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Overexpression of Phenylalanine Ammonia-Lyase in Transgenic Roots of *Coleus blumei* Alters Growth and Rosmarinic Acid Synthesis

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Summary

Most natural phenolic compounds in plants are derived from *trans*-cinnamic acid, formed by the nonoxidative deamination of L-phenylalanine by phenylalanine ammonia-lyase (EC 4.3.1.24). Although a strict line between primary and secondary metabolism cannot be drawn, phenylalanine ammonia-lyase is considered to play a pivotal role in channelling carbon flux from primary metabolism to phenolic synthesis. The objective of this work is to evaluate the impact of phenylalanine ammonia-lyase activity on phenolic production in *Coleus blumei*. Transgenic roots of *C. blumei*, harbouring the *Arabidopsis thaliana PAL1* gene, under the control of the CaMV 35S promoter, along with empty vector and wild-type roots, were regenerated. Transgenic root lines had disparate phenylalanine ammonia-lyase activities ranging from 67 to 350 %, compared to wild-type roots. Growth rates significantly differed, with the lowest in transgenic roots exerting augmented phenylalanine ammonia-lyase activity. Transgenic roots with high phenylalanine ammonia-lyase activity had lower growth rates, lower amounts of total phenolics, rosmarinic acid (the major phenolic compound in *C. blumei*) and chlorogenic acid, but increased amounts of caffeic acid. There was no increase in total phenolics and rosmarinic acid content after feeding transgenic roots with casein enzymatic hydrolysate and L-tyrosine. This shows that augmented phenylalanine ammonia-lyase activity inhibits growth and phenolic metabolism, and the probable regulator of these processes is *trans*-cinnamic acid.

Key words: caffeic acid, *Coleus blumei*, overexpression, phenolics, phenylalanine ammonia-lyase, rosmarinic acid, transgenic roots

Introduction

Phenylpropanoids are major components of plant specialized metabolism. They have a wide variety of functions such as defense against herbivores and microbes, or as structural components of cell walls, pigments, protectors from UV light or signalling molecules. They are major biologically active components of human diet and much interest has been attracted for medicinal use and for use in cosmetic and perfume industries. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.24) is the first and the key enzyme in the regulation of overall carbon flux

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Abbreviations: CAF: caffeic acid; CHA: chlorogenic acid; GA: gallic acid; GLA: glufosinate ammonium; IAA: indole-3-acetic acid; LB: Luria-Bertani medium; MS: Murashige and Skoog medium; MS-1NAA: Murashige and Skoog medium supplemented with 1 mg/L of α-naphthaleneacetic acid; NAA: α-naphthaleneacetic acid; PAL: phenylalanine ammonia-lyase; RA: rosmarinic acid; *t*-CA: *trans*-cinnamic acid

into the phenylpropanoid pathway (*1*). PAL catalyses biotransformation of L-phenylalanine (L-Phe) to *trans*-cinnamic acid (*t*-CA) and ammonia, and it is one of the most studied plant enzymes. The membrane-associated cytochrome P450 cinnamate 4-hydroxylase (C4H; EC 1.14. 13.11) anchors PAL and 4-coumarate:CoA ligase (4CL; EC 6.2.1.12) to the endoplasmic reticulum, and this complex is known as the core of the phenylpropanoid pathway. Numerous reports show that increases in PAL activity and in phenolic compounds occur in response to different stimuli. Manipulation of *in vivo* PAL activity with a specific inhibitor of PAL reduced the formation and the accumulation of phenolics (*2*). Furthermore, the metabolic impact of PAL mutations and heterologous PAL overexpression implied a positive correlation between PAL activity and phenolic biosynthesis (*1*,*3*–*6*).

Coleus blumei is a typically ornamental plant with colourful foliage. It is a member of the mint family (Lamiaceae) and valued as a medicinal plant in India, Indonesia and Mexico. The most prominent secondary metabolite in *C. blumei* is rosmarinic acid (RA), an ester of caffeic acid (CAF) and 3,4-dihydroxyphenyllactate. Rosmarinic acid has various biological activities (*e.g.* antioxidant, antimutagen, anti-inflammatory, antiviral and antibacterial), and it is used in the pharmaceutical, food and cosmetic industries (*7*). RA is a natural polyphenolic carboxylic acid and the enzymes involved in its biosynthetic pathway have been elucidated in suspension cultures of *Anchusa officinalis* and *C. blumei* (*7*,*8*). Its biosynthesis starts from L-Phe, which is converted to 4-coumaroyl-CoA by core phenylpropanoid pathway enzymes. In parallel, the other precursor for RA is formed from L-tyrosine (L-Tyr). The first step is the transamination of L-Tyr to 4-hydroxyphenylpyruvate by tyrosine aminotransferase (TAT; EC 2.6.1.5), which is then reduced to 4-hydroxyphenyllactate by hydroxyphenylpyruvate reductase (4-HPPR; EC 1.1.1.237). Rosmarinic acid synthase (RAS; EC 2.3.1. 140) catalyses the formation of 4-coumaroyl-4'-hydroxyphenyllactate, and the resulting ester is afterwards hydroxylated by two cytochrome P450 monooxygenases to form rosmarinic acid. PAL activity always precedes RA accumulation, and a positive correlation between PAL activity and RA biosynthesis was confirmed in several studies in cell cultures of Boraginaceae and Lamiaceae species (*9*,*10*).

In the present study we have determined whether overexpression of heterogenous *PAL1* gene from *Arabidopsis thaliana* leads to an increase in PAL activity and how it affects growth, total phenolic accumulation, and synthesis of major phenolics in *Coleus blumei*.

Materials and Methods

Bacterial strains, plasmids and media

Subcloning of DNA fragments was carried out using *E. coli* strain TOP10 (Invitrogen, Carlsbad, CA, USA). The binary vector pGPTV-*AtPAL1* carries the *PAL1* gene of *A. thaliana* (*AtPAL1*) under the control of the CaMV 35S promoter and the *nos* terminator. The binary vector pGPTV-C carries the CaMV 35S promoter and *nos* terminator, without the inserted *AtPAL1* gene. Both vectors carried a *nos* promoter-driven *bar* gene, encoding resistance to glufosinate ammonium (GLA). Vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by transformation. Bacteria were grown aerobically in Luria-Bertani (LB) or YEB medium: 5 g/L of beef extract and sucrose, 1 g/L of yeast extract and peptone, supplemented with kanamycin (30 mg/L). *E. coli* was cultured at 37 °C and *A. tumefaciens* at 28 °C. Plant material was cultured on a medium supplemented with Murashige and Skoog (MS) salts (*11*), 10 mg/L of thiamine, 1 mg/L of pyridoxine, 1 mg/L of nicotinic acid, 100 mg/L of *myo*-inositol and 20 g/L of sucrose. Media were solidified with 8 g/L of agar. Depending on the experiment, plant growth regulators (α-naphthaleneacetic acid, NAA or indole-3-acetic acid, IAA), 0.8 g/L of casein enzymatic hydrolysate and 5 mg/L of L-Tyr were added before autoclaving, and the antibiotics and GLA were added to the cooled medium, after autoclaving. The pH (5.7) was adjusted before autoclaving.

Construction of plant expression vectors

Multiple alignment of amino acid sequences of *A. thaliana* PAL1 and other known PAL proteins from the Laminaceae plants (*Salvia miltiorrhiza*: GenBank no. EF462460, *Agastache rugosa*: GenBank no. AF326116) and Boraginaceae (*Lithospermum erythrorhizon*: GenBank no. D83076) showed that AtPAL1 is highly similar to the PAL proteins involved in RA synthesis. The full-length cDNA of the *PAL1* gene of *A. thaliana* (*AtPAL1*) was obtained from Arabidopsis Biological Resource Center, The Ohio State University, Columbus, OH, USA (clone U10120, GenBank no. AY079363) and cloned into the pGPTV binary vector (*12*). The *AtPAL1* gene was amplified by PCR using the primers P1*Xba*I (GCTCTAGAATGGAGATTAACGGGGCACA) and P2*Sac*I (CGAGCTCTTAACATATTGGAATGGGgGCTC) into which the restriction enzyme sites *Xba*I and *Sac*I were introduced (underlined). In addition, a *Sac*I restriction site on 3´-end of *AtPAL1* gene was removed by changing the nucleotide T to C (lowercase in primer P2*Sac*I). The Expand High Fidelity PCR System was used. Cycling conditions were: an initial denaturation for 5 min at 95 °C, followed by 25 cycles of 45 s at 95 °C, 45 s at 58 °C and 90 s at 72 °C, and an extension of 7 min at 72 °C. The amplified PCR product was digested with *Xba*I and *Sac*I, gel-purified and ligated into the binary plasmid pGPTV. Plasmid pGPTV-*AtPAL1* was recovered. After digestion of pGPTV with *Xba*I and *Sac*I, blunt ends were formed with the aid of the Klenow fragment, and after self ligation, the empty vector pGPTV-C was recovered. Plasmid constructions were confirmed by restriction analysis, PCR and DNA sequencing, and transferred into *Agrobacterium tumefaciens* strain GV3101, using electroporation. Transformants were analyzed for the presence of the plasmids with restriction enzymes and PCR.

Genetic transformation and root regeneration

Root induction, transformation and transgenic tissue selection of *Coleus blumei* were performed according to the methods described by Bauer *et al.* (*13*,*14*). The leaves excised from *Coleus* plants grown *in vitro*, were cut into one-cm squares and used for transformation experiments. For inoculation, *Agrobacterium tumefaciens* GV3101 (pMP90) harbouring pGPTV-*AtPAL1* or pGPTV-C plasmid was

grown for two days at 28 °C with agitation, in liquid YEB medium, supplemented with 100 mg/L of rifampicin and 30 mg/L of kanamycin. An aliquot (500 µL) was subcultured in 30 mL of fresh YEB medium and cultured until the A_{600} reached 0.6. Bacteria were recovered by centrifugation, resuspended in 30 mL of liquid MS medium and used for transformation. Explants were immersed in the agrobacterial suspension for 15 min, blotted on sterile filter paper and placed upside-down on MS medium, supplemented with 1 mg/L of NAA (MS--1NAA). Three days after the infection, explants were transferred to liquid MS-1NAA medium, containing 1 mg/L of GLA (for the selection of transformation events) and 250 mg/L of cefotaxime and 100 mg/L of vancomycine (to inhibit the growth of agrobacteria). After 7 days, explants were subcultured on solid medium of the same composition. Induced roots that grew into the medium were cut from the leaves and subcultured three times on MS medium supplemented with 0.2 mg/L of IAA and 0.2 mg/L of GLA for 4 weeks, and further on MS-1NAA medium without GLA, cefotaxime and vancomycine (*14*). Thirteen lines carrying the *AtPAL1* transgene (lines denoted with P) and 7 transgenic lines with only the CaMV 35S promoter (lines denoted with K) were selected and further cultured on MS-1NAA medium. The wild-type roots (WT) were induced from leaf explants on MS-1NAA medium, without GLA and antibiotics.

Root cultivation

Stocks of root clones were maintained on solid MS--1NAA medium, at 24 °C, in the dark and subcultured at 4-week intervals. For experiments, roots were cultured in 100-mL Erlenmeyer flasks, containing 30 mL of liquid MS-1NAA medium. In some experiments, 0.8 g/L of casein enzymatic hydrolysate and/or 5 mg/L of L-Tyr were added to the MS-1NAA medium. Roots were incubated on an orbital shaker at 80 rpm. For subculture, approx. 0.15 g of root tissue (2–15 mm long tips) from a 4-week--old shake flask was transferred to fresh liquid medium. Roots cultured in liquid media were incubated at 20 °C in the dark and subcultured at 4-week intervals.

Molecular characterization of transgenic root lines

Total genomic DNA was isolated from the tips of GLA-resistant roots, using the DNeasy plant mini kit (QIAGEN, Hilden, Germany). The presence of T-DNA in transgenic roots was detected by PCR for the *bar* gene and/or part of CaMV 35S promoter and the *AtPAL1* gene. Plasmid DNA from the appropriate *Agrobacterium* strain was used as a positive control, and DNA isolated from wild-type roots as a negative control. To confirm the aseptic nature of the roots, the *virB10* gene was amplified by PCR (negative result confirmed the decontamination). Oligonucleotide primers for the *bar* gene were 5´GGTCTGCACCATCGTCAACC and 5´CATCAGATCTCGGTGACGGGC; for the CaMV 35S-*AtPAL1* fragment 5´CACTGACGTAAGGGATGACGCAC and 5´CGAGCTCTTAACATATTGGAATGGGGGCTC; and for the *virB10* gene 5'CAATCCCGATCAAGTCGTGCGC and 5'AGACGCCAACCTCGTGAAACCG. PCR amplification of the target sequences was performed in 50 µL of the following reaction mixture: 0.2 mM of each dNTP, Go*Taq* buffer, 3 mM $MgCl_2$, 10 pmol of each primer 1.0 U Go*Taq* polymerase and 50 ng of plant DNA. The *bar* gene was amplified in all transgenic root lines. In addition, the CaMV 35S-*AtPAL1* fragment was amplified in root lines transformed by *AtPAL1*. The PCR conditions were: an initial denaturation at 95 °C for 5 min followed by 30 cycles at 95 °C for 45 s, at 60 °C for 45 s and at 72 °C for 2 min; final extension was at 72 °C for 7 min. PCR products were analyzed on 1 % (by mass per volume) agarose gel.

Growth analysis

Roots were harvested from the culture medium four weeks after inoculation, washed with distilled water and blotted dry with paper towels. Fresh mass was determined and the growth rate was calculated as follows:

$$\text{Growth rate}=(\text{Final fresh mass}-\text{Initial fresh mass})/\text{Initial fresh mass} \quad /1/$$

Tissue was lyophilized and the yield of dry tissue was measured. Dry tissue was homogenized in a mortar. A mass of 20 mg of homogenized tissue was used for protein extraction and 50 mg for the determination of phenolics.

Determination of soluble phenols

Phenolics were extracted from 50 mg of dry pulverized tissue with 2 mL of 70 % ethanol in an ultrasonic bath at 70 °C for 20 min. The total phenol concentration was determined spectrophotometrically according to the Folin–Ciocalteu colorimetric method (*15*), using gallic acid (GA) as the standard and expressing the results as GA equivalents. The reaction was performed by mixing 20 µL of ethanol extract, 1.58 mL of water, 0.1 mL of Folin-Ciocalteu reagent and 0.3 mL of sodium carbonate (200 g/L). After 2 h at 37 °C, absorbance was measured at 765 nm and compared to the GA calibration curve. Data represent the mean of three independent measurements.

Determination of phenolic acids

Ethanol extracts were centrifuged at 10 000×*g* for 15 min and after filtration (0.45 µm pore filter) diluted with acidified (0.01 % H_3PO_4) 50 % methanol (1:5). Identification and quantitative determination of rosmarinic acid, chlorogenic acid (CHA) and caffeic acid were performed by HPLC on a PerkinElmer Series 200 chromatograph system consisting of a vacuum degasser, quaternary pump, autosampler and diode array detector. Hypersil ODS column, 250×46 mm, particle size 5 µm was used. The solvent for separation was 50 % methanol in water acidified with 0.01 % H_3PO_4. The flow rate was 0.85 mL/min and the detection wavelength was 300 nm. Authentic RA, CHA and CAF were used as reference substances.

Protein extraction and PAL activity assay

Methods for protein extraction and the PAL activity assay are described elsewhere (*16*). Soluble proteins were extracted with 2 mL of 200 mM borate buffer (pH=8.5), containing 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 20 000×*g* at 4 °C for 30 min. The supernatant was desalted on a PD-10 column and the protein

content of the filtrate was quantified with the Bradford reagent, using bovine serum albumin as a standard (*17*).

For PAL activity the assay reaction mixture was incubated at 40 °C for 3 h, while the change in the absorbance at 280 nm was monitored (*t*-CA production). The specific PAL activity was expressed as the amount of *t*-CA synthesized in one second per mg of protein (pkat/mg; kat=mol/s).

Statistical analysis

One way ANOVA, followed by the Duncan New Multiple Range Test was used to evaluate the significance of differences within the parameters. The results were analyzed using STATISTICA v. 7.0 software (StatSoft Inc, Tulsa, OK, USA), with p=0.05.

Results

Generation of C. blumei transgenic roots

To determine the role of PAL in the accumulation of phenolics in roots, the *AtPAL1* gene, under the control of the CaMV 35S promoter or empty vector (without an insert), was introduced into *C. blumei* by *A. tumefaciens*. Up to 20 roots were induced per leaf explant. Induced roots were subjected to selection medium with IAA that enabled better elongation and supported faster decontamination of the roots from agrobacteria (*14*). Nearly 98 % of induced roots ceased to grow on the selection medium. The surviving roots were further cultured on solid MS-1NAA medium. Genomic DNA was extracted from root lines and subjected to PCR with transgene-specific primers to confirm the insertion of the foreign genes. The PCR for the *bar* gene, plus the CaMV 35S promoter and *AtPAL1* gene produced 495-bp and 2286-bp DNA fragments, respectively (Fig. 1). No DNA band was detected in wild-type roots, and no *virB10* gene was amplified, which confirmed that root cultures were not contaminated with agrobacteria. We established 13 lines with the *AtPAL1* transgene, seven empty vector transgenic lines (with the CaMV 35S promoter alone), and one wild-type root line.

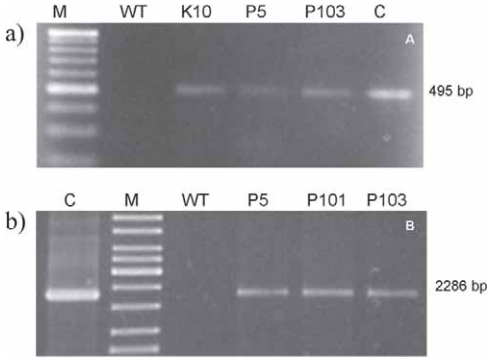

Fig. 1. PCR amplification of *bar* (a) and *AtPAL1* (b) transgenes in roots transformed with *AtPAL1* (P5, P101 and P103) and empty vector (K10), and in wild-type (WT) roots. C: positive control, pGPTV-*AtPAL1* plasmid, M: molecular marker

PAL activity in transformed root lines

PAL activity was measured to determine the effect of *AtPAL1* transgene on enzyme activity. No significant PAL activity was detected in crude protein extracts, so extracts were desalted to eliminate low molecular mass substances (M_r<1000). Fig. 2 shows recoverable enzyme activities in transgenic root lines, ranging from 67 to over 350 % of the control, wild-type root line. Six root lines

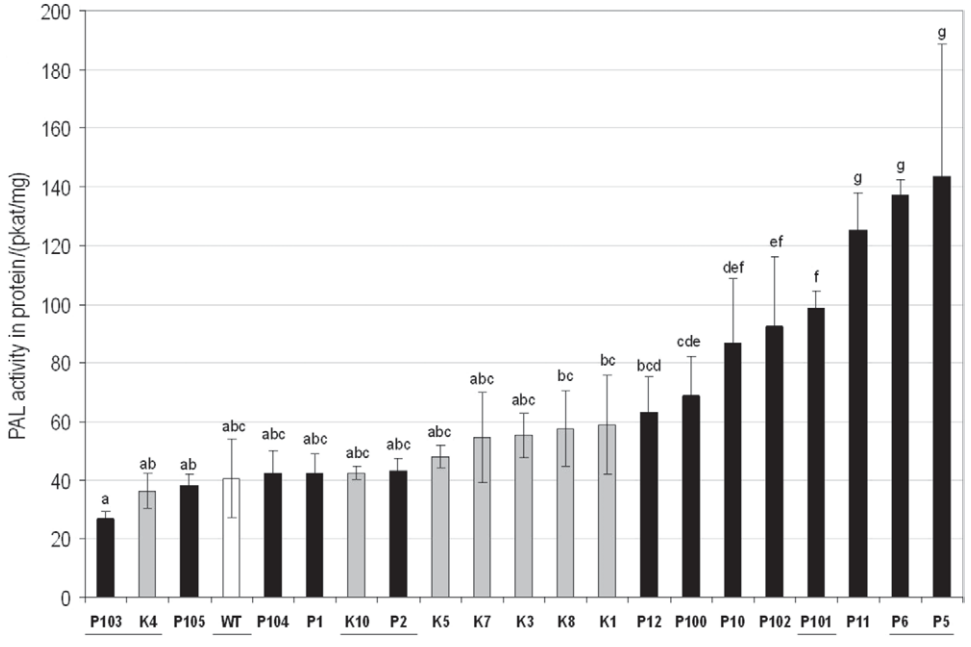

Fig. 2. Specific activities of PAL in *C. blumei* transgenic roots transformed with the *A. thaliana PAL1* gene (black bars), empty vector (grey bars) and wild type roots (white bar). Roots were grown in liquid MS medium, supplemented with 1 mg/L of NAA and harvested after 28 days. Underlined root lines were selected for further experiments. Bars represent the means±S.D. of three independent measurements. Different letters denote statistical differences (p≤0.05)

with the *AtPAL1* transgene (P10, P102, P101, P11, P6 and P5) had significantly (p=0.05) higher PAL activity, compared to the empty vector transgenic and wild-type root lines. The highest PAL activity was detected in line P5 with 143.43 pkat/mg of protein. Two root lines with the *AtPAL1* transgene (P103 and P105) had reduced PAL activity (Fig. 2). For further experiments, root lines that covered the range of PAL activities (WT, K4, K10, P2, P5, P6, P101, P103) were selected.

Growth of transgenic roots

The colour, thickness and ramification of different root lines did not vary. During the culture period, roots were yellowish with brown tips, and at the end of the subculture they turned brown. Growth rates varied considerably: *e.g.* from 2.93 to 24.11 for lines P5 and P103, respectively. In general, lines with higher PAL activity grew slower. Lines P5 and P6 had the highest PAL activity, and they grew three times slower than the wild type roots and 8 times slower than line P103, with the lowest PAL activity (Fig. 3a).

Phenolics in roots

Despite the high variation in PAL activity, accumulation of total phenolics was not significantly different between root lines. Roots accumulated from 31 to 41 mg of phenolic compounds, expressed as gallic acid equivalents, per g of dry tissue. Lines P5 and P6 accumulated the lowest amount of total phenolics (Fig. 3b). Accumulation of RA (the major phenolic acid in *C. blumei*), CAF (which plays a central role in the biochemistry of Lamiaceae species), and CHA (an antioxidant widespread in fruits and leaves of plants) was measured by HPLC. The most abundant phenolic compound in all root lines was RA (Table 1). Lines P5 and P6, which had the highest PAL activity, accumulated at least 30 % less RA (13.26 and 12.75 mg/g of dry mass, respectively) than the wild-type and other transgenic roots, but accumulated significantly higher amounts of CAF. All other lines, regardless of whether they were transformed or wild-type, accumulated similar amounts of RA (from 19.45 to 23.36 mg/g of dry mass). The highest RA accumulation was in the empty vector-transformed line K10. All lines carrying the *AtPAL1* transgene (especially the ones with elevated PAL activity) accumulated more CAF than the wild-type and empty vector-transformed lines. In addition, lines P5 and P6 accumulated notable amounts of unidentified compounds (peaks 1 and 6) and lower amount of another unidentified compound (peak 4, Fig. 4), compared to other lines. For example, wild-type roots accumulated

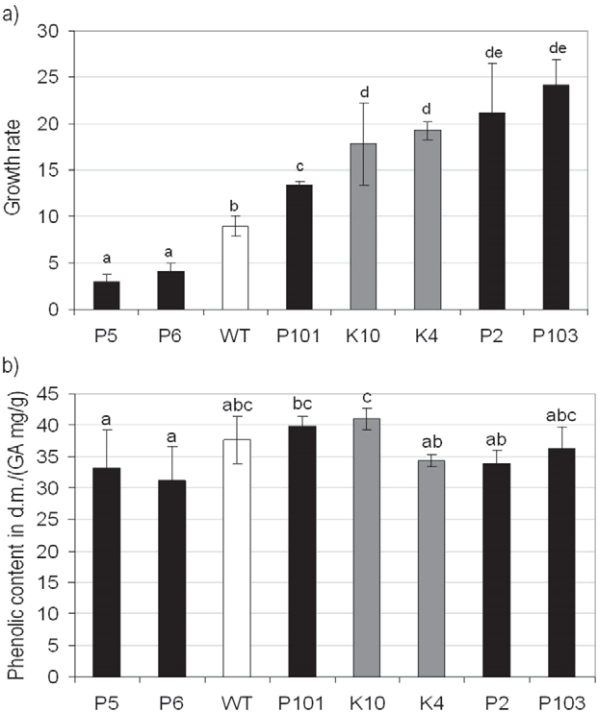

Fig. 3. Growth rates (a) and total phenolic content (b) of *C. blumei* transgenic roots transformed with the *AtPAL1* gene (black bars), empty vector (grey bars) and wild-type roots (white bar). Roots were grown in liquid MS medium, supplemented with 1 mg/L of NAA and harvested after 28 days. Growth was calculated according to Eq. 1. Bars represent the means±S.D. of three independent measurements. Different letters denote statistical differences (p≤0.05)

Table 1. Accumulation of rosmarinic acid (RA), caffeic acid (CAF) and chlorogenic acid (CHA) in *C. blumei* transgenic roots transformed with the *AtPAL1* gene (P2, P5, P6, P101, P103), in roots transformed by the empty vector (K4, K10) and in wild-type roots (WT). Roots were grown in liquid MS medium, supplemented with 1 mg/L of NAA and harvested after 28 days

Root	RA in d.m.	*w*(RA of total phenolics)	CAF in d.m.	CHA in d.m.
line	mg/kg	%	mg/kg	mg/kg
K10	(23356.75±993.70)^{c}	(56.94±2.09)^{a}	(208.66±56.20)^{a}	(31.66±7.11)^{c}
P103	(20612.50±1344.98)^{bc}	(56.84±1.77)^{a}	(298.64±45.43)^{bc}	(12.26±2.57)^{a}
K4	(20449.23±1960.45)^{bc}	(59.53±4.32)^{a}	(260.21±43.06)^{ab}	(20.78±1.32)^{b}
WT	(19438.49±3048.45)^{b}	(51.46±4.09)^{b}	(265.36±48.14)^{ab}	(29.31±7.79)^{bc}
P101	(19579.76±288.18)^{b}	(49.04±1.27)^{b}	(334.97±49.31)^{bc}	(22.78±7.19)^{b}
P2	(19448.12±2164.61)^{b}	(57.24±3.01)^{a}	(301.40±18.63)^{bc}	(21.13±2.65)^{b}
P5	(13260.99±2501.08)^{a}	(40.13±3.85)^{c}	(421.90±39.96)^{d}	(8.74±4.25)^{a}
P6	(12752.60±2867.04)^{a}	(40.55±2.44)^{c}	(343.93±27.36)^{c}	(11.24±3.37)^{a}

Data represent the means±S.D. of three independent measurements; letters in superscripts indicate significant difference at p≤0.05; d.m.=dry mass

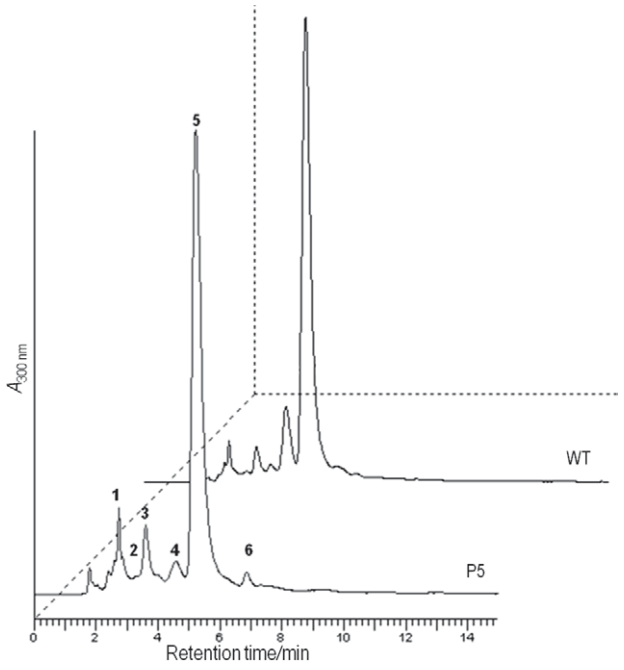

Fig. 4. HPLC analysis of wild-type (WT) and *AtPAL1*-transformed *C. blumei* roots (P5). Roots were grown in liquid MS medium, supplemented with 1 mg/L of NAA, harvested after 28 days and extracted in 70 % ethanol. Peak 2 is chlorogenic acid, peak 3 caffeic acid, peak 5 rosmarinic acid. Peaks 1, 4 and 6 are unidentified compounds

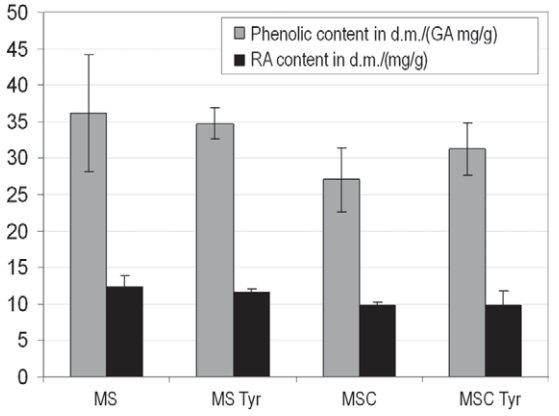

Fig. 5. Total phenolics and rosmarinic acid (RA) in *C. blumei* transgenic root line P5, transformed with *AtPAL1*. Roots were grown in liquid MS medium, supplemented with 1 mg/L of NAA (MS), and MS medium supplemented with 1 mg/L of NAA and 0.8 g/L of casein enzymatic hydrolysate (MSC). After 28 days, the media were replaced with fresh MS or MSC medium supplemented with, or without 5 mg/L of L-Tyr. Roots were harvested 3 days after the medium replacement. Bars represent the means±S.D. of three independent measurements. Different letters denote statistical differences ($p \leq 0.05$)

8 times less of the compound expressed in peak 6 than line P5 (Fig. 4). It is important to note that line P103, with the lowest PAL activity, accumulated similar amounts of RA, compared to WT and P101 roots with elevated PAL activity. All lines accumulated low amounts of CHA (Table 1, Fig. 4). The fraction of RA in total phenolics varied from 40.13 % in line P5 to 59.53 % in line K4. Lines with the highest PAL activity accumulated the lowest RA, relative to total phenolics (Table 1).

In order to eliminate the deficiency of RA precursors as a reason for lower RA accumulation, we cultured line P5 (with augmented PAL activity) in MS-1NAA medium supplemented with casein enzymatic hydrolysate (mixture of all amino acids) and with L-Tyr. There was no significant difference in total phenolics and RA content between the roots exposed to casein hydrolysate or after the addition of L-Tyr (Fig. 5). The addition of L-Tyr to the MS-1NAA medium was correlated with a significant drop in CAF accumulation (from 64.86 to 34.27 mg/kg of dry mass), and with a reduction in CHA (from 29.11 to 16.77 mg/kg of dry mass), while its addition to the medium with casein hydrolysate did not significantly affect CAF and CHA accumulation.

Discussion

Plants synthesize a large number of different phenolics that are crucial for many important functions. Non-oxidative deamination of phenylalanine, catalyzed by PAL, is generally considered to represent a first and key point controlling carbon flux into the phenylpropanoid pathway (*1,4*). Among four *Arabidopsis thaliana PAL* genes, *PAL1* and *PAL2* were shown to have a functional specialization in phenolic biosynthesis (*6,18*). In addition, *PAL1* was shown to be involved in phenolic biosynthesis in *A. thaliana* roots (*19*) and was chosen for overexpression in this study.

The *AtPAL1* gene, under the control of the CaMV 35S promoter, was introduced into *C. blumei*. The produced transgenic roots had disparate PAL activities, ranging from 67 up to 350 % of the wild type levels. There are numerous reports that indicate positive relationships between PAL activity and phenolic accumulation, but also there are puzzling data concerning this correlation. Overexpression of PAL failed to alter the isoflavone content in soybean (*20*), and higher PAL activity induced by PAL overexpression in tobacco was associated with a higher accumulation of CHA, but not the flavonoid glycoside rutin (*1*). Liu *et al.* (*21*) reported higher genistein accumulation after PAL overexpression in tobacco, but some of the specimens with increased PAL activity had significantly decreased genistein content. They obtained similar results for lettuce (*21*). In addition, our former study showed that a strong induction of PAL in *C. blumei* hairy roots, using a yeast elicitor and methyl jasmonate treatment, does not always lead to higher RA accumulation (*16*).

In the present study, *C. blumei* roots with augmented PAL activities had decreased amounts of total phenolics, RA and CHA, but increased amounts of CAF. RA is synthesized from L-Phe and L-Tyr *via* two parallel pathways with the final products joined by an ester bond and hydroxylated to form RA (*7*). If PAL overexpression reroutes the majority of the shikimic acid towards L-Phe, it is possible that L-Tyr becomes limiting factor for RA synthesis in the lines with increased PAL activity. But even after nutrient medium modification (addition of L-Tyr and/or casein enzymatic hydrolysate), there was no increase in total phenolics or RA content. Although

in the majority of cases, induction of PAL activity led to an increase in phenylpropanoid accumulation, the overexpression of PAL may be a wrong strategy for enhancing phenolic biosynthesis in *C. blumei*. Apparently, after PAL overexpression, a blockage occurred in the phenylpropanoid pathway. It is quite possible that the augmented PAL protein is suppressed with its own product (*t*-CA) and did not operate in transgenic cells, which could explain the necessity for desalting protein extracts before PAL activity measurements. *t*-CA is described as a competitive inhibitor of PAL, it suppresses transcription of the *PAL* gene and induces the synthesis of a proteinaceous inactivator of PAL (*3*,*22*). Achnine *et al.* (*23*) emphasize that associations between PAL and C4H probably help to reduce the size of the cellular *t*-CA pool and diminish potential feedback inhibition of PAL by *t*-CA. The addition of *t*-CA reduced PAL activity by 40–50 %, inhibited the taxane pathway and reduced taxol by 90 % (*24*). An interesting feature is that low concentrations (0.5–1 pM) of *t*-CA and *p*-coumaric acid weakly enhance PAL activity (*25*), which could explain numerous reports that emphasize the correlation between an increase in PAL and an increase in phenolic compounds, in response to various stimuli.

The growth rates of *C. blumei* transgenic roots significantly differed, with the lowest growth in roots with high PAL activity. Slower and stunted growth was related to posttranscriptional silencing of *PAL* in transgenic tobacco, where PAL activity was 5 to 20 times lower than in the control, wild-type plant (*3*), but also in transgenic tobacco with an 8-fold increase in PAL activity (*26*). To eliminate suppressor activity of *t*-CA, part of it could be converted to *cis*-cinnamic acid, which has lower inhibitory effect on PAL, but suppresses root growth (*27*,*28*). Indeed, *C. blumei* transgenic roots with high PAL activity showed strong growth suppression, which could be caused by *cis*-cinnamic acid synthesized from overproduced *t*-CA. In addition, there was an elevated accumulation of caffeic acid in *C. blumei* roots with high PAL activity. Caffeic acid is a strong activator of PAL (*29*), and perhaps its higher accumulation compensated for some of the inhibitory effect of *t*-CA.

Conclusion

To date, the overall effects of genetic alteration are not well understood, especially in terms of determining the effects of excessive expression, differential regulation and compartmentalization of enzymes. The hypothesis that PAL activity is a rate-determining step in RA biosynthesis is only partially true, since it seems that RA accumulation is probably affected by intermediate products, which have key roles in the channeling of metabolite synthesis.

In conclusion, overexpression of AtPAL1 in *C. blumei* transgenic roots substantially altered the growth and phenolic production. Our experiments indicate that PAL-catalyzed conversion of L-Phe to *t*-CA is not a limiting step in RA biosynthesis, and imply a possible crucial role for *t*-CA, perhaps as a signal molecule for regulating not only metabolite flux, but also plant growth.

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