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Involvement of an *ent*-copalyl diphosphate synthase in tissue-specific accumulation of specialized diterpenes in *Andrographis paniculata*

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ABSTRACT

Ent-labdane-related diterpene (*ent*-LRD) specialized (i.e. secondary) metabolites of the medicinal plant kalmegh (*Andrographis paniculata*) have long been known for several pharmacological activities. However, our understanding of the *ent*-LRD biosynthetic pathway has remained largely incomplete. Since *ent*-LRDs accumulate in leaves, we carried out a comparative transcriptional analysis using leaf and root tissues, and identified 389 differentially expressed transcripts, including 223 transcripts that were preferentially expressed in leaf tissue. Analysis of the transcripts revealed various specialized metabolic pathways, including transcripts of the *ent*-LRD biosynthetic pathway. Two class II diterpene synthases (*ApCPS1* and *ApCPS2*) along with one (*ApCPS1′*) and two (*ApCPS2′* and *ApCPS2″*) transcriptional variants that were the outcomes of alternative splicing of the precursor mRNA and alternative transcriptional termination, respectively, were identified. *ApCPS1* and *ApCPS2* encode for 832- and 817-amino acids proteins, respectively, and are phylogenetically related to the dicotyledons *ent*-copalyl diphosphate synthases (*ent*-CPSs). The spatio-temporal patterns of *ent*-LRD metabolites accumulation and gene expression suggested a likely role for *ApCPS1* in general (i.e. primary) metabolism, perhaps by providing precursor for the biosynthesis of phytohormone gibberellin (GA). However, *ApCPS2* is potentially involved in tissue-specific accumulation of *ent*-LRD specialized metabolites. Bacterially expressed recombinant ApCPS2 catalyzed the conversion of (E,E,E)-geranylgeranyl diphosphate (GGPP), the general precursor of diterpenes to *ent*-copalyl diphosphate (*ent*-CPP), the precursor of *ent*-LRDs. Taken together, these results advance our understanding of the tissue-specific accumulation of specialized *ent*-LRDs of medicinal importance.

1. Introduction

Plants produce a large and diverse array of specialized (i.e. secondary) metabolites that have applications as pharmaceuticals, pesticides, flavours and fragrances [1]. Several specialized metabolites are directly utilized as drugs; however, many are leading models for the development of semisynthetic and synthetic drugs [2]. Specialized metabolites are biosynthesized in plants in tissue-specific, organ-specific, and developmentally-specific ways and also in response to pathogen attack and environmental perturbation; involving highly complex and sophisticated biosynthetic pathways [1]. A comprehensive knowledge of the metabolic pathways and their regulation shall be useful to overcome low product yield of the specialized metabolites in plants, following pathway engineering and molecular breeding approaches [3,4].

Labdane-related diterpenes (LRDs), with almost 7000 known members comprise a large superfamily of plant compounds with a broad range of biological activities [5,6]. LRDs possess a characteristic skeleton with a basic decalin structure and an additional C6 skeleton that is either acyclic or constitutes a ring structure with/without an oxygen atom [6,7]. LRDs are derived from the general diterpene precursor (E,E,E)-geranylgeranyl diphosphate (GGPP) following two sequential biosynthetic cyclization and/or rearrangement reactions catalyzed by diterpene synthases (diTPSs) [8]. The first step is a protonation-initiated cyclization of GGPP by class II diTPS, mostly by copalyl diphosphate synthase (CPS) that leads to the formation of a bicyclic labdadienyl/copalyl diphosphate (CPP) with a specific stereochemistry (*ent*, *syn*, *syn-ent* or (+)/nor-

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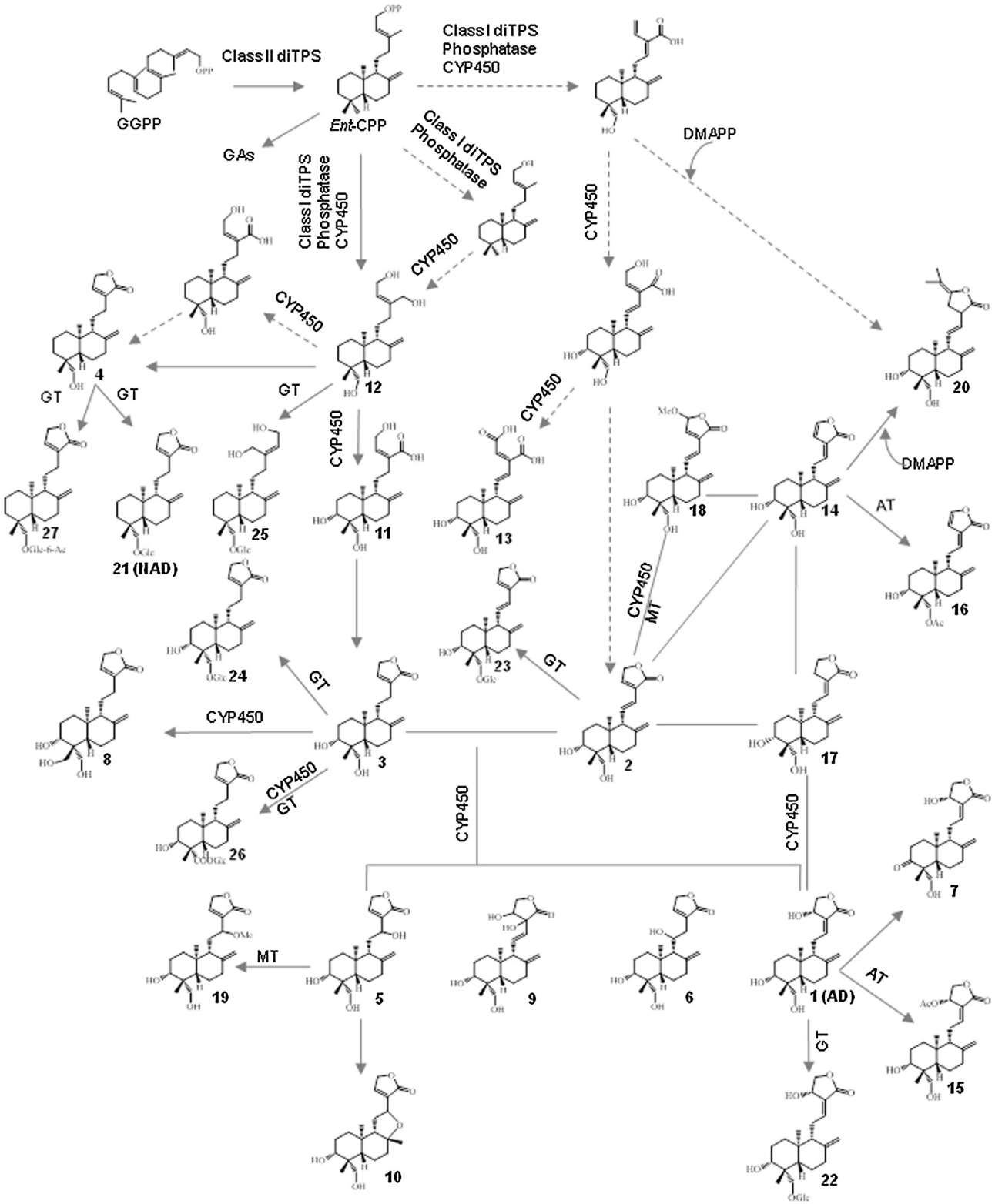

Fig. 1. A proposed model for the biosynthesis of bioactive *ent*-LRDs in kalmegh. The pathway is drawn based on common architecture of LRD biosynthesis and the known structures of *ent*-LRDs isolated from kalmegh, including two abundant *ent*-LRDs, andrographolide (AD) and neoandrographolide (NAD). Nomenclatures of the *ent*-LRDs are mentioned in Fig. S1. Broken arrows represent steps with hypothetical pathway intermediates. Probable biosynthetic steps for the formation of compound 20 are presented in Fig. S2. GGPP- geranylgeranyl diphosphate, *ent*-CPP- *ent*-copalyl diphosphate, DMAPP – dimethylallyl diphosphate, GA – gibberellin, CYP450 – cytochrome P450 monooxygenase, GT – glycosyltransferase, AT – acyltransferase, MT – methyltransferase.

mal). The most common variant is *ent*-CPP. Although, (+)- and *syn*-CPP-producing enzymes were also reported, so far enzyme that produces *syn-ent*-CPP has not been identified. However, diterpene natural products with *syn-ent* stereochemistry were recognized from plants of the *Calceolaria* genus [6]. Beside CPSs, other class II diTPS activities that produce LRDs with endocyclic double bond or oxygen-containing LRDs are also reported [7,9,10]. CPP is further cyclised and/or rearranged in a diphosphate ionization-