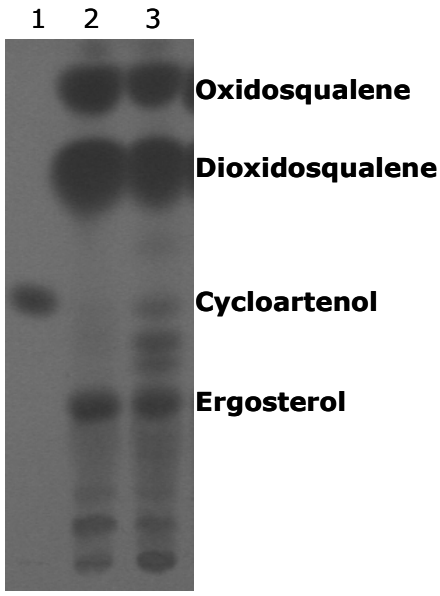

Fig. 4.3. TLC analysis of hexane extracts from transformed yeast strain GIL77. TLC was developed with benzene/acetone (19:1). 1, cycloartenol standard; 2, extract of GIL77 transformed with empty vector (pYES2) as control; 3, extract of GIL77 transformed with pYES2-*RsCAS*.

Lipid extract from the new and largest spot was analyzed by GC-MS. A total ion chromatogram (TIC) of pYES2-RsCAS transformant gave a single product peak with the same retention time as authentic cycloartenol (Fig. 4.4). The MS-spectrum of this peak further confirmed this identification (Fig. 4.5). Application of a similar expression method to pYES2-*KcCAS* from *K. candel* also resulted in a single product peak on TIC, but with significantly lower catalytic efficiency than was the case for pYES2-*RsCAS*, but the retention time and fragmentation pattern were identical to those for pYES2-*RsCAS*, identifying the chemical structure of the reaction product as cycloartenol

(Fig.4.4 and Fig. 4.5).

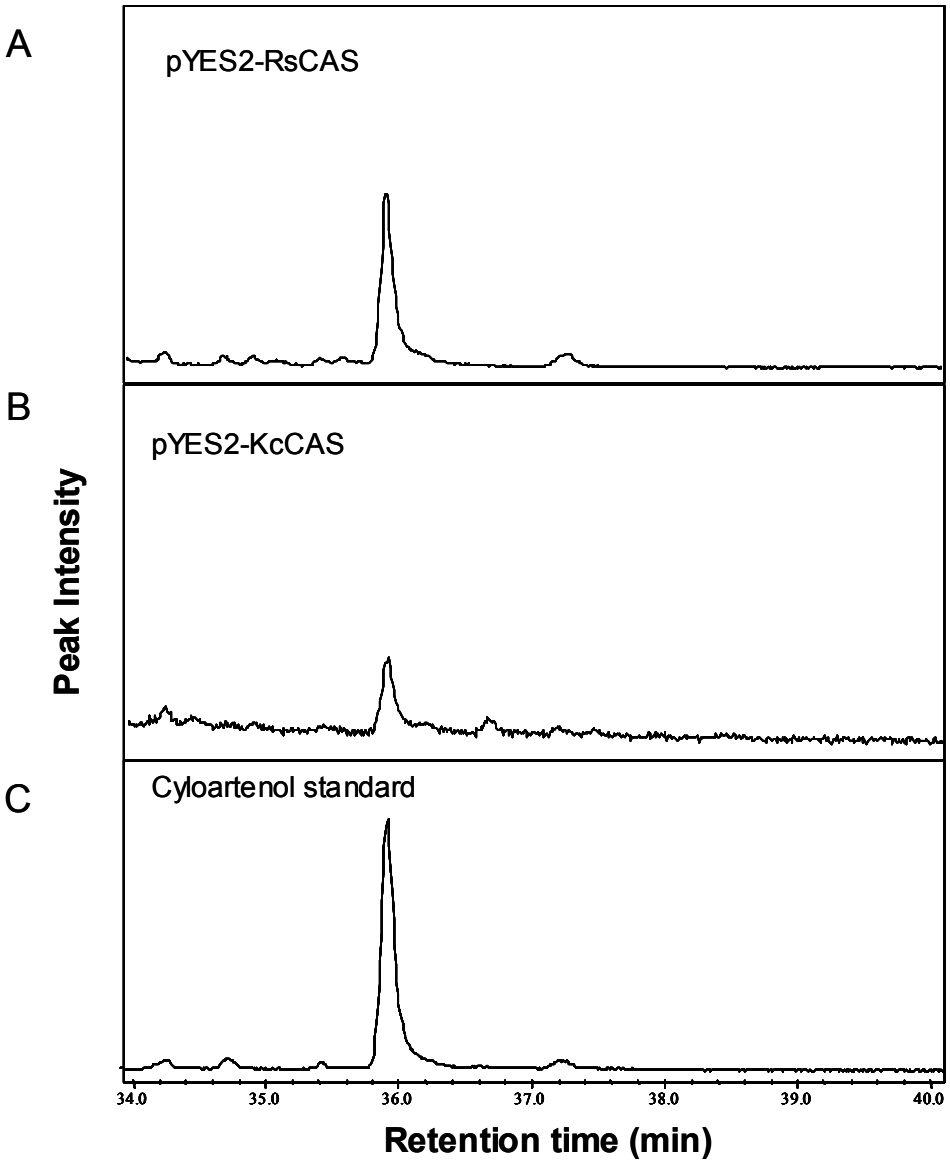

Fig. 4.4. GC-MS profile of extracts from transformed strain GIL77. TIC chromatogram of pYES-*RsCAS* (A), pYES2-*KcCAS* (B) and authentic cycloartenol standard (C), were shown respectively.

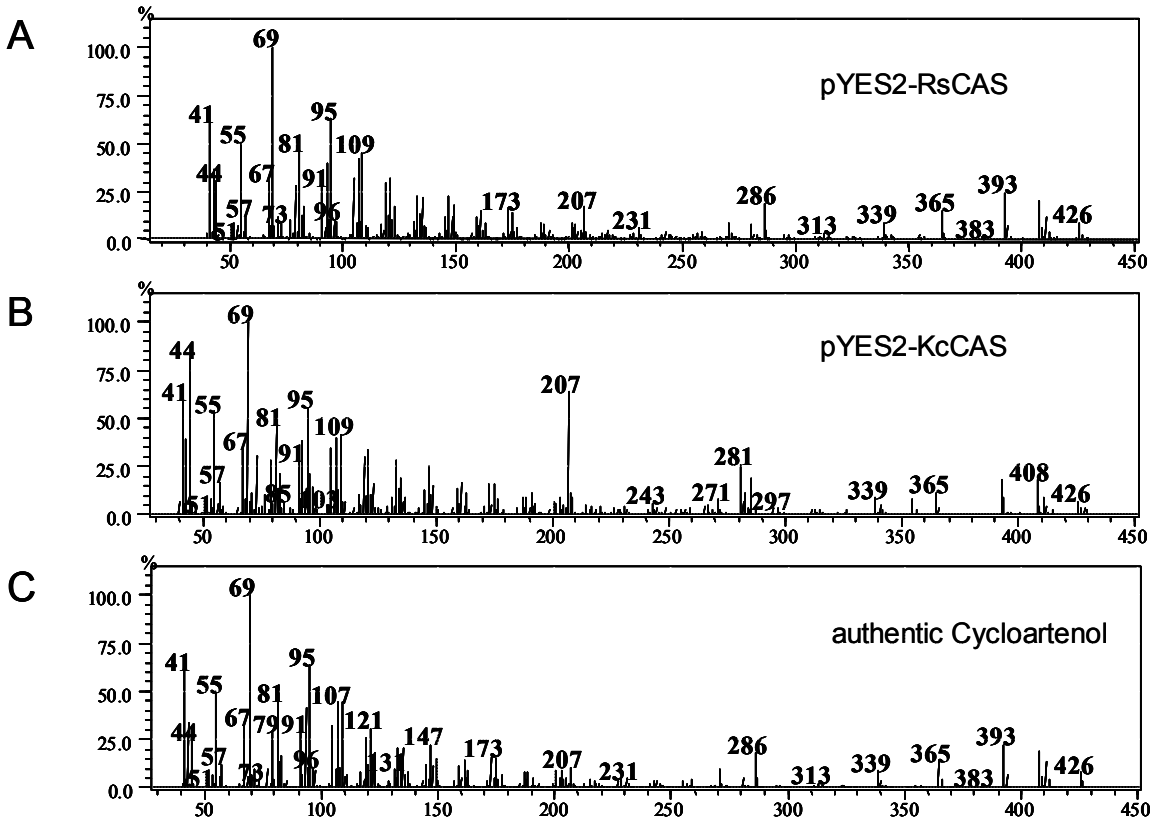

Fig. 4.5. GC-MS analysis of *RsCAS* and *KcCAS* products. EI-mass spectra were shown in *RsCAS* (A), *KcCAS* (B), and authentic cycloartenol (C).

Phylogenetic analysis of KcCAS and RsCAS in phytosterols OSCs

To determine the relationship of *KcCAS* and *RsCAS* to other OSCs responsible for phytosterol biosynthetis, phylogenetic analysis was constructed. As Fig. 4.6 shows that *KcCAS* and *RsCAS* join with the dicot CAS cluster. As shown there, there are three distinct branches of plant sterols; dicot CAS, monocot CAS, and lanosterol synthases and putative OSC (uncharacterized OSC). The present study indicates that *KcCAS* and *RsCAS* form a new branch in the dicot CAS cluster, suggesting that both genes are new members of dicot CAS among plant OSCs.

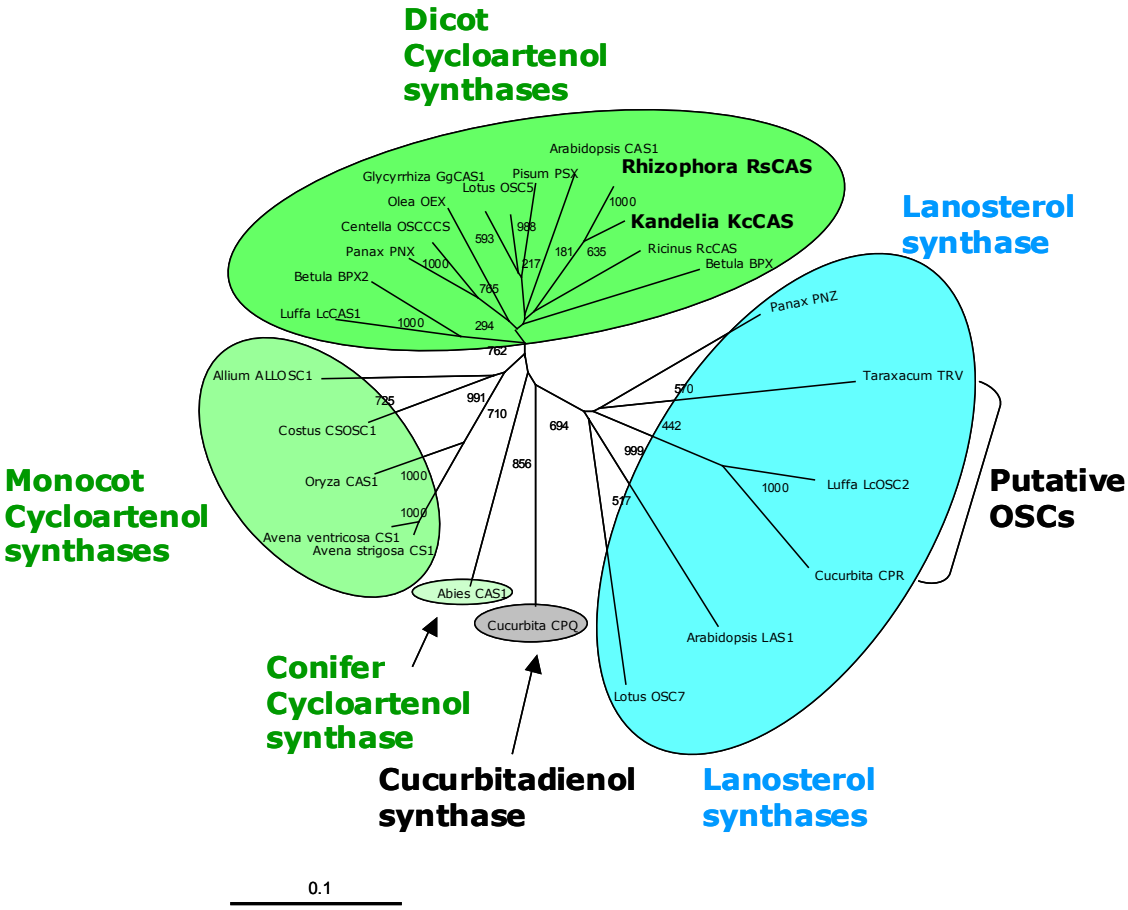

Fig. 4.6. Phylogenetic tree of *RsCAS* and *KcCAS* in the plant sterol OSCs.

A number of mutagenesis studies on the role of amino acid residues in cycloartenol synthase (CAS1) have revealed the significance of Tyr410, His477, and Ile481 (Fig. 4.7) in the product specificity of cycloartenol (Hart et al., 1999; Herrera et al., 2000; Meyer et al., 2002). Among these catalytically important amino acid residues, Tyr410 has been found to be the key to the catalytic difference between cycloartenol synthase and lanosterol synthase (Herrera et al., 2002). The amino acid residues corresponding to Tyr410 for cycloartenol synthase are conserved in both *RsCAS* and *KcCAS* (indicated by ♦ in Fig. 4.2), reinforcing the catalytic importance of this residue.

Several lines of study indicate that the sterol is a modulator of membrane permeability (Sietsma and Haskins, 1968; Sietsma, 1971). Fortification of the cell membrane with sterols appeared to increase the tolerance of external stresses of the organisms (Haskin, 1965; Tilman and

Bean, 1970). In this context, our *in vitro* study showed that triterpene, as well as phytosterols, was incorporated into the lipid bilayer of liposome (data not shown), suggesting that terpenoid is also a modulator of membrane permeability. Studies on gene expression of mangrove OSCs under different salt concentrations are in progress, and might give more insight into the physiological roles of phytosterols and terpenoids in mangrove plant species.

			*			Δ ◆	
Dicot CAS	*Arabidopsis* CAS1	406	KMQGYNGSQ	414	463	RHISKGAWPFSTADHGWPISDC	484
	Betula BPX	416	KMQGYNGSQ	424	473	RHISKGAWPFSTADHGWPISDC	494
	Betula BPX2	415	KMQGYNGSQ	423	472	RHISKGAWPFSTADHGWPISDC	493
	Ricinus RcCAS	408	KMQGYNGSQ	416	465	RHISKGAWPFSTADHGWPISDC	486
	***Kandelia* KcCAS**	407	KMQGYNGSQ	415	464	RHISKGAWPFSTADHGWPISDC	485
	***Rhizophora* RsCAS**	407	KMQGYNGSQ	415	464	RHISKGAWPSSTADHGWPISDC	485
	Pisum PSX	406	KMQGYNGSQ	414	463	RHISKGAWPFSTADHGWPISDC	484
	Lotus OSC5	406	KMQGYNGSQ	414	463	RHISKGAWPFSTADHGWPISDC	484
	Glycyrrhiza GgbAS1	406	KMQGYNGSQ	414	463	RHISKGAWPFSTADHGWPISDC	484
	Olea OEX	246	KMQGYNGSQ	254	303	RHISKGAWPFSTADHGWPISDC	324
	Centella OSCCCS	406	KMQGYNGSQ	414	463	RHISKGAWPFSTADHGWPISDC	484
	Panax PNX	406	KMQGYNGSQ	414	463	RHVSKGAWPFSTADHGWPISDC	484
	Luffa LcCAS1	414	KMQGYNGSQ	422	471	RHISKGAWPFSTADHGWPISDC	492
Monocot CAS	*Allium* ALLOSC1	411	KMLGYNGSQ	419	466	CHISKGAWPFSTADHGWPISDC	487
	Avena strigosa CS1	407	KMQGYNGSQ	415	464	RHISKGAWPFSTADHGWPISDC	485
	A. ventricosa CS1	407	KMQGYNGSQ	415	463	RHISNGAWPFSTADHGWPISDC	484
	Oryza CAS1	405	KMQGYNGSQ	413	462	RHISKGAWPFSTADHGWPISDC	483
	Costus CSOSC1	406	KMRGYNGSQ	414	463	RHISKGAWPFSTADHGWPISDC	484
Conifer CAS	*Abies* CAS1	406	KMQGYNGSQ	414	463	RHISNGAWPFSTRDHGWPISDC	484
LAS	*Arabidopsis* LAS1	406	KMQGYNGSQ	414	463	RHPCKGGWGFSTGDNPWPVSDC	484
	Panax PNZ	419	KMKGYNGSQ	427	476	RHISKGGWPFSTPDNGWPVSDC	497
	Lotus OSC7	407	KMQGYGGSQ	415	464	RHISKGSWGFSTPDNGWPVSDC	485
Putative OSC	*Taraxacum* TRV	404	KMQGYNGSQ	412	461	RHISRGGWPFSTPDNGWPVSDC	482
	Luffa LcOSC2	406	KMQGYNGSQ	414	463	RHISKGGWPFSTPDNGWPVSDC	484
	Cucurbita CPR	405	KMQGYHGSQ	413	464	RHNSKGGWPFSTPDNAWPVSDC	485

Fig. 4.7. Multiple amino acid sequence around the importance of the amino acid residues in specificity product of cyloartenol for phytosterol biosynthesis. ∗, Δ, and ♦ symbols denote the amino acid residues whose mutation in *Arabidopsis* CAS1. CAS, cycloartenol synthase; LAS, lanosterol synthase; CDS, cucurbitadienol synthase.

Mangrove Phytosterols

Table 4.1 summarizes the phytosterol composition from leaves and roots of *K. candel* and *R. stylosa*. β-Sitosterol is the major phytosterol component of both species in leaves and roots, as could be expected for higher plants including mangrove species (Nes and Heftmann, 1981; Hogg and Gillan, 1984; Ghosh et al, 1985; Koch et al., 2003)

Concentration of cycloartenol, a precursor for phytosterol biosynthesis, is usually low in the plant tissue (van der Heijden et al., 1989; Husselstein-Muller et al., 2001). Our study also found low concentration of cycloartenol in the leaf of both of *R. stylosa* and *K. candel*, suggesting that both genes are responsible for the genesis of cycloartenol in these plants. It is yet to come to demonstrate the involvement of cycloartenol synthase gene in the phytosterol biosynthesis of plant kingdom.

Table 4.1. Phytosterols composition (%) of mangrove leaves and roots

Isoprenoids	*Kandelia candel*		*Rhizophora stylosa*	
	Leaves	Roots	Leaves	Roots
Campesterol	9.0 ± 0.3	20.4 ± 0.2	6.6 ± 0.2	9.4 ± 0.7
Stigmasterol	13.4 ± 0.2	48.6 ± 0.3	6.5 ± 0.1	12.5 ± 0.9
β-sitosterol	69.5 ± 0.4	23.6 ± 0.3	80.7 ± 0.3	80.3 ± 0.6
Lanosterol	3.2 ± 0.2	5.7 ± 0.1	1.0 ± 0.1	
Cycloartenol	4.8 ± 0.4	1.7 ± 0.1	5.2 ± 0.0	2.7 ± 0.7

Phytosterols in the lipid extracts were analysed by GC-FID. Data are expressed as the mean ± SE. Data on the phytosterols are expressed as the mean of triplicate analyses

Chapter V

Effect of Salt Stress on mRNA Level and Terpenoid Concentration in *Kandelia candel* (L.) Druce and *Bruguiera gymnorrhiza* (L.) Lamk.

Part 1: Expression of four oxidosqualene cyclase mRNAs and isoprenoids concentration under salt stress in *Kandelia candel* (L.) Druce and *Bruguiera gymnorrhiza* (L.) Lamk.

Introduction

Mangroves are woody plants that grow at the interface between land and sea in tropical and subtropical area. Mangroves are salt tolerant and have mechanisms to take up water against strong osmotic potentials (Tomlinson, 1986). Mangroves are also rich sources of pentacyclic triterpenoids and phytosterols (isoprenoids) (Ghosh et al., 1985; Basyuni et al., 2007b). According to their morphological characteristics in salt management, mangrove plants fallen into two major groups (Scholander et al., 1962). The first group is the salt-secreting species that have either salt glands or salt hairs to remove excess salt. The second is non-secreting species that do not have such morphological features for excretion of excess salt (Scholander et al., 1962; Tomlinson, 1986). *Kandelia candel* (L.) Druce and *Bruguiera gymnorrhiza* (L.) Lamk. (Rhizophoraceae) are common mangrove species in Okinawa, Japan and belonging to the latter group. Both species are therefore ideal model for studying the molecular mechanisms of salinity tolerance at cellular level.

The mechanisms of plant cells to cope with the salt stress and the molecular strategies are not well understood (King, 1991; Yeo, 1998; Munns, 2005). Although the mechanisms of salt tolerance of the plants appeared to be complex and variable, several lines of mechanisms have been proposed: adjustment of the osmotic pressure by accumulation of small molecules of osmolytes such as glycine-betaine or sugar alcohols (Popp, 1984; Fan et al., 1993; Bohnert et al., 1995; Sakamoto & Murata, 2000); salt extrusion across the plasma membrane using ion transporters (Dupont, 1992; Allen et al., 1995; Shi et al., 2000; Kura-Hotta et al., 2001); accumulation of salt in vacuole using tonoplast transporter (Blumwald & Poole, 1987; Matoh et al., 1989; Gaxiola et al., 1999; Mimura et al., 2003).

A number of studies have shown that salinity stress increased the expression of a number of genes and protein levels (Sugihara et al. 2000; Yamada et al. 2002a; Banzai et al. 2002; Yamada et al. 2002b; Ueda et al. 2002; Ashihara et al. 2003). Our previous study also showed that the salt

stress induced a change in the concentration of terpenoids in mangrove roots and leaves (Oku et al. 2003). However, the relationship between salt stress and terpenoid biosynthesis is yet to be worked out. Oxidosqualene cyclases (OSCs) catalyze the cylization of 2,3-oxidosqualene, a common precursor of both triterpenoids and phytosterols biosynthesis (Abe et al., 1993; Haralampidis et al., 2002). cDNAs of β-amyrin synthase (βAS) and lupeol synthase (LUS) have already been isolated from *B*. *gymnorrhiza* (Basyuni et al., 2007c). In addition, two cDNAs for multifunctional triterpene synthase and cyloartenol synthase, have also been cloned from *K*. *candel* (Basyuni et al., 2006; Basyuni et al., 2007a). Molecular cloning of these genes provides useful tools for studying the salt stress related regulation of isoprenoids biosynthesis in mangrove plants. Thus, the aim of this chapter is to study the relationship between terpenoid biosynthesis and salt stress, although it is yet to come to give rationale for the increased concentration of terpenoid in salt stressed mangroves.

Experimental procedures

Chemicals

Authentic standard of β-amyrin, α-amyrin, lupeol, lupenone and cyclaortenol were purchased from Extrasynthese, Genay, France, whereas β-sitosterol, stigmasterol and campesterol were purchased from Tama Biochemical (Tokyo, Japan), cholesterol from Wako Pure Chemical Industries (Osaka, Japan) and lanosterol from Sigma-Aldrich (USA). Oligonucleotide primers were synthesized by Hokkaido System Science (Hokkaido, Japan).

PCR and sequence analysis for actin gene

PCR was performed with a PTC-200 Peltier Thermal Cycler (MJ Research). The PCR reaction products were separated by SeaKem^{R} GTG agarose (BMA), purified by Suprec^{TM}-01 (Takara Bio Inc.), ligated to TOPO 10 (Invitrogen), and introduced into electrocompetent *E. coli* (Invitrogen) by Gene Pulser Xcell^{TM} (Bio-Rad). Plasmid DNA was extracted by GenElute^{TM} Plasmid Miniprep Kit (Sigma-Aldrich). Sequencing was carried out by ABI PRISM^{TM} 3100-Avant Genetic Analyzer (Applied Biosystems) using Bigdye^{R} Terminator ver. 1.1/3.1 Cycle Sequencing Kit (Applied Biosystems).

Salt tolerant experiments

Propagules of *K. candel* and *B. gymnorrhiza* were collected in Okukubi river (Okinawa, Japan) and planted in Wagner pots with sand and varied salinity under natural sunlight in glass house. A seawater solution was prepared by dissolving the commercial salt powder (Red sea salt, Houston, TA, USA) to make 0, 0.5, 1.5, 2 and 3% (equal to sea water level) salinity concentration according to manufacturer's protocol. Salinity in this study is mass ratio of salt powder against solution (Fofonoff and Lewis, 1979; Gieskes, 1982). Salt concentration in each pot treatment was checked once a week during the experiments by Salinity hand-held refractometer S/Mill-E (Atago Co., Ltd, Tokyo, Japan), and adjusted whenever needed. Five to six plants per treatments were grown for 5 months. After 5 months of cultivation, the plants were harvested, washed, and the

leaves and roots were snap frozen in liquid nitrogen at once after collection and stored at -80 ºC for RNA analysis and isoprenoids.

Total RNA extraction and cDNA synthesis

Total RNA from leaves and roots of *K. candel* and *B. gymnorrhiza* (Fig. 5.1) were extracted using CTAB method as described in our previous report (Basyuni et al., 2007b) with minor modifications to improve the yield and quality of total RNA. Total RNA was dissolved into 20 µl of DEPC treated water (approximately 2-5µg/µl in concentration). The first-strand cDNA was synthesized from 2-5µg of total RNA with Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) with 10 mM dNTP in a total volume of 20 µl by incubating for 5 min at 65 ºC, 1 h at 50 ºC, and 5 min at 85 ºC according to the manufacturer's instruction. After first-strand cDNA synthesis, cDNA was treated with 2 units of RNase H to remove the RNA and used for quantitative real-time reverse transcription PCR (RT-PCR).

Cloning of actin gene fragment

Total RNA from roots of *K. candel* and leaves of *B. gymnorrhiza* were reverse-transcribed with 2.5 µM oligo $(dT)_{20}$ primer to produce a cDNA in total volume of 20 µl by incubating for 5 min at 65 ºC, 1 h at 50 ºC, and 5 min at 85 ºC, and using cloned AMV First-Strand cDNA synthesis Kit (Invitrogen) according to manufacturer's protocol. The resulting cDNA mixture was diluted with 50 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and directly used as template for the following PCRs. Two degenerate oligonucleotide primers (actinF and actinR) corresponding to the highly conserved amino acid sequence of diverse actin genes (Jin et al., 1999) were used to amplify the core fragment of actin gene (ActinF= 5'-TACCCNATYGARCACGG-3' and ActinR= 5'-TTRATRTC DCKRACRATTTC-3' (10 ng)) using Ex Taq™ HS DNA polymerase (Takara Bio Inc.) and 0.2 mM dNTP in a final volume of 50 µl. PCR amplification was carried out for 2 min at 94 ºC, followed by 35 cycles of 30 sec at 94 ºC, 30 sec at 55 ºC and 30 sec at 72 ºC, with a final extension of 8 min at 72 ºC. Two PCR products (405 and 401 bp) were separated using 1 % agarose SeaKem® GTG® agarose (BMA) and purified by Suprec ™-01 filter (Takara Bio Inc.). The purified

fragments were ligated to a plasmid vector of TOPO 10 (Invitrogen) and propagated in *E. coli*, by Gene Pulser Xcell™ (Bio-Rad) and sequenced by ABI PRISM™ 3100-Avant Genetic Analyzer (Applied Biosystems) with Bigdye® Terminator ver. 1.1/3.1 Cycle Sequencing Kit (Applied Biosystems). DNA sequence revealed the presence of two types of core fragments (*KcAct1* and *BgAct1*). The number of clones sequenced for *KcAct1* and *BgAct1* gene was 4 and 2, respectively.

Based on the sequences of above two types of core fragment, specific primers to each gene were designed to amplify the 3'- ends of cDNAs by RACE method (Frohman et al., 1988) using GeneRacer™ Kit (Invitrogen). The primers used for 3'-RACE were as follows: KcACT-S1 (5'-GCCATGTACGTTGCCATCCA-3'), KcACT-S2 (5'-CCACCACTGCTGAGCGTGAA-3'); BgAC T-S1 (5'-ATGTACGTTGCCATTCAGGC-3'), BgACT-S2 (5'-GATGAAGATACTCACTG AGC -3').

The PCR products of the 3'- ends of each gene were cloned into a TOPO 10 plasmid vector, and propagated in *E. coli* for sequencing. In the case of 3'-RACE, four clones of each gene were sequenced in both strands. The partial DNA sequence of *KcAct1* and *BgAct1* were obtained.

Real-time reverse transcription PCR (RT-PCR)

Real-time reverse transcription PCR was used to quantitate the mRNA level in this study. The PCR primers were designed based on our previous and present study as shown in Table 5.1 (Basyuni et al., 2006; 2007a;b).

Table 5.1. Description for the real-time reverse transcription PCR (RT-PCR) primers of mangrove roots and leaves

Plant gene	Accesion No.		Primer sequence 5'-3'	Length (bp)	Tm (°C)
K. candel KcAct1	Present study	KcACT-F1	AGCATCAGGCATCCATGAGAC	242	60
		KcACT-R3	TGCTGAGAGATGCCAGAATG		
B. gymnorrhiza BgAct1	Present study	BgACT-F1	GGAATGGAAGCAGCAGGCATTC	122	60
		BgACT-R1	CCTGGGAACATGGTTGAAC		
K. candel KcMS	AB257507	KcMS-F3	GGCTACTGCTGGCAAGACTTA	173	60
		KcMS-R3	CCAGTGCAGTATGTATCAAA		
K. candel KcCAS	AB292609	KcCAS-F1	GCTACATGGTTTGGCATAAA	194	60
		KcCAS-R1	GCAGTATTCACCACGTGGGATC		
B. gymnorrhiza BgbAS	AB289585	BgbAS-F1	ACAAAGAGATAATGGTGGTTG	131	60
		BgbAS-R1	GCCCAGCATGAATAAGAGCC		
B. gymnorrhiza BgLUS	AB289586	BgLUS-F1	CCAAACACAACGAGACGATG	89	62
		BgLUS-R2	TTGATCGGTTTCCCTCAAGA		

The PCR amplification was carried out in a 20 µl total volume containing 1.2 µl of template DNA, 0.2 µM each primer, 2.5 mM dNPT mixture, 0.1 µl 100 diluted SYBR Green I (Takara Bio Inc.) and 5 units Takara Ex Taq™ HS (Takara Bio Inc.). The real-time RT-PCR was performed

using iCycler (Bio-Rad, Tokyo, Japan) and cycled 35 times as follows: 30 sec denaturation at 94 ºC, 30 sec annealing at 60 ºC or 62 ºC, and 30 sec polymerization at 72 ºC. The real time RT-PCR product on 3% agarose gel electrophoresis showed discrete-sized single band as predicted from template sequence. Each data point represents the average of five to six independent measurements and the error bars indicate the standard errors of individual experiments. To quantitate the expression level, standard curve was constructed by performing PCR with serially diluted known concentrations of template DNA for genes of interest (*KcMS*, *KcCAS*, *BgbAS* and *BgLUS*). *KcAct1* and *BgAct1*were internal control to normalize the PCR efficiency as described previously (Larionov et al., 2005). Amplification specificity was checked by melting curve analysis.

Similarity and phylogenetic analysis of amino acid sequence of plant actin genes

The amino acid sequences were aligned and similarity scores were obtained using the FASTA ver. 3.4t26 (Pearson and Lipman, 1988) of the DNA Data Bank of Japan (Mishima, Shizuoka, Japan). The best score of results are shown in Table 5.2. Phylogenetic analysis of deduced amino acid alignment from plant actin genes was conducted with CLUSTAL W ver. 1.83 (Thomson et al., 1994) of the DNA Data Bank of Japan (Mishima, Shizuoka, Japan) followed by drawing with TreeView, ver. 1.6.6 (Page, 1996) based on a neighbor-joining method. Bootstrap analysis with 1000 replications was used to assess the strength of the nodes in the tree (Felsenstein, 1985). The DDBJ/GenBank/EMBL accession numbers of the sequence of used this analysis are as follows: AF061018 (*Scherffelia dubia*), AF061019 (*Coleochaete scutata*), AF061020 (*Mesostigma viride*), X16280 (*Oryza sativa RAc1*), U76191(*Pisum sativum PEAc9*), AF386514 (*Pyrus communis*), AB032361 (*Mimosa pudica* actin isoform B), AF288226 (*Setaria italica*), AY742219 (*Saccharum officinarum Ssactin*), (*Bruguiera gymnorrhiza BgAct1*), (*Kandelia candel KcAct1*), BT003847 (*Arabidopsis thaliana Act-12*), AY360221 (*Ricinus communis RcACT*), AF112538 (*Malva pusilla Act1*), EF418792 (*Populus trichocarpa*), AY825362 (*Chorispora bungeana CbACTIN2*), AY653160 (*Eucommia ulmoides ACT1*), AF172094 (*Picea rubens*), DQ252512 (*Solanum tuberosum*).

Analysis of isoprenoids

The leaves (2-3 leaves) or roots (3 to 5 g in wet weight) of *K. candel* and *B. gymnorrhiza* (Fig. 5.1) were powdered in liquid N_2, added with cholesterol as internal standard and extracted with 25 times volume of chloroform-methanol (2:1 by volume) (CM21). The method of isoprenoid analysis has been the same throughout this study as described in the foregoing chapter.

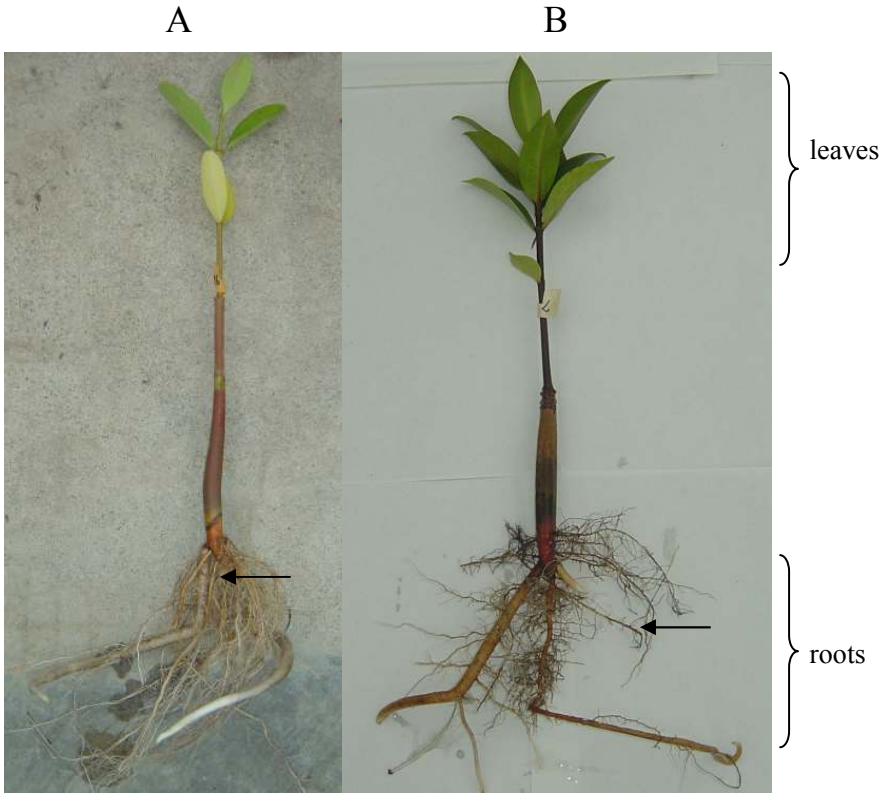

Fig. 5.1. Established seedlings by *K. candel* (A) and *B. gymnorrhiza* (B) showing the classification of plant parts used (roots are indicated by arrow) in the present study.

The absolute content (concentration) of isoprenoids was calculated from the ratio of the peak area of the respective compound to that of the internal standard cholesterol.

Identification of isoprenoids

The chemical structures of isoprenoids were mainly identified by comparison of their retention time on GC-FID column with those of authentic standards. Columns used and temperature

programs has been described in the foregoing chapter.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (MRT). The value of $P < 0.05$ was selected as a limit of statistical significance. All statistical analyses were performed using the SAS 9.1 statistical software program (SAS Institute Inc. Cary, NC, USA)

Results and Discussion

Cloning of actin gene fragment from K. candel and B. gymnorrhiza

The actin gene of *K. candel* (*KcAct1*) *and B. gymnorrhiza* (*BgAct1*) for internal standard were amplified based on the highly conserved regions of known plant actin genes, as described previously (Jin et al., 1999). The core sequence of *KcAct1* and *BgAct1* (405 and 401 bp in length, respectively) were cloned into a TOPO 10 vector (Invitrogen). Four clones for *KcAct1* and two clones for *BgAct1* were extended to 3'-end by 3'-RACE method as described previously (Frohman et al., 1988).

The partial sequence of *KcAct1* and *BgAct1* were 1083 and 944 bp, respectively. These DNA sequences encoded 277 amino acids residues for both cases. *KcAct1* and *BgAct1* shared 97.6% identities in their amino acid sequence, and 88.6% in their DNA sequence.

The deduced amino acid sequence of *KcAct1* and *BgAct1* showed significant similarity to known plant actin genes (Table 5.2). *KcAct1* showed high similarity (93.9-96.0%) to the actin gene from a variety of plant species. The similarities of *BgAct1* with plant actin genes were also high (95-96.6%). These results showed that both *KcAct1* and *BgAct1* encoded actin gene, and can be reasonably used as internal standard for real-time PCR.

To observe the relationship of *KcAct1* and *BgAct1* to other plant actin genes, unrooted phylogenetic analysis was constructed. Phylogenetic tree shows that *KcAct1* and *BgAct1* join with the *Oryza sativa* RAc1 cluster (Fig. 5.2). The present study indicates that *KcAct1* and *BgAct1* form a new branch in cluster, suggesting that both genes are new members of plant actin genes.

Table 5.2. The similarity of amino acid sequences between *KcAct1* and *BgAct1* (377 bp) with plant actin genes. The percent similiarties were obtained using PASTA ver. 4.t26 (Pearson and Lipman, 1988)

Plant gene	DDBJ/EMBL/GenBank accession No.	*KcAct1 (%)*	*BgAct1* (%)
Solanum tuberosum Actin-like mRNA	DQ252512	96.0	96.6
Eucommia ulmoides Actin1	AY653160	95.3	96.3
Arabidopsis thaliana Actin-12	P53497	95.0	95.3
Pisum sativum actin PEAc9	U76190	94.6	95.0
Mimosa pudica Actin	AB032361	94.3	95.3
Chorispora bungeana Actin2	AY825362	94.3	95.6
Picea rubens Actin	AF172094	94.3	96.0
Gossypium hirsutum Actin11	AY305732	93.9	95.6
Stevia rebaudiana Actin	AF548026	93.9	95.6
Malva pusilla Act1	AF112538	93.9	95.6
Populus trichocarpa ACT	EF418792	93.9	95.6
Ricinus communis RcACT	AY360221	93.9	95.6
Saccharum officinarum Scactin	AY742219	93.9	95.0

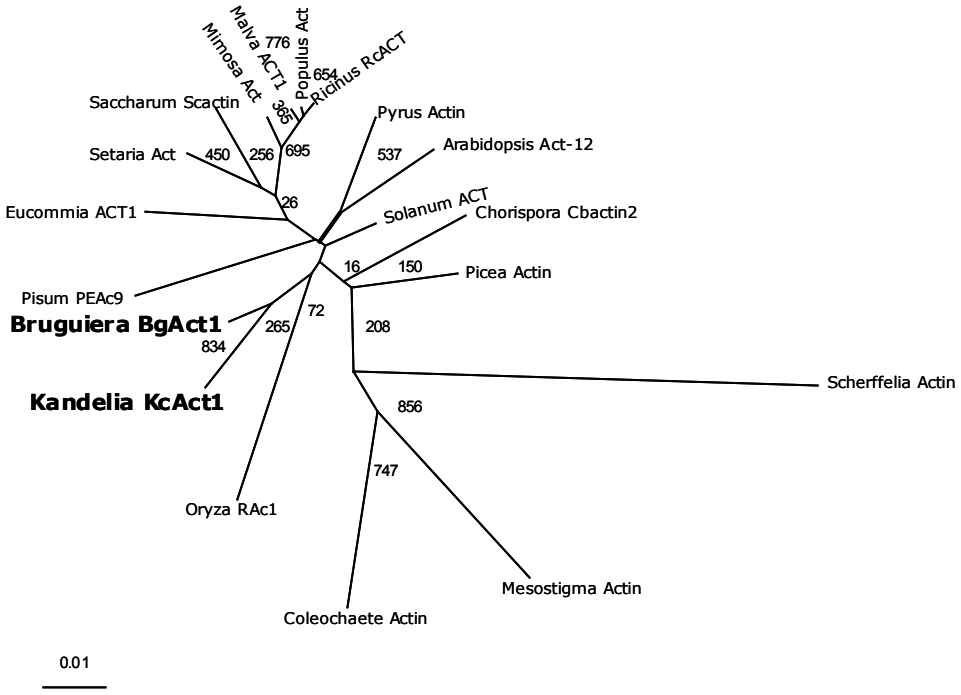

Fig. 5.2. Phylogenetic tree of *KcAct1* and *BgAct1* in the plant actin genes.

mRNA expression of four OSCs gene in roots and leaves of K. candel and B. gymnorrhiza

Understanding a halophyte plant's responses to salt stress mechanism will require a comprehensive observation of stress-induced changes in gene expression. The responses of gene expression in salt-tolerant halophyte mangroves may lead to clarification of the mechanism that allows these plants to cope with salt stress. Real time reverse transcription PCR (RT-PCR) was conducted to determine the expression level of OSCs under salt stressed conditions. Gene expressions studied were *KcMS* multifunctional triterpene synthase and *KcCAS* cycloartenol synthase from *K. candel* and *BgbAS* β-amyrin synthase and *BgLUS* lupeol synthase from *B. gymnorrhiza* mRNAs.

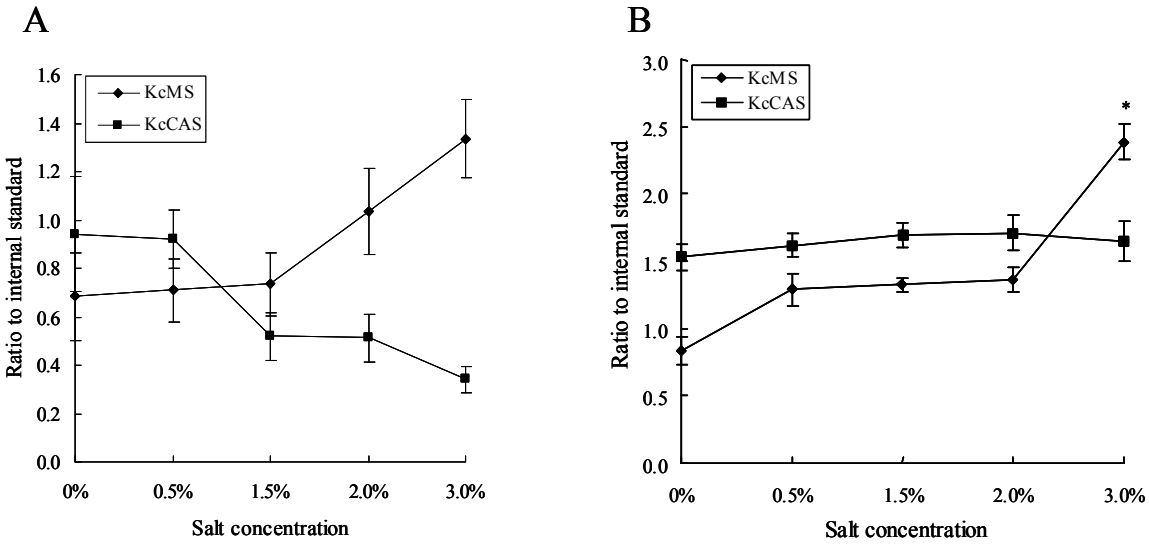

Fig. 5.3. Effect of salt stress on mRNA levels of *KcMS* multifunctional triterpene synthase (♦) and *KcCAS* cyloartenol synthase (■) in the leaves (A) and roots (B) of *K. candel*. *KcAct1* from *K. candel* was used an internal control to normalize the PCR. The values are the means of four independent measurements (n=5 ± SE). The asterisk indicates a statistically significant difference from 0% at $P < 0.05$ using Duncan's multiple range test.

It is important to note that OSCs in the isoprenoid pathway are the branching point to flux the biosynthesis flow toward either phytosterols or variety of triterpenoids. The relative level of *KcMS* mRNA was found to increase with salt stress in roots and leaves of *K. candel*. However,

statistically significant difference was found only in 3% (2.39) treatment of root compared to control (0.83) (Fig. 5.3). These results demonstrated that expression of *KcMS* gene was increased by salt stress.

In contrast to *KcMS* mRNA, the level of *KcCAS* mRNA was largely constant with increasing salt concentration in the roots, and decreased in the leaves. It has been reported that salt stress had no effect on the mRNA level of *GgCAS* cyloartenol synthase in *Glycyrrhiza glabra* relatively constant as well as squalene synathse (Hayashi et al., 2003).

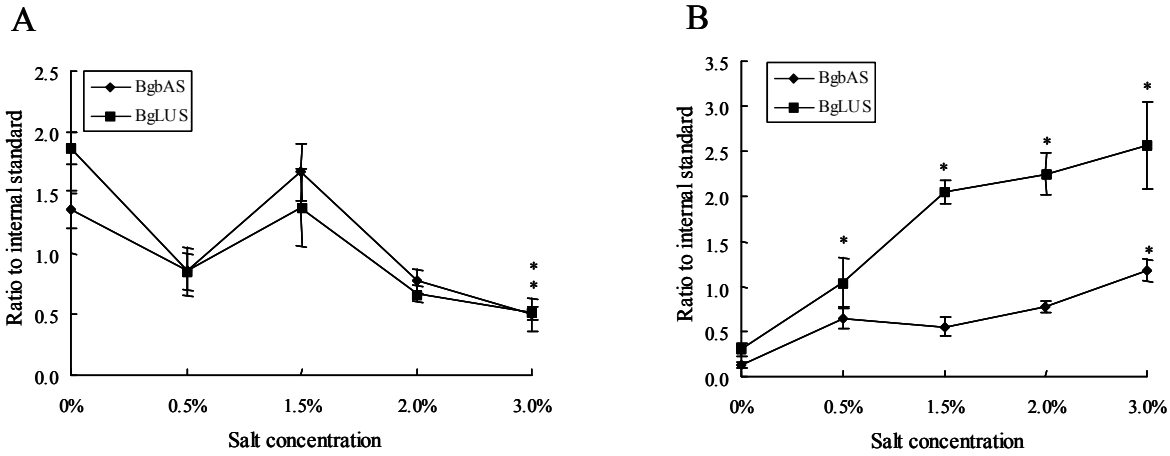

Fig. 5.4. Effect of salt stress on mRNA levels of two triterpene syntheses of *BgbAS* β-amyrin synthase (♦) and *BgLUS* lupeol synthase (■) in the leaves (A) and roots (B) of *B. gymnorrhiza*. *BgAct1* from the same plant was used an internal control to normalize the PCR. The values are the means of four independent measurements (n= 6 ± SE). The asterisk indicates a statistically significant difference from 0% at $P < 0.05$ using Duncan's multiple range test. .

The mRNA level of *BgLUS* was higher than *BgbAS* in both leaves and roots of *B. gymnorrhiza* in the present study. All salt treatment significantly increased the mRNA level of *BgLUS*. In case of *BgbAS*, statistically significant difference was only noted for 3% treatment (Fig. 5.4). In contrast to this observation, salt stress significantly decreased the expression of both genes in the leaves of *B. gymnorrhiza* (Fig. 5.4).

Our observation on mRNA expression of OSC genes are in well agreement with other studies. A number of triterpene synthase genes have been reported to be differentially expressed in a variety of plant tissues. A putative β-amyrin synthase *CabAS*, associated with asiaticoside in *Centella asiatica* was found to decrease in the time course of mRNA levels (Kim et al., 2005). The β-amyrin synthase *AsbAS1*, involved in the in the biosynthesis of anti-fungal triterpene saponins in *Avena strigosa*, is strongly expressed in roots, being consistent with the localization of avenacin A-1 in this organ (Haralampidis et al., 2001). In *Ricinus communis*, high expression levels of *RcLUS* lupeol synthase were observed only in glaucous stems, whereas *RcCAS* cyloratenol synthase was expressed in leaves, roots, and stems (Guhling et al., 2006). A putative lupeol synthase of *Medicago truncatula* was reported to be expressed in roots, leaves and nodules, but not in stem tissues. The transcripts of the multifunctional triterpene synthase *LjAMY2* from *Lotus japonicus* was detected only in roots, whereas expression of the β-amyrin synthase *PSY* (*Pisum sativum*) was only localized in the stem and root tissues (Iturbe-Ormatxe et al., 2003).

Our previous study suggested that terpenoid plays a role in the protection of mangrove from salt stress (Oku et al., 2003). Furthermore, triterpenoids existed in greater proportion in the outer parts of the root, and this may be the additional evidence for the protective roles of triterpenoids in mangrove (Basyuni et al., 2007b). In addition to these findings, a number of studies suggested a protective role of terpenoid to external stresses, such as the exodermis of barley roots against pathogen fungi (Reissinger et al., 2003). The size of maize primary roots was increased against salt stress (Karahara et al., 2004). The endodermis of alkali grass was shown to be an effective barrier to Na^+ (Peng et al., 2004).

Since root is the primary tissue to be subjected the salinity of the soils, the mechanisms for salt tolerance may be more clearly pronounced in the roots. It has been reported that the part of root, the endodermis, and to some extent the exodermis, represent apoplastic barriers that control the uptake and radial transport of water and solutes by the root. Furthermore, these layers have several additional functions such as mechanical protection and defenses against external factors (Lux et al., 2004). Roots have remarkable ability to control their own Na^+ and Cl^- concentrations. Genes for salt tolerance expected to function in roots would be involved in the control of ion uptake and

transport, and the regulation of ion homeostasis and water status (Munns, 2005).

Effect of salt stress on the isoprenoids composition

The present result has extended our previous data on the lipid composition and its relevance to salt tolerance, and describes the isoprenoids composition of two mangrove leaves and roots under different salt concentration. The isoprenoids content identified in the mangrove leaves and roots from *K. candel* and *B. gymnorrhiza* are shown in Tables 5.3 to 5.10. The terpenoids largely fall into three types of carbon skeleton: lupane (lupeol, lupenone); oleanane (β–amyrin, taraxerol); ursane (α-amyrin). The triterpenoids and phytosterol each mainly consisted of 5 compounds. The phytosterol components in the mangroves leaves and roots are campesterol, stigmasterol, β-sitosterol, lanosterol and cyloartenol.

Table 5.3. Effect of salt stress on the isoprenoids concentration of *K. candel* leaf

Isoprenoids	Concentration (μg/g)				
	0% (*n*=4)	0.5% (*n*=4)	1.5%(*n*=4)	2.0% (*n*=5)	3.0% (*n*=3)
Campesterol	19.8 ± 1.1	13.4 ± 1.2[a]	14.0 ± 0.1[a]	14.1 ± 1.6[a]	13.0 ± 0.4[a]
Stigmasterol	37.5 ± 3.3	31.8 ± 3.7	25.8 ± 1.4[a]	36.5 ± 3.1	23.6 ± 1.0[a]
β-sitosterol	112.4 ± 3.3	88.9 ± 4.4[a]	85.1 ± 2.7[a]	87.2 ± 1.8[a]	82.0 ± 3.4[a]
Lanosterol	13.3 ± 1.3	12.0 ± 1.6	10.3 ± 1.0	13.8 ± 1.7	9.8 ± 0.9
Taraxerol	5.1 ± 1.3	6.5 ± 0.6	6.5 ± 0.1	5.6 ± 0.8	5.5 ± 0.4
β-Amyrin	293.0 ± 1.0	317.1 ± 2.5[a]	329.8 ± 3.7[a]	351.4 ± 3.1[a]	356.7 ± 1.7[a]
Cycloartenol	21.0 ± 1.6	16.5 ± 2.3	21.0 ± 1.0	29.0 ± 0.4[a]	18.9 ± 1.5
Lupeol	161.8 ± 0.9	175.4 ± 3.4[a]	184.8 ± 3.2[a]	194.9 ± 3.6[a]	207.6 ± 1.2[a]
α-Amyrin	102.9 ± 0.6	104.5 ± 1.9	105.9 ± 1.0	109.8 ± 1.2[a]	116.1 ± 1.1[a]

Isoprenoids in the lipid extracts were analysed by GC-FID. Data are expressed as the mean of duplicate analyses ± SE
[a]Significantly different from 0% at P <0.05 using Duncan's multiple range test

Table 5.4. Effect of salt stress on the isoprenoids proportion of *K. candel* leaf

Isoprenoids	Proportion (%)				
	0% (*n*=4)	0.5% (*n*=4)	1.5%(*n*=4)	2.0% (*n*=5)	3.0% (*n*=3)
Campesterol	2.6 ± 0.0	1.8 ± 0.1[a]	1.8 ± 0.0[a]	1.7 ± 0.2[a]	1.6 ± 1.1[a]
Stigmasterol	5.7 ± 0.3	4.1 ± 0.4	3.7 ± 0.2[a]	4.4 ± 0.4	2.8 ± 1.2[a]
β-sitosterol	16.3 ± 0.7	11.7 ± 0.5[a]	11.3 ± 0.3[a]	10.4 ± 0.3[a]	9.8 ± 0.7[a]
Lanosterol	1.7 ± 0.1	1.5 ± 0.2	1.3 ± 0.1	1.7 ± 0.2	1.2 ± 0.2
Taraxerol	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.0	0.7 ± 0.1	0.7 ± 0.0
β-Amyrin	40.2 ± 0.3	41.4 ± 0.4[a]	42.1 ± 0.3[a]	42.1 ± 0.2[a]	42.8 ± 0.3[a]
Cycloartenol	2.2 ± 0.4	2.2 ± 0.3	2.7 ± 0.1	3.5 ± 0.1[a]	2.3 ± 0.5
Lupeol	17.0 ± 0.4	22.9 ± 0.5[a]	23.1 ± 0.4[a]	23.3 ± 0.5[a]	24.9 ± 0.2[a]
α-Amyrin	13.4 ± 0.2	13.6 ± 0.4	13.2 ± 0.2	13.3 ± 0.2	13.9 ± 0.5

Isoprenoids in the lipid extracts were analysed by GC-FID. Data are expressed as the mean of duplicate analyses ± SE
[a]Significantly different from 0% at P <0.05 using Duncan's multiple range test

Table 5.5. Effect of salt stress on the isoprenoids concentration of *K. candel* root

Isoprenoids	Concentration (µg/g)				
	0% (n=4)	0.5% (n=4)	1.5%(n=4)	2.0% (n=5)	3.0% (n=5)
Campesterol	37.7 ± 1.8	54.2 ± 3.4[a]	40.6 ± 1.6	43.3 ± 2.9	31.4 ± 1.5
Stigmasterol	103.1 ± 2.8	104.2 ± 4.5	113.9 ± 3.6	109.6 ± 5.0	79.1 ± 5.5[a]
β-sitosterol	42.2 ± 3.3	62.4 ± 4.5[a]	55.7 ± 3.3[a]	53.3 ± 3.7[a]	58.4 ± 1.4[a]
Lanosterol	7.4 ± 1.8	7.0 ± 2.0	11.4 ± 1.6	6.8 ± 1.4	3.4 ± 0.2
Taraxerol	5.8 ± 1.0	8.7 ± 2.2	12.7 ± 0.9[a]	15.2 ± 2.0[a]	7.2 ± 0.9
β-Amyrin	6.6 ± 0.5	11.1 ± 0.9	11.3 ± 0.9	12.8 ± 1.9[a]	14.1 ± 2.3[a]
Lupenone	2.4 ± 0.5	3.2 ± 0.6	3.8 ± 0.8	4.5 ± 0.9	3.0 ± 0.3
Cycloartenol	3.9 ± 0.7	5.5 ± 1.0	7.1 ± 0.9[a]	7.2 ± 0.8[a]	3.4 ± 0.4
Lupeol	4.9 ± 0.8	7.7 ± 0.9	9.8 ± 0.5[a]	10.7 ± 0.9[a]	19.5 ± 2.6[a]
α-Amyrin	2.9 ± 0.7	3.1 ± 0.2	3.8 ± 0.2	5.1 ± 0.9[a]	5.8 ± 0.7[a]

Isoprenoids in the lipid extracts were analysed by GC-FID. Data are expressed as the mean of duplicate analyses ± SE
[a]Significantly different from 0% at P <0.05 using Duncan's multiple range test

Table 5.6. Effect of salt stress on the isoprenoids proportion of *K. candel* root

Isoprenoids	Proportion (%)				
	0% (n=4)	0.5% (n=4)	1.5%(n=4)	2.0% (n=5)	3.0% (n=5)
Campesterol	16.9 ± 0.5	20.2 ± 0.5[a]	15.0 ± 0.3[a]	16.7 ± 0.6	14.8 ± 0.6[a]
Stigmasterol	46.2 ± 0.3	39.2 ± 1.1[a]	42.3 ± 1.0[a]	41.5 ± 1.3[a]	37.0 ± 1.6[a]
β-sitosterol	19.0 ± 2.0	23.4 ± 0.5[a]	20.7 ± 0.5	18.1 ± 1.7	27.5 ± 1.0[a]
Lanosterol	3.3 ± 0.8	2.5 ± 0.7	4.2 ± 0.5	3.4 ± 1.3	1.6 ± 0.1
Taraxerol	2.6 ± 0.4	3.2 ± 0.7	4.7 ± 0.1[a]	5.5 ± 0.5[a]	3.4 ± 0.3
β-Amyrin	2.9 ± 0.2	4.1 ± 0.1	4.2 ± 0.2	4.5 ± 0.6	6.5 ± 1.6[a]
Lupenone	1.1 ± 0.2	1.2 ± 0.2	1.4 ± 0.3	2.0 ± 0.7	1.5 ± 0.2
Cycloartenol	1.7 ± 0.3	2.0 ± 0.3	2.6 ± 0.2	3.0 ± 0.5[a]	1.6 ± 0.2
Lupeol	2.2 ± 0.3	2.9 ± 0.4	3.7 ± 0.3	3.8 ± 0.2	9.4 ± 1.5[a]
α-Amyrin	1.3 ± 0.3	1.3 ± 0.1	1.4 ± 0.0	1.8 ± 0.3	2.8 ± 0.4[a]

Isoprenoids in the lipid extracts were analysed by GC-FID. Data are expressed as the mean of duplicate analyses ± SE
[a]Significantly different from 0% at P <0.05 using Duncan's multiple range test

Tables 5.3 to 5.6 summarize isoprenoid composition from leaves and roots of *K. candel*. As shown in the tables, the concentration and relative proportion (percentage) of terpenoids (β-amyrin, lupeol and α-amyrin) in the leaves and roots significantly increased with salinity.

A similar experiment was carried out with propagules of *B. gymnorrhiza*. Tables 5.7 to 5.10 show the isoprenoids composition of leaves and roots maintained under the same conditions as described for *K. candel*. The medium salt stress to high salt stress (1.5 - 3%) caused significant increase in the concentration and proportional of terpenoids (β-amyrin and lupeol) in the roots. However the prominent terpenoids in the leaves decreased significantly with salinity. Similarly, a salt-dependent change was also noted to rather lesser extent. The concentration and proportion of β-amyrin and lupeol increased in all salt-stressed groups compared with the control (0%).

Membrane lipids are known to play important roles of the cellular ability to cope with saline

conditions and also have vital functions in the tolerance to several physiological stressors (Brown and Dupont, 1989; Sudha and Ravishankar, 2002; Shepherd and Griffiths, 2006). The concentration and relative of proportion triterpenoids (β- and α- amyrin and lupeol) increased with salinity in the seedling of *K. candel* (roots and leaves) and *B. gymnorrhiza* (roots), suggesting that terpenoids may play an important role in the adaptation of mangroves to salt stress. Furthermore, salt stress modulated the terpenoid concentration not only the root, but also in the leaf. Several studies have demonstrated that salt stress induced the specific genes in the isoprenoid biosynthesis, such as the *gcpE* gene in barley root (Campos et al., 2001), *lmo1451* of *Listeria monocytogenes* (Begley et al., 2002), suggesting the involment of terpenoids in the salt-stress reponse (Ueda et al., 2002).

The predominant free sterol of both species was campesterol, stigmasterol and β-sitosterol. Significant differences were found between control and salt-treated groups. All free sterols appeared to decrease with an increasing salinity in the leaves. The decrease in sterols (including free sterols) and phospolipid percentages with increasing salinity has been reported in root plasma membrane of salt marsh grass (*Spartina patens*) (Wu et al., 1998).

Table 5.7. Effect of salt stress on the isoprenoids concentration of leaf of *B . gymnorrhiza*

Isoprenoids	Concentration (μg/g)				
	0% (*n* =4)	0.5% (*n* =4)	1.5%(*n* =4)	2.0% (*n* =4)	3.0% (*n* =4)
Campesterol	20.8 ± 0.9	20.4 ± 0.8	27.0 ± 3.3[a]	33.9 ± 2.2[a]	29.7 ± 1.2[a]
Stigmasterol	38.1 ± 1.4	36.2 ± 3.5	31.9 ± 2.6	44.4 ± 1.2	43.7 ± 1.5
β-sitosterol	105.7 ± 1.1	87.2 ± 1.9[a]	96.2 ± 3.5[a]	143.8 ± 4.1[a]	124.3 ± 3.2[a]
Lanosterol	39.7 ± 2.2	41.3 ± 3.2	31.8 ± 0.8	49.0 ± 4.4[a]	52.3 ± 1.5[a]
β-Amyrin	396.6 ± 2.0	300.6 ± 4.1[a]	246.4 ± 4.2[a]	295.4 ± 2.7[a]	193.4 ± 3.4[a]
Lupenone	148.4 ± 2.4	140.7 ± 4.2	227.2 ± 2.5[a]	341.5 ± 2.7[a]	280.7 ± 4.2[a]
Lupeol	1562.3 ± 1.8	1124.8 ± 4.1[a]	865.5 ± 4.1[a]	1409.5 ± 2.1[a]	1157.9 ± 1.9[a]

Isoprenoids in the lipid extracts were analysed by GC-FID. Data are expressed as the mean of duplicate analyses ± SE
[a]Significantly different from 0% at P <0.05 using Duncan's multiple range test

Table 5.8. Effect of salt stress on the isoprenoids proportion of leaf of *B . gymnorrhiza*

Isoprenoids	Proporsional (%)				
	0% (*n* =4)	0.5% (*n* =4)	1.5%(*n* =4)	2.0% (*n* =4)	3.0% (*n* =4)
Campesterol	0.9 ± 0.0	1.2 ± 0.0	1.8 ± 0.2[a]	1.5 ± 0.1[a]	1.6 ± 0.1[a]
Stigmasterol	1.7 ± 0.1	2.1 ± 0.2	2.1 ± 0.2	1.9 ± 0.0	2.3 ± 0.1[a]
β-sitosterol	4.6 ± 0.1	5.0 ± 0.1	6.3 ± 0.[a]	6.2 ± 0.2[a]	6.6 ± 0.2[a]
Lanosterol	1.7 ± 0.1	2.4 ± 0.2[a]	2.1 ± 0.0[a]	2.1 ± 0.2[a]	2.8 ± 0.1[a]
β-Amyrin	17.2 ± 0.1	17.2 ± 0.2	16.1 ± 0.3[a]	12.7 ± 0.1[a]	10.3 ± 0.2[a]
Lupenone	6.4 ± 0.1	8.0 ± 0.2[a]	14.9 ± 0.2[a]	14.7 ± 0.1[a]	14.9 ± 0.2[a]
Lupeol	67.6 ± 0.2	64.2 ± 0.4[a]	56.7 ± 0.2[a]	60.8 ± 0.2[a]	61.5 ± 0.2[a]

Isoprenoids in the lipid extracts were analysed by GC-FID. Data are expressed as the mean of duplicate analyses ± SE
[a]Significantly different from 0% at P <0.05 using Duncan's multiple range test

Table 5.9. Effect of salt stress on the isoprenoids concentration of root of *B . gymnorrhiza*

Isoprenoids	Concentration (μg/g)				
	0% (n=5)	0.5% (n=5)	1.5%(n=5)	2.0% (n=4)	3.0% (n=5)
Campesterol	39.8 ± 0.6	38.8 ± 1.5	32.7 ± 1.5[a]	32.5 ± 0.7[a]	37.3 ± 2.4
Stigmasterol	106.0 ± 1.8	113.8 ± 3.4	82.7 ± 2.8[a]	89.0 ± 1.8[a]	100.9 ± 2.1
β-sitosterol	34.1 ± 1.0	32.3 ± 1.7	30.6 ± 1.3	31.4 ± 1.7	44.0 ± 3.1[a]
Lanosterol	7.8 ± 0.8	5.3 ± 0.5	9.8 ± 1.4	7.1 ± 0.7	8.2 ± 0.8
β-Amyrin	6.6 ± 0.8	7.2 ± 0.9	7.8 ± 0.3	10.0 ± 0.5[a]	12.8 ± 0.7[a]
Lupeol	2.8 ± 0.3	3.6 ± 0.3	5.0 ± 0.8[a]	4.8 ± 0.4[a]	5.7 ± 0.4[a]

Isoprenoids in the lipid extracts were analysed by GC-FID. Data are expressed as the mean of duplicate analyses ± SE
[a]Significantly different from 0% at $P < 0.05$ using Duncan's multiple range test

Table 5.10. Effect of salt stress on the isoprenoids proportion of root of *B . gymnorrhiza*

Isoprenoids	Proportion (%)				
	0% (n=5)	0.5% (n=5)	1.5%(n=5)	2.0% (n=4)	3.0% (n=5)
Campesterol	20.6 ± 0.7	19.4 ± 0.4	19.4 ± 0.4	18.6 ± 0.6[a]	17.6 ± 0.7[a]
Stigmasterol	52.8 ± 0.3	56.1 ± 1.0[a]	49.1 ± 1.3[a]	50.6 ± 1.1	47.7 ± 1.1[a]
β-sitosterol	17.7 ± 0.2	16.2 ± 0.7	18.1 ± 0.2	17.9 ± 0.7	20.7 ± 1.1[a]
Lanosterol	4.0 ± 0.4	2.6 ± 0.3	5.8 ± 0.8[a]	4.1 ± 0.4	3.9 ± 0.4
β-Amyrin	3.4 ± 0.4	3.6 ± 0.5	4.6 ± 0.0[a]	5.6 ± 0.4[a]	6.0 ± 0.3[a]
Lupeol	1.1 ± 0.1	1.8 ± 0.2	3.0 ± 0.5[a]	3.2 ± 0.5[a]	2.7 ± 0.2[a]

Isoprenoids in the lipid extracts were analysed by GC-FID. Data are expressed as the mean of duplicate analyses ± SE
[a]Significantly different from 0% at P <0.05 using Duncan's multiple range test

The change in plant sterol composition (namely, the increase in campesterol and decrease in sitosterol) observed in our study does not provide evidence to support the notion that "molar planar" plant sterols (e.g. campesterol) is required to stabilize membranes and reduce membrane permeability, as suggested by Grunwald (1974), Douglas (1985), Mansour et al. (1994), and Wu et al. (2005). However that theory appeared to be supported in the case of *B. gymnorrhiza*. Contrary to this proposal, more "less planar" free sterol (e.g. β-sitosterol) was increased with salt concentration in both species (Table 5.3 to 5.10). β-sitosterol has been reported to be the most efficient sterol in reducing water permeability among the all the sterols investigated, including stigmasterol, campesterol, cholesterol, and cyloartenol (Schuler et al., 1991). Our present study was in well agreement with previous reports that the increased β-sitosterol in the roots both species may play an important role in regulating water permeability (Wu et al., 1998; Mansour et al., 2002). The β-sitosterol probably plays a similar role in plant cell membrane to that of cholesterol in mammalian cells to regulate membrane fluidity and hence the permeability (Hartmann, 1998).

A number of studies have demonstrated that the sterol is a modulator of membrane

permeability (Sietsma, 1971), membrane enzyme activities (Sandstrom and Cleland, 1989), membrane ion absorption (Hodges et al., 1972; An et al., 2003), and membrane fluidity (Hasono, 1992; Hartmann, 1998). To gain more insight into structural and functional roles of terpenoids, we used simple and well-defined *in vitro* membrane models (liposomes) and our *in vitro* study showed that triterpene, as well as phytosterols, was incorporated into the lipid bilayer of liposome (Basyuni et al., 2007b), suggesting that terpenoid is also a modulator of membrane permeability. Triterpenoid alcohols have been structurally distinguished from phytosterols, even though they share common biosynthetic pathway and hence the similar chemical structure. However, there has been no scientific rationale to functionally distinguish these compounds as the structural membrane lipid. Furthermore, fortification of the cell membrane with sterols appeared to increase the tolerance of the organisms against external stresses. Changes in the membrane lipid composition and properties represent an important factor in the adaptation to high salt concentration (Russell et al., 1995). These observations strongly suggested that the terpenoids contributes to the salt tolerance of this plant probably by changing the property of the cell membrane.

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