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A phenylalanine ammonia-lyase ortholog (*Pk*PAL1) from *Picrorhiza kurrooa* Royle ex. Benth: Molecular cloning, promoter analysis and response to biotic and abiotic elicitors

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ABSTRACT

Picrorhiza kurrooa Royle ex Benth. is a highly reputed medicinal herb utilised in the preparation of a number of herbal drug formulations, principally due to the presence of novel monoterpene iridoid glycosides kenned as picrosides. Phenylalanine ammonia-lyase catalyses an important rate-limiting step in phenylpropanoid pathway and supplies precursors like cinnamic acid, vanillic acid, ferulic acid, etc., to a variety of secondary metabolites including picrosides. The imperilled status of *P. kurrooa* coupled with lack of information regarding biogenesis of picrosides necessitates deciphering the biosynthetic pathway for picrosides. In the present study, a PAL gene, designated *Pk*PAL1 was isolated from *P. kurrooa*. The cDNA is 2312 bp in length, consisting of an ORF of 2142 bp encoding for a 713 amino acid protein having a predicted molecular weight of 77.66 kDa and an isoelectric point of pH 6.82. qRT-PCR analysis of various tissues of *P. kurrooa* showed that *Pk*PAL1 transcript levels were highest in the leaves, consistent with picroside accumulation pattern. Using Genome walking, a 718 bp promoter region was also isolated resulting in identification of distinct *cis*-regulatory elements including TGA-element, TGACG-motif, CGTCA-motif, etc. qRT-PCR indicated up-regulation of *Pk*PAL1 by methyl jasmonate, salicylic acid, 2,4-dicholorophenoxy acetic acid and UV-B elicitations that corroborated positively with the identified *cis*-elements within the promoter region. Moreover, altitude was found to have a positive effect on the *Pk*PAL1 transcript levels, driving the expression of *Pk*PAL1 abundantly. Based on docking analysis, we identified eight residues as potentially essential for substrate binding in *Pk*PAL1.

1. Introduction

Picrorhiza kurrooa Royle ex Benth. (Plantaginaceae) is a highly endangered, perennial medicinal herb, endemic to the North Western Alpine Himalayas and found at an altitude of 2800–4800 m (Bhat et al., 2012b). *P. kurrooa* is highly valued in Ayurvedic system of medicine and has been used traditionally to treat liver ailments, dyspepsia, chronic diarrhoea and upper respiratory tract ailments. In modern medicine, it is being used in the treatment of hepatic disorders, gastric troubles, anaemia, asthma and pregnancy related problems. The biological activity of *P. kurrooa* is attributed to novel monoterpene iridoid derived secondary metabolites known as picrosides which include picroside-I, picroside-II and other metabolites like picroside-III, picroside-IV, apocynin, androsin, catechol, and kutkoside (Stuppner and Wagner, 1989). Picrosides have been shown to exhibit a vast array of pharmacological properties that include hepato-protective, anti-inflammatory, immuno-modulatory, cardio-protective, neuro-protective and anti-carcinogenic effects (Girish and Pradhan, 2012; Meng et al., 2012; Rajkumar et al., 2011; Sidiq et al., 2011).

Despite numerous medicinal properties attributed to picrosides, their biosynthetic pathway is unknown. Picrosides are basically monoterpene iridoid glycosides, biosynthesised by the isoprenoid pathway through the precursor geranyl diphosphate (Gahlan et al., 2012). Although some recent work has been initiated mainly aimed at unravelling the pathway of picroside biosynthesis (Bhat et al., 2012a, 2013, 2014; Gahlan et al., 2012; Kawoosa et al., 2010; Kumar et al., 2013; Pandit

Abbreviations: RACE, rapid amplification of cDNA ends; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; MeJA, methyl jasmonate; SA, salicylic acid; 2,4-D, 2,4-dicholorophenoxy acetic acid; TSS, transcriptional start site; UV-B, ultraviolet-B; UTR, untranslated region; *ges*, geraniol synthase; *g10h*, geraniol 10-hydroxylase; *cpr*, cytochrome P450 reductase; *pal*, phenylalanine ammonia-lyase; *c4h*, cinnamoyl 4-hydroxylase; *c3h*, coumaric acid 3-hydroxylase; *caomt*, caffeic acid o-methyltransferase.

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et al., 2012; Singh et al., 2013), however, various biosynthetic steps leading to the formation of picrosides still remain largely unresolved. The prevailing concept is that the precursor iridotrial is cyclized to a limited number of core structures, which are subsequently decorated with functional groups, through a series of oxidation and cyclization steps followed by condensation of glucose moiety, leading to the formation of a common core precursor known as catalpol (Kumar et al., 2013). The catalpol backbone is further modified by esterification with different phenolic acids including cinnamic acid, vanillic acid, p-coumaric acid, caffeic acid, benzoic acid or ferulic acid, etc., at R1, R2 and R3 positions, respectively, resulting in the formation of picrosides and other associated metabolites (Fig. 1).

Esterification of catalpol with different phenolic acids is one of the final and crucial steps in the biosynthesis of picrosides. Pertinently, the first step necessary for the synthesis of the phenolic skeletons and other phenylpropanoids in higher plants is catalysed by a crucial enzyme known as phenylalanine ammonia-lyase (PAL, EC 4.3.1.5). PAL is commonly considered the principal enzyme in the biosynthesis of phenolic compounds. PAL catalyses the deamination of the L-phenylalanine, giving rise to trans-cinnamic acid and ammonium (Dixon and Paiva, 1995). Cinnamic acid is further modified by an array of enzymes, leading to the production of phenolic acids including vanillic acid, benzoic acid, ferulic acid etc. as well as numerous flavonoids and isoflavonoids (Dixon and Paiva, 1995). PAL links primary and secondary metabolism and is also a rate-limiting step of phenylpropanoid metabolism (MacDonald and D'Cunha, 2007). PAL is one of the most extensively studied enzymes in plants with reference to its crucial function in the biosynthesis of various secondary metabolites. Research on PAL has attracted quite a lot of attention, primarily because PAL plays a key role in connecting plant primary metabolism and phenylpropanoid metabolism and is also involved in the biosynthesis of signalling molecules. PAL genes have been identified from different angiosperm species, such as *Petroselinum crispum*, *Phyllostachys edulis*, and *Rhus chinensis* (Gao et al., 2012; Ma et al., 2013).

In *P. kurrooa*, final modification of the catalpol is dependent on the PAL enzyme for supplying the necessary precursors since biosynthesis of phenolic moieties is tightly regulated by phenylalanine ammonia-lyase. However, not a single full-length PAL isoform has been reported from *Picrorhiza* so far. Moreover, many studies have indicated that PAL influences the biosynthesis of plant secondary metabolites including alkaloids, flavonoids, and phenolic acids, and the synthesis of these metabolites increases with the increase in activity of PAL (Jin et al., 2013). However, the influence of PAL on the growth of *P. kurrooa* has not yet

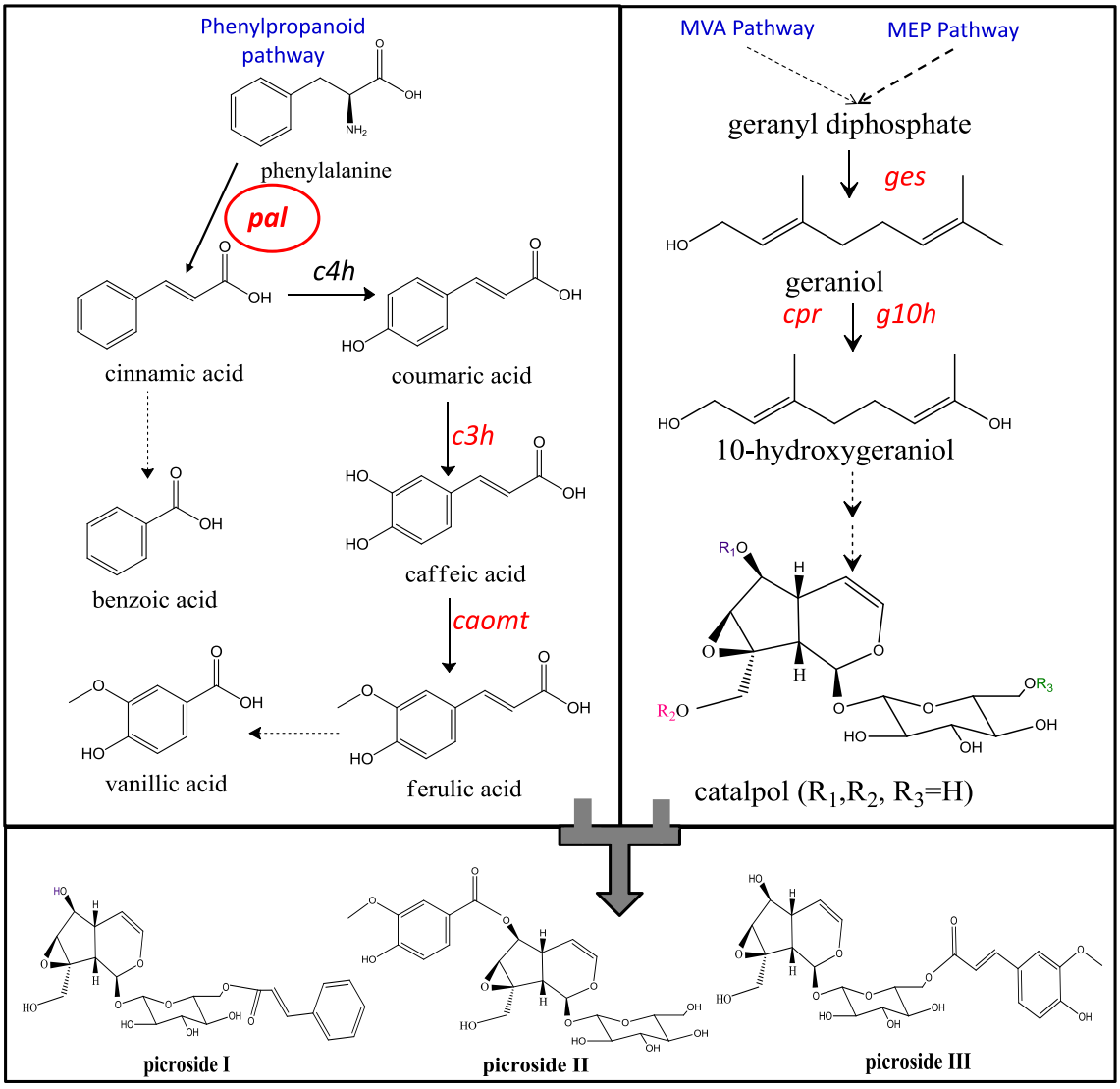

Fig. 1. Picroside biosynthetic pathway in *Picrorhiza kurrooa*. Picrosides are iridoid glycosides, synthesised both from a common precursor known as catalpol, via cytoplasmic MVA and plastidic MEP pathways. Catalpol backbone is modified by esterification of R1, R2 or R3 groups, with various phenolic moieties like cinnamic acid, vanillic acid, p-coumaric acid, caffeic acid, and benzoic acid, resulting in the formation of P-I, P-II and other metabolites in *P. kurrooa*. *ges*: Geraniol synthase; *g10h*: Geraniol 10-hydroxylase; *cpr*: Cytochrome P450 reductase; *pal*: Phenylalanine ammonia-lyase; *c4h*: Cinnamoyl 4-hydroxylase; *c3h*: Coumaric acid 3-hydroxylase; *caomt*: Caffeic acid o-methyltransferase. Dashed lines represent multiple steps.