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Cryptic Regiospecificity in Deprotonation Step of Triterpene Biosynthesis Catalyzed by New Members of Lupeol Synthase

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Abstract. Two new lupeol synthase cDNAs, *OEW* and *TRW*, have been cloned from *Olea europaea* and *Taraxacum officinale*, respectively. Both of them show only 58% amino acid sequence identity with the known *Arabidopsis thaliana* lupeol synthase (*LUP1*). Feeding experiments of [1,2-$^{13}C_2$] acetate in yeast transformants with *OEW* and *TRW* revealed that these new lupeol synthases strictly discriminate terminal two methyl groups of lupenyl cation, and abstract proton exclusively from (*Z*)-methyl of 2,3-oxidosqualene which originates from C-6 of mevalonate during the final deprotonation step in lupeol formation. The results are in sharp contrast to the one with LUP1 which does not differentiate these methyl groups and abstracts proton from both methyl groups in equal ratio. These facts indicate the presence of two types of lupeol synthase in plants which clearly differ in sequence as well as in deprotonation mechanism.

Keywords: Oxidosqualene cyclase, triterpene synthase, lupeol

Cyclization of 2,3-oxidosqualene into sterols and triterpenes is one of the most complex and fascinating chemical reactions found in nature. The enzyme oxidosqualene cyclases (OSCs) are widely distributed among various eukaryotic organisms that catalyze this remarkable transformation from acyclic precursor into varieties of cyclic compounds.[1] In plants, besides sterols, a large number of nonsteroidal triterpenes are formed as the cyclization products of OSCs, and their derivatives constitute one of the most abundant class of natural products in terms of structural diversity, biological activity, and potential for new drug development.[2] Despite the early mechanistic hypothesis postulated by Ruzicka et al. as the famous "biogenetic isoprene rule",[3] detailed studies on the mechanism leading to various tetra- and pentacyclic triterpene carbon frameworks have been hampered by scarce availability of these interesting emzymes. Recent cloning of plant triterpene synthase cDNAs has certainly opened up a frontier in this research field, allowing in depth studies on the mechanism leading to each specific cyclization product. Two triterpene synthase cDNAs, lupeol synthase (*LUP1*) from *Arabidopsis thaliana*[4] and β-amyrin synthase (*PNY*) from *Panax ginseng*[5] have become available and our domain swapping study between these two OSCs has demonstrated that a rather limited region of the polypeptide sequence is responsible for product specificity.[6]

Furthermore, [1,2-$^{13}C_2$] acetate feeding experiments revealed that in the LUP1 catalyzed reaction, deprotonation occurs on either of the two terminal methyl groups of lupenyl cation in equal ratio, while in the PNY reaction, these methyl groups are strictly discriminated in the ring expansion step that forms the E-ring (Scheme 1).[6]

In the course of our extensive search for new plant OSCs, we have cloned two highly homologous (78.3% amino acid identity of the deduced products) triterpene synthase cDNAs named *OEW* from *Olea europaea* and *TRW* from *Taraxacum officinale*.[7] Functional expression of these clones in yeast demonstrated both of them to encode lupeol synthase proteins, although they showed diminished level of sequence identity (58% amino acid identities) with *LUP1*.[4] This rather low level of identity is surprising considering that they have the same function as lupeol synthase even though they derived from different plant species. Therefore, a question arose

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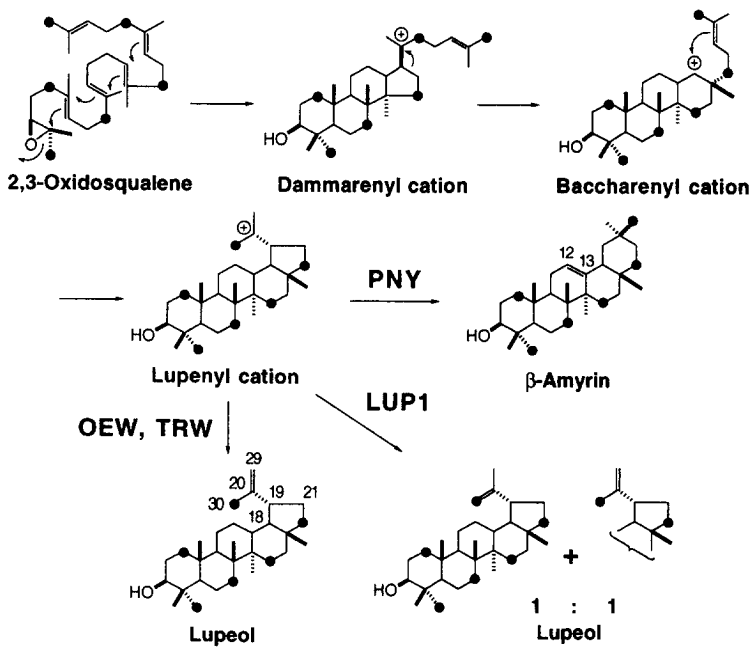

Scheme 1. Cyclization mechanism and the fate of carbon atom derived from C-2 of mevalonate by each triterpene synthases. (Dots indicate the carbons originate from C-2 of mevalonate)

whether there actually exist two types of lupeol synthase in nature. Here we report that two new lupeol synthases, namely OEW and TRW, strictly control the final deprotonation process during lupeol formation and unlike LUP1 reaction, no scrambling of methyl groups takes place, thus demonstrating that these clones represent a new family of lupeol synthase.

In order to see whether methyl group scrambling observed for LUP1 is also taking place in the reactions of OEW and TRW, feeding of [1,2-$^{13}C_2$] acetate was carried out. As C-6 of mevalonate originates from intact incorporation of acetate, the ^{13}C NMR signal of any methyl group derived from this carbon should appear with an accompanying doublet while one from C-2 of mevalonate will be a singlet. OEW and TRW were both expressed in yeast mutant GIL77 which lacks lanosterol synthase[8] with plasmid pYES2 under control of the *GAL1* promoter as described.[5] The production of lupeol was observed during the culture of the transformant yeast with galactose induction.[7] [1,2-$^{13}C_2$] Sodium acetate (85 mg, 90% atom ^{13}C, MSD ISOTOPES) diluted with non-labelled sodium acetate (165 mg) was fed to the culture (1000 mL) of the GIL77 transformant with either *OEW* or *TRW* during the galactose induction and resting period.[6] After alkaline treatment, lupeol (ca. 5 mg) was extracted with hexane and purified by silica gel column chromatography for ^{13}C NMR measurement. The lupeol specimen obtained from the *OEW* transformant showed a labelling pattern expected from the mevalonate pathway.[9] Surprisingly, a signal due to C-30 at 19.3 ppm appeared as an enriched singlet with no accompanying doublet indicating that this carbon derived from C-2 of mevalonate, thus from the (*E*)-methyl of 2,3-oxidosqualene (Fig. 1). On the other hand, an *exo*-methylene signal due to C-29 at 109.3 ppm was accompanied with a doublet (J=72.5 Hz) indicating its mevalonate C-6 origin, thus from the (*Z*)-methyl of 2,3-oxidosqualene (Fig. 1). Accordingly, a quarternary olefinic signal due to C-20 at 151.0 ppm appeared with only one set of doublet signals (J=72.5 Hz) (Fig. 1). These findings indicate that the final deprotonation takes place *exclusively* from the (*Z*)-methyl of 2,3-oxidosqualene, which is derived from C-6 of mevalonate, and hence no scrambling of methyl groups is occurring (Scheme 1). The result with TRW was exactly the same as

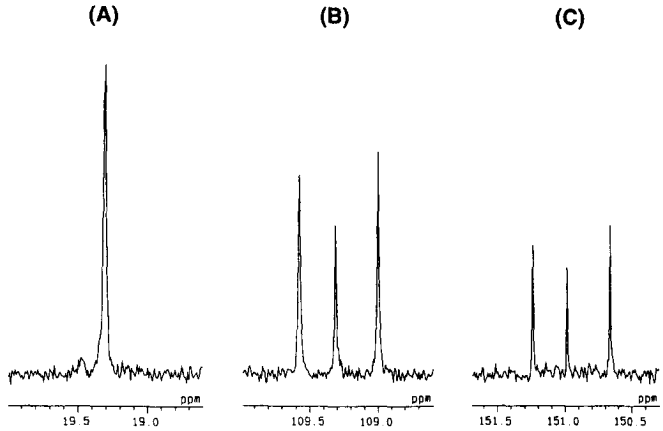

Figure 1. Partial ^{13}C NMR spectra (100 MHz, $CDCl_3$) of lupeol obtained from OEW, incubated with [1,2-$^{13}C_2$] acetate. (A) C-30 (19.3 ppm), (B) C-29 (109.3 ppm), (C) C-20 (151.0 ppm).

observed with OEW, in that the signal at 19.3 ppm was an enriched singlet while the signal at 109.3 ppm appeared with an accompanying doublet (J=72.4 Hz) (data not shown).

The above results are in sharp contrast to the previous finding with LUP1, where both the C-29 and C-30 signals were accompanied by doublets demonstrating the scrambling of methyl groups in the final deprotonation.[6] The exact reason for the observed difference is not clear at present, however, it could be argued that LUP1 lupeol synthase forms a rather loose active site allowing the isopropyl group of lupenyl cation to rotate before deprotonation, while these methyl groups are held tightly by OEW and TRW proteins. The observed specificity of deprotonation from the (Z)-methyl of 2,3-oxidosqualene during lupeol formation is in good accordance with the previous finding from incorporation experiment of [2-^{14}C]-mevalonate into betulinic acid, betulin, and lupeol in *Menyanthes trifoliata*,[10] establishing the wide occurence of this mechanism in higher plants.

Production of both β-amyrin and lupeol by some chimeric enzymes constructed with PNY and LUP1 had led us to assume that the same amino acid residue might be responsible for the final proton abstraction in β-amyrin and lupeol synthases.[6] However, the present result is contrary to our expectation. Specific deprotonation from the (Z)-methyl, and not from (*E*)-methyl, of 2,3-oxidosqualene, indicates that the putative amino acid residue responsible for the final proton abstraction in these lupeol synthases is likely to lie in an opposite side of the active site with respect to the one in β-amyrin synthase. (Z)-Methyl of 2,3-oxidosqualene, thus of baccharenyl cation, is expected to reside in a distal side from 12 position (12α proton is abstracted in β-amyrin synthase to form C12-C13 double bond[11]) at nascent lupenyl cation stage as no rotation of the isopropyl group could be assumed before deprotonation. Therefore, the amino acid residue in charge of the final proton abstraction in OEW and TRW lupeol synthases might be different from the one in β-amyrin synthase.

Comparison of the deduced amino acid sequences of these lupeol and β-amyrin synthases has suggested that OEW and TRW form a distinct lupeol synthase group which is more remote from β-amyrin synthase in terms of sequence identity (63.7% identity between OEW and PNY, 63.6% between TRW and PNY, and 70.2% between LUP1 and PNY).[7] From the above results, it is obvious that there actually exist two types of lupeol synthase, which could be differentiated by sequence as well as by deprotonation mechanism. As OEW and TRW exhibited more specific enzyme function, that is regiospecific deprotonation at the termination of the cyclization, this group can be regarded as more authentic form of lupeol synthase than LUP1 from *A. thaliana*.

The facts that LUP1 produces some minor products including β-amyrin[4] and shares higher sequence identity with β-amyrin synthase than with either of OEW and TRW, might suggest that LUP1 is situated between the

lupeol synthase of OEW and TRW type and β-amyrin synthase in the course of evolution. The presence of two types of lupeol synthase demonstrates that even with the lower sequence identity, the enzymes can still exhibit the same function. A similar situation has also been reported for monoterpene synthases from *Abies grandis* and *Mentha spicata*.[12] *A. grandis* (-)-(4*S*)-limonene synthase clone shows 63-65% amino acid sequence identity with other monoterpene synthases from the same plant which produce different monoterpene products, while it shows only 30% identity with *M. spicata* (-)-(4*S*)-limonene synthase clone. This would imply the presence of other triterpene synthases with similar degree of sequence diversity yet producing the same product. As shown in our chimeric studies, product specificity of triterpene synthases is governed by a subtle difference of sequence in a restricted region on whole polypeptide chain.[6] Therefore, even though two types of lupeol synthase show rather low level of sequence identity, the amino acid residues indispensable for lupeol formation must be well conserved.

The experiments described here were successful in distinguishing two mechanistically different types of lupeol synthase, which otherwise would have been difficult. cDNA cloning of other types of triterpene synthase and mechanistic studies are now underway to elucidate the divergence of triterpene synthases and their complex cyclization mechanisms.

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