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# Functional identification of a *Lippia dulcis* bornyl diphosphate synthase that contains a duplicated, inhibitory arginine-rich motif

Matthew C. Hurd, Moonhyuk Kwon, Dae-Kyun Ro*

*University of Calgary, Department of Biological Sciences, Calgary, AB, T2N 1N4, Canada*

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ABSTRACT

*Lippia dulcis* (Aztec sweet herb) contains the potent natural sweetener hernandulcin, a sesquiterpene ketone found in the leaves and flowers. Utilizing the leaves for agricultural application is challenging due to the presence of the bitter-tasting and toxic monoterpene, camphor. To unlock the commercial potential of *L. dulcis* leaves, the first step of camphor biosynthesis by a bornyl diphosphate synthase needs to be elucidated. Two putative monoterpene synthases (*LdTPS3* and *LdTPS9*) were isolated from *L. dulcis* leaf cDNA. To elucidate their catalytic functions, *E. coli*-produced recombinant enzymes with truncations of their chloroplast transit peptides were assayed with geranyl diphosphate (GPP). *In vitro* enzyme assays showed that *LdTPS3* encodes bornyl diphosphate synthase (thus named *LdBPPS*) while *LdTPS9* encodes linalool synthase. Interestingly, the *N*-terminus of LdBPPS possesses two arginine-rich ($RRX_8W$) motifs, and enzyme assays showed that the presence of both $RRX_8W$ motifs completely inhibits the catalytic activity of LdBPPS. Only after the removal of the putative chloroplast transit peptide and the first $RRX_8W$, LdBPPS could react with GPP to produce bornyl diphosphate. LdBPPS is distantly related to the known bornyl diphosphate synthase from sage in a phylogenetic analysis, indicating a converged evolution of camphor biosynthesis in sage and *L. dulcis*. The discovery of LdBPPS opens up the possibility of engineering *L. dulcis* to remove the undesirable product, camphor.



## 1. Introduction

Terpenoids (also known as isoprenoids) constitute the largest class of plant metabolites with over 55,000 known structures and are implicated in many roles from hormones and energy capture to herbivore deterrents and pollinator attraction [1,2]. Additionally, terpenes are commonly used in pharmaceuticals, cosmetics, fragrances, agriculture and industrial products, many of which constitute food, perfumery, and pharmaceutical industries [3,4]. In plants, the immense structural diversity of terpenes is derived from the common precursor isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP) from the cytosolic mevalonate pathway and the chloroplastic non-mevalonate (or methylerythritol phosphate) pathway [5]. In the cytosol, IPP and its isomer DMAPP are synthesized from acetyl-CoA while in the chloroplast IPP and DMAPP are synthesized from glyceraldehyde-3-phosphate and pyruvate. Prenyltransferases catalyze the condensation of IPP onto DMAPP to yield various prenyl diphosphates of different lengths, such as C10 geranyl diphosphate (GPP), C15 farnesyl diphosphate (FPP), and C20 geranylgeranyl diphosphate (GGPP). These prenyl diphosphate backbones are rearranged by an enzyme class called terpene synthases (TPS). Most commonly, TPSs initiate catalysis by diphosphate cleavage (Class I TPS) or protonation of the double-bond (Class II TPS) to produce a highly reactive carbocation which undergoes rearrangements via hydride shifts, methyl shifts, Wagner-Meerwein rearrangements, or ring closures, followed by deprotonation or nucleophilic attack to quench the carbocation [5].

The enormous structural diversity of terpenoids has benefited human society in food and pharmaceuticals. One example is terpenoid-based, no- or low-calorie sweeteners. Due to growing demands for zero-calorie sugar substitutes, ethnobotanical documents were searched for sources, resulting in the rediscovery of *L. dulcis* as the source of the ultra-sweet sesquiterpene ketone, hernandulcin, in its leaves and flowers [6]. Remarkably, hernandulcin is 1000 times sweeter than sugar in sensory tests. Additional to the hernandulcin, the main volatile constituent of *L. dulcis* is camphor, a bitter-tasting and toxic monoterpene, which was found to encompass 53% (*w*/*w*) of the essential oil in *L. dulcis* leaves [7].

* Corresponding author. University of Calgary, Department Biological Sciences, 2500 University Dr. NW, Calgary, AB, T2N 1N4, Canada.
*E-mail address:* daekyun.ro@ucalgary.ca (D.-K. Ro).

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Camphor is a known toxin, especially in children, and was branded as unsafe by the US Food and Drug Administration in products containing camphorated oil in excess of 11%. While a low yield of endogenous hernandulcin is a major challenge for extraction and commercialization, camphor contamination is also a limitation to both the safety and quality of a hernandulcin-based sweetener using *L. dulcis*.

The exact ecological role of camphor is not known, but it may play a defensive role in plant. For example, camphor accumulates in young white spruce (*Picea glauca*) and is believed to deter snowshoe hare from herbivory [8]. Camphor was also found to be toxic to four species of beetles that are pests to stored grains [9], and it exhibited antitussive properties in Guinea pigs [10]. Xu et al. discovered that camphor-induced activation of the Transient Receptor for Potential Vanilloid subtype 1 channel (TRPV1) led to a rapid desensitization, which may be responsible for eliciting camphor's analgesic effects [11].

The biosynthesis of camphor was first elucidated by feeding assays using soluble sage (*Salvia officinalis*) protein extracts where neryl diphosphate was converted to borneol [9]. However, after considering the endogenous phosphatase activity, Croteau and Karp discovered that the biosynthesis of camphor proceeds via a bornyl diphosphate intermediate followed by hydrolysis to borneol [12] (Fig. 1). A monoterpene synthase (mono-TPS) named bornyl diphosphate synthase (SoBPPS) was found to catalyze the first step of camphor biosynthesis from GPP to bornyl diphosphate in sage [13]. The diphosphate intermediate is anomalous in monoterpene biosynthesis, such that the cleaved diphosphate from GPP is reincorporated, yielding bornyl diphosphate. Due to a highly reactive secondary carbocation intermediate, Whittington et al. hypothesized that the active site conformation prevents a nearby water to quench the carbocation, thus preventing hydration [14]. Additionally, the cleaved diphosphate is stabilized within the active site in a conformation that facilitates reincorporation via highly favorable electrostatic interactions between negatively charged diphosphate and positively charged carbocation [14].

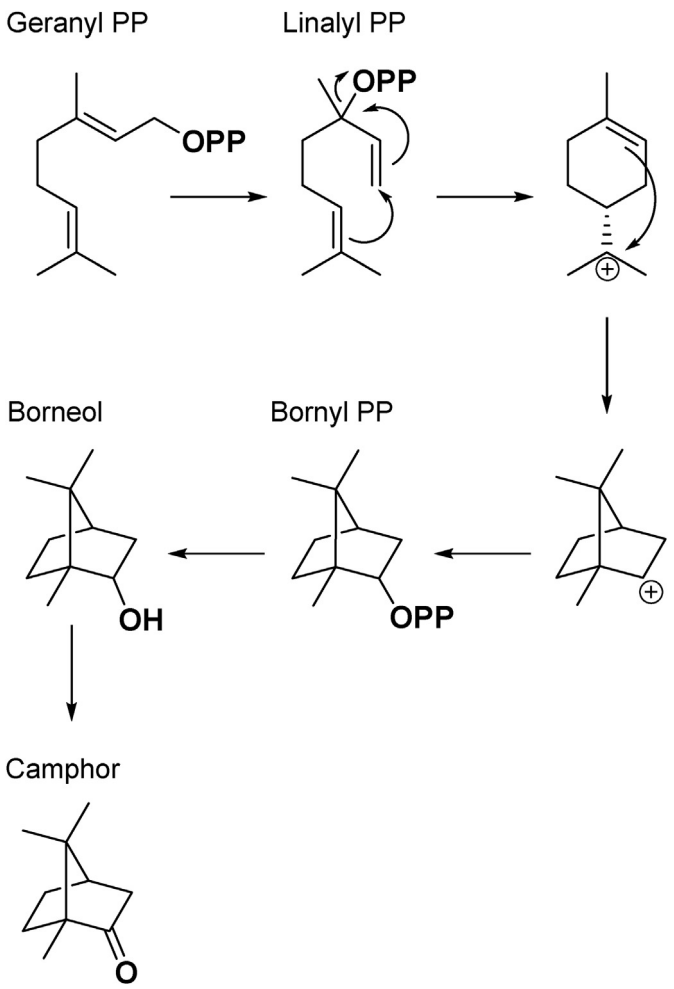


**Fig. 1. Reaction mechanism of BPPS.**
Reaction mechanism of SoBPPS involves an isomerization of GPP to linalyl diphosphate and the subsequent diphosphate cleavage and rearrangement of the carbocation. The secondary carbocation is then quenched by the diphosphate to yield bornyl diphosphate.

Although *SoBPPS* has been identified from sage [13], it is well accepted that the catalytic function of TPSs cannot be predicted by sequence comparison [15], and therefore the activity test of each TPS is necessary to determine the catalytic nature. Here, we describe the function of *L. dulcis* cDNA encoding bornyl diphosphate synthase (*LdBPPS*), which catalyzes the first dedicated reaction of camphor biosynthesis in *L. dulcis*. LdBPPS is distantly related to the known SoBPPS from sage in the phylogenetic analysis. Intriguingly, this enzyme contains a duplicated $RRX_8W$ motif in its *N*-terminus, and its effect on the enzyme activity is explored here. Identification of LdBPPS will help engineer a pleasantly sweet and safe *L. dulcis* cultivar.

## 2. Materials and methods

### 2.1. Plant propagation and cDNA construction

*Lippia dulcis* seeds (Chiltern Seeds, Oxfordshire, UK) were germinated and grown in a phytochamber at 21 °C for 16 h light/8 h dark cycle in semi-wet soil. Young leaves were flash frozen in liquid $N_2$ and ground to fine powder. Total RNA was extracted from 100 mg ground *L. dulcis* leaves using Trizol© following the manufacturer's protocol. cDNA was synthesized from 1 to 2 μg total RNA using M-MuLV reverse transcriptase, according to the manufacturer's protocol (NEB). For metabolite analysis, approximately 100 mg young, fresh leaf was flash frozen in liquid nitrogen and ground to fine powder. The ground leaf material was extracted for 4 h at room temperature in dichloromethane with slight agitations. The dichloromethane extract was then injected for metabolite analysis in a gas-chromatography mass spectrometry (GC-MS).

### 2.2. Candidate gene identification and sequence alignment

454 GS-FLX Titanium and Illumina sequencing reads from *L. dulcis* leaves have previously been reported and were available online (www.phytometasyn.ca) [16,17]. To identify candidate monoterpene synthases, *SoBPPS* sequence was used as a query to blast against the transcriptome assemblies. Three putative monoterpene synthases (*LdTPS2*, *LdTPS3* and *LdTPS9*) were identified with predicted chloroplast transit peptides, according to the ChloroP algorithm [18]. *LdTPS2* was determined to be the previously-characterized geraniol synthase (*LdGES*) [19].

### 2.3. Plasmid construction

*LdTPS3* and *LdTPS9* were subcloned into pBluescript II SK (+) from *L. dulcis* young leaf cDNA. *LdTPS3* was PCR-amplified using a forward primer 5′-GACCGGATCCAACATGGCTGCATTCAATATTATAATGC-3′ and a reverse primer 5′-GACCTCTAGACTAAAGGGGCTCAAAGAACAAT-3′.

Truncated monoterpene synthase candidates were cloned into *E. coli* overexpression plasmid pET28a utilizing *C*-terminal HIS-tag.

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