cDNA ISOLATION AND GENE EXPRESSION OF KAURENE OXIDASE FROM *MONTANOA TOMENTOSA* (ZOAPATLE)

Nemesio Villa-Ruano[1], Martha G. Betancourt-Jiménez[1] and Edmundo Lozoya-Gloria[1]*

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ABSTRACT

The *ent*-kaurenoic acid (KA) is the precursor of gibberellins in all plants. In a previous report, we proposed the biosynthetic conversion of KA into grandiflorenic acid (GF) in "zoapatle" (*Montanoa tomentosa*), which is the most important bioactive diterpene of this Mexican medicinal plant. It is known that KA is the product of *ent*-kaurene oxidase enzyme (KO), being so a key enzyme for all plants. This time, we show the isolation of a full-length *ent*-kaurene oxidase cDNA from *Montanoa tomentosa* (MtKO-cDNA). Expression analyses of MtKO-cDNA were compared with endogenous levels of KA in leaves of different ages. Obtained data shown that KA is mainly synthesized in the mesophyll of young leaves and accumulated later on in old leaves including glandular trichomes. However, *MtKO* gene expression was clear in young leaves but low or null in old tissues and trichomes. The detection of low levels of MtKO transcript in glandular trichomes suggests that these structures may have some role in KA biosynthesis. The high concentration of KA in the whole leaf suggests a possible defense mechanism based on the production and accumulation of such diterpene. The present investigation is a contribution to the characterization of the metabolic pathway of *M. tomentosa* pharmacological diterpenes.

Keywords: ent-kaurene oxidase, *ent*-kaurenoic acid, glandular trichomes, grandiflorenic acid, *Montanoa tomentosa*.

RESUMEN

El ácido *ent*-kaurenoico (KA) es el precursor de las giberelinas en todas las plantas. Previamente propusimos la conversión biosintética del KA en ácido grandiflorénico (GF) en el "zoapatle" (*Montanoa tomentosa*), que es el diterpeno bioactivo más importante de esta planta medicinal Mexicana. Se sabe que el KA es el producto de la enzima *ent*-kaureno oxidasa (KO) considerada como una enzima clave para todas las plantas. Aquí reportamos el aislamiento de un ADNc completo de la *ent*-kaureno oxidasa de *Montanoa tomentosa* (MtKO-cDNA). El análisis de la expresión del gen correspondiente se comparo con los niveles endógenos de KA en hojas de diferen-

[1]Departamento de Ingeniería Genética, Centro de Investigación y de Estudios Avanzados del IPN, Unidad Irapuato. Km. 9.6 Libramiento Norte Carretera Irapuato-León, P.O. Box 629, C.P. 36821 Irapuato, Guanajuato, México.
*Tel. +52 462 6239659, Fax. +52 462 6245849, E-mail: HYPERLINK "mailto:elozoya@ira.cinvestav.mx" elozoya@ira.cinvestav.mx

tes edades. Los datos obtenidos sugieren que el KA es sintetizado principalmente en el mesófilo de las hojas jóvenes y acumulado posteriormente en hojas viejas y tricomas glandulares. Sin embargo, la expresión del gen MtKO fue clara en tejidos jóvenes y escasa o nula en las hojas viejas así como en los tricomas. La detección de bajos niveles del transcrito de la MtKO en los tricomas glandulares, sugiere que estas estructuras podrían estar involucradas en la biosíntesis del KA. La presencia de altas concentraciones de KA en la hoja entera sugiere un posible mecanismo de defensa basado en la producción y la acumulación de este diterpeno. La presente investigación es una contribución a la caracterización de la vía metabólica de los diterpenos farmacológicos de *M. tomentosa*.

INTRODUCTION

Montanoa tomentosa (zoapatle) is a Mexican medicinal plant that belongs to the Compositae family, used for common disorders in women´s health. The pharmaceutical bioactive compounds are KA ([4α]-kaur-6-en-18-oic acid) and GF ([4α]-kaura-9 [11]-16-dien-18-oic acid). In roots besides KA and GF, monoginoic acid (MO) (13-methyl-[4 α]-norkaur-15-en-18-oic acid) is the most abundant but less studied related diterpene (Enriquez *et al.*, 1997). GF and KA have been associated with uterotonic activities in female rats (Campos-Bedolla *et al.*, 1997) and the aqueous crude extract of zoapatle was proposed to have aphrodisiac effects in male rats (Carro-Juarez *et al.*, 2004). Recently, the biosynthetic relationship among these diterpenes was studied in the plant itself (Villa-Ruano *et al.,* 2009), opening the possibility for future biotechnological manipulation.

It is known that KA is the precursor of all gibberellins that are diterpene plant hormones biosynthesized by complex pathways controlling growth and development (Yamaguchi, 2008). Recently, gibberellins and abscisic acid (ABA) where involved in the phytochrome-mediated germination response (Seo *et al.,* 2009). KA has been found in glandular trichomes of *M. tomentosa* leaves (Robles-Zepeda *et al.*, 2009), and this as well as other diterpenes and hormones such as gibberellic acid (GA) among others (Bari & Jones, 2009), may be related to ecological roles as plant defense substances specifically against bacteria (Zgoda-Pols *et al*, 2002), protozoos (Vieira *et al.,* 2005), fungi (Cotoras *et al*, 2004), insects (Topcu and Gören, 2007) and viruses (Zhu *et al.,* 2005).

The KO, a P450 enzyme, catalyzes the transformation of *ent*-kaurene into KA in three oxidation steps (Helliwell *et al.*, 1999), and due to its central role in the biosynthesis of gibberellins and other diterpenes with relevant ecological and pharmacological roles; it is considered as a key enzyme in plants. In the present work we report the identification and isolation of a full-length cDNA of KO from *Montanoa tomentosa* (MtKO-cDNA). *MtKO* gene expression was studied by quantifying MtKO-mRNA transcript abundance in young shoots, mesophyll and glandular trichomes at different leaf ages. KA content in different leaf ages was also determined to compare it with MtKO gene expression.

MATERIALS AND METHODS

Plant material

New, young, mature, and old leaves of *Montanoa tomentosa* including the apex, as well as young shoots were taken from plants grown at 23-27 °C and 16 h/8 h light/dark photoperiod for two years in an experimental greenhouse at Cinvestav-IPN Unidad Irapuato in México. Dr. Jerzy Rzedowszki from the Instituto de Ecología UNAM, Michoacán México, previously certified these plants.

Nucleic acid extraction

Total RNA extraction was carried out as described in the Trizol protocol (Invitrogen ™), starting from 100 mg of fresh tissue previously grounded in a mortar in the presence of liquid nitrogen. A phenol: chloroform: isopropanol (25:24:1, v/v/v) extraction was done and the RNA integrity was confirmed by agarose 1% gel electrophoresis after staining with ethidium bromide. RNA from glandular trichomes was obtained according with Yerguer *et al* (1992). Briefly, trichomes from frozen plant tissue were specifically broken by strong vortexing with powdered dry ice, and sieved from larger tissue fragments for collecting glandular heads. This method preserves the integrity of active enzymes and nucleic acids from glandular trichomes avoiding contamination with mesophyll cells material. The non-sieved remaining tissue was confirmed as naked mesophyll without trichomes by microscopy, and grounded in the presence of liquid N_2 for RNA extraction as it is described above.

Isolation and molecular cloning of full length MtKO-cDNA by RT-PCR

All the retro transcription reactions were made from 10 μg of total RNA using the reverse transcriptase enzyme SuperScript II (Invitrogen ™). Degenerated primers 5'-GGGAATYTRYTGCARTTGAAGGAGAAG-3' and 5'-CCCTCTTYCCNSCTCCRAACGCCAT-3' were used to amplify a 1200 bp internal fragment of a putative MtKO-cDNA from total RNA. These oligonucleotides were designed according to conserved domains of putative P450 plant enzymes available in the NCBI Gen Bank such as *Stevia rebaudiana* (AAY42951), *Arabidopsis thaliana (*AAC39507), *Pisum sativum* (AAP69988), *Cucurbita maxima (*AAG41776), and *Fragaria grandiflora (*AAG41776). PCR running conditions to obtain this amplicon were as follows: 4 min at 94 °C of initial denaturation, 30 cycles at 94 °C for 30 sec, 57 °C for 30 sec, and 72 °C for 1 min 20 sec polymerization, finishing with 5 min at 72 °C. Full-length MtKO-cDNA was obtained by completing the 5' and 3'- ends according with the manual of the Gen RACER Kit (Invitrogen™). Specific oligonucleotides to get the 5'-end were 5'-GGCGTTTGACGGTCTTGTGATAATC-3' and 5'-GGTTTTATCAGCTGTGAGGACC-3', and for the 3'-end were 5'-GGGACATCCAAAGTGTGTGTGGG-3' and 5'-GGGAGCCTATTATCGAATCATCAG-3'. In both cases, the used conditions were 5 cycles at 94 °C for 30 sec, 63 °C for 30 sec, and 72 °C for 1 min; followed by 25 cycles at 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 1 min, finishing with an extension of 72 °C for 4 min. All PCR products were individually ligated to the TOPO TA cloning vector (Invitrogen ™). Finally, the complete cDNA was amplified from the cloned one using 5'-ATGGATACCCTCACCGGATTC-3' as forward primer and 5'-TCAATTTCTGGGCTTTATTAAGGC-3' as reverse primer in a PCR reaction consisting of 4 min at 94 °C of initial denaturation, 35 cycles at 94 °C for 30 sec, 57 °C for 30 sec, and 72 °C for 1 min 40 sec, finishing with 5 min at 72 °C. Resulted PCR products were sequenced by triplicate on ABI PRISM 3700 instruments (ABI, Foster City, CA). The identity of these sequences was edited and compared with available sequences in the Gen Bank using the Blast X program.

MtKO gene expression analysis

Gene expression was analyzed by semi-quantitative RT-PCR using the protocol of SuperScript II (Invitrogen ™) for synthesizing the cDNA pools. Specific oligonucleotides to perform the PCR reactions were as described above. The β-actin gene expression was assayed as a constitutive and positive control in the transcription analysis of *MtKO* gene. Oligonucleotides used to amplify β-actin transcripts were 5'-GCAGACGGTGAGGATATCC-3' as forward primer and 5'-CAGCAAGATCCAAACGAAG-3' as reverse primer, and oligonucleotides for MtKO transcript amplification were

those used to amplify the full length MtKO-cDNA in the same PCR conditions as described above. PCR products were analyzed by agarose 1% gel electrophoresis after staining with ethidium bromide.

Metabolite extraction and analysis

KA was extracted, identified, and quantified by GC-MS according to Villa-Ruano *et al.*, 2009. Identity of this diterpene was confirmed by their respective retention time and spectra as follows: KA: *m/z* 374 (M^+ 86% rel. int.), 359 (97), 331 (66), 257 (100), 241 (68), 143 (29), 91 (30), 73 (81). Abietic acid was used as internal standard; all the assays were performed in triplicate.

RESULTS AND DISCUSSION

MtKO-cDNA nucleotide sequence and comparison to other sequences

Full-length MtKO-cDNA was successfully completed at both 5' and 3' ends having 1542 bp coding for 514 aa. Total amino acid sequence was analyzed and translated with ExPASY software (http:ca.expasy.org/tools/dna.html). When compared specifically to the *Stevia reabaudiana* KO (AAY42951) aa sequence, a high amino acid (87%) homology was shown. Important and conserved catalytic domains of P450 enzyme regions were found in the MtKO sequence (Davidson *et al.,* 2004) (Figure 1). Amino acid sequence com-

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Mt   1 MDTLTGILTTVNQTIGIDGMAVVLIVALSFWYLRTFAFNHSRRSNNLPPVPEVPGVPLLG 60
       MD +TG+LT-----I-I-G-AV-L-VAL-FWYL+++------+SN+LP-VPEVPGVPLLG
Sr   1 MDAVTGLLTVPATAITIGGTAVALAVALIFWYLKSYTSARRSQSNHLPRVPEVPGVPLLG 60

Mt  61 NLLQLKEKKPYMTFTRWAETYGPIYSIKTGATSMVVVSSNEIAKEALVTRFQSISTRNLS 120
       NLLQLKEKKPYMTFTRWA-TYGPIYSIKTGATSMVVVSSNEIAKEALVTRFQSISTRNLS
Sr  61 NLLQLKEKKPYMTFTRWAATYGPIYSIKTGATSMVVVSSNEIAKEALVTRFQSISTRNLS 120

Mt 121 KALKVLTADKTMVAMSDYNDYHKTVKRHILTSFLGPNAQKKHRIHRDTMIENVSNQLYEF 180
       KALKVLTADKTMVAMSDY+DYHKTVKRHILT+-LGPNAQKKHRIHRD-M++N+S-QL+EF
Sr 121 KALKVLTADKTMVAMSDYDDYHKTVKRHILTAVLGPNAQKKHRIHRDIMMDNISTQLHEF 180

Mt 181 VKNSTEQEEVDLRKIFQSELFGLAMRQALGKDVESLYVEDLNLTMNRDEIFQVLVVDPMM 240
       VKN+-EQEEVDLRKIFQSELFGLAMRQALGKDVESLYVEDL-+TMNRDEIFQVLVVDPMM
Sr 181 VKNNPEQEEVDLRKIFQSELFGLAMRQALGKDVESLYVEDLKITMNRDEIFQVLVVDPMM 240

Mt 241 GSIDVDWRDFFPYLKWVPNKKFENTIQNMHIRREAVMKALIKEHKNRIASGEKLNSYIDY 300
       G+IDVDWRDFFPYLKWVPNKKFENTIQ-M+IRREAVMK+LIKE+K-RIASGEKLNSYIDY
Sr 241 GAIDVDWRDFFPYLKWVPNKKFENTIQQMYIRREAVMKSLIKENKKRIASGEKLNSYIDY 300
             ----------------A-----------------
Mt 301 LLSDAHTLTDQQLLMSIWEPIIESSDTTMVTTEWAIYELAKNPKIQDRLYRDIQSVCGSD 360
       LLS+A-TLTDQQLLMS+WEPIIESSDTTMVTTEWA+YELAKNPK+QDRLYRDI+SVCGS+
Sr 301 LLSEAQTLTDQQLLMSLWEPIIESSDTTMVTTEWAMYELAKNPKLQDRLYRDIKSVCGSE 360
         ---------B----------                              ----
Mt 361 KITEENLSQLPYITAIFHETLRRHSPVPIIPLRHVHEDTVLGGYHVPAGTELAVNIYGCN 420
       KITEE+LSQLPYITAIFHETLRRHSPVPIIPLRHVHEDTVLGGYHVPAGTELAVNIYGCN
Sr 361 KITEEHLSQLPYITAIFHETLRRHSPVPIIPLRHVHEDTVLGGYHVPAGTELAVNIYGCN 420
       ----------C---------          --------------D-----------
Mt 421 MEKNVWEDPEEWNPERFMKEKETIDFQRTMAFGGGKRVCAGSLQALLISCVGIGRMVQEF 480
       M+KNVWE+PEEWNPERFMKE-ETIDFQ+TMAFGGGKRVCAGSLQALL-+-+GIGRMVQEF
Sr 421 MDKNVWENPEEWNPERFMKENETIDFQKTMAFGGGKRVCAGSLQALLTASIGIGRMVQEF 480

Mt 481 EWKLKDMAQEDVNTIGLTTQMLRPLRALIKPRN 514
       EWKLKDM-QE+VNTIGLTTQMLRPLRA+IKPRN
Sr 481 EWKLKDMTQEEVNTIGLTTQMLRPLRAIIKPRN 514
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Figure 1. Amino acid sequence alignment of KO enzymes from *M. tomentosa* (Mt) and *Stevia reabaudiana* (Sr). Middle sequence shows identical, equivalent (+) or different (-) amino acids between both proteins. Important and conserved catalytic domains of P450 enzyme regions are indicated under dotted lines. A domain is associated to substrate binding, B domain includes the EXXR sequence involved in positioning the haem-binding pocket, C domain contains the highly conserved PERF motif and D domain has the FXXGXRVCAG arrangement which is the active site associated to the Fe++ haem-binding region.

parison of all reported plant KOs was carried out with Protein BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ClustalW software from EMBL-EBI was used to search for similarities and the resulted dendogram is shown in Figure 2 indicating that MtKO is quite similar to *S. rebaudiana* and *Lactuca sativa* enzymes. The high homology of this cDNA with corresponding sequences of *Arabidopsis thaliana* and *Stevia reabaudiana* predicts the same oxidation function to produce KA from *ent*-kaurene (Richman *et al.,* 1999). This MtKO-cDNA, is the second one isolated from an Asteraceae.

MtKO gene expression

Expression analysis of MtKO gene was performed by semi-quantitative RT-PCR assays in different tissues and at several leaf stages. MtKO gene expression was observed in mesophyll and shoots (Figure 3A) and in new, young, mature and even in old mesophyll tissues (Figure 3B). In trichomes low expression levels were detected, mainly in new and young tissues, but apparently it was no expression in mature and old tissues (Figure 3A and 3B). Interestingly, an inverse relationship among MtKO gene expression and KA concentration was found in new, young, mature and old leaves (Figure 3B and 3C). High levels of MtKO transcript but low KA concentration in new and young leaves, may suggest a quick exchange of this precursor into other tetracyclic diterpenes as gibberellins, which could be required to leaf expansion and stem elongation (Olszewski *et al.,* 2002). So, KA may be

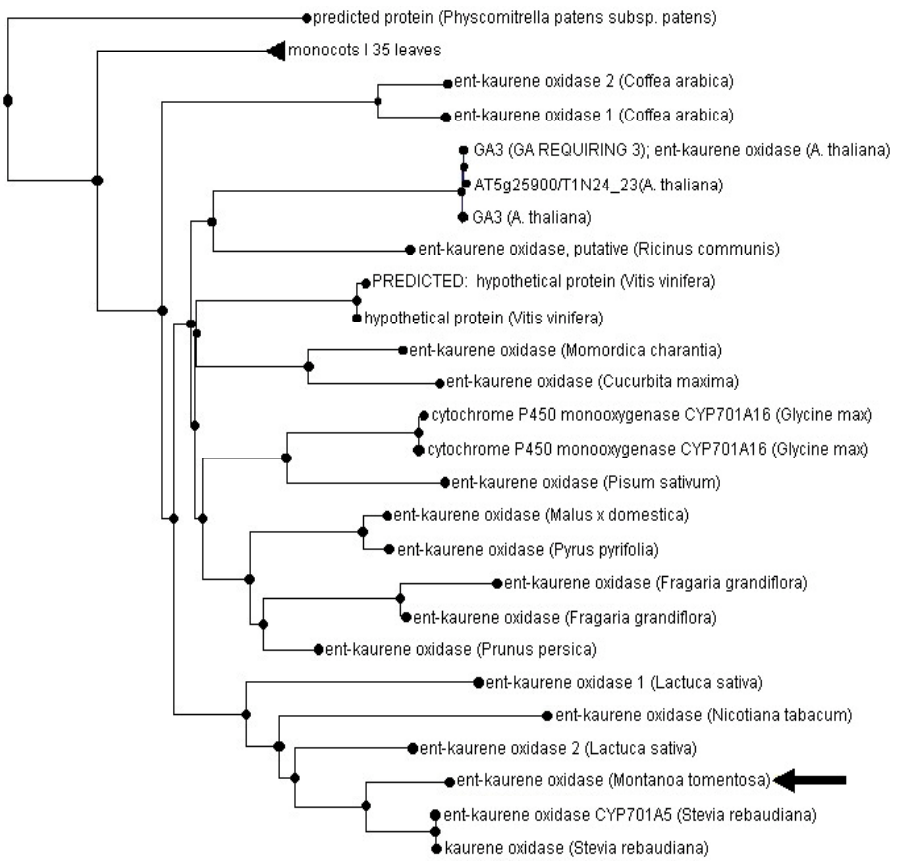

Figure 2. Dendogram of all KO amino acid sequences currently reported. Position of MtKO is indicated at the bottom with the arrow. MtKO is closely related to the KO of *Stevia rebaudiana* and *Lactuca sativa.*

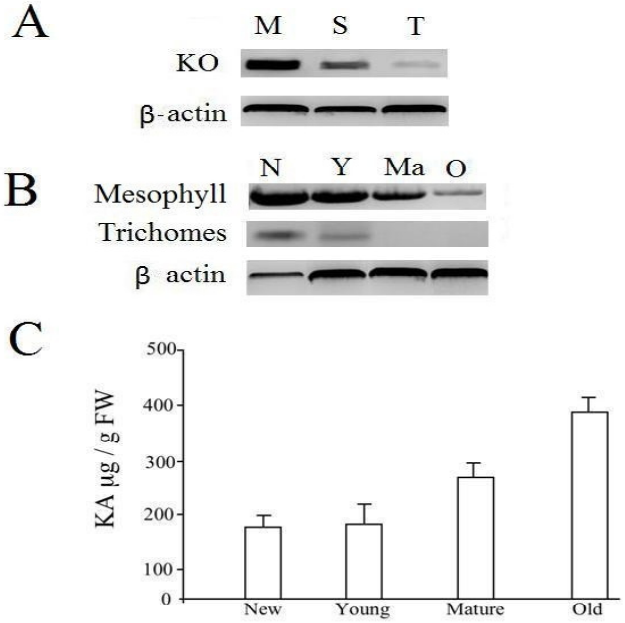

Figure 3 (**A**) Semi-quantitative PCR of *MtKO* gene expression in: M, leaf mesophyll; S, shoots and T, trichomes. (**B**) *MtKO* gene expression in: N, new; Y, young; Ma, mature and O, old tissues obtained from the aforementioned developmental stages. β-actin is the constitutive gene expressed as control. (**C**) KA abundance in *M. tomentosa* leaves of different developmental stages. Assays were made at least by triplicate. Bars indicate standard deviation.

used to produce GF, which is actively biosynthesized in these tissues (Villa-Ruano *et al.,* 2009) and accumulation of these diterpenes in mature and old tissues could be related to a defense mechanism. It is not yet clear if these results may be due to an active transport of KA from young to old tissues, or to a continuous accumulation of KA in the same tissue. Inactivation or posttranslational modification of the MtKO protein may also be involved. Further experiments are necessary to check these possibilities. Despite the existence of two KO sequences from Asteraceae plants (Richman *et al,*. 1999; Sawada *et al,*. 2008), there is not any information about their compartment expression and *in vitro* activities as reported here.

Initial detection of KA in glandular trichomes (Robles-Zepeda *et al*, 2009), suggested that this diterpene could be partially biosynthesized in these structures. In our work, most of the MtKO-mRNA was observed in the mesophyll, but it was also found in the trichomes. Although a very specific trichome purification procedure was used and we afforded high purity preparations, which decreases mesophyll contamination; we cannot discard that the trichome signal could be due to contamination with mesophyll cells. The efficiency of this trichome purification method was demonstrated in geranium when ω5 fatty acids were found exclusively in trichomes and not in the trichome-free supporting tissue, indicating that the most likely site of ω5 biosynthesis was the trichome (Yerger *et al.,* 1992). However, although the amplification power of PCR procedure is far beyond the finest metabolite analysis, it is not discarded an actively KA transport from mesophyll cells to trichomes.

The present work contributes to the knowledge of pharmacological diterpenes metabolism from *M. tomentosa*, specifically of KA. The proposal of a microsomal KA-C9 (11) desaturase enzyme (Villa-Ruano *et al.,* 2009) and the gene expression of KO in *M. tomentosa* as shown here to produce some of the most bioactive diterpenes in pharmacology and ecology like GF and KA respectively, suggests that these compounds play an important function in the plant physiology.

CONCLUSIONS

This work reports the isolation of a full-length cDNA from *Montanoa tomentosa* that encodes a putative *ent*-kaurene oxidase (MtKO) enzyme involved in kaurenoic acid biosynthesis.

The MtKO gene expression pattern studied in green tissues including shoots showed that this gene is mainly expressed in young tissues.

Higher levels of KA were observed in old leaves than in young leaves showing an inverse relationship with MtKO gene expression, suggesting that accumulation of this compound in old leaves might be based in its transport from young to old tissues or from accumulation throughout leaf development. Accumulation of KA and the decreased amount of MtKO-mRNA in old tissue seems to be the result of the overall diterpenes metabolism in zoapatle plants.

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