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Research article

The isolation and functional characterization of three liverwort genes encoding cinnamate 4-hydroxylase

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ABSTRACT

The plant phenylpropanoid pathway is responsible for the synthesis of a wide variety of secondary metabolites. The second step in phenylpropanoid synthesis is carried out by the cytochrome P450 monooxygenase enzyme cinnamate 4-hydroxylase (C4H), which catalyzes the *p*-hydroxylation of *trans*-cinnamic acid to *p*-coumarate. Genes encoding C4H have been characterized in many vascular plant species, but as yet not in any bryophyte species. Here, a survey of the transcriptome sequences of four liverwort species was able to identify eight putative *C4Hs*. The three liverwort *C4H* genes taken forward for isolation and functional characterization were harbored by *Plagiochasma appendiculatum* (*PaC4H*) and *Marchantia paleacea* (*MpC4H1* and *MpC4H2*). When the genes were heterologously expressed in yeast culture, an assay of enzyme activity indicated that PaC4H and MpC4H1 had a higher level of activity than MpC4H2. The favored substrate (*trans*-cinnamic acid) of all three liverwort C4Hs was the same as that of higher plant C4Hs. The co-expression of *PaC4H* in yeast cells harboring *PaPAL* (a *P. appendiculatum* ene encoding phenylalanine ammonia lyase) allowed the conversion of L-phenylalanine to *p*-coumaric acid. Furthermore, the expression level of *PaC4H* was enhanced after treatment with abiotic stress inducers UV irradiation or salicylic acid in the thallus of *P. appendiculatum*. The likelihood is that high activity *C4Hs* evolved in the liverworts and have remained highly conserved across the plant kingdom.

1. Introduction

The plant phenylpropanoid pathway is responsible for the synthesis of many important secondary metabolites; its core reactions involve the enzymes phenylalanine ammonia lyase (PAL; EC 4.3.1.5), cinnamate 4-hydroxylase (C4H; EC 1.14.13.11) and 4-coumarate:coenzyme A ligase (4CL; EC 6.2.1.12) (Hahlbrock and Scheel, 1989). These three enzymes direct carbon flux to the flavonoids, lignins, hydroxycinnamic esters and coumarins (Fig. 1), compounds which contribute to plants' structural components, pigments, antioxidants, signaling molecules, UV protection, antibiotics and anti-insect defense (Morant et al., 2003; Taylor and Grotewold, 2005; Weisshaar and Jenkins, 1998). C4H, a member of the CYP73 group of cytochrome P450 monooxygenases, acts together with NADPH-P450 reductase and NADPH to catalyze the first oxidative reaction in the pathway, namely the conversion of *trans*-cinnamic to *p*-coumaric acid. The isolation of *C4Hs* has been hampered by the lability of the P450s and their tendency to be membrane-bound. The first *C4H* to be isolated was obtained from *Helianthus tuberosus* (the Jerusalem artichoke) by Teutsch et al. (1993), but since this time, *C4H* cDNA and gDNA sequences have been acquired from a number of other plant species. Some species have proven to harbor only a single copy of *C4H* (Bell-Lelong et al., 1997; Koopmann et al., 1999), whereas others harbor a small gene family (Betz et al., 2001; Chen et al., 2007a; Kawai et al., 1996; Ni et al., 2014; Potter et al., 1995). While the physiological substrate of C4H is *trans*-cinnamic acid, it can also process a number of other substrates (Schalk et al., 1997a, 1997b; Urban et al., 1994): these all share a planar structure, a negatively charged side chain and a size of less than two aromatic rings (Schoch et al., 2003).

Growing concerns over limited resources and associated environmental problems are encouraging the development of sustainable processes for the production of economically significant compounds. The liverworts, a class of primitive land plants, produce a range of hydrophobic terpenoids and aromatic compounds,

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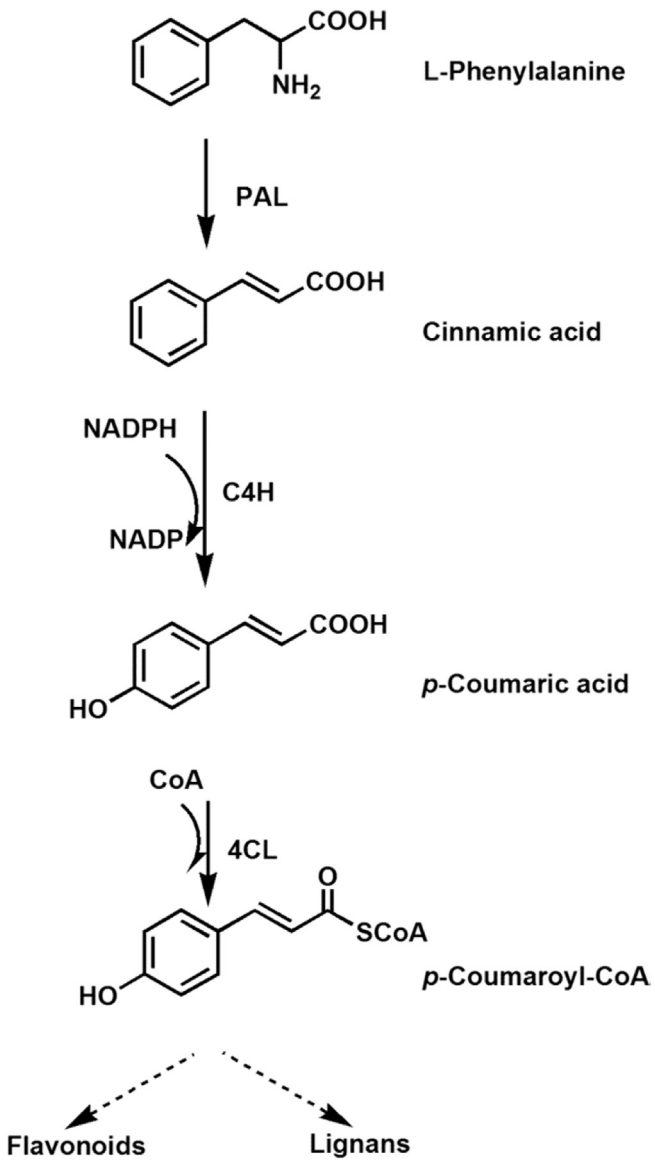

Fig. 1. The phenylpropanoid pathway in liverworts. C4H catalyzes the hydroxylation of *trans*-cinnamic to *p*-coumaric acid. PAL: phenylalanine ammonia lyase, C4H: cinnamate 4-hydroxylase, 4CL: 4-coumarate:coenzyme A ligase. These steps are common in the synthesis of flavonoids, monolignols and lignans.

many of which have been associated with useful properties (Asakawa et al., 2012; Lou et al., 2002; Qu et al., 2007; Xie et al., 2010). Genes encoding both PAL and 4CL have been cloned and characterized from the liverwort species *Plagiochasma appendiculatum* (Gao et al., 2015; Yu et al., 2014), but as yet none have been described for *C4Hs*. Here, the transcriptome sequences of four liverwort species were exploited to identify eight putative *C4H* genes, and three of these were isolated and functionally characterized. The expression of *C4H* gene in *P. appendiculatum* was also measured with the abiotic stress inducers treatment.

2. Materials and methods

2.1. Plant material, reagents and nucleic acid extraction

P. appendiculatum and *Marchantia paleacea* thallus were grown under a 12 h photoperiod. Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Total RNA, extracted from two month old snap-frozen tissue using a CTAB-based protocol (Gambino et al., 2008), was used as the template for cDNA synthesis based on a PrimeScript™ RT Master Mix (Takara, Otsu, Japan), following the supplier's protocol.

2.2. Cloning and sequence analysis of liverwort C4H cDNAs

Inspection of the transcriptome sequences from *P. appendiculatum* (SRP073827), *M. paleacea* (SRP078650), *Marchantia emarginata* (SRP078649) and *Conocephalum japonicum* (SRP078647) revealed eight distinct sequences as putatively encoding C4H. A phylogenetic analysis, based on the neighbor-joining method implemented in MEGA v4.0 software (Tamura et al., 2007), was used to select three of these, one harbored by *P. appendiculatum* (designated *PaC4H*) and two by *M. paleacea* (*MpC4H1* and *MpC4H2*), for functional characterization. Their full length cDNA sequences were amplified using the primer pairs PaC4H-F/R, MpC4H1-F/R and MpC4H2-F/R (sequences given in Table 1). Their deduced polypeptide sequences were aligned with those of other plant C4Hs with the help of DNAMAN v7 software (Lynnon Biosoft, Quebec, Canada).

2.3. Recombinant protein expression and microsomes preparation

The pYeDP60 expression vector and yeast strain WAT11 were used to heterologously express each of the three liverwort *C4H* genes. The three open reading frames (ORFs) were amplified from their corresponding cDNA clone using primer pairs PaC4H-DPF/R, MpC4H1-DPF/R, MpC4H2-DPF/R (Table 1), each primer pair with a BamHI and EcoRI site. After digestion, the resulting fragments were ligated into the appropriate pYeDP60 cloning site. The constructs were introduced into WAT11 using the polyethylene glycol-LiOAc method (Gietz et al., 1992). Transformants were selected using an SD-Ade drop-out medium (SD medium without adenine). Positive clones were verified by PCR. The methods used to culture yeast cells, to induce heterologous expression and to prepare microsomes were as described by Urban et al. (1994), with minor modifications. Buffer A was insteaded by 100 mM potassium phosphate (pH 8.0), 400 mM sucrose, 14 mM 2-mercapto-ethanol, 1 mM PMSF, 1× protein inhibitor cocktail and ultra-centrifugation at 100,000 g was used for 90 min. The pellets were finally re-suspended in 100 mM potassium phosphate pH 8.0, 400 mM sucrose, 0.5 mM glutathione, 10% (v/v) glycerol. Each sample was divided into 50 μL aliquots and kept at −80 °C until required.

Table 1
Primer sequences used in this research.

Primer name	Primer sequences (5′-3′)
PaC4H-F	GATCTCGTCTTCTCTTGGGCTC
PaC4H-R	GTATAGTGCTCACAACCTCGGT
MpC4H1-F	AGGGTTGAGTGGGGTTCT
MpC4H1-R	CAGTCGGAAAACTCTGCT
MpC4H2-F	GCCTGCTAAGTGGGAAAG
MpC4H2-R	AAAGGAGTCCGGATCAGA
PaC4H-DPF	CGGGATCCATGGGGACCGGGTTCTCGAC
PaC4H-DPR	CGCGGTCTCGAATTCTCAGTCTGATCTCGGTTTGA
MpC4H1-DPF	CGCCGTCTCGGATCCATGCTCACCCTGCAAAATGT
MpC4H1-DPR	GGAATTCTCAATCGGCCCTGGGTTTCA
MpC4H2-DPF	CGGGATCCATGGTAGATCAATTAGAAAG
MpC4H2-DPR	CGCGGTCTCGAATTCTCAGTCCTTCCTATCTTTAA
PaPAL-TRPF	ATAAGAATGCGGCCGCATGGCGGCGATGGTGATGGA
PaPAL-TRPR	CGACTAGTTCACACATCAATTGGGATGG
PaC4H-LEUF	ATAAGAATGCGGCCGCATGGGGACCGGGTTCTCGAC
PaC4H-LEUR	CGACTAGTTCAGTCTGATCTCGGTTTGA
PaC4H-RTF	GAGCACTGGAGAAGAATGAG
PaC4H-RTR	GCTGCAACCTTCTTCTGATG