Oxidosqualene Cyclases from Cell Suspension Cultures of *Betula platyphylla* var. *japonica*: Molecular Evolution of Oxidosqualene Cyclases in Higher Plants

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***Betula platyphylla* var. *japonica* is a rich source of triterpenoid as it contains dammarane type triterpenes in the leaves, and lupane type and oleanane type triterpenes in the bark. Four oxidosqualene cyclase cDNAs (*BPX*, *BPX2*, *BPW* and *BPY*) were cloned by homology based PCR methods from cell suspension cultures of *B. platyphylla* var. *japonica*. Open reading frames consisting of 2274, 2304, 2268 and 2340 bp were ligated into yeast expression plasmid pYES2 under the control of *GAL1* promoter and introduced into lanosterol synthase deficient (*erg7*) *Saccharomyces cerevisiae* strain GIL77. Analyses of *in vitro* enzyme activities and/or accumulated products in the transformants demonstrated that they encode cycloartenol synthase (*BPX* and *BPX2*), lupeol synthase (*BPW*) and β-amyrin synthase (*BPY*) proteins. Phylogenetic tree was constructed for all the known oxidosqualene cyclases (OSCs) including the clones obtained in this study, revealing that OSCs having the same enzyme function form respective branches in the tree even though they derive from different plant species. Intriguing correlation was found between reaction mechanism and molecular evolution of OSCs in higher plants.**

Key words cycloartenol synthase; lupeol synthase; β-amyrin synthase; *Betula platyphylla* var. *japonica*; molecular evolution

Triterpenoid is a large class of isoprenoidal natural products present in higher plants. More than eighty different skeletal types of triterpenes are known as natural products, which are elaborated at the cyclization step of a common substrate, 2,3-oxidosqualene (Fig. 1).[1] Following structural modifications on cyclization products, such as oxygenation, esterification and glycosylation, provide further structural diversity. The reactions of skeleton formation catalyzed by oxidosqualene cyclases (OSCs) are important and interesting not only from mechanistical viewpoint but also from biological and evolutional viewpoints as they include cycloartenol synthase of primary metabolism for phytosterol biosynthesis.[2] In order to obtain insights in the reaction mechanism of oxidosqualene cyclization, purification and cDNA cloning of triterpene synthases have been extensively carried out. Up to now, eleven triterpene synthase and five cycloartenol synthase cDNAs have been cloned from dicotyledonous plant.[3—7] They are β-amyrin synthases from *Panax ginseng* (*PNY*, *PNY2*),[5,8] *Pisum sativum* (*PSY*)[9] and *Glycyrrhiza glabra* (*GgbAS1*),[10] lupeol synthases from *Olea europaea* (*OEW*) and *Taraxacum officinale* (*TRW*),[11] isomultiflorenol synthase from *Luffa cylindrica.* (*LcIMS1*),[12] and multifunctional triterpene synthases from *Arabidopsis thaliana* (*LUP1*[13] and *YUP8H12R.43*[14]) and *P. sativum* (*PSM*).[9] Successful cloning of these cDNAs clearly proved that triterpene skeleton is produced by respective triterpene synthase of different product specificity and that triterpene synthases are distinct proteins from cycloartenol synthase. However, as none of plant species so far tested yielded more than two distinct product specific triterpene synthase clones, 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.[15] It is of great interest to know whether there exist corresponding numbers of product specific triterpene synthases in one plant species.

As we reported in our previous publication,[11] phylogenetic tree analysis indicated that triterpene synthase clones had diverged from ancestral cycloartenol synthase gene. In addition, *LUP1* lupeol synthase from *A. thaliana* with low product specificity did not join a lupeol synthase branch of *OEW*, *TRW* and *MtN18* from *Medicago truncatula*.[16] In order to establish the presence of two distinct lupeol synthase branches in the tree, search for *LUP1* type lupeol synthase clones from different plants should be pursued. Furthermore, to obtain more detailed insights for the molecular evolution of plant OSCs, triterpene synthase clones producing other skeletal types but lupane and oleanane are needed. Mechanistically, yet unknown synthase clone producing tetracyclic dammarane skeleton would be the most valuable, since tetracyclic dammarenyl cation must be on the way to pentacyclic triterpenes like lupeol and β-amyrin in triterpene synthase catalyzed reaction. Addition of water to this cationic intermediate would produce dammarenediol while further D-ring expansion and fifth ring closure are required for pentacyclic triterpene formation. Therefore, the location of dammarane producing clones in the tree would provide a key clue whether mechanistically more complex pentacyclic triterpene synthases evolved from tetracycle synthase clones by gaining additional enzyme functions or tetracycle synthase clones diverged just by loss of functions of the already existing pentacycle synthases.

In this context, we have attempted cDNA cloning of triterpene synthases from Japanese birch, *Betula platyphylla* var. *japonica*, by employing homology based PCR method that was successful in our previous studies.[5] The white bark of this plant is known to contain high amount of betulin having lupane skeleton,[17] while the leaves are rich source of dammarane triterpenes like betulafolienetriol although all the dammerenoids in this plant possess 3α-hydroxyl group. In the present study, cell suspension cultures were induced and served as the source of cDNAs, as no information is available

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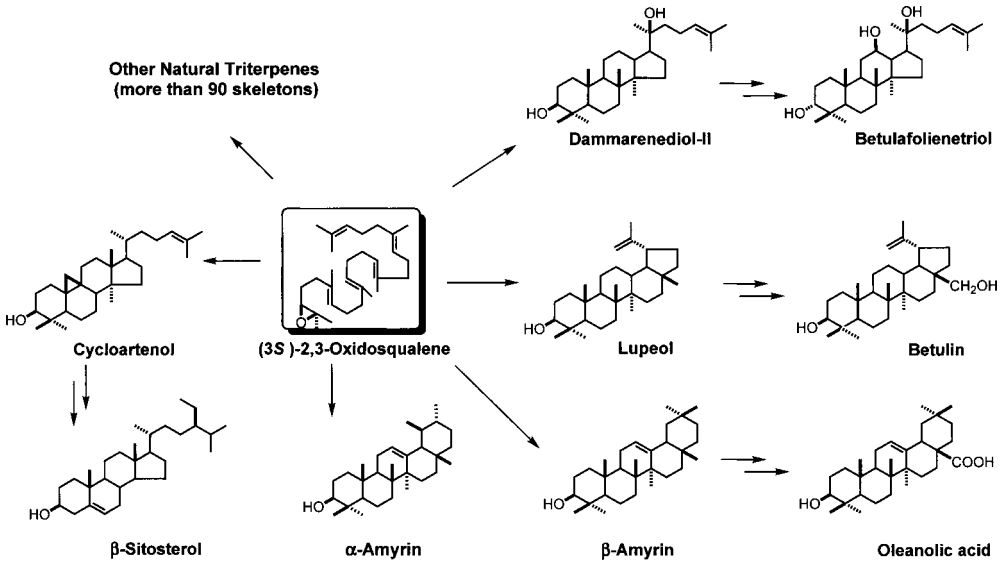

Fig. 1. Oxidosqualene Cyclization in Higher Plants and Triterpenes in *B. platyphylla* var. *japonica*

for the timing and the site/organ of triterpene biosynthesis in the intact plant.

MATERIALS AND METHODS

Plant Cell Culture Callus was induced from the leaf stem of *B. platyphylla* var. *japonica*. It was sub-cultured every month on MS medium[18] supplemented with 1% agar, 3% sucrose, 1 μM naphthaleneacetic acid (NAA) and 1 μM *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea under 16 h light per day at 25 °C. The cell suspension cultures were maintained on the same medium without agar under dark.

PCR and Sequence Analysis PCR was performed by Robo Cycler™ Gradient 40 (Stratagene). The PCR reaction products were separated by agarose gel electrophoresis, purified by a Wizard PCR Preps Kit (Promega), and ligated into pT7Blue (Novagen). Resulting plasmid was propagated in *E. coli* NovaBlue (Novagen), and isolated by GFX™ Micro Plasmid Prep Kit (Amersham Pharmacia Biotech). Sequencing was carried out by DNA Sequencer Model 4000 (Li-Cor) using Thermo Sequenase Cycle Sequencing Kit (Aloka).

Preparation of mRNA and cDNA Mixture Total RNA was prepared from 30 d suspension cultures of *B. platyphylla* var. *japonica* by phenol-SDS method, and used as a template to synthesize the cDNA mixtures using Superscript II (BRL) and an oligo dT primer RACE32 (5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTT-3′) following the manufacturer's protocol. The resulting 20 μl cDNA solution was diluted to 100 μl with TE buffer and used as a template for the following PCRs.

Cloning of OSCs Based on the highly conserved regions of the known OSC enzymes, four primers, 161S (5′-GAYGGIGGITGGGGIYTICA-3′), 463S (5′-MGICAYATHWSIAARGGIGCITGG-3′), 603A (5-CCCAISWICCITMCCAISWICCRTC-3′) and 711A (5′-CKRTAYTCICCIARIGCCCADATIGGRAA-3′) were synthesized. PCR mixture (total volume 100 μl) consisting of cDNA mixture (3 μl), primer (162S and 711A, 1 μg each), dNTP (0.2 mM each) and buffer (supplied by manufacturer), was kept at 94 °C for 5 min, then 1 unit of Ex. Taq DNA polymerase (Takara Shuzo) was added. PCR was carried out with the program; denaturing at 94 °C for 1 min, annealing at 42 °C for 2 min, and extension at 72 °C for 3 min (total of 30 cycles), following by a 10 min extension at 72 °C. PCR product (1800 bp) was subcloned to the plasmid vector. Ten colonies were picked up, and nucleotide sequence was determined. Eight of ten clones were identical to each other, and named as *BPW*. The other two clones were independent clones, and their full length clones were named as *BPX* and *BPX2*, respectively.

The second PCR was carried out with 463S and 556A primers (1 μg each) using the first PCR product as a template. PCR conditions were the same as above. PCR product (450 bp) was subcloned to the plasmid vector. Sixteen colonies were picked up and sequenced. They included partial clones of *BPW* (12 clones), *BPX2* (3 clones) and one new clone. The full length clone of this new clone was designated as *BPY*.

According to the sequence of these four clones, specific primers for each clone were designed to obtain the 3′ and 5′ end sequences. RACE PCRs were carried out following the method described in our previous paper with some modifications.[5] Used primers were as follows. For 3′-RACE; BPX-1S (5′-GCAACATATGAGCTCACAGGATCCTATCCC-3′), BPX-2S (5′-ATCGGGAAGAAGAAGTAGGCGTCTGTATCA-3′), BPX2-1S (5′-5′-GGGAAATCGGTAGATGTGA-3′), BPX2-2S (5′-GTTCTAGCATCCACAAGGCATG-3′), BPW-1S (5′-TTCCCAGCATGGGAGCCTCAAA-3′), BPW-2S (5′-ATATAGAAGGCAACCGTGCAAA-3′), BPY-1S (5′-TCTTTGCGGACATTGTCA-3′) and BPY-2S (5′-AATGCTGCTCAGTTCC-3′). For 5′-RACE; BPX-5RT (5′-GGATAGGATCCTGTGAGCTCATATGTTGCG-3′), BPX-1A (5′-CCTCAAAGACATGACTGTTGG-3′), BPX-2A (5′-AATTGCTCCTTGCCCATCAATAGC-3′), BPX2-5RT (5′-ACCGATTTCCCAACTGTTTCTG-3′), BPX2-1A (5′-TATGCTGCATCACAGTGTCCA-3′), BPX2-2A (5′-ACAGCTCCCATTCCATCCTCAG-3′), BPW-5RT (5′-CAACGCTTCCAATGCAAAGGTA-3′), BPW-1A (5′-TCCTCCTTTGCAACTGTAC-3′), BPW-2A (5′-TAGCCCCATCTTCACCATCTTCA-3′), BPY-5RT (5′-GAAGGAACTGAGCAGCA-3′), BPY-1A (5′-GTCTCCAGAAGGGTTGTCCTTGA-3′) and BPY-2A

(5′-CACTTTCGCGCTCTTGCACAAG-3′).

Cloning of Full-Length cDNAs To obtain the full-length clones, two specific primers for each clone with specific restriction enzyme sites at each ends were designed. Used primers were as follows; BPX-KpnI-N (5′-ATAGGGTACCATGTGGAAGCTGAAGATCG-3′), BPX-XhoI-C (5′-CATTCTCGAGTTAGGGAGCCTGCAATACC-3′), BPX2-KpnI-N (5′-AACGGTACCATGTGGAAGTTGAAGATCG-3′), BPX2-EcoRI-C (5′-AAGGAGAATTCTCAGAGGGCCTTC-3′), BPW-KpnI-N (5′-GATGGTACCATGTGGAAGTTGA-3′), BPW-BamHI-C (5′-CAGGTAGGATCCTCATGCAAATAGC-3′), BPY-KpnI-N (5′-ACTAGGGTACCATGTGGAGGCTTAAGA-3′), and BPY-XhoI-C (5′-GTACTCGAGTTATTTATACTTC-3′).

With each set of primers and cDNA pool as template, PCRs were performed with the same condition as our previous paper.[5] The obtained full length cDNA clones, *BPX*, *BPX2*, *BPW* and *BPY*, were sequenced in both strands, and their sequences were submitted to DDBJ sequence-database.[19]

Each of the 2.3 kb PCR products was digested with appropriate restriction enzyme sites and ligated into the corresponding sites of pYES2 (Invitrogen) to construct the plasmid $pOSC_{BPX}$, $pOSC_{BPX2}$, $pOSC_{BPW}$ and $pOSC_{BPY}$.

Expression in *Saccharomyces cerevisiae* and Preparation of Crude Enzyme Solutions Each plasmid was transferred to *S. cerevisiae* strain GIL77,[5], using Frozen-EZ Yeast Transformation II™ kit (ZYMO RESEARCH). The transformants were inoculated in 20 ml synthetic complete medium without uracil (SC-U), containing ergosterol (20 μg/ml), hemin (13 μg/ml) and Tween 80 (5 mg/ml), and incubated at 37 °C for 2 d. Then, media were changed to SC-U with the same supplements and 2% galactose in place of glucose. Cells were incubated at the same condition for one day, harvested by centrifugation at 500×***g*** for 5 min, resuspended in 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.4, containing 0.45 M sucrose, 1 mM EDTA and 1 mM dithiothreitol), and broken by vortex with acid-washed glass beads (Sigma, #G-8772). Cell homogenates were centrifuged at 18400×***g*** for 20 min, and the supernatant served as the enzyme preparation.

***In Vitro* Triterpene Synthase Assay** Radio labelled substrate, (3*S*)-[^{14}C]-2,3-oxidosqualene (26 Ci/mol) was biosynthetically prepared by feeding [1-^{14}C] sodium acetate (Amersham Life Science, CFA.13) to *S. cerevisiae* mutant GL7 (lanosterol synthase deficient) following the reported methods[20] with some modifications.

(3*S*)-[^{14}C]-2,3-oxidosqualene (0.17 nmol, 10000 dpm in 10 μl ethyleneglycol monomethylether), 100 μl enzyme fraction (described above), and 890 μl phosphate buffer (100 mM potassium phosphate, pH 7.4, containing 0.1% Triton-X100) were incubated at 30 °C for 3 h. Reaction products were extracted with 1 ml of hexane, separated by silica gel TLC (Merck #11798) (benzene : acetone = 19 : 1), and analyzed by an Imaging Plate Analyzer (BAS1500, Fuji Film) (data not shown). Authentic triterpene monoalcohols that had been spotted on both side of TLC were visualized with phosphomolybdic acid staining. The corresponding spots were scraped off the plate and extracted with acetone. After addition of non-labeled carriers, reaction products were subjected to HPLC analysis on ODS-120T column (4.6×200 mm, Tosoh, with 95% CH_3CN, 1.5 ml/min, at 40 °C). The peaks corresponding to lupeol, β-amyrin and cycloartenol were collected separately and radioactivities counted with a liquid scintillation counter.

Liquid Chromatography-Atmospheric Pressure Chemical Ionization (LC-APCI)-MS Analysis of the OSC Products Accumulated in GIL77 Transformants The transformants were cultured for 2 d and expression of OSC was induced at the same condition as described above. Then cells were collected and resuspended in the same volume of 0.1 M potassium phosphate buffer (pH 7.0) supplemented with 2% glucose and hemin (13 μg/ml) and further incubated for 24 h at 30 °C. Cells were collected and refluxed with 2 ml of 20% KOH/50% EtOH *aq.* for 5 min. After extraction with the same volume of hexane, the extract was concentrated and applied onto TLC plate (Merck #11798) which was developed with benzene : acetone = 19 : 1. The band corresponding to triterpene monoalchohol was scraped off and extracted with acetone. The extract was concentrated and applied to LC-APCI-MS (LCQ, Thermo Quest). HPLC was carried out at the same condition described above. All triterpene monoalcohols gave the base peak ion at *m*/*z* 409 $[M+H-H_2O]^+$ in APCIMS analysis (data not shown). For rigorous identification of triterpene products, MS/MS spectrum was measured (*m*/*z* 409 as the parent ion), which gave characteristic pattern for each triterpene as shown in Figs. 2, 3 and 4.

Phylogenetic Analysis The phylogenetic tree was constructed from the amino acid sequences of all the known plant OSCs by the Neighbor-Joining method[21] followed by drawing with Tree View.[22] The accession numbers of the sequences on EMBL, GenBank and DDBJ sequence-data banks used in this analysis are as follows, *CAS1* cycloartenol synthase (*A. thaliana*); U02555, *PSX* cycloartenol synthase (*P. sativum*); D89619, *PNX* cycloartenol synthase (*P. ginseng*); AB009029, *GgCAS1* cycloartenol synthase (*G. glabra*); AB025968, *LcCAS1* cycloartenol synthase (*L. cylindrica*); AB033334, *LUP1* lupeol synthase (*A. thaliana*); U49919, *MtN18* putative lupeol synthase (*Medicago. truncatula*); Y15366, *OEW* lupeol synthase (*O. europaea*); AB025343, *TRW* lupeol synthase (*T. officinale*); AB025345, *PNY* β-amyrin synthase (*P. ginseng*); AB009030, *PNY2* β-amyrin synthase (*P. ginseng*); AB014057, *PSY* β-amyrin synthase (*P. sativum*); AB034802, *GgbAS1* β-amyrin synthase (*G. glabra*); AB037203, *PSM* mixed amyrin synthase (*P. sativum*); AB034803, *YUP8H12R.43* multifunctional triterpene synthase (*A. thaliana*); AC002986, *LcIMS1* isomultiflorenol synthase (*L. cylindrica*); AB058643, *PNZ* putative oxidosqualene cyclase (*P. ginseng*); AB009031, *TRV* putative oxidosqualene cyclase (*T. officinale*); AB025346, *LcOSC2* putative oxidosqualene cyclase (*L. cylindrica*); AB033335.

RESULTS AND DISCUSSIONS

cDNA Cloning Growth of *B. platyphylla* var. *japonica* cells reached maximum on day 30 after transfer to new medium (data not shown). Thirty days old cells were harvested and extracted with chloroform. Triterpene mono- and di-alcohol fractions were analyzed by LC-APCI-MS. Appreciable amount of lupeol (not quantitated) was detected in chloroform extract while dammarenediol was not detected even with selective ion monitoring (data not shown). Thirty

days old cells were used for the extraction of RNA. As reported in our previous paper,[5] nested PCR was required to obtain the fragment cDNA encoding OSCs from *P. ginseng* hairy roots. In this study, however, single PCR with primers 162S and 711A gave a DNA fragment of expected size (*ca.* 1.8 kb) from *B. platyphylla* cDNA mixtures. This fragment was subcloned in *E. coli*, and ten colonies were picked up for nucleotide sequencing. Eight of ten clones were identical to each other showing 77% sequence identity to lupeol synthase clones *OEW* (*O. europaea*) and *TRW* (*T. officinale*).[11] Its full length cDNA was named as *BPW*. The other two clones were not identical each other but both of them showed high sequence identities (79 and 83%) to cycloartenol synthase cDNA clone *PSX* (*P. sativum*),[4] and their full length clones were named as *BPX* and *BPX2*, respectively. In order to obtain another clones, nested PCR was carried out with additional primers 463S and 556A. PCR products (450 bp in length) were obtained and subcloned. Sixteen clones were picked up and sequenced. They included *BPW* (twelve clones), *BPX* (one), *BPX2* (two) and one new clone. This new clone has 86% sequence identity to β-amyrin synthase clone *PNY* (*P. ginseng*).[5] Its full length clone was designated as *BPY*. Based on the sequences of these fragments, the 3′- and 5′-ends clones were obtained by RACE method.[19]

Expression of cDNAs in *ERG7* Deficient *S. cerevisiae* Mutant GIL77 Based on the obtained sequences, the primers, corresponding to the sequences of the 5′- and 3′-termini and incorporating the appropriate restriction endonuclease sites, were synthesized to obtain the full-length clones. PCR products (*ca.* 2.3 kb in length) were digested with corresponding restriction enzymes, and ligated to the downstream of *GAL1* promoter of pYES2 to construct the plasmids

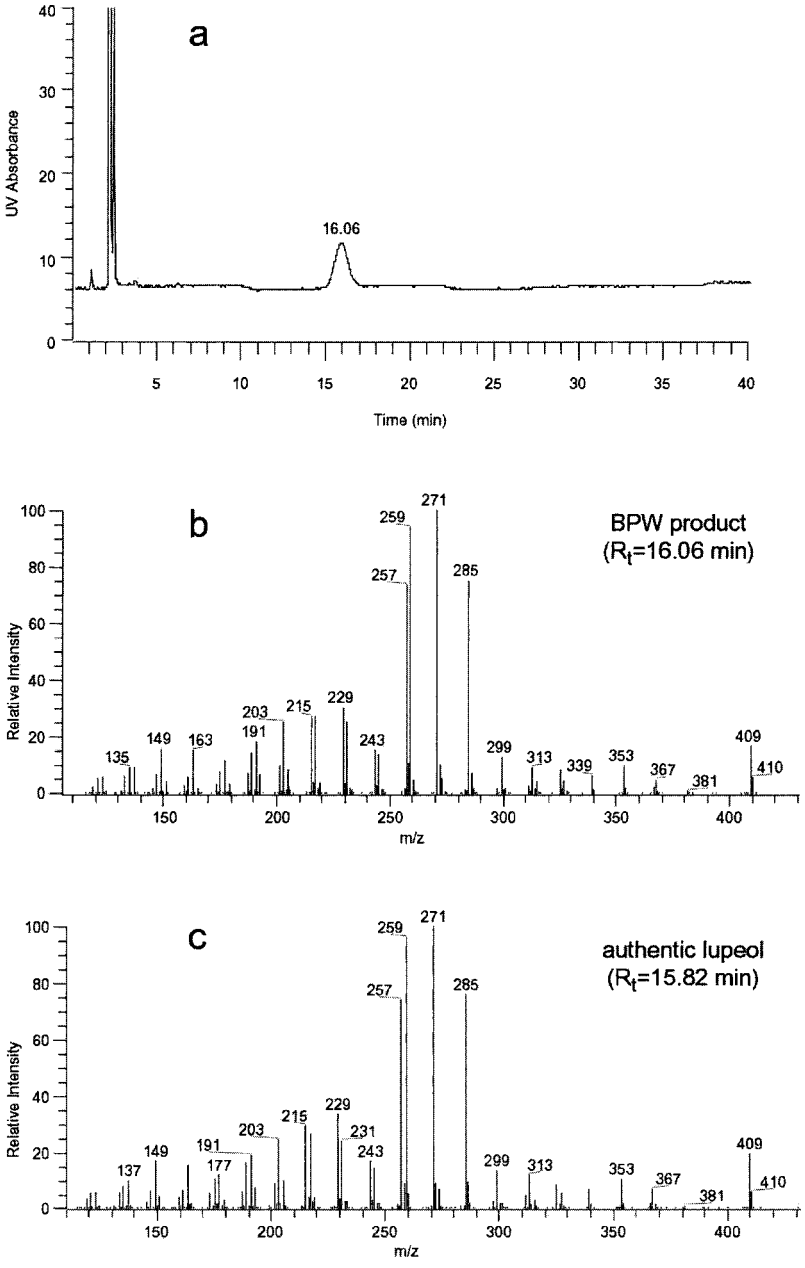

Fig. 2. LC-APCI-MS Analysis of the Product by $pOSC_{BPW}$

The HPLC profile (monitored by UV absorption at 202 nm) of triterpene mono-alcohol fraction obtained from yeast GIL77/$pOSC_{BPW}$ is illustrated (a), as are the MS/MS fragmentation patterns for the triterpene product with $t_R = 16.06$ min (b), and for authentic lupeol (c).

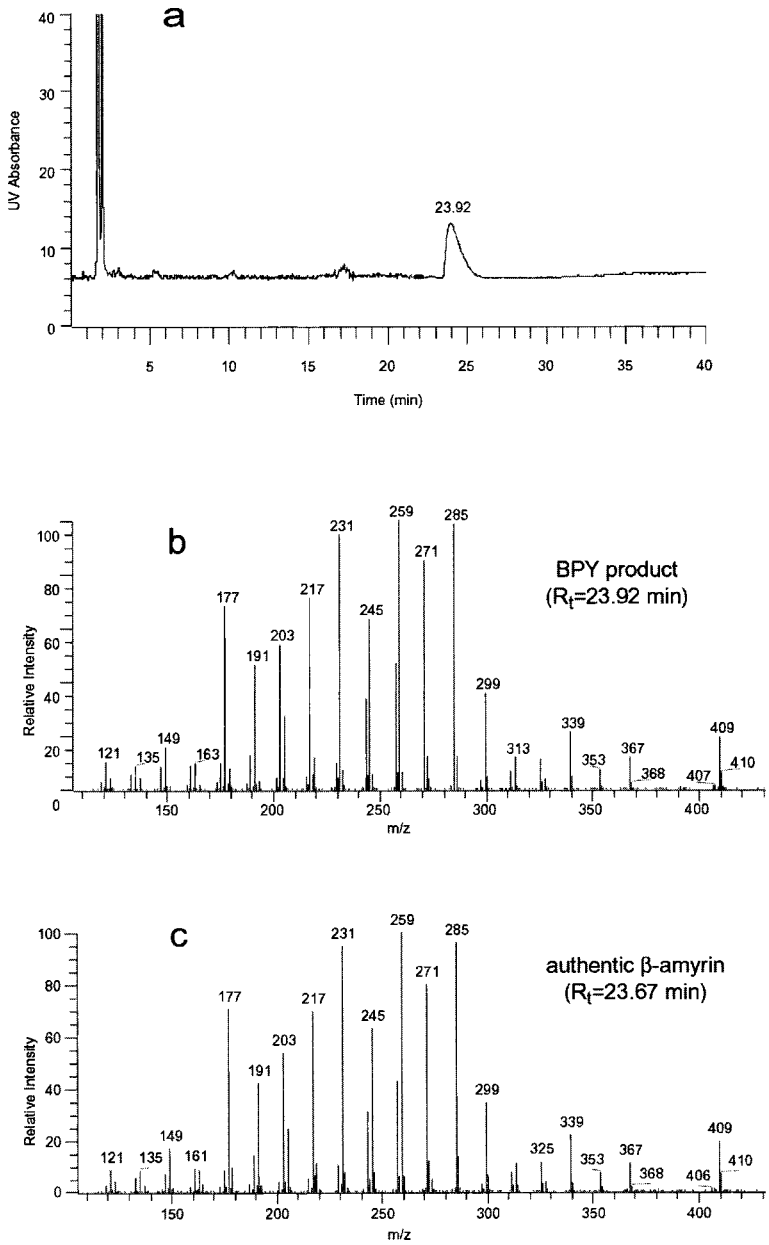

Fig. 3. LC-MS Analysis of the Product by $pOSC_{BPY}$

The HPLC profile (monitored by UV absorption at 202 nm) of triterpene mono-alcohol fraction obtained from yeast GIL77/$pOSC_{BPY}$ is illustrated (a), as are the MS/MS fragmentation patterns for the triterpene product with t_R=23.92 min (b), and for authentic β-amyrin (c).

$pOSC_{BPX}$, $pOSC_{BPX2}$, $pOSC_{BPW}$ and $pOSC_{BPY}$. The resulting plasmids were transferred to the *S. cerevisiae* strain GIL77, a lanosterol synthase deficient mutant.[5] The transformants were cultured and *GAL1* promoter was induced by replacing the carbon source from glucose to galactose. Cells were harvested by centrifugation and disrupted by vortexing with glass beads. Supernatants obtained by centrifugation served as the crude enzyme preparations. *In vitro* enzyme activity was assayed employing [^{14}C]-labeled oxidosqualene as the substrate. Reaction products were separated by TLC and radioactive spots corresponding to triterpene monoalcohol were detected in all clones by radioautogram (data not shown). Further separation of these products was attained by HPLC. Radioactivities derived from *BPX* and *BPX2* were associated exclusively in the peak corresponding to cycloartenol (their enzyme activities were estimated to be 13.1 and 11.9 pkat/g proteins, respectively), while those from *BPW* and *BPY* clones were found in the peaks that correspond to lupeol (0.226 pkat/g protein) and β-amyrin (0.192 pkat/g protein), respectively. These results indicated that *BPX* and *BPX2* encode cycloartenol synthase while *BPW* and *BPY* products are lupeol and β-amyrin synthases, respectively. In order to further confirm their enzyme functions, the accumulated products in the transformed yeasts were analyzed. Cells were harvested and disrupted by boiling with 20% KOH/50% ethanol *aq*. The resulting suspensions of disrupted cells were extracted with hexane and separated by TLC. Band corresponding to triterpene monoalcohol, that was not detected (by phosphomolybdic acid staining) in negative control with void vector, was recovered with EtOH and analyzed by LC-APCI-MS. As shown in Figs. 2, 3 and 4, products accumulated in transformants with *BPX*, *BPX2*, *BPW* and *BPY* were iden-

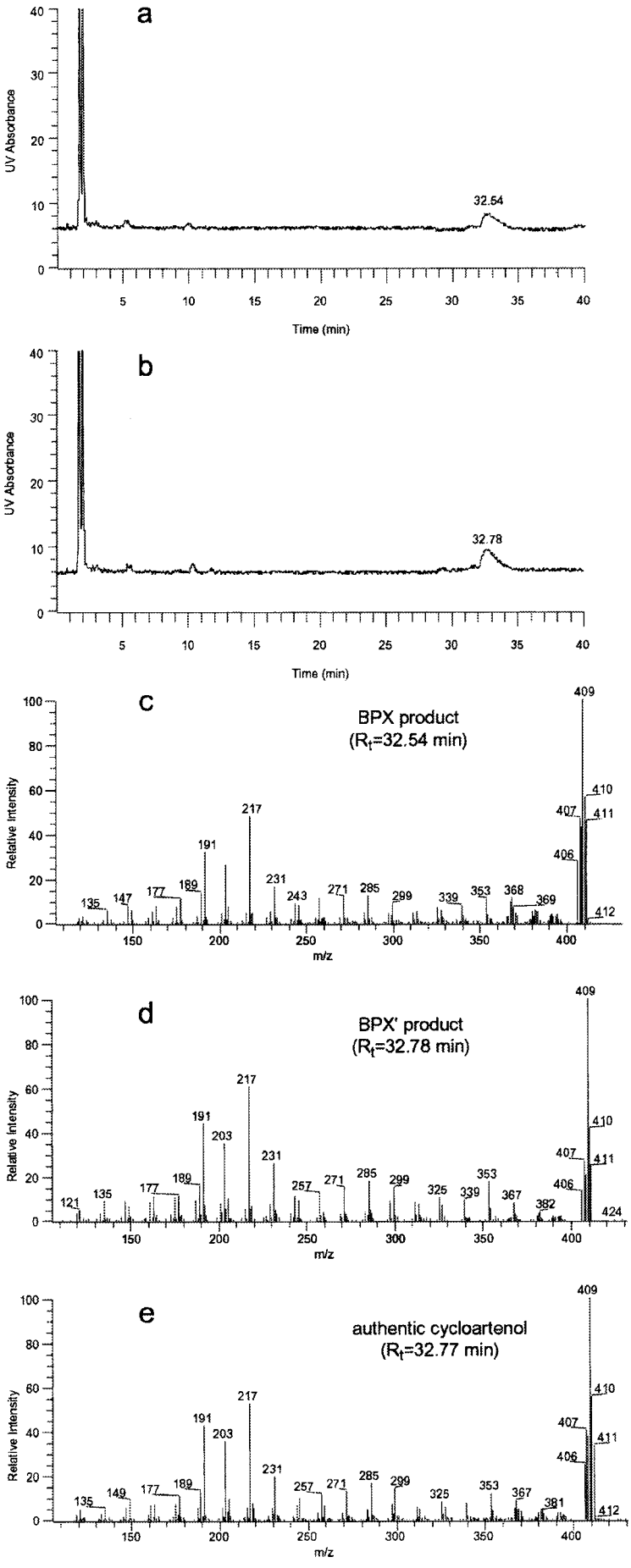

Fig. 4. LC-MS Analysis of the Products by $pOSC_{BPX}$ and $pOSC_{BPX2}$

The HPLC profile (monitored by UV absorption at 202 nm) of triterpene mono-alcohol fraction obtained from yeast GIL77/$pOSC_{BPX}$ (a) and GIL77/$pOSC_{BPX2}$ (b) are illustrated, as are the MS/MS fragmentation patterns for the triterpene product from yeast GIL77/$pOSC_{BPX}$ (c) with t_R=32.54 min and from GIL77/$pOSC_{BPX2}$ (d) with t_R=32.78 min, and for authentic cycloartenol (e).

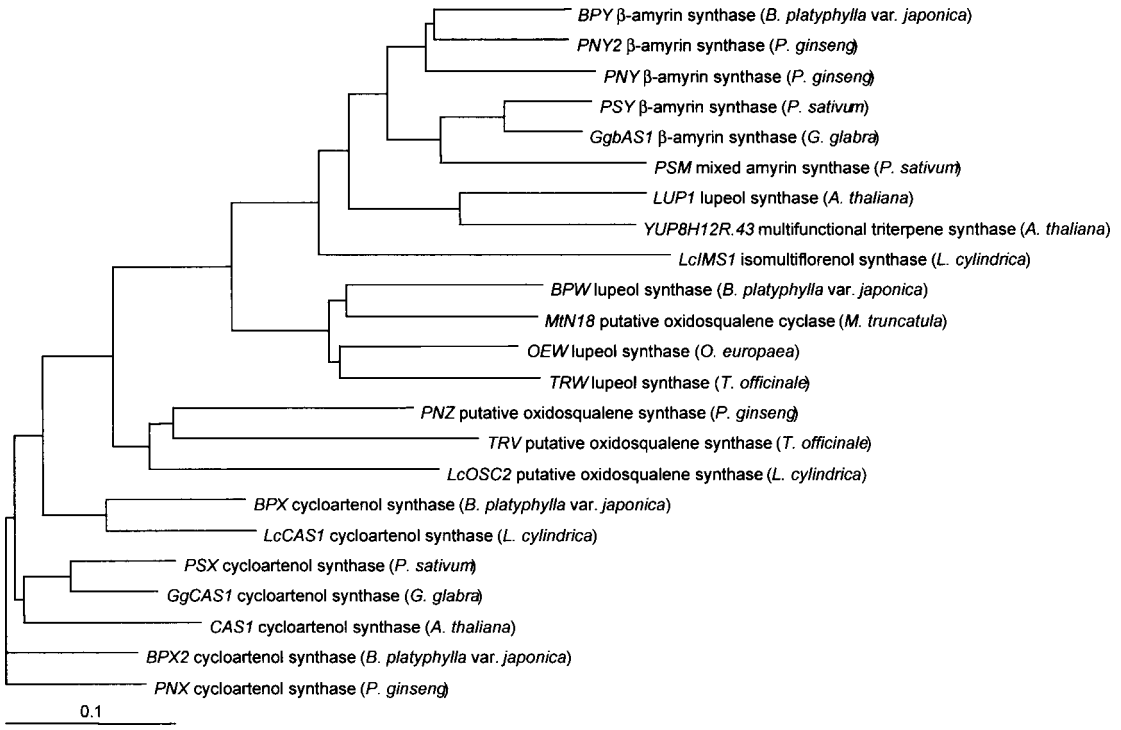

Fig. 5. Phylogenetic Tree of Plant OSCs

Distances between each clone and group are calculated with the program CLUSTAL W. The indicated scale represents 0.1 amino acid substitution per site.

tified as cycloartenol, cycloartenol, lupeol and β-amyrin, respectively. These results rigorously established that both *BPX* and *BPX2* encode cycloartenol synthase for sterol biosynthesis, while *BPW* and *BPY* code for distinct pentacyclic triterpene synthases for lupeol and β-amyrin, respectively.

Two Cycloartenol Synthases in One Plant *BPX* and *BPX2* consist of 2304 and 2274 nucleotide open reading frames that encode 768 and 758 amino acid long proteins, respectively. These cycloartenol synthase clones show high sequence identities to cycloartenol synthase clones *LcOSC1* (83%) from *L. cylindrica*[6] and *PNX* (80%) from *P. ginseng*.[5] Southern blot analysis suggested that there are more than two copies of cycloartenol synthase gene in the genome of *G. glabra*.[7] Successful cloning of two cDNAs of cycloartenol synthase (*BPX* and *BPX2*) in this study clearly proves the presence and expression of more than two cycloartenol synthase genes in one plant species. As cycloartenol is a common precursor for phytosterols, indispensable components of plasma membrane, it is reasonable to assume the presence of multiple copies of cycloartenol synthase gene to compensate otherwise fatal mutation in one of the genes. Besides mere backup system, however, they are supposed to be expressed independently, that is, one is constitutively expressed for phytosterol production and the other is differentially expressed depending on the different environmental conditions to produce some particular sterols for distinct functions. Regulation and physiological function of these two cycloartenol synthases in this plant are interesting issues for future studies.

Molecular Evolution of Triterpene Synthases *BPW* and *BPY* are composed of 2268 and 2340 nucleotide open reading frames that encode 756 and 780 amino acid long proteins. *BPW*, a lupeol synthase, shows high sequence identities to the known lupeol synthases *OEW* (78%) from *O. europaea* and *TRW* (75%) from *T. officinale*, but not so high identity (58%) to *A. thaliana* lupeol synthase *LUP1*. Enzyme function of LUP1 is different from the former ones as it produces not only lupeol but also some minor triterpenes.[13,23,24] *BPY*, a β-amyrin synthase, shows more than 80% sequence identity to the known β-amyrin synthases such as *PNY* (82%) and *PNY2* (84%) from *P. ginseng*.

A long standing question whether triterpene synthases giving different products are distinct proteins or they are post-translationally generated from one gene product has not been answered yet. So far, there has been no report on the purification of more than one triterpene synthase proteins with different product specificity from one plant species. Successful cloning of lupeol synthase (*BPW*) and β-amyrin synthase (*BPY*) in this study clearly demonstrates that even in one plant species there could be more than two distinct triterpene synthases.

We already reported the phylogenetic analysis for thirteen different plant OSCs and proposed the presence of two branches of lupeol synthase gene.[11] Reconstruction of phylogenetic tree (Fig. 5), by incorporating the sequence data of newly obtained OSC clones, reveals that *BPW* situates in a branch of authentic lupeol synthases, while *BPY* in a β-amyrin branch, as expected. This tree provides some interesting insights for molecular evolution of plant OSCs. Clones having the same enzyme function form one branch in the tree even though they derive from different plant species. Respective enzyme functions must have differentiated in an ancestral plant prior to the evolution of individual plant species. In addition to the product specific (monofunctional) enzymes, existence of multiproduct (multifunctional) triterpene synthases has been demonstrated. They are,

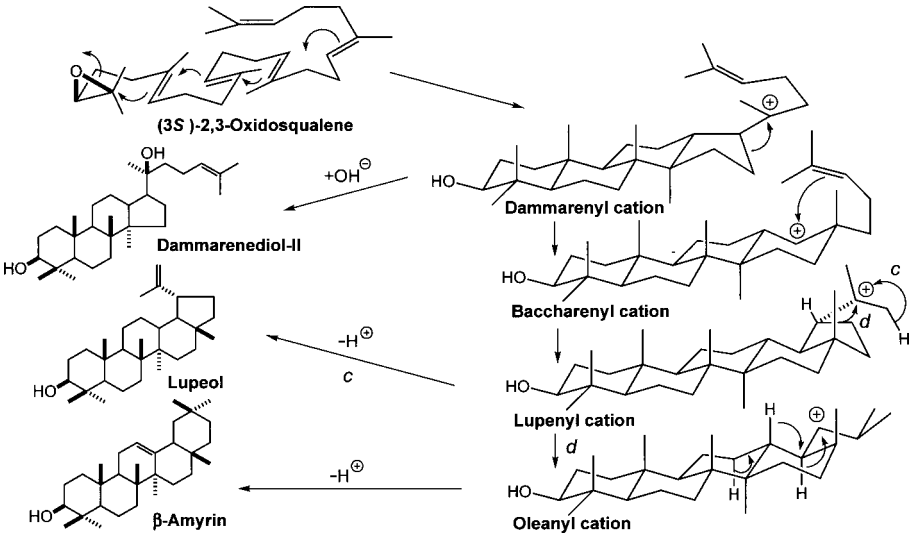

Fig. 6. Cyclization of Oxidosqualene to Dammarenediol-II, Lupeol and β-Amyrin

LUP1[13] and *YUP8H12R.43*[14] from *A. thaliana* and *PSM* from *P. sativum*.[10] Multiproduct nature is most prominent in *YUP8H12R.43* clone as it produces at least nine different cyclic triterpenes when expressed in yeast. These multifunctional clones are scattered around a β-amyrin branch of the tree, but they are located at relatively far from the branching point. This might imply that these multifunctional enzymes are at the evolutional transition state from one product specific synthase to another monofunctional enzyme. It is very interesting to note that both mono- and multifunctional triterpene synthases contribute to elaborate the huge structural diversity of triterpenes found in the plant kingdom.

Although no conclusive discussion for the order of OSC evolution could be drawn from this unrooted phylogenetic tree, pentacyclic triterpene synthases for secondary metabolism, such as lupeol synthase and β-amyrin synthase, must have evolved in this order from cycloartenol synthase for sterol biosynthesis, if we assume the cycloartenol synthase gene for the primary metabolism to be the progenitor. There is one big mechanistic difference in the reactions catalyzed by cycloartenol synthase and by other triterpene synthases. That is a manner of substrate fixation. For the production of cycloartenol, oxidosqualene must be held in *pre*-chair-boat-chair conformation in the active site of the enzyme to achieve the stereochemistry of the product. On the other hand, all chair conformation is mandatory for the formation of other cyclic triterpenes like lupeol and β-amyrin. During the course of evolution, active site structure of cycloartenol synthase protein must have changed so as to fix the same acyclic substrate in a different conformation to evolve into triterpene synthase proteins. As shown in Fig. 6, cyclization for pentacyclic triterpenes is initiated by protonation on epoxide and opening of epoxide drives a series of electrophilic addition reactions to give dammarenyl cation. Cyclizations first diverge at this intermediate stage to give different products. If this carbocation is quenched by water, dammarenediol is produced without rearrangement. If the reaction proceeds further to expand the fourth ring and form new ring, then lupenyl cation is produced. Quenching of lupenyl cation by elimination of a methyl proton leads to lupeol formation, while the expansion of the fifth ring leads to all six-membered pentacyclic oleanyl cation, and further 1,2-hydride shifts and deprotonation at C-12 gives β-amyrin. Complexity of the cyclization mechanisms, thus, increases from dammarenediol synthesis to lupeol synthesis and then to β-amyrin synthesis. It is very interesting to note that this order of complexity well correlates to the positions of the respective triterpene synthases in the tree.

In the tree, there is a branch composed of putative OSC clones *PNZ* from *P. ginseng*,[5] *TRV* from *T. officinale*[11] and *LcOSC2* from *L. cylindrica* (unpublished data) between cycloartenol and lupeol synthase branches. From the above discussion, it is highly expected that *PNZ*, *TRV* and *LcOSC2* represent yet unidentified dammarenediol synthase. Unfortunately, none of these clones exhibited enzyme activity in yeast expression system employed in this study. Two possibilities could account for the absence of enzyme activity of these clones. One is that the present expression system is not suitable for these potential triterpene synthase proteins. The other is that they might be pseudogenes and their translates have fatal mutation(s) to function as active enzymes. Further investigations are underway to resolve this question.

Although dammarenediol synthase activity was demonstrated in microsomal fraction prepared from *P. ginseng* hairy root cultures,[25] our previous attempts for cDNA cloning of this enzyme have been unsuccessful so far. The sequence of dammarenediol synthase might be so much different even in the conserved regions found in other known OSCs that the homology based PCR method did not work well, or the level of mRNA might be quite low in the cultured cells used for RNA extraction. Further attempts for cloning of dammarenediol synthase from *B. platyphylla* var. *japonica* as well as from *P. ginseng* are in progress by changing the sequence of PCR primers and the timing of RNA extraction.

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