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ORIGINAL PAPER

RNAi suppression and overexpression studies of hydroxyphenylpyruvate reductase (*HPPR*) and rosmarinic acid synthase (*RAS*) genes related to rosmarinic acid biosynthesis in hairy root cultures of *Coleus blumei*

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Abstract Hydroxyphenylpyruvate reductase (HPPR) and rosmarinic acid synthase (RAS) have been described as enzymes involved in rosmarinic acid (RA) biosynthesis. In this study, elucidating the role of HPPR and RAS in the formation of RA was pursued. For this purpose, hairy root lines of *Coleus blumei* carrying *HPPR* or *RAS* RNAi suppression and overexpression constructs were established using *Agrobacterium rhizogenes*-mediated transformation. Transgenic lines were evaluated for RA accumulation. Hairy root lines showing undetectable HPPR or RAS mRNA levels displayed strongly reduced RA contents with reduction of up to 92 % compared to control hairy root lines. An increase of RA levels up to 176 % was shown in overexpression hairy root lines with equally increased *HPPR* transcript levels while *RAS* overexpression resulted in co-suppression effects with reduced RA levels. These observations support the participation of HPPR and RAS in RA biosynthesis.

Keywords *Coleus blumei* · RNAi · Overexpression · Rosmarinic acid · Hydroxyphenylpyruvate reductase · Rosmarinic acid synthase

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Introduction

Rosmarinic acid (RA), an ester of caffeic acid and 3.4-dihydroxyphenyllactic acid, is one of the most common caffeic acid esters found in the plant kingdom (Petersen et al. 2009). Hydroxyphenylpyruvate reductase (HPPR) and rosmarinic acid synthase (RAS) have been detected and described as enzymes involved in the biosynthesis of RA in *Coleus blumei* Benth. (syn. *Solenostemon scutellarioides* (L.) Codd, Lamiaceae; Fig. 1; Petersen and Alfermann 1988; Häusler et al. 1991; Petersen 1991, 1997; Petersen et al. 1993, 2009) and other species of this family (e.g. *Melissa officinalis*; Weitzel and Petersen 2011). HPPR is responsible for the NAD(P)H-dependent reduction of 4-hydroxyphenylpyruvate, which results from the transamination of L-tyrosine, to D-4-hydroxyphenyllactate (pHPL). pHPL is then *trans*-esterified with a 4-coumaroyl moiety from 4-coumaroyl-CoA by RAS which yields an ester (4-coumaroyl-4′-hydroxyphenyllactate) that is subsequently hydroxylated to RA by cytochrome P450 monooxygenase reactions (Petersen et al. 1993; Petersen 1997).

Addition of a fungal elicitor from *Pythium aphanidermatum* to suspension cultures of *Coleus blumei* enhances RA accumulation as well as HPPR and RAS activities while the addition of methyl jasmonate increased HPPR activity but left RAS activity largely unaffected (Szabo et al. 1999). Kim et al. (2004) purified the HPPR protein for peptide sequencing and isolated a cDNA encoding a protein with HPPR-activity from *Coleus blumei* (EMBL accession number AJ507733; *CbHPPR*). The RAS protein was similarly purified for peptide sequencing from suspension cultures of *Coleus blumei* and the RAS cDNA cloned by Berger et al. (2006) (EMBL accession number AM283092; *CbRAS*).

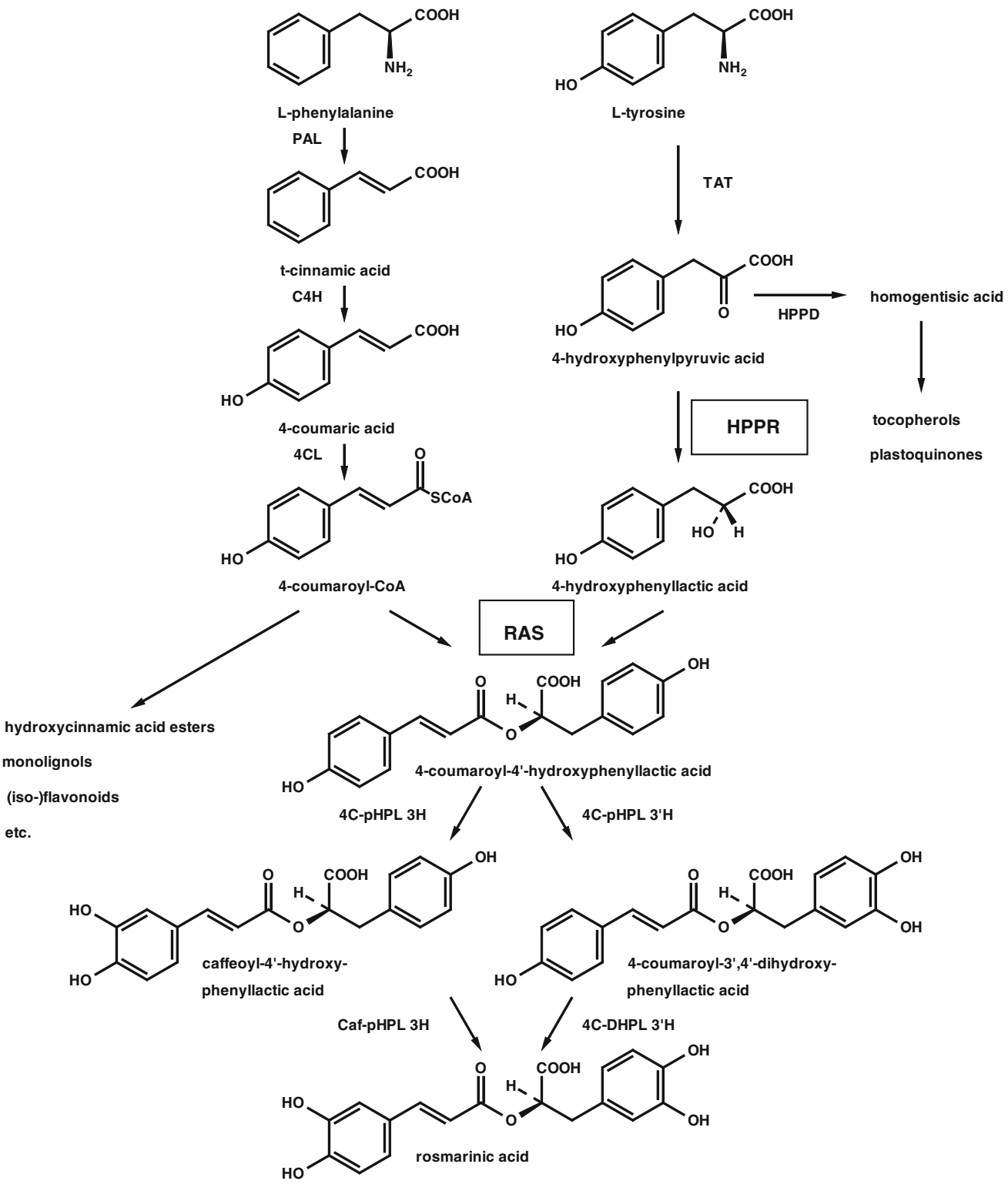

Fig. 1 Biosynthetic scheme for rosmarinic acid in *Coleus blumei* and pathways using common precursors. *PAL* phenylalanine ammonia-lyase, *C4H* cinnamic acid 4-hydroxylase, *4CL* 4-coumarate:coenzyme A ligase, *TAT* tyrosine aminotransferase, *HPPR* hydroxyphenylpyruvate reductase, *HPPD* hydroxyphenylpyruvate dioxygenase, *RAS* hydroxycinnamoyl-CoA:hydroxyphenyllactate HCT ("rosmarinic acid synthase"), *4C-pHPL 3/3′-H* 4-coumaroyl-4′-hydroxyphenyllactate 3/3′-hydroxylase(s), *Caf-pHPL 3H* caffeoyl-4′-hydroxyphenyllactate 3′-hydroxylase, *4C-DHPL 3′H* 4-coumaroyl-3′,4′-dihydroxyphenyllactate 3-hydroxylase. Enzymes in *boxes* have been silenced or overexpressed in this study

HPPR belongs to the family of D-isomer-specific 2-hydroxyacid dehydrogenases which was first described by Grant (1989). HPPR is active as a homodimer. The monomer consists of 313 amino acid residues and has a molecular mass of 34 kDa. When Kim et al. (2004) obtained the HPPR sequence from *Coleus blumei*, it displayed high sequence similarities to D-isomer-specific 2-hydroxyacid dehydrogenases from *Arabidopsis thaliana* and *Oryza sativa* with unassigned functions at that time. Investigations by Timm et al. (2008) identified the orthologous gene from *A. thaliana* as a gene encoding the cytosolic hydroxypyruvate reductase (HPR2) which prefers NADPH as cosubstrate over NADH. HPR2 is an enzyme of photorespiration in addition to the peroxisomal NADH-dependent hydroxypyruvate reductase (HPR1). The peroxisomal HPR1 has been biochemically characterised from many plant species already starting as early as 1954 (Stafford et al. 1954). The cytosolic HPR2 was first isolated from spinach and pea by Kleczkowski et al. (1988, 1991) who also postulated its role as an overflow enzyme for the reduction of hydroxypyruvate leaked from peroxisomes in photorespiration (Kleczkowski and Randall 1988; Kleczkowski and Edwards 1989). A role of HPR2 together with isocitrate dehydrogenase in NADPH/NADP cycling in non-photosynthetic tissues has been proposed by Igamberdiev and Kleczkowski (2000) from their investigations of barley endosperm in developing seeds. HPPR from *Coleus blumei* has been crystallised and the structure solved. Co-crystallisation yielded the structure together with the cosubstrate $NADP^+$ and this structure was used for docking experiments with pyruvate, hydroxypyruvate, phenylpyruvate, 4-hydroxyphenylpyruvate (pHPP) and 3.4-dihydroxyphenylpyruvate (DHPP) (pdb-codes 3ba1, 3baz; Janiak et al. 2010). The HPPR structure from *Coleus blumei* showed the highest structural similarities to human glyoxylate reductase/hydroxypyruvate reductase (pdb-code 2gcg; Booth et al. 2006) and glycerate dehydrogenase from *Hyphomicrobium methylovorum* (pdb-code 1gdh; Goldberg et al. 1994). A purified heterologously expressed HPPR from *Coleus blumei* (CbHPPR) was tested with aromatic substrates such as phenylpyruvate, pHPP and DHPP as well as with substrates lacking the aromatic ring such as pyruvate, hydroxypyruvate, glyoxylate and oxoisocaproate. Hydroxypyruvate proved to be the best substrate for HPPR (Janiak 2007). This and the high sequence similarities of *CbHPPR* to the *HPR2* sequence from *A. thaliana* raised some doubts about the involvement of CbHPPR in RA biosynthesis.

RAS belongs to the superfamily of BAHD acyltransferases as defined by St. Pierre and De Luca (2000) and D'Auria (2006). Hydroxycinnamoyltransferases (HCTs) from this family catalyse the transfer of a 4-coumaroyl or caffeoyl moiety from an activating coenzyme A thioester to shikimate (hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase = HST) or quinate (hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase =HQT) as acceptors resulting in the formation of 4-coumaroylshikimate, caffeoylshikimate, 4-coumaroylquinate or chlorogenate (Hoffmann et al. 2003; Niggeweg et al. 2004). Down-regulation of HCTs preferring shikimate as substrate affected both the amount and composition of lignin in *A. thaliana*, *Nicotiana benthamiana*, *Pinus taeda* and *Medicago sativa* (Hoffmann et al. 2004, 2005; Wagner et al. 2007; Shadle et al. 2007). It was therefore suggested that HST is involved in the formation of monolignols via an intermediate shikimate ester stage. On the other hand, chlorogenic acid levels as well as pathogen and UV resistance were affected after down-regulation of a HCT that preferentially uses quinate as acceptor (Niggeweg et al. 2004). RAS (hydroxycinnamoyl-CoA:hydroxyphenyllactate HCT) shows a substrate specificity for (hydroxy)phenyllactates and does not accept shikimate or quinate as substrates (Berger et al. 2006; Sander and Petersen 2011). Thus, RAS seems to be specific for RA biosynthesis in *Coleus blumei* despite the high sequence similarities between RAS and HST/HQT sequences. Interestingly, CbRAS and CbHST isolated from accordingly transformed *E. coli* utilised a wide variety of acceptor substrates that may not be present in the plant's metabolism (Sander and Petersen 2011).

In order to get better insight into the physiological importance of HPPR and RAS for RA biosynthesis, we set out to separately downregulate the levels of the HPPR and the RAS proteins in *Coleus blumei* hairy root cultures using the RNAi technique and to analyse the impact on the formation of RA. In parallel, *HPPR* and *RAS* were overexpressed under control of the constitutive CaMV (cauliflower mosaic virus) 35S-promoter.

Materials and methods

Construction of HPPR and RAS RNAi and overexpression plasmids

Primer synthesis and sequencing services were provided by Eurofins MWG Operon (Ebersberg, Germany).

The pHANNIBAL vector (Wesley et al. 2001) was used for composing the RNAi constructs. A 639 bp fragment of the *Coleus blumei HPPR* cDNA sequence (introduced into the expression vector pET-15b; Kim et al. 2004) was amplified by PCR with the primer pairs HPPR1-f/HPPR2-r and HPPR3-f/HPPR4-r (Table 1) with the following PCR mixture and program: 1 µl plasmid as template, 5 µl GoTaq 5× buffer, 2.5 µl 25 mM $MgCl_2$, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 0.5 µl 10 mM dNTP mix, 0.1 µl GoTaq polymerase (5 U/µl, Promega, Mannheim, Germany), 15 µl H_2O; 60 s 95 °C; 36 cycles:

Table 1 Sequences of PCR primers used in this work, restriction sites are underlined

Primer name (introduced restriction site)	Sequence (5′ → 3′)
HPPR1-f (*Eco*RI)	CAGAATTCGAACGCCGGTGCC
HPPR2-r (*Kpn*I)	CCGGTACCTCTGGTTCCCTTTCG
HPPR3-f (*Bam*HI)	CAGGATCCGAACGCCGGTGCC
HPPR4-r (*Hin*dIII)	GCGAAGCTTCTCTGGTTCCCTTTCG
RAS1-f (*Eco*RI)	ATGAATTCATTTCCACATACCCTCTC
RAS2-r (*Kpn*I)	ATGGTACCATCGATCGACGATCGC
RAS3-f (*Bam*HI)	ATGGATCCATTTCCACATACCCTCTC
RAS4-r (*Hin*dIII)	TATAAGCTTATCGATCGACGATCGC
HPPR-*Xho*I-f	ATACTCGAGATGGAGGCGATTGGC
HPPR-*Bam*HI-r	TATGGATCCTCAAACTACTGGAGTTAAGAG
RAS-*Eco*RI-f	TATGAATTCATGAAGATAGAAGTCAAAG
RAS-*Bam*HI-r	GATGGATCCTCAAATCTCATAAAACAAC
HST-*Xho*I-f	ATCTCGAGATGAAAATCCACGTGAGAGATTC
HST-*Bam*HI-r	TAGGATCCTCAAATCTGATAGAGCAGCTTC
rolA-f	CCGGACTAAACGTCGCCGGC
rolA-r	GAACGTCCCGGTCGGGCTTG
rolB-f	TCTCACTCCAGCATGGAGCC
rolB-r	GAGAGTCGCAGGGTTAGGTC
rolC-f	ATG CCT CAC CAA CTC ACC AG
rolC-r	GACAAGCAGCGATGAGCTAG
virC-f	ATCATTTGTAGCGACT
virC-r	AGCTCAAACCTGCTTC
actin-f	CTTATGTTGCCCTGGACTATGAGC
actin-r	GGCAACGGAATCTCTCAGCTCC
35S-f	TCGACGAATTAATTCCAATCC
35S-r	GATGCAATTAGTCCTGAATC
nptII-f	AGG CTA TTC GGC TAT GAC TG
nptII-r	ATC GGG AGC GGC GAT ACC GTA

45 s 95 °C, 45 s 58 °C, 90 s 72 °C; last cycle 5 min 72 °C. After restriction digest with the appropriate restriction enzymes and purification, the first fragment was ligated into pHANNIBAL into the restriction sites *Eco*RI and *Kpn*I in sense direction, the second fragment into the *Bam*HI and *Hin*dIII sites in antisense direction. After subcloning in *E. coli* JM109, plasmid isolation and sequencing for verification of the sequence and the correct orientation, the expression cassette was excised with *Not*I and ligated into the binary vector pART27 (Gleave 1992) opened with *Not*I (Fig. 2).

The RAS RNAi cassette was constructed using the same methods as above. A 645 bp internal *RAS* fragment for sense and antisense cloning was amplified using the primer pairs RAS1-f/RAS2-r (with a *Eco*RI and a *Kpn*I restriction site, respectively; Table 1) and RAS3-f/RAS4-r (with restriction sites *Bam*HI and *Hin*dIII; Table 1) and the pET-15b vector containing the full *CbRAS* orf as template (Berger et al. 2006).

The pHANNIBAL vector was also used for constructing the HPPR and RAS overexpression cassettes. The vector was digested with the restriction enzymes *Xho*I and *Bam*HI for HPPR and *Eco*RI and *Bam*HI for RAS and thus the intron was removed. The full open reading frames of the *Coleus blumei HPPR* or *RAS* were amplified by PCR with the primer pairs HPPR-*Xho*I-f/HPPR-*Bam*HI-r or RAS-*Eco*RI/RAS-*Bam*HI (Table 1) as described above but with an annealing temperature of 56 and 58 °C, respectively. The PCR products were digested with appropriate restriction enzymes, purified and ligated into pHANNIBAL. *E. coli* JM109 was transformed with this plasmid for multiplication. The plasmids were isolated and the expression cassettes excised with *Not*I and ligated into pART27 (Fig. 2).

Transformation of *Agrobacterium rhizogenes*

The *A. rhizogenes* strain LBA15834 was grown for 36–48 h in 10 ml liquid YMB medium (Singleton et al. 2002) under shaking at 110 rpm at 26 °C. Bacterial suspension (2–4 ml) was centrifuged for 5 min at 10,000*g*. The pellet was resuspended in 100 µl 20 mM $CaCl_2$ and 3 µl pART27 vector preparation harbouring one of the RNAi or overexpression cassettes were added. The bacteria were frozen for 5 min in liquid nitrogen, followed by incubation at 37 °C for 25 min. The bacteria were again sedimented for 2 min at 3,000*g*. The pellet was resuspended in ca. 50 µl remaining supernatant YMB medium. The bacteria were spread on YMB agar plates with spectinomycin (100 µg/ml) for selection and incubated at 26 °C for 36–48 h. Positive clones were confirmed by plasmid isolation and restriction enzyme digest.

For control plant transformation, *A. rhizogenes* LBA15834 was used as such or transformed with the original pART27 vector.

Transformation of plant material

Sterile plant cultures of *Coleus blumei* grown on hormone-free CB-medium (CB2OH; Petersen and Alfermann 1988) originated from commercially available seeds and had been cultivated in our laboratory for more than 10 years. Leaves

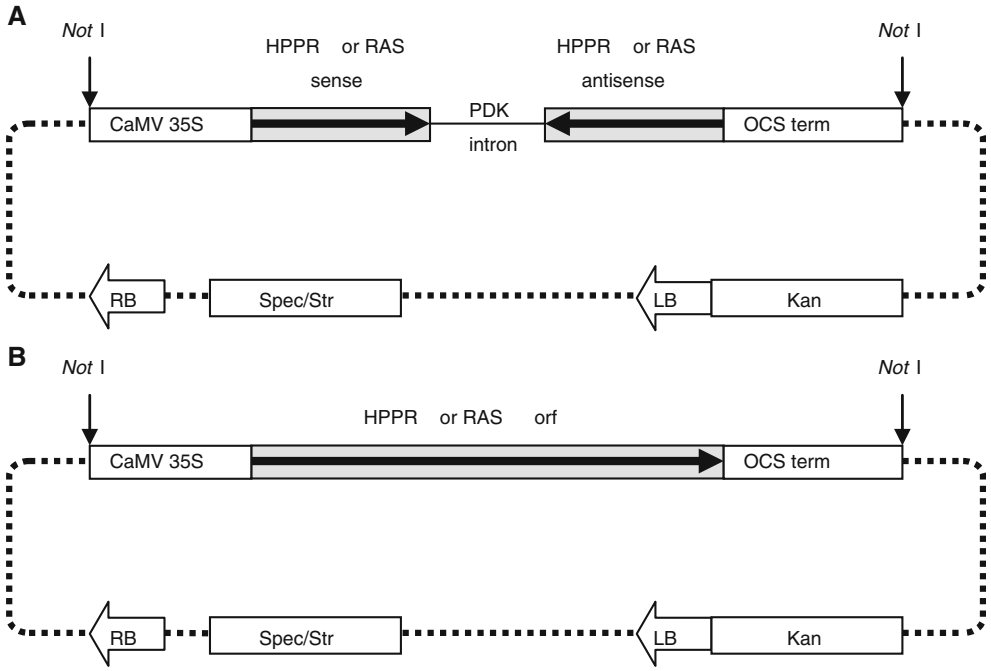

Fig. 2 Vector constructs [based on pHannibal (Wesley et al. 2001) for expression cassette and pART27 (Gleave 1992) for transfer] used for RNAi suppression (**a**) and overexpression (**b**) of HPPR or RAS, respectively. *CaMV 35S* cauliflower mosaic virus 35S promoter, *PDK intron* intron 2 of pyruvate orthophosphate dikinase, *OCS term* terminator of the octopine synthase gene, *Kan* kanamycin resistance gene, *LB* left border, *Spec/Str* spectinomycin/streptomycin resistance gene, *RB* right border

were cut from the plant and the lower leaf surface was punctured and wetted with 10 µl transformed (pART27, pART27-HPPR-RNAi, pART27-HPPR-overexpression, pART27-RAS-RNAi, pART27-RAS-overexpression) or untransformed *A. rhizogenes* suspension (grown over night) under sterile conditions. The leaves were kept on CB2OH agar plates without antibiotics for 48–52 h. Then the agrobacteria were washed off with Cefotaxim solution (500 µg/ml) and the leaves were further kept on CB2OH agar plates with Cefotaxim (500 µg/ml) to prevent growth of agrobacteria. Single hairy roots of an approximate length of 10 mm were cut from the leaves and placed on CB2OH agar plates with Cefotaxim (500 µg/ml) and kanamycin (60 µg/ml) for selection of positive transformants. Kanamycin resistant transformants were regularly washed with Cefotaxim solution for several weeks to remove remaining agrobacteria. The agrobacteria-free hairy roots were transferred to liquid CB2OH medium (50 ml in 100 ml Erlenmeyer flasks). The liquid-cultivated hairy roots were maintained by transferring 0.5 g root material into 50 ml fresh CB2OH medium every 14 days. The roots were cultivated on a rotary shaker (110 rpm) in the dark at 26 °C.

DNA and RNA extraction and cDNA synthesis

Nucleic acids were isolated from 12 day-old hairy roots separated from their medium and frozen and powdered in liquid nitrogen. DNA for PCR reactions was isolated by the slightly modified protocol of Murray and Thompson (1980). RNA was isolated according to Chomczynski and Sacchi (1987). The RNA concentration was determined spectrophotometrically and a defined amount was transcribed into cDNA with the RevertAid® First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany).

PCR

PCR reactions for verification of the presence or absence of genes in hairy root cultures were performed according to the manufacturer's protocol for GoTaq polymerase (Promega, Mannheim, Germany). Elongation times used were 1 min per 1,000 bp. For genomic DNA as template 36 PCR cycles were run and for cDNA 28 cycles. For expression analysis, PCR reactions with limited cycle numbers were performed with the following cycle numbers: *CbHPPR* 28, *CbHPR1* 29, *CbRAS* 36, *CbHST* 36, *actin* 28. PCR amplifications (see Table 1 for primer sequences) were applied to show the absence of *Agrobacterium* in hairy root cultures (absence of *virC*; Sawada et al. 1995) and the presence of the stably integrated bacterial *rolA/B/C*-genes. It was also used to verify the expression of *CbHPPR* (HPPR-*Xho*I-f/HPPR-*Bam*HI-r), *CbHPR1* (EMBL accession number A1EGU2; HPR-*Nde*I-f/HPR-*Bam*HI-r), *CbRAS* (RAS-*Eco*RI-f/RAS-*Bam*HI-r) and *CbHST* (HST-*Xho*I-f/HST-*Bam*HI-r) with

actin as internal control (actin-f/actin-r; Al-Qsous et al. 2004).

Southern blot analysis

Genomic DNA used for Southern blotting was isolated from hairy roots according to Rogers and Bendich (1985). 30–35 µg DNA were digested with 100 U *Eco*RI for at least 16 h. The digested DNA was electropherographed on a 0.6 % agarose gel. The gel was treated for 20 min in 0.25 M HCl (depurination), for 2 × 20 min in 0.5 M NaOH, 1.5 M NaCl (denaturation) and for 2 × 20 min in 0.1 M Tris–HCl pH 7.0, 1.5 M NaCl (neutralisation). The DNA was blotted onto a positively charged nylon membrane by capillary transfer in 10× SSC (saline sodium citrate) buffer. After UV-crosslinking, the membrane was hybridised with a DIG-labelled probe over night at 68 °C according to Engler-Blum et al. (1993). The PCR DIG-Probe Synthesis Kit (Roche, Mannheim, Germany) was used for preparing the digoxigenin-labelled probes according to the manufacturer's protocol. A ~700 bp fragment of the CaMV 35S promoter of the pHANNIBAL vector (primers 35S-f/35S-r; Table 1) or a ~700 bp fragment of the *nptII* gene of the pART27 vector (primers nptII-f/nptII-r; Table 1) were amplified and used as probe. Washes were done at 65 °C in the buffers suggested in the optimised protocol by Engler-Blum et al. (1993). This protocol was also essentially used for chemoluminescence detection with CSPD (disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3.3.1.1$^{3'7}$]decan}-4-yl)phenylphosphate).

RA extraction and HPLC analysis

12 day-old hairy roots were separated from the medium, frozen in liquid nitrogen, ground to a fine powder and lyophilised for 24–48 h. 20 mg were mixed with 1 ml 70 % ethanol and treated in an ultrasonic bath at 70 °C for 10 min twice with intermediate vigorous mixing. After centrifugation at 15,800*g* for 5 min, the supernatant was diluted 1:10 with 45 % methanol/0.01 % H_3PO_4 and analysed by HPLC.

HPLC analysis was performed isocratically with 45 % methanol/0.01 % H_3PO_4 on an Equisil ODS column (250 × 4 mm, precolumn 20 × 4 mm, particle size 5 µm; Dr. A. Maisch GmbH, Ammerbuch, Germany) at a flow rate of 1 ml/min. The detection wavelength was 333 nm. Authentic RA was used for identification and quantification.

Statistical analysis

Statistical analysis was performed with the Tuckey test of the SAS® 9.1 program package.

Results and discussion

RNAi and overexpression hairy root cultures of *Coleus blumei*

The vector pHANNIBAL (Wesley et al. 2001) was used to construct the hairpin expression cassettes for HPPR and RAS. A 639 bp sequence in the centre of *HPPR* (bp 158–796) or a 645 bp central sequence of *RAS* (bp 376–1,020) were ligated into pHANNIBAL in sense and antisense direction on either side of the *PDK* (pyruvate orthophosphate dikinase) intron. The intron between the sense and antisense fragments facilitates the formation of a hairpin structure of the RNA transcribed under control of the CaMV 35S promoter and results in an intron-enhanced silencing (Smith et al. 2000). The RNAi-cassette was released from the vector with *Not*I and ligated into the transformation vector pART27 (Gleave 1992). This vector carries the left (LB) and right border (RB) sequences for the transfer of the T-DNA into plant cells, the spectinomycin/streptomycin resistance gene for selection in bacteria and the kanamycin resistance gene for selection in plants (Fig. 2). Similarly, the *HPPR* or *RAS* overexpression cassettes with the 35S promoter followed by the complete open reading frames were constructed in pHANNIBAL. During this procedure the *PDK* intron was eliminated. Each of the overexpression cassettes was ligated into the *Not*I site of pART27 (Fig. 2).

The RNAi and overexpression plasmids were transferred into *A. rhizogenes* LBA15834. The most suitable combination of *Coleus blumei* sterile plant lines and *A. rhizogenes* strains (TR105, LBA9402, LBA15834) had been determined before (data not shown). Hairy roots were established on sterile punctured leaves of *Coleus blumei* by incubation with appropriately transformed *A. rhizogenes*. Selection was performed with kanamycin (60 µg/ml) in hormone-free CB-medium. Liquid-cultivated hairy root cultures in hormone-free CB2OH-medium were established for further analysis.

Since RA is accumulated in all plant tissues of *Coleus blumei* (Szabo 1993), roots are suitable organs for experiments aiming at suppression and overexpression effects. RA accumulation has been shown in hairy root cultures of other Lamiaceae species as well, e.g. *Coleus forskohlii* (Li et al. 2005). After transformation of plant material with *A. rhizogenes*, roots are readily regenerated on hormone-free medium which avoids the establishment of an elaborated regeneration protocol. Hairy roots have previously been used to show RNAi-effects on enzymes of lignan biosynthesis in *Linum perenne* and *L. corymbulosum* or isoflavonoids in pea (Hemmati et al. 2007; Bayindir et al. 2008; Kaimoyo and Van Etten 2008).

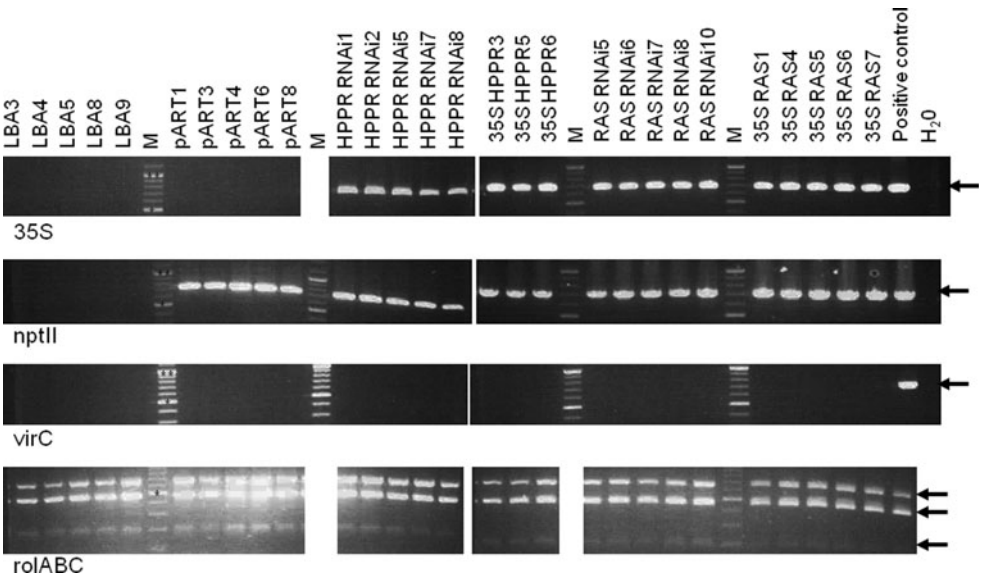

Fig. 3 PCR amplification with limited cycle numbers of partial sequences of agrobacterial genes (*virC*, ~700 bp) and transferred genes (*rolA*, ~200 bp; *rolB*, ~650 bp; *rolC* ~450 bp; *nptII*, ~700 bp; 35S, ~700 bp) with genomic DNA from hairy roots of *Coleus blumei*. Marker sizes: * 500 bp, ** 1,000 bp. *Arrows* indicate the expected amplification product(s)

In addition to the HPPR-RNAi, HPPR-overexpression, RAS-RNAi and RAS-overexpression hairy root lines, control hairy root cultures were established with empty pART27 vector and with untransformed *A. rhizogenes* LBA15834.

Further analysis (see below) was performed with five hairy root lines each of *Coleus blumei* established without pART27 (LBA 3, 4, 5, 8, 9), with empty pART27 (pART 1, 3, 4, 6, 8), pART27 with HPPR-RNAi (HPPR RNAi 1, 2, 5, 7, 8), pART27 with RAS-RNAi (RAS RNAi 5, 6, 7, 8, 10), pART27 with RAS-overexpression (35S RAS 1, 4, 5, 6, 7) and three lines for HPPR-overexpression (35S HPPR 3, 5, 6).

Basic characterisation of hairy root lines

The absence of agrobacteria from the hairy root cultures was proven by PCR-amplification of a partial sequence of the *virC* gene, which is present in the Ri-plasmid outside of the transferred T-DNA of *A. rhizogenes*. None of the above-mentioned established controls and transgenic lines showed amplification of *virC* and thus all hairy root lines were free of agrobacteria (Fig. 3).

The presence of the T-DNA was shown by amplification of partial sequences of the agrobacterial *rol*-genes (*rolA*, *rolB*, *rolC*). These genes are necessary for tumour/hairy root induction. All hairy root cultures showed amplification of all three *rol*-genes and thus were proven to be real hairy and not adventitious roots (Fig. 3). This is of relevance, since Bulgakov et al. (2004, 2005) had shown that the presence or absence of the *rol* genes influences plant secondary metabolism.

Amplification of a part of the *nptII* gene (neomycin phosphotransferase conferring kanamycin resistance) was observed in all hairy root cultures except the control cultures without pART27 vector. This proves the presence of the T-DNA of the pART27 vector (Fig. 3).

PCR-amplification of a stretch of the 35S-promoter showed the presence of the expression cassette from pHANNIBAL in all but the control cultures (Fig. 3). This can be taken as proof for the presence of the HPPR-RNAi, RAS-RNAi, HPPR-overexpression and RAS-overexpression constructs in the transformed hairy root lines.

The number of transformation events per hairy root line was determined by Southern blot analysis. Probes directed against the *nptII* and the 35S promoter regions were used. The *nptII*-probe detected multiple transformation events in all cultures except those established without pART27. Consequently multiple hybridising bands were detected with the 35S-probe in all cultures except the control cultures without pART27 and those with pART27 without the pHANNIBAL expression cassette. The number of transformation events per line could be estimated between 2 and 12 (online resource 1). Such multiple transformation events are routinely reported in *Agrobacterium*-mediated plant transformation (see e.g. Thomashow et al. 1980; Radchuk et al. 2005; Zhang et al. 2008).

Expression of HPPR and HPR1 in hairy root lines

The effects of RNAi suppression and overexpression of *HPPR* were monitored in 12 day old liquid-cultivated hairy root cultures by PCR (with reduced cycle numbers) of the complete open reading frame of *HPPR* using cDNA. A 150 bp fragment of the *actin* gene was amplified as a control. Figure 4 shows comparatively equal loading demonstrated by the *actin* band. The optimal number of PCR cycles was determined to be 28 cycles. Similar amplification rates for *HPPR* were observed in the control hairy root lines LBA and

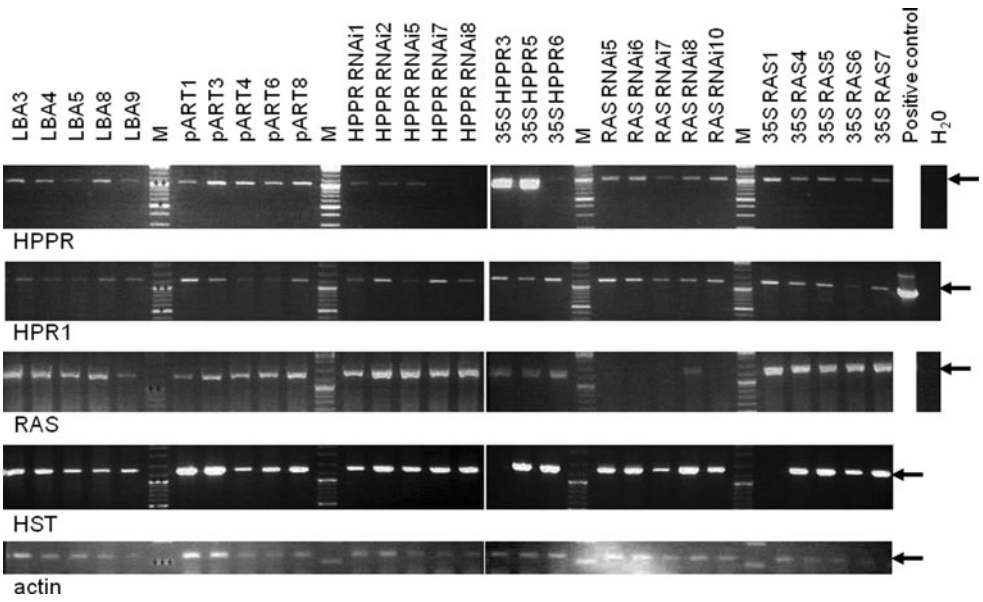

Fig. 4 PCR amplification with limited cycle numbers of *CbHPPR* (~950 bp), *CbHPR1* (~1,200 bp), *CbRAS* (~1,300 bp) and *CbHST* (~1,300 bp) with cDNA from hairy roots of *Coleus blumei*. *Actin* (~150 bp) was amplified as "housekeeping gene". Marker sizes: * 500 bp, ** 1,000 bp, ***100 bp. *Arrows* indicate the expected amplification product

pART and in the lines transformed with *RAS* RNAi and *RAS* overexpression constructs (Fig. 4). This shows the expression of the internal *HPPR* gene of the plant material and furthermore excludes strong secondary effects of HPPR suppression or overexpression on other genes of the same pathway. In three out of five *HPPR* RNAi lines *HPPR* cDNA could still be amplified, although to a slightly lower extent than in the control lines. This shows a reduction but not a complete destruction of the *HPPR* mRNA. Hairy root lines *HPPR* RNAi 7 and 8 did not show an amplification product and thus were strongly or completely suppressed with respect to *HPPR* expression (Fig. 4). A reduced but not fully suppressed transcript level is often found in RNAi experiments, for example in opium poppy for codeinone reductase (Allen et al. 2004) or salutaridinol 7-*O*-acetyltransferase (Kempe et al. 2009) or in tomato or tobacco for chalcone synthase (Schijlen et al. 2007) and chalcone isomerase (Nishihara et al. 2005).

The overexpression lines 35S HPPR 3 and 5 showed strong overexpression while the expression level in line 35S HPPR 6 was significantly reduced. The reduction of gene expression in overexpression lines has been explained by co-suppression effects especially after the incorporation of multiple copies of a foreign gene (Napoli et al. 1990; Banyai et al. 2010). An overdosage of a specific mRNA formed from multiple gene copies results in a complete destruction of the mRNA by processes similar to RNAi-silencing.

The peroxisomal HPR1 is the main enzyme of photorespiration which is supported in its action by its cytoplasmic counterpart HPR2 (Kleczkowski et al. 1988, 1991; Timm et al. 2008). The latter enzyme from *A. thaliana* (UniProtKB Q9CA90) has high sequence similarities to HPPR (similarity 87 %, identity 76 % on amino acid basis) and thus might be able to act in photorespiration as well as RA biosynthesis although RA accumulation has not been described from this species. We have shown that the recombinant HPR2 from *A. thaliana* also accepts pHPL, the substrate of HPPR, while HPPR also acts on non-aromatic substrates such as hydroxypyruvate (Janiak 2007; Hücherig 2010). The mRNA levels of the main photorespiratory peroxisomal enzyme HPR1 were also monitored by PCR in our hairy roots to test the expression of photorespiratory genes in heterotrophic plant tissues. The *HPR1* gene is expressed to a low extent in all hairy root lines (Fig. 4).

Expression of RAS and HST in hairy root lines

All control hairy root lines as well as those carrying *HPPR* RNAi and overexpression constructs showed the expression of *RAS* cDNA in comparatively equal amounts (Fig. 4). This represents the expression of the internal *RAS* gene in the *Coleus blumei* hairy roots. The *RAS* expression levels in *RAS* RNAi lines were undetectable in four out of five lines while one line (RAS RNAi 8) showed a reduced expression (Fig. 4).

All hairy root lines carrying the *RAS* overexpression construct showed reduced or normal levels of *RAS* expression which can be explained by co-suppression effects (Fig. 4). Similar effects have been shown for other overexpression experiments with genes involved in plant secondary metabolism, e.g. for chalcone synthase in *Petunia* (Napoli et al. 1990), and termed co-suppression or homology-dependent gene silencing (Meyer and Saedler 1996). In *Agrobacterium tumefaciens*-transformed

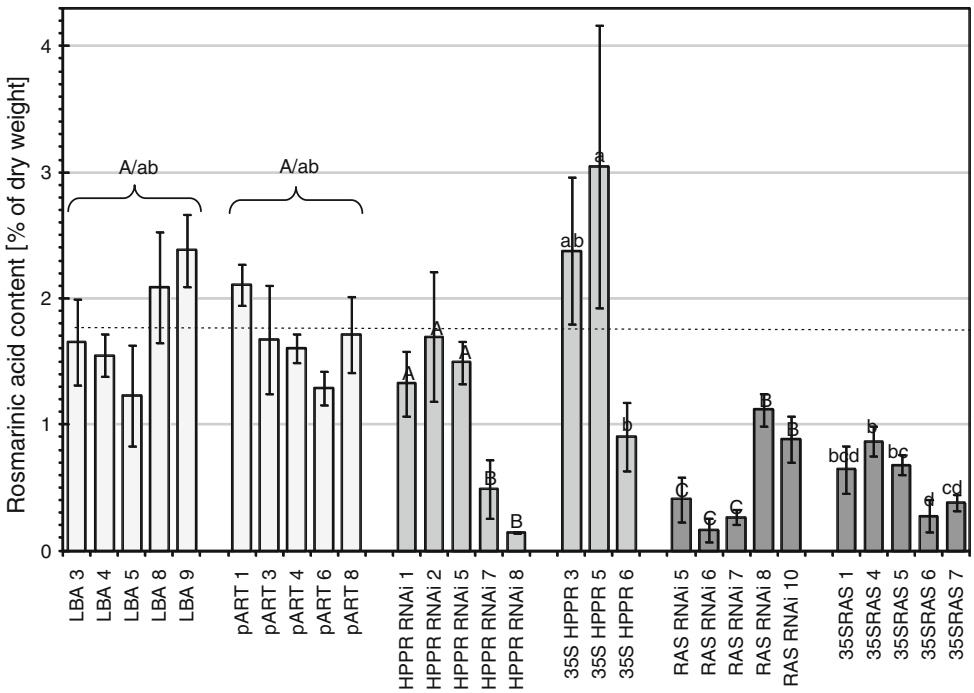

Fig. 5 Rosmarinic acid content [% of dry weight] in hairy root lines after 12 days of cultivation: *LBA* transformed with *A. rhizogenes* LBA15834, *pART* transformed with empty pART27, *HPPR RNAi* pART27 with *HPPR* RNAi cassette, *RAS RNAi* pART27 with *RAS* RNAi cassette, *35S HPPR* pART27 with *HPPR* overexpression cassette, *35S RAS* pART27 with *RAS* overexpression cassette. The *dotted line* indicates the mean value of control hairy root lines. The *bars* are means from three independent harvests with standard deviations. Statistical analysis was performed with Tuckey's test using the SAS program package; values with *differing letters* are significantly different

Artemisia annua plants the co-suppression effect was clearly seen in a reduced expression level of the farnesyl pyrophosphate synthase (*FPS*) gene and a reduced artemisinin content especially in plant lines with multiple integration of the *FPS* gene (Banyai et al. 2010).

The expression levels of the *CbHST* gene (coding for hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase) which is related to *RAS* (Petersen et al. 2009) was also analysed for comparison (Fig. 4). With two exceptions, *CbHST* expression was present in all lines to a higher extent than *CbRAS* expression showing that this gene, as a gene involved in the formation of lignin precursors, is more or less constitutively expressed. Due to the considerable sequence similarities (similarity 73 %, identity 56 % on amino acid basis) between *CbRAS* and *CbHST*, a suppression of *HST* in a *RAS* RNAi-silenced hairy root line might have been possible. Our expression results, however, show that *HST* expression remains unaffected.

Rosmarinic acid content in hairy roots lines

Control hairy root lines (LBA, pART) showed an accumulation of RA around 1.73 % of the dry weight (Fig. 5). Slightly lower RA contents (1.3–1.7 %) were measured in the hairy root lines HPPR RNAi 1, 2 and 5 that still showed expression of *HPPR*. The hairy root lines without detectable *HPPR* expression had significantly reduced RA levels at 0.49 and 0.14 % of the dry weight. This means that the hairy root line RNAi HPPR 8 only accumulated a residual RA amount of 8 % of the control lines. The residual RA level present in the hairy roots might be explained by a very low amount of HPPR protein still present in the cells. Similar observations have been made for lignan accumulation in hairy roots of *Linum perenne* by Hemmati et al. (2007) after RNAi-mediated suppression of pinoresinol/lariciresinol synthase.

The *HPPR* overexpression line with reduced *HPPR* mRNA level displayed a reduced RA content of 0.9 % of the dry weight. *HPPR* overexpression lines 35S HPPR 3 and 5 with increased *HPPR* mRNA levels showed highly increased RA contents of up to 3 % of the root dry weight corresponding to 176 % of the control cultures.

These prominent effects of *HPPR* RNAi suppression or overexpression on the RA content strongly supports a role of HPPR in RA biosynthesis although a participation in other physiological processes (photorespiration, NADP/NADPH cycling) cannot be excluded. It must be taken into account that only one single gene involved in RA biosynthesis had been overexpressed. The activity of HPPR thus seems to be a limiting point in RA biosynthesis.

Strongly reduced RA contents between 0.16 and 0.41 % were observed in hairy root lines RAS RNAi 5, 6 and 7 where mRNA levels were undetectable. The hairy root line RAS RNAi 6 only reached 9 % of the RA amounts observed in controls. Although *RAS* expression was also not measurable in line 10, the RA level was only reduced to 0.89 % here. The highest RA content (but lower than in control hairy root lines) was determined in hairy root line RAS RNAi 8 with detectable, but reduced *RAS* mRNA level.

RAS overexpression hairy root lines all showed reduced RA contents compared to control lines (between 0.28 and

0.87 % of the root dry weight) which can be explained by co-suppression effects. A failure in overexpression had already been visible in the expression analysis (Fig. 4).

The effects of RNAi suppression and overexpression of two enzymes of RA biosynthesis, HPPR and RAS, on the accumulation of RA in hairy roots of *Coleus blumei* support their role in this biosynthetic pathway although higher RA levels could not be achieved by RAS overexpression due to co-suppression effects. Moreover, it must be taken into account that the availability of the precursors might be limiting and not the activity of the ester-forming enzyme. The simultaneous overexpression of more than one gene involved in a metabolic pathway (e.g. the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*) resulted in increases in the total alkaloid content (Peebles et al. 2011). Further investigations should try to clarify whether the gene/enzyme described as HPPR can also participate in other biosynthetic pathways, e.g. photorespiration in photosynthetically active tissues or NADP/NADPH cycling in heterotrophic tissues, and thus would be an example for dual function in "primary" as well as "secondary" metabolism.

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