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HPPR encodes the hydroxyphenylpyruvate reductase required for the biosynthesis of hydrophilic phenolic acids in *Salvia miltiorrhiza*

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[ABSTRACT] *Salvia miltiorrhiza* is a medicinal plant widely used in the treatment of cardiovascular and cerebrovascular diseases. Hydrophilic phenolic acids, including rosmarinic acid (RA) and lithospermic acid B (LAB), are its primary medicinal ingredients. However, the biosynthetic pathway of RA and LAB in *S. miltiorrhiza* is still poorly understood. In the present study, we accomplished the isolation and characterization of a novel *S. miltiorrhiza* Hydroxyphenylpyruvate reductase (HPPR) gene, *SmHPPR*, which plays an important role in the biosynthesis of RA. *SmHPPR* contained a putative catalytic domain and a NAD(P)H-binding motif. The recombinant *SmHPPR* enzyme exhibited high HPPR activity, converting 4-hydroxyphenylpyruvic acid (pHPP) to 4-hydroxyphenyllactic acid (pHPL), and exhibited the highest affinity for substrate 4-hydroxyphenylpyruvate. *SmHPPR* expression could be induced by various treatments, including SA, GA_3, MeJA and Ag^+, and the changes in *SmHPPR* activity were correlated well with hydrophilic phenolic acid accumulation. *SmHPPR* was localized in cytoplasm, most likely close to the cytosolic NADPH-dependent hydroxypyruvate reductase active in photorespiration. In addition, the transgenic *S. miltiorrhiza* hairy roots overexpressing *SmHPPR* exhibited up to 10-fold increases in the products of hydrophilic phenolic acid pathway. In conclusion, our findings provide a new insight into the synthesis of active pharmaceutical compounds at molecular level.

[KEY WORDS] *Salvia miltiorrhiza*; Hydrophilic phenolic acids; Rosmarinic acid; Llithospermic acid B; Biosynthesis pathway

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Introduction

Salvia miltiorrhiza Bunge (*DanShen* in Chinese, Labiatae), a well-known herb in traditional Chinese medicine, is widely used in China, Japan, America, and European countries to treat a number of cardiovascular and cerebrovascular diseases [1]. The plant contains two primary classes of active pharmaceutical ingredients: lipid-soluble tanshinones and water-soluble phenolic acids, including rosmarinic acid (RA), an ester of caffeic acid, and 3, 4-dihydroxyphenyllactic acid, and its derivative lithospermic acid B (LAB) [2]. In Chinese folk medicine, the plant is usually prepared by extracting the dried *S.miltiorrhiza* root and rhizome with hot water; the hydrophilic phenolic acids of the plant have attracted attention because they are the main components in the resultant water decoction.

The biosynthesis of hydrophilic phenolic acids in plant is a very complex process composed of many distinct enzymatic steps, and several of the biosynthetic genes involved in this process have been cloned [3] (Fig. 1). The RA biosynthesis pathway was first elucidated in suspension cultures of *Coleus blumei* (Lamiaceae). The initial substrates, L-phenylalanine and L-tyrosine, are converted to 4-coumaroyl-CoA and 4-hydroxyphenyllactic acid (pHPL) via the phenylpropanoid

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pathway and the tyrosine-derived pathway, respectively. Tyrosine is transaminated by tyrosine aminotransferase (TAT), with 2-oxoglutarate as the cosubstrate, to 4-hydroxyphenylpyruvic acid (pHPP), which is then reduced tothecorresponding pHPL by hydroxyphenylpyruvate reductase (HPPR) [3-4]. HPPR catalyzes the last step of the tyrosine-derived pathway and competes for its substrate with 4-hydroxyphenylpyruvate dioxygenase (HPPD), which catalyzes the side-branch leading to the synthesis of tocopherol (Vitamin E) [5-8]. *CbHPPR* (EMBL accession number AJ507733) cloned from the suspension cells of *C. blumei* encodes a protein of 313 amino acid residues that belongs to the family of D-isomer- specific 2-hydroxyacid dehydrogenases. Heterologously expressed CbHPPR has been shown to catalyze the NAD(P)H-dependent reduction reactions of 4-hydroxyphenylpyruvate to 4-hydroxyphenyllactate and 3, 4-dihydroxyphenylpyruvate to 3, 4-dihydroxyphenyllactate [3-4]. Other enzymes involved in the biosynthesis of RA have also been researched in several other species of Lamiaceae and Boraginaceae [5, 7, 9-10]. A number of genes that participate in this metabolic process have been isolated as well [11]. However, neither the RA pathway nor the enzymes which involves is well characterized in *S. miltiorrhiza*, and it is also uncertain how RA is further metabolized into LAB. The present study was designed to investigates these issues.

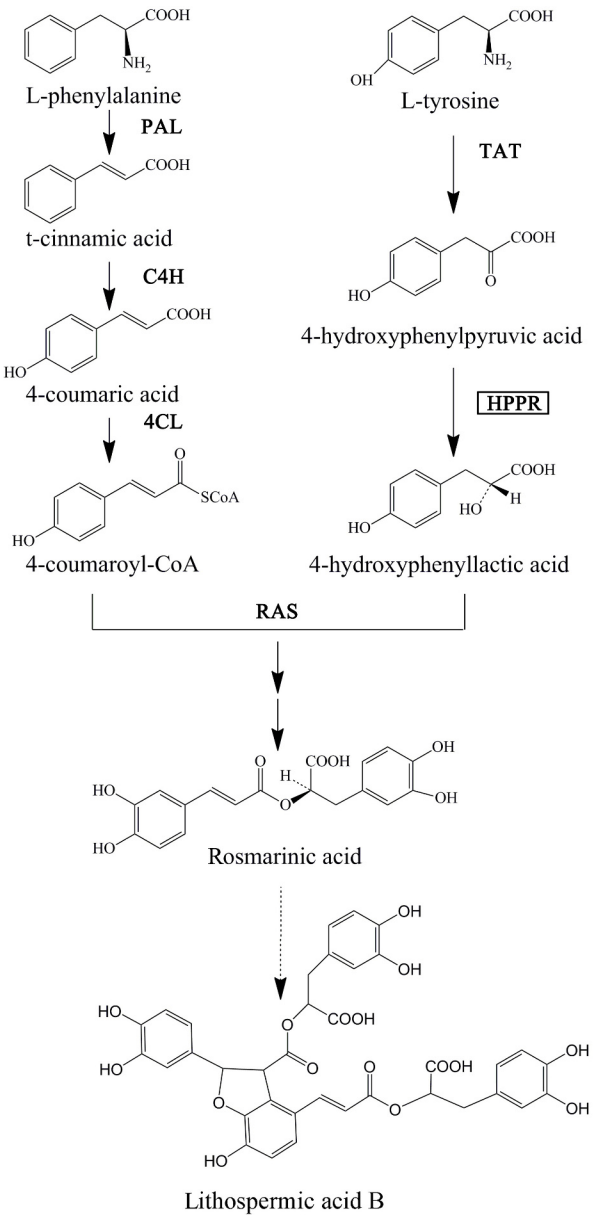

Fig. 1 Proposed biosynthesis pathway of hydrophilic phenolic acids in *Salvia miltiorrhiza*. The proposed enzymes involved are as follows: TAT, tyrosine aminotransferase; HPPR, 4-hydroxyphenylpyruvate reductase; RAS, rosmarinic acid synthase,hydroxycinnamoyl-CoA: hydroxyphenyllactatehydroxycinnamoyltransferase; PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid CoA-ligase. The double arrows before rosmarinic acid indicate that it is not formed by a one-step reaction. A dotted arrow indicates that a reaction is unclear and the enzyme involved in that step has not been identified

The accumulation of secondary metabolites, including various elicitors and signal molecules, is thought to aid plants in resisting stresses [12]. Methyl jasmonate (MeJA) is a signal compound thought to play an integral role as a second messenger in the elicitation process leading to the accumulation of secondary metabolites [13]; this compound has therefore been commonly used as an elicitor to explore the regulatory mechanisms underlying the biosynthesis of metabolites such as phenolic acid and alkaloids [14-16]. In *Lithospermum erythrorhizon* cell suspension cultures, elicitation by MeJA leads to a 10-fold stimulation of RA accumulation and a strong transient increase in HPPR activity, which was well correlated with the induction of RA accumulation [14]. In *C. blumei* suspension cultures, a fungal elicitor enhances RA accumulation by 3-fold, and the specific activities of PAL and rosmarinic acid synthase (RAS) are coordinately induced [15]. To gain new insights into the molecular mechanisms underlying RA and LAB biosynthesis in *S. miltiorrhiza*, we have undertaken differential display analyses of genes after the addition of MeJA [17] and Ag^+ [18] to *S. miltiorrhiza* hairy root cultures. Ag^+ does not stimulate RA accumulation but dramatically enhances LAB content, which reveals that a potential biosynthetic route from RA to LAB is extremely active when exposed to Ag^+.

In the present study, we cloned a *S. miltiorrhiza* hydroxyphenylpyruvate reductase, *SmHPPR*, and described its critical role in the biosynthesis of hydrophilic phenolic acids. Our data indicated that the recombinant *SmHPPR* enzyme exhibited high HPPR activity, reducing pHPP to pHPL. The expression of *SmHPPR* could be rapidly induced with abiotic treatment, concomitantly inducing LAB and RA biosynthesis. Cytosolic-localized *SmHPPR* might function similarly to the cytosolic NADPH-dependent hydroxypyruvate reductase (HPR) active in photorespiration. The overexpression of