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Research article

Functional characterization of a Mg^{2+}-dependent *O*-methyltransferase with coumarin as preferred substrate from the liverwort *Plagiochasma appendiculatum*

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ABSTRACT

Coumarins (1,2-benzopyrones), which originate via the phenylpropanoid pathway, are found ubiquitously in plants and make an essential contribution to the health of the plant. Some natural coumarins have been used as human therapeutics. However, the details of their biosynthesis are still largely unknown. Scopoletin is derived from either esculetin or feruloyl CoA according to the plant species involved. Here, a gene encoding a *O*-methyltransferase (PaOMT2) was isolated from the liverwort species *Plagiochasma appendiculatum* (Aytoniaceae) through transcriptome sequencing. The purified recombinant enzyme catalyzed the methylation of esculetin, generating scopoletin and isoscopoletin. Kinetic analysis shows that the construct from the second Met in PaOMT2 had a catalytic efficiency for esculetin (K_{cat}/K_m) of about half that of the full length PaOMT2, while the K_ms of two enzymes were similar. The catalytic capacities of the studied protein suggest that two routes to scopoletin might co-exist in liverworts in that the enzyme involved in the methylation process participates in both paths, but especially the route from esculetin. The transient expression of a PaOMT2-GFP fusion in tobacco demonstrated that PaOMT2 is directed to the cytoplasm.

1. Introduction

Coumarins are derived from 1,2-benzopyrones and may be classified as simple coumarins, 7-oxygenated coumarins, furanocoumarins and pyranocoumarins (Estévez-Braun and González, 1997; Murray, 1991; Murray et al., 1982). These molecules are widely distributed in plants, both in the free form and as glycosides, and are commonly found in families such as the Umbelliferae/Apiaceae and Rutaceae (Dewick, 2009). They contribute vitally to the health of plants being involved in processes such as defense against phytopathogens, response to abiotic stresses, regulation of oxidative stress, and probably hormonal regulation (Bourgaud et al., 2006). Coumarins also receive attention for their diverse bioactivities. Some natural coumarins have been used as human therapeutics. Dicoumarol is a prominent example with blood anticoagulant properties (Mueller, 2004), and methoxsalen is used medically to facilitate skin repigmentation in vitiligo and other conditions (Dewick, 2009).

Scopoletin is a prevalent methoxylated coumarin, and has been reported from many plants, e.g. tobacco (Fraissinet-Tachet et al., 1998; Maier et al., 2000), sunflower (Cabello-Hurtado et al., 1998; Gutierrez et al., 1995) and rubber tree (Giesemann et al., 1986; Silva et al., 2002). According to the literature, it has two hypothesized biosynthetic pathways, with methylation occurring either before or after lactonization (Fig. 1). Coumarins originate from the general phenylpropanoid pathway in plants, along with many other compounds, including lignins. Despite their importance for plants and their pharmacological activity, major details of their biosynthesis are still largely unknown. Tracer experiments conducted with *Lavandula officinalis* indicated that umbelliferone is derived from 4-coumaric acid which is converted from cinnamic acid by cinnamate 4-hydroxylase (Teutsch et al., 1993). The 4-coumaric acid is *ortho*-hydroxylated to 2,4-dihydroxycinnamic acid, followed by trans/cis isomerization in the side-chain and then lactonization to form umbelliferone. Esculetin and scopoletin, with additional oxygen substituents on the aromatic ring, appear to be derived by modification of umbelliferone (Dewick, 2009). Umbelliferone is readily incorporated by *Daphne mezereum*, thus making

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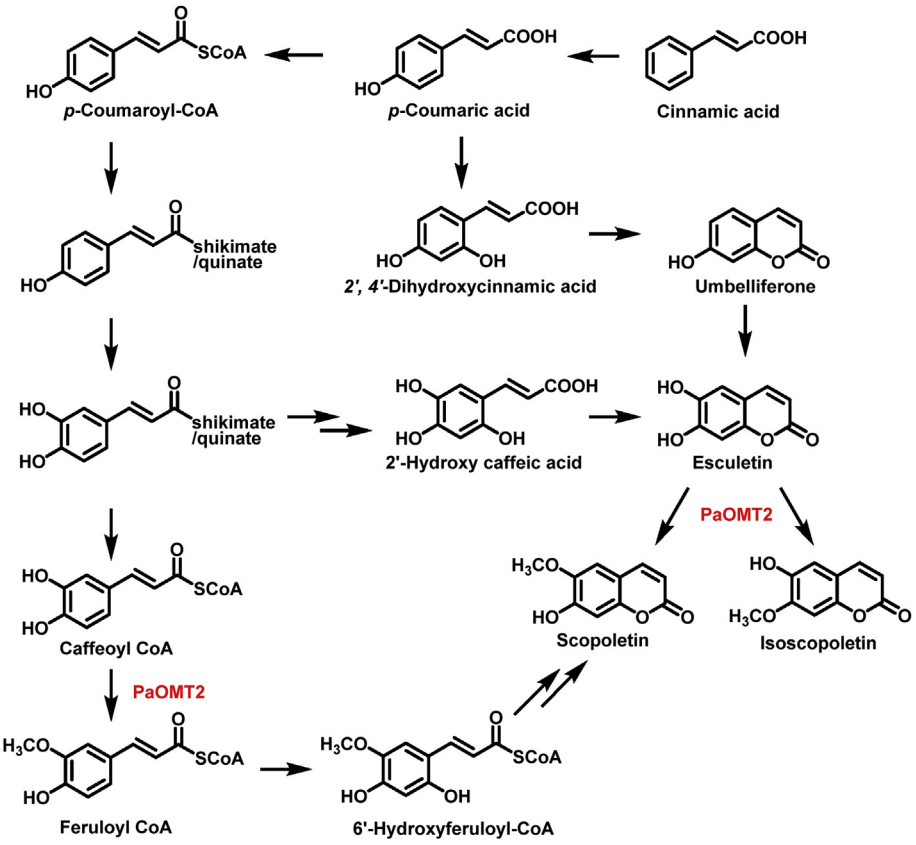

Fig. 1. Proposed two routes of biosynthesis of scopoletin via the phenylpropanoid pathway from esculetin and feruloyl CoA.

esculetin a likely precursor for the synthesis of scopoletin (Brown, 1986). Another biosynthetic pathway, forming scopoletin via feruloyl CoA has been established recently in *Arabidopsis thaliana* (Kai et al., 2006). T-DNA insertion mutants within the gene encoding CYP98A3, which catalyzes 3′-hydroxylation of *p*-coumarate, give rise to a large decrease in both scopoletin and scopolin contents, confirming their synthesis from feruloyl CoA in Arabidopsis. In addition, two 2-oxoglutarate-dependent dioxygenases which exhibited *ortho*-hydroxylation activity toward feruloyl CoA to form scopoletin were found to be involved in coumarin formation in sweet potato (Matsumoto et al., 2012). Thus scopoletin is formed from either esculetin or feruloyl CoA depending on the plant species. A wide range of *O*-methyltransferases involved in secondary metabolism have been characterized from several plant species, including MsCCoAOMT (CCoAOMT from *Medicago sativa*, AAC28973.1), McPFOMT (PFOMT from *Mesembryanthemum crystallinum*, AY145521.1) and SmCOMT (COMT from *Selaginella moellendorffii*, GQ166949.1) (Ferrer et al., 2005; Ibdah et al., 2003; Weng et al., 2011). Some of them, like SOMT-9 (OMT from *Glycine max*, TIGR Accession number: TC178411) and McPFOMT (Kim et al., 2006; Ibdah et al., 2003) convert esculetin to its corresponding methylated products, but with a much lower catalytic efficiency compared with the optimal substrate.

As basal higher plants, liverworts produce a great number of natural products via the phenylpropanoid pathway, including coumarins (Jung et al., 1994), lignin (Espiñeira et al., 2011) and flavonoids (Lou et al., 2002). Here, we describe the isolation and functional characterization of a cation-dependent OMT-encoding gene from the liverwort species *Plagiochasma appendiculatum*. The recombinant enzyme (denoted PaOMT2) converted esculetin to scopoletin and isoscopoletin with a much higher conversion rate compared to the enzyme PaOMT1 (Xu et al., 2015), although they share a high sequence identity. The sequence basis of the substrate selectivity of PaOMT1 and PaOMT2 was investigated. A subcellular localization experiment demonstrated that PaOMT2 was directed to the cytoplasm.

2. Materials and methods

2.1. Plant materials and chemicals

P. appendiculatum plants were grown in a greenhouse under a 12-h-light/12-h-dark photoperiod and a temperature of about 25 °C. They were propagated via gemmae and sexual crossing. *Nicotiana benthamiana* plants were grown on soil in a conditioned growth chamber at 24 °C/22 °C and under a 12-h/12-h light/dark regime for 5–6 weeks. Unless otherwise noted, all substrates (esculetin, phenylpropanoids, flavonoids and their methylated products), SAM and other chemicals were purchased from either Chengdu Must Bio-technology (Chengdu, China) or Sigma-Aldrich (St. Louis, USA). Caffeoyl aldehyde, caffeoyl alcohol, 5-hydroxyconiferyl aldehyde and 5-hydroxyconiferyl alcohol were prepared as described previously (Kim et al., 2004; Sun et al., 2013). Caffeoyl CoA was synthesized from caffeic acid using liverwort 4-coumarate CoA ligase (Gao et al., 2015), and purified using an LC-18 SPE tube (Supelco, Bellefonte, USA) (Beuerle and Pichersky, 2002). The product was checked for quality by spectrophotometric assay.

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was extracted using a CTAB-based method from two