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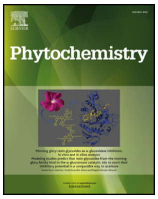

A cytochrome P450 monooxygenase responsible for the C-22 hydroxylation step in the *Paris polyphylla* steroidal saponin biosynthesis pathway

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ABSTRACT

Polyphyllins are the major steroidal saponin components of *Paris polyphylla*, the main source plant of the common Chinese herbal medicine Paridis Rhizoma with strong pharmacological activity and extremely high economic value and great market prospects. However, the production of polyphyllins in plants is limited, and their biosynthesis pathway has not been reported. The downstream hydroxylation step was particularly unclear. To clarify the enzymes and intermediates involved in the downstream steps of polyphyllin biosynthesis, we performed a comparative transcriptome analysis and discovered a cytochrome P450 gene that encodes a protein with monooxygenase activity. Heterologous expression in *Saccharomyces cerevisiae* demonstrated that it encodes an enzyme that catalyzes the formation of 22(R)-hydroxycholesterol from cholesterol. The relative gene expression measured by RT-PCR and polyphyllin contents measured by HPLC in *P. polyphylla* roots at different ages confirmed that this gene is involved in polyphyllin biosynthesis. To our best knowledge, this is the first report on the cloning of a CYP450 enzyme gene from the steroidal saponin pathway of higher plants.

1. Introduction

Paris polyphylla SMITH var. *yunnanensis* (FRANCH.) HARA is the main source plant of the common Chinese herbal medicine called Paridis Rhizoma. The major active components of this plant are steroidal saponins called polyphyllins, which account for about 80% of the total number of active compounds (Jagmohan et al., 2014). Studies have shown that polyphyllins have strong pharmacological effects, including antitumor activity (Man et al., 2011; Yan et al., 2009), immuno-stimulating properties (Zhang et al., 2007), anthelmintic activity (Wang et al., 2010) antimicrobial activity (Qin et al., 2012) and protective effects on ethanol- or indomethacin-induced gastric mucosal lesions (Matsuda et al., 2003). The Chinese medicine “Yunnan BaiYao”, “GongXueNing” and “ReDuQing”, which are made from the plant's rhizome as the main raw material have clinically significant effects in the treatment of functional uterine bleeding, neurodermatitis, surgical inflammation and tumors, giving it extremely high economic value and great market prospects (Wu et al., 2004). At present, the wild plant is the only source of Paridis Rhizoma. However, the seeds of *P. polyphylla* have secondary physiological dormancy (Ye et al., 2011) and the plant's reproductive rate is low (Chen et al., 2007). Moreover, the natural growth rate of rhizomes is also very slow, so that it usually takes 10–15 years from seeds to medicinal products, which severely restricts the production of Paridis Rhizoma, resulting in a large market gap. Thus, improving the availability of the plant resources of *P. polyphylla* or finding ways to efficiently accumulate or produce polyphyllins through biotechnological means is an urgent problem that needs to be solved.

The sapogenins constituting the steroidal saponins of *P. polyphylla* are dominated by diosgenin and pennogenin, with diosgenin being the main component (Jagmohan et al., 2014). In the 1960s, steroidal sapogenins began to receive extensive attention as raw materials for the synthesis of steroidal contraceptives and hormonal drugs (Guclu and Mazza, 2007). Later on, increasing numbers of interesting physiological activities of steroidal saponins or sapogenins have been found (Guclu and Mazza, 2007; Liu et al., 2015). Furthermore, about 300 different steroidal hormone drugs are synthesized starting from diosgenin, and the global demand for diosgenin is second only to that of antibiotics, which prompted the manufacturing of diosgenin to become the pharmaceutical industry's second-largest production field (Qiu et al., 2011).

There are two upstream pathways for the biosynthesis of steroidal saponins (Fig. 1): the cytoplasmic mevalonate (MVA) pathway and the plastidic 2-C-methyl-D-erythritol- methl-D-erythritol-4-phospate (MEP)

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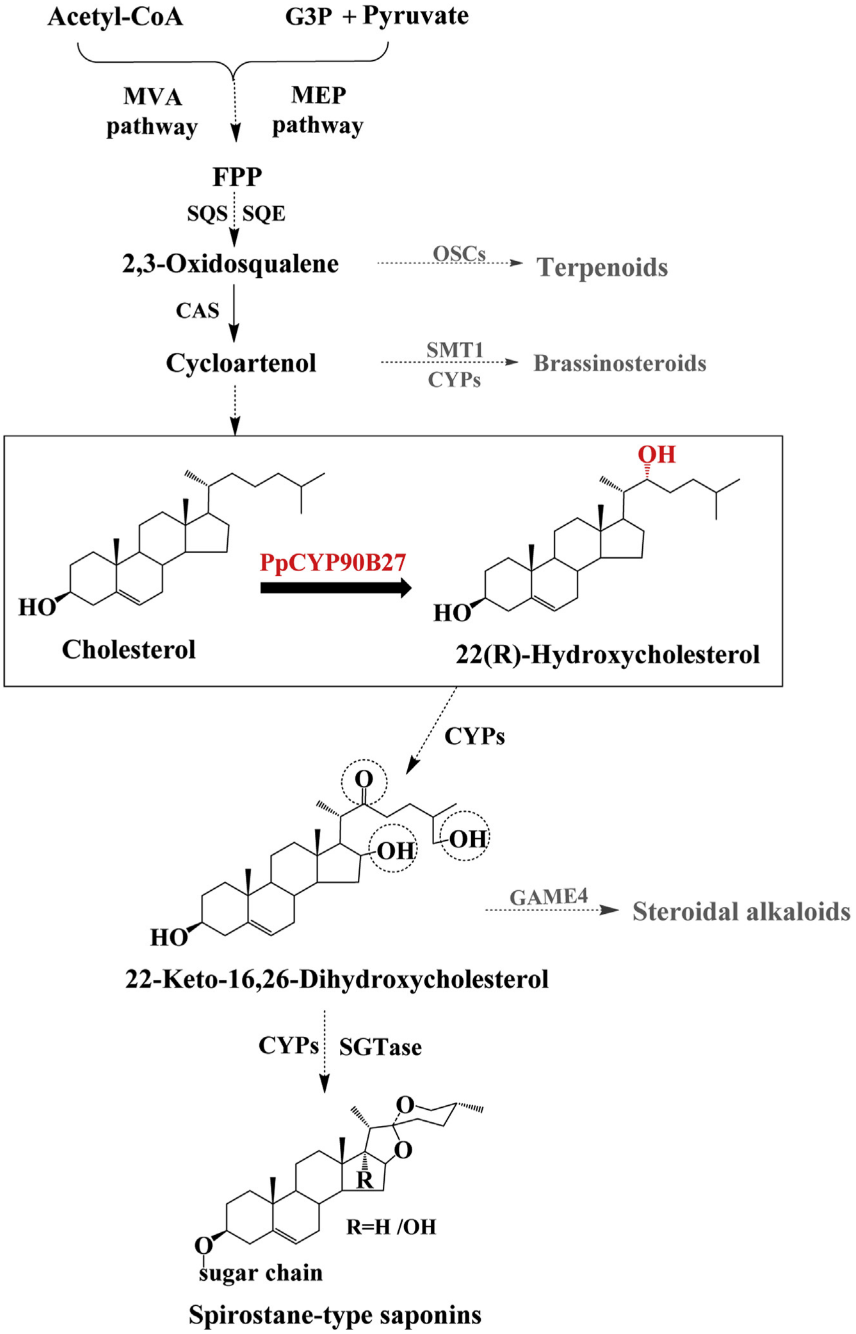

Fig. 1. Biosynthetic pathway of spirostane-type saponins in plants. The solid- and dashed lines indicate single- and multiple catalytic steps, respectively. The enzyme shown in red was identified in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pathway, whereby the MVA pathway plays a leading role (Wang et al., 2015). Early work on the biosynthesis pathways of steroidal saponins had focused on the Dioscoreaceae, and cholesterol was proved to be the precursor of diosgenin in 1965 (Eich, 2008). In higher plants and algae, the alkylated substrate cycloartenol undergoes a series of modifications, including oxidations and reductions, to form cholesterol (Cardenas et al., 2015; Lu et al., 2014; Wang et al., 2013). The side-chain of cholesterol undergoes a cyclization to form a hemiketal after C-22, C-26, and C-16 hydroxylation and other reactions, further generating steroidal sapogenins with a spiroketal structure, such as diosgenin (Ohnishi et al., 2009).

At present, the research on the downstream pathway of biosynthesis of steroids in plants is mainly focused on steroidal alkaloids and brassinosteroids. It was reported that silencing the *GAME4* gene via RNA interference in tomato and potato resulted in a decrease of the production of steroid alkaloids and the accumulation of the steroidal saponin uttroside B. Similarly, downregulation of the *GAME11* gene via virus-induced gene silencing (VIGS) was also shown to lead to the accumulation of an array of cholestatin-type steroidal saponins (Itkin et al., 2013). The same study also suggested that the biosynthetic pathways of steroidal saponins and -alkaloids partially overlap and branch, whereby GAME4 plays an important role. The genes encoding the C27 sterol C-22 hydroxylase mainly belong to the CYP85 clade in the phylogenetic tree of plant CYP450s (Ghosh, 2017). These include *AtCYP90B1* (Fujita et al., 2006), *LeCYP90B3* and *LeCYP724B2* (Ohnishi et al., 2006), which are responsible for the early C-22 hydroxylation in the brassinosteroid biosynthesis pathway, as well as *VcCYP90B27*, which is responsible for the C-22 hydroxylation in the steroidal alkaloid