Arch. Biol. Sci., Belgrade, 67 (1), 193-199, 2015 DOI:10.2298/ABS140917024V

FUNCTIONAL CHARACTERIZATION OF *ENT*-KAURENE OXIDASE, MTKO, FROM *MONTANOA TOMENTOSA* (ZOAPATLE)

Nemesio Villa-Ruano[1,*], Edmundo Lozoya-Gloria[2], Martha G. Betancourt-Jiménez[2], Tony Herlt[4], Yesenia Pacheco-Hernández[3] and Carlos J. Castro-Juarez[1]

[1] *Universidad de la Sierra Sur. Guillermo Rojas Mijangos S/N, C.P. 70805, Ciudad Universitaria* Miahuatlán de Porfirio Díaz, Oaxaca México
[2] *Centro de Investigación y de Estudios Avanzados del IPN, Unidad Irapuato,* Km 9.6 Libramiento Norte Carretera Irapuato-León, C.P. 36821 Irapuato, Guanajuato, México
[3] *Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional-Unidad Oaxaca,* Hornos 1003, Col. Noche Buena, CP71230 Sta Cruz Xoxocotlán, Oaxaca, México
[4] *Research School of Chemistry, Australian National University,* Canberra ACT, 0200, Australia

[*]Corresponding author: necho82@yahoo.com.mx

Abstract - Kaurene oxidases are P450 proteins that catalyze the conversion of *ent*-kaurene into kaurenoic acid, the final enzymatic product with a wide range of pharmacological properties. We describe the functional characterization of an *ent*-kaurene oxidase (EC 1.14.13.78) isolated from *Montanoa tomentosa* after heterologous expression in *Saccharomyces cerevisiae*, as well as the detection of the enzymatic activity in the plant itself. In the presence of NADPH and FAD, the microsomal fraction from transformed INVS*c*1 cells, *ent*-kaurene produced *ent*-kaurenoic acid, which was confirmed by GC-MS analyses. The kinetic parameters for *ent*-kaurene using 0.5 mg of microsomal protein were $K_{m\ app}$= 80.63±1.2 μM and $V_{max\ app}$= 31.80±1.8 $\mu mol^{-1}mg^{-1}h^{-1}$. Optimal temperature and pH were 30°C and 7.6, respectively. Similar kinetic parameters were observed when leaf microsomes from *M. tomentosa* were assayed under the same conditions as for yeast microsomes. This result strongly suggests that *ent*-kaurene oxidase activity is present in leaf microsomes. The enzymatic activity was competitively inhibited by paclobutrazol, with IC_{50}=43.9 μM, implying that MtKO is resistant to inhibition by azolic-type compounds. This study confirmed the biochemical detection of *ent*-kaurene oxidase activity in the plant, and the heterologous functionality of a cDNA with an *ent*-kaurene oxidase identity from *M. tomentosa* (zoapatle).

Key words: ent-kaurene oxidase; yeast expression; leaf microsomes; kinetic characterization; *Montanoa tomentosa*

Received: September 17, 2014; **Accepted** November 24, 2014

INTRODUCTION

Kaurenoic acid (KA) is a natural occurring compound with a wide range of biological activities. In rats, KA causes a higher and more prolonged hypoglycemic effect than glibenclamide (Bresciani et al., 2004). The possible uses of *ent*-kaurene derivatives to treat alimentary disorders such as obesity and diabetes have been already described (Kim et al., 2006). Therefore, biotechnology approaches for the synthesis of KA represent an alternative to get its controlled production for pharmacological aims. KA also plays

an important function in plants as a key intermediary between primary and secondary diterpene metabolism. This diterpene is commonly associated with the biosynthesis of gibberellins (Helliwell et al., 1999), steviol glycosides (Humphrey et al., 2006) and the uterotonic grandiflorenic acid (Villa-Ruano et al., 2009).

Kaurene oxidases (KO) are directly involved in the biosynthesis of KA (Helliwell et al., 1999), with only a few KO from plants and fungi models kinetically characterized so far (Helliwell et al., 1999; Morrone et al., 2010; Wang et al., 2012). Because of the pharmacological importance of KA, we describe the biochemical characterization of the cDNA with an *ent*-kaurene oxidase identity (MtKO), which was previously isolated from *Montanoa tomentosa* (Villa-Ruano et al., 2010). This plant produces and accumulates high endogenous levels of KA in its aerial organs (Villa-Ruano et al., 2009). Therefore, we also describe the enzymatic activity in the leaf microsomes from the same plant.

MATERIALS AND METHODS

MtKO heterologous expression

The open reading frame (ORF) of MtKO was amplified by RT-PCR, according to Villa-Ruano et al. (2010), using the Platinum® DNA polymerase high fidelity (Invitrogen TM). The full cDNA sequence was cloned into the pCR®8/GW/TOPO vector (Invitrogen TM) in accordance with the manual instructions. The generated construction was designed as TOPO8-MtKO, and then it was used to transform the *Escherichia coli* TOP10 F' strain through heat shock (42°C for 2 min). The construction was replicated and extracted according to the Health-Care® Miniprep kit's instructions (General-Electric) and subsequently analyzed in an ABI PRISM 3700 instrument sequencer (ABI, Foster City, CA). Specific *att*-LR recombination between TOPO8-MtKO (100 µg) and PYESDEST52 (Invitrogen TM) was performed by LR Clonase II enzyme mix (Invitrogen TM) in order to generate the PYES-MtKO construction. This construction was replicated, extracted and finally sequenced as previously described. The construction was inserted into the *Saccharomyces cerevisiae* INVSc1 strain by the lithium acetate procedure (Rose et al., 1990). Both yeast selection and induction of the recombinant protein were carried out according to the respective manual with slight modifications: the SC-minimal medium containing 2% glucose was refreshed once at 24 h and then replaced at 45 h by the SC-minimal medium containing 2% galactose (Sigma-Aldrich Co) for 74-76 h. The cells were harvested by centrifugation, washed with sterile water and resuspended in 1 mL of 50 mM Tris-HCl, 1 mM EDTA, pH 7.4, containing 0.6 M sorbitol 0.1 mM dithiothreitol and 0.4 mM phenylmethylsulfonyl fluoride (PMSF). This buffer was previously degassed in a bath-sonicator (Bransonic® ultrasonic cleaner 1510) for 20 min (Chung et al., 2004).

Obtainment of microsomal fractions

The yeast cells were disrupted with acid-washed glass beads (G-9268, 425-600 µm 0.5 mm diameter, Sigma-Aldrich Co.) at 4°C using a manual Mini-Bead-Beater-8 (Biospec®). The lysate was centrifuged at 12 000 g for 10 at 4°C min and ultracentrifuged at 190 000 g for 90 min at 4°C to obtain microsomal fractions. Microsomes from the leaves of *M. tomentosa* were extracted according to Villa-Ruano et al. (2009). Both microsomal preparations were resuspended in 2 mL of degassed Na-phosphate buffer (pH 7.2) containing 1 mM EDTA, 1 mM L-ascorbic acid, 1 mM $Na_2S_2O_5$, 200 µM PMSF and 10% glycerol.

Enzymatic assays

Enzymatic reactions were performed in 10 mL screw-capped glass tubes containing 500 µL (0.5 mg) of microsomal protein from *M. tomentosa* leaves or yeast, 100 µM NADPH and 1 µM FAD. The reactions were initiated by adding 50 µM *ent*-kaurene dissolved in absolute MeOH and incubated for 1 h at 30°C. After this time, the reactions were stopped with a volume of acetone and the enzymatic products were extracted three times with hexane:ethyl acetate (90:10 v/v), then concentrated to 100 µL and finally derivatized with an equivalent volume of N,O-Bis(trimethyl-

silyl)trifluoroacetamide-1%-trimethylchlorosylane (BSTFA+1%TMCS). The *ent*-kaurene diterpene was chemically synthesized in Dr. Mander's Lab at ANU or obtained by the free endosperm enzymatic method (Graebe et al., 1969; Robinson and West, 1970). The optimal pH and temperature for this enzyme were investigated in accordance with Villa-Ruano et al. (2009). Apparent kinetic parameters for the conversion of *ent*-kaurene into KA were calculated from a saturation curve (10-500 µM *ent*-kaurene) and plotted as a non-linear regression graph using the Solver® software in triplicate experiments (Villa-Ruano et al., 2009). The effect of paclobutrazol (Sigma-Aldrich Co.) on MtKO activity was assessed by its addition in different concentrations (10-500 µM) to enzymatic assays. Results of inhibitory activity of paclobutrazol were expressed in a Lineweaver-Burk plot. The IC50 of paclobutrazol was determined with 50 µM *ent*-kaurene and calculated by linear regression using the GraphPad Prism 6.05 software.

Feeding experiments using yeast cells

The possible metabolism of *ent*-kaurene by intact INVS*c*1 cells was studied by feeding experiments. 100 µM *ent*-kaurene were directly added to 5 mL of SC-minimal medium containing 2% galactose and transformed INVS*c*1 yeast cells (OD_{600}=0.9). The cells were incubated for 48 h at 30°C. The enzymatic products were recovered three times with an equivalent volume of hexane: ethyl acetate (90:10 v/v), concentrated to 100 µL under N_2 stream and finally derivatized with BSTFA+1%TMCS.

GC-MS analyses

Analyses of the enzymatic products were performed in a Hewlett Packard 6890 II series GC-MS equipped with a HP-1 capillary column (30 m X 0.25 mm I.D. covered with a 0.25 µm of dimethylpolysiloxane). Mobile phase was helium at 1 mL min^{-1} flow rate. Injector temperature was maintained at 200°C. The oven temperature was 150°C during 3 min, followed by an increase of 4°C and finally held at 300°C for 20 min. Authentic KA was purified from *M. tomentosa* leaves by HPLC and then derivatized with BSTFA+1%TMCS for its use as a reference standard (Villa-Ruano et al., 2009). The spectrum of trimethyl sylanized KA (TMS-KA) at 70 eV was compared with that reported by Villa-Ruano et al. (2009). The Kovats index (KI) of unidentified compounds was calculated using a mix of *n*-alkanes C_8-C_{20}, C_{21}-C_{40} (Sigma-Aldrich Co.).

RESULTS AND DISCUSSION

According to our results, *ent*-kaurene was metabolized by both leaf and yeast microsomes for producing KA as the main enzymatic product (Figs. 1B, 1D). Microsomes without *ent*-kaurene and cofactors were unable to produce KA (Figs. 1C, 1E). These findings clearly demonstrated that MtKO-cDNA is a bona fide *ent*-kaurene oxidase. The mass spectrum of the main enzymatic product was m/z 374 (M+ 86% rel. int.) 359 (97) 331 (66) 257 (100) 241 (68) 143 (29) 91 (30) 73 (81), which was equivalent to the TMS-KA previously reported by Villa-Ruano et al. (2009). In addition, the peak nearest to TMS-KA (23.25 min) partially matched with the mass spectra and KI (2256) of the *ent*-kaurenal, which was previously reported by Helliwell et al. (1999) [286.3(M+; 99%) 271(10.5%) 255(34.5%) 243(62.5%) 225(27.3%) 215 (12.5%) 198 (33.6%) 187 (39.2%) 161 (40.3%)]. Because we used an HP-1 column, as Helliwell et al. (1999) did for GC-MS analyses, the observed KI and mass spectrum could suggest that *ent*-kaurenal is present as an enzymatic product. On the other hand, TMS-kaurenol was not clearly identified under our enzymatic reaction conditions, followed by derivatization. These results could be related to the rapid oxidization of *ent*-kaurene into *ent*-kaurenal/KA.

Interestingly, the results of feeding experiments using intact INVS*c*1 cells containing the PYES-MtKO construction resulted in small yields of KA (Fig. 2A). These results suggested that hydrophobic *ent*-kaurene easily diffuses across the yeast membranes and is transformed into KA, subsequently to be excreted to the medium. This process could be a consequence of KA's potential toxicity to yeast. When non-transformed INVS*c*1 cells and yeast free medium were assessed by feeding experiments there was no evidence

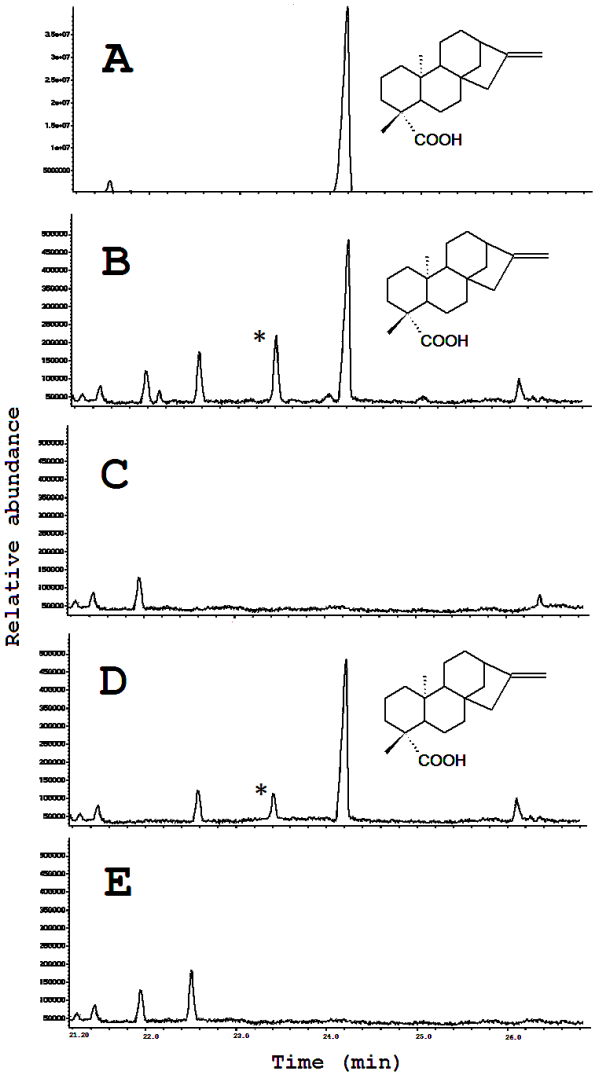

Fig. 1. TICs from GC-MS showing the metabolites originated by the MtKO activity. A – TIC of authentic *ent*-kaurenoic acid isolated from *Montanoa tomentosa*. B – TIC from enzymatic assays using microsomes from transformed yeasts with the addition of *ent*-kaurene. C – TIC from enzymatic assays using microsomes from transformed yeast without the addition of *ent*-kaurene. D – TIC from enzymatic assays using microsomes from the leaves of *M. tomentosa* with the addition of *ent*-kaurene. E – TIC from enzymatic assays using microsomes from leaves of *M. tomentosa* without the addition of *ent*-kaurene. *Peak with a putative *ent*-kaurenal identity.

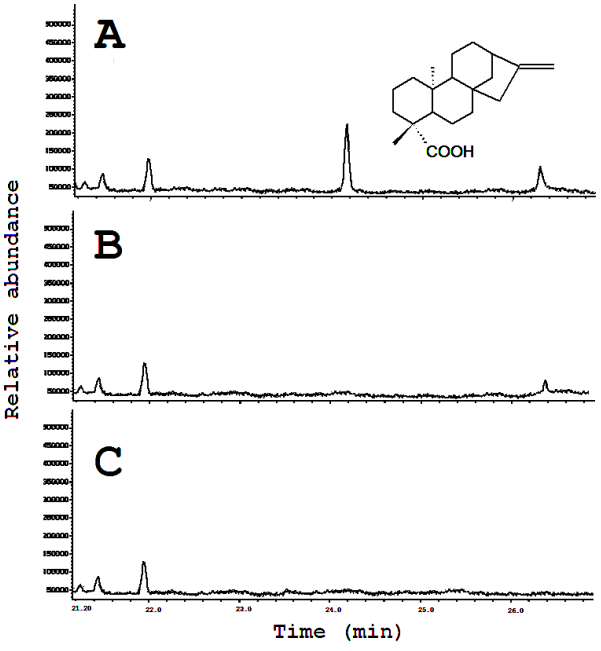

Fig. 2. TICs from feeding experiments using *ent*-kaurene. A – metabolites recovered from feeding experiments using intact INVSc1 containing the PYES-MtKO construction. B – metabolites recovered from the medium containing non-transformed INVSc1 cells. C – metabolites recovered from the yeast free medium.

of *ent*-kaurene metabolism (Figs. 2B, 2C). A similar behavior has been observed in other *in vivo* experiments using *S. cerevisiae* as expression system for *ent*-kaurene oxidase enzymes (Helliwell, et al., 1999; Humphrey et al., 2006). This property could be of interest for biotechnological aims because of the yeast's ability for excreting high-purity KA directly to the culture medium.

Previous studies demonstrated that *ent*-kaurene oxidase 2 from rice (OsKO2) depends on the simultaneous expression of a P450 reductase to be functional in the *Pichia pastoris* system (Ko et al., 2008). The *ent*-kaurene oxidases from *Arabidopsis thaliana*, *Stevia rebaudiana* and *M. tomentosa* did not seem to require such biochemical support, at least in the *S. cerevisiae* system (Helliwell et al., 1999; Humphrey et al., 2006).

The effects of temperature and pH on MtKO activity confirmed a functional range of 25-32°C and 7.0-8.0 respectively for this protein. However, optimal parameters were found at 30°C and 7.6 (Fig.

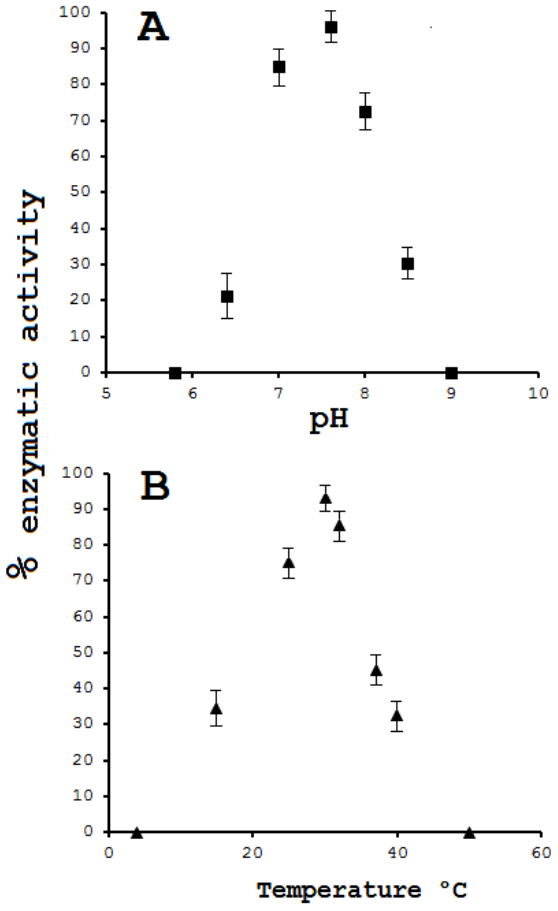

Fig. 3. Effect of temperature and pH on the MtKO activity. A – pH. B – temperature; 100% enzymatic activity was ~32 μmol^{-1}mg^{-1}h^{-1}. Bars represent the standard deviation of experiments performed in triplicate.

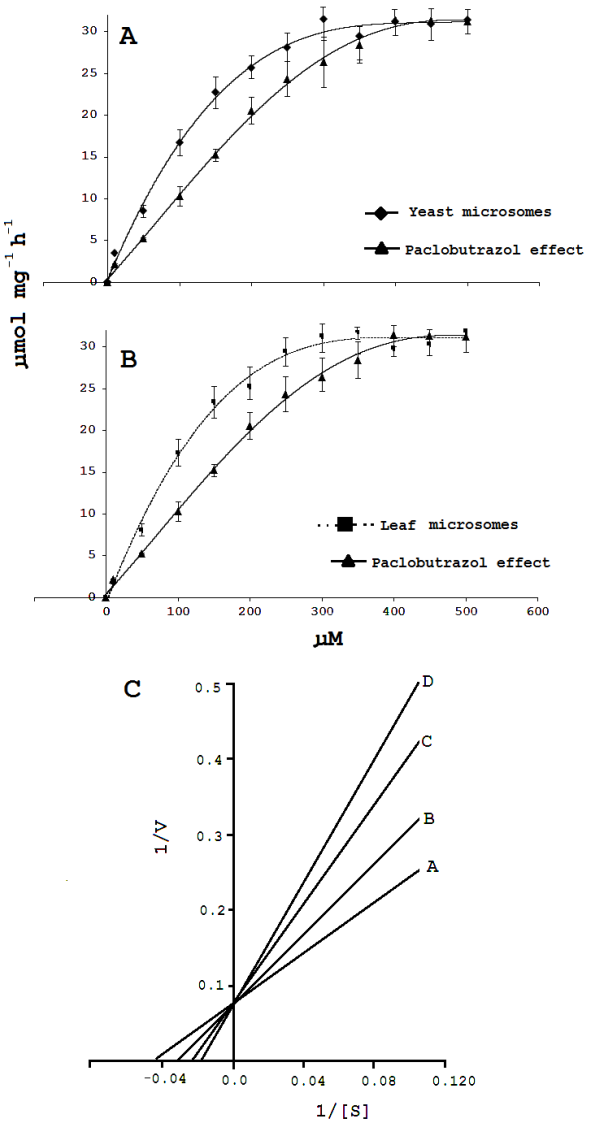

Fig. 4. MtKO enzymatic activity assessed at 10-500 μM *ent*-kaurene using microsomes (0.5 mg) from the leaves of *Montanoa tomentosa* (A) and transformed INVS*c*1 cells (B). C Linewaver-Burk plot showing the standard activity of the MtKO (A) against different concentrations of paclobutrazol (B, 40 μM; C, 100 μM; D, 200 μM).

3). The results of the analyzed data by saturation curves showed a $K_{m\ app}$= 80.63±1.2 μM and a $V_{max\ app}$= 31.80±1.8 μmol^{-1}mg^{-1}h^{-1} (Fig. 4). Surprisingly the $K_{m\ app}$ of MtKO was considerably higher than those of the orthologous proteins from *Gibberella fujikuroi* and *A. thaliana* (Ashman et al., 1990; Morrone et al., 2010). This result could suggest the possible ability of this protein to receive multiple substrates, but further studies are required to demonstrate it. The saturation point of MtKO was around 32 μmol mg^{-1} h^{-1}, exhibiting its efficiency in KA generation in a short period of time. This fact is another interesting property for biotechnological application.

Some related P450 oxide-reductases, such as the abietadiene hydroxylase and the abietadienol hydroxylase from *Abies grandis* as well as the KA desaturase enzyme of *M. tomentosa,* exhibited lower enzymatic activities than MtKO (Funk and Croteau, 1994; Villa-Ruano et al., 2009). The high enzymatic activity of MtKO could explain the prominent

endogenous levels of KA in the aerial parts of *M. tomentosa*. According to our results, microsomes isolated from the leaves of *M. tomentosa* showed a similar activity, properties and kinetic parameters to those of yeast microsomes when *ent*-kaurene was assayed. This supports the premise that MtKO is functionally active in both systems.

After the addition of paclobutrazol to both microsomal systems, a clear inhibition was observed (Fig. 4A, 4B). The Lineweaver-Burk plot demonstrated that paclobutrazol engendered a competitive inhibition on MtKO activity (Fig. 4C). Previous inhibitory tests on CYP701B1 from *Physcomitrella patens* showed an IC_{50}=64 μM for uniconazole (Miyazaki et al., 2011). Curiously, MtKO protein had an IC_{50}=43.9 μM for paclobutrazol, exhibiting 100-times more resistance to azolic compounds than the KO from *A. thaliana* (Miyazaki et al., 2011). Despite these findings, the resistance of MtKO cannot be directly compared with that of CYP701B1, because of differences in the design of the kinetic experiments for both enzymes.

Today, there are few works on the heterologous characterization of KO, including those of some fungi and known plant models (Ashman et al., 1990; Morrone et al., 2010; Wang et al., 2012). This is, however, the first one showing the kinetic detection of a KO in the leaves of a medicinal plant that accumulates high amounts of kaurenoids (Villa-Ruano et al., 2009). Further studies on *ent*-kaurene oxidases isolated from medicinal plants that dynamically produce *ent*-kaurene, isokaurene and *ent*-beyerene derivatives could reveal interesting multifunctional and multi-substrate properties.

Acknowledgments - This work was supported by the Grant CB-2010-151144Z from Conacyt-México. We specially thank Allison Tai Rosewicz for reviewing the MS.

Authors' contributions

N. Villa-Ruano, E. Lozoya-Gloria, MG. Betancourt-Jiménez and Y. Pacheco-Hernández contributed in the molecular cloning, yeast expression and partial biochemical characterization of MtKO. T. Herlt contributed in the chemical synthesis of the *ent*-kaurene. C.J Castro-Juárez contributed in the enzymatic synthesis of the same diterpene precursor and in the detection of the *ent*-kaurene oxidase activity in the leaf microsomes from *M. tomentosa*. All the authors contributed in the organization of this article.

Conflict of interest disclosure

The authors declare no conflict of interest.

REFERENCES

Ashman, P.J., Mackenzie, A. and *P.M. Bramley* (1990). Characterization of *ent*-kaurene oxidase activity from *Gibberella fujikuroi*. *Biochem. Bisphys. Acta* **1036**:151-157.

Bresciani, L.F.V., Yunes, R.A., Bürguer, C., De Oliveira, L.E., Bof, K.L. and *V. Cechinel-Filho* (2004). Seasonal variation of kaurenoic acid, a hypoglycemic diterpene present in *Wedelia paludosa* (*Acmela brasiliensis*) (Asteraceae). *Z. Naturforsh.* **59c**, 229-232.

Chung, W.G., Sen, A., Wang-Buhler, J.L., Yang, Y.H., Lopez, N., Merrill, G.F., Miranda, C.L., Hu, C.H. and *D.R. Buhler* (2004). cDNA-directed expression of a functional zebrafish CYP1A in yeast. *Aquatic Toxicol.* **70**, 111-121.

Funk, C. and *R. Croteau* (1994). Diterpenoid resin acid biosynthesis in conifers: characterization of two cytochrome P-450 dependent monooxygenases and an aldehyde dehydrogenase involved in abietic acid biosynthesis. *Arch. Biochem. Biophys.* **308**, 258-266.

Graebe, J.E. (1969). The enzymatic preparation of 14C-Kaurene. *Planta (Berl.)* **85**: 171-174.

Helliwell, C.A., Poole, A., Peacock, W.J. and *E.S. Dennis* (1999). Arabidopsis *ent*-kaurene oxidase catalyzes three steps of gibberellin biosynthesis. *Plant Physiol.* **119**, 507-510.

Humphrey, T.V., Richman, A.S., Menassa, R. and *J.E. Brandle* (2006). Spatial organization of four enzymes from *Stevia rebaudiana* that are involved in steviol glycoside synthesis. *Plant Mol. Biol.* **61**, 47-62.

Kim, S., Na, M.K., Oh, H., Jang, J.P., Sohn, C.B., Kim, B.Y., Oh, W.K and *J.S. Ahn* (2006). PTP1B inhibitory activity of kaurane diterpenes isolated from *Siegesbeckia glabrescens*. *Enzyme Inhib. Med. Chem.* **21**, 379-383.

Ko, K.W., Lin, F., Katsumata, T., Sugai, Y., Miyazaqui, S., Kawaide, H., Okada, K., Nojiri H. and *H. Yamane* (2008). Functional identification of a rice *ent*-kaurene oxidase, OsKO2, using

the *Pichia pastoris* expression system. *Biosci. Biotechnol. Biochem.* **72**, 3285-3288.

Miyazaki, S., Katsumata, T., Natsume, M. and *H. Kawaide* (2011). The CYP701B1 of *Physcomitrella patens* is an *ent*-kaurene oxidase that resists inhibition by uniconazole-P. FEBS Lett. **585**, 1879-1883.

Morrone, D., Chen, X., Coatest, R.M. and *R.J. Peters* (2010). Characterization of the kaurene oxidase CYP701A3, a multifunctional cytochrome P450 from gibberellin biosynthesis. *Biochem. J.* **431**, 337-344.

Robinson, D.R. and *C.A. West* (1970). Biosynthesis of cyclic diterpenes in extracts from seedlings of *Ricinus communis*. II. Conversion of geranylgeranyl pyrophosphate into diterpene hydrocarbons and partial purification of the cyclization enzymes. *Biochemistry* **9**, 80-89.

Rose, M.D., Winston, F. and *P. Hieter* (1990). Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Press, New York.

Villa-Ruano, N., Betancourt-Jiménez, M.G. and *E. Lozoya-Gloria* (2009). Biosynthesis of uterotonic diterpenes from *Montanoa tomentosa* (zoapatle). *J. Plant Physiol.* **166**, 1961-1967.

Villa-Ruano, N., Betancourt-Jiménez, M.G. and *E. Lozoya-Gloria* (2010). cDNA isolation and gene expression of kaurene oxidase from *Montanoa tomentosa* (zoapatle). *Rev. Latinoam. Quim*. **38**, 21-28.

Wang, Q., Hillwig, M.L., Wu, Y. and *R. Peters* (2012). A rice *ent*-kaurene oxidase paralog diverted to a more specialized diterpenoid metabolism. *Plant Physiol.* **158**, 1418-1425.