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Cloning and characterisation of rosmarinic acid synthase from *Melissa officinalis* L.

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ABSTRACT

Lemon balm (*Melissa officinalis* L.; Lamiaceae) is a well-known medicinal plant mainly due to two groups of compounds, the essential oil and the phenylpropanoid derivatives. The prominent phenolic compound is rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. RA shows a number of interesting biological activities. Rosmarinic acid synthase (RAS; 4-coumaroyl-CoA:hydroxyphenyllactic acid hydroxycinnamoyltransferase) catalyses the ester formation. Cell cultures of *M. officinalis* have been established in order to characterise the formation of RA in an important diploid medicinal plant. RAS activity as well as the expression of the *RAS* gene are closely correlated with the accumulation of RA in suspension cultures of *M. officinalis*. The *RAS* cDNA and gene (*MoRAS*) were isolated. The *RAS* gene was shown to be intron-free. MoRAS belongs to the BAHD superfamily of acyltransferases. Southern-blot analysis suggests the presence of only one *RAS* gene copy in the *M. officinalis* genome. The enzyme was characterised with respect to enzyme properties, substrate preferences and kinetic data in crude plant extracts and as heterologously synthesised protein from *Escherichia coli*.

1. Introduction

Lemon balm (*Melissa officinalis* L.) is a well-known medicinal plant from the family Lamiaceae. Therapeutic properties of *M. officinalis* are sedative, carminative, antispasmodic, antibacterial, antiviral, antifungal, anti-inflammatory and antioxidative, mainly due to the content of essential oil and the presence of phenolic acid esters (Petersen and Simmonds, 2003). Preparations of lemon balm are used to treat infections of *Herpes simplex* (Wölbling and Leonhardt, 1994; Mazzanti et al., 2008). The main active phenolic compound is rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxphenyllactic acid (Scarpati and Oriente, 1958). RA can be found in all plant organs of *M. officinalis*. Its content in leaves is about 6% of the dry wt. (Parnham and Kesselring, 1985). RA putatively serves as a constitutively accumulated defence compound and as protection against herbivores.

The biosynthetic pathway leading to RA has been well established on the enzymatic level in *Coleus blumei* Benth. (syn. *Solenostemon scutellarioides* (L.) Codd, *Plectranthus scutellarioides* (L.) R. Br.; Lamiaceae; Petersen et al., 1993; Petersen, 1997). Both species, *M. officinalis* and *C. blumei*, belong to the subfamily Nepetoideae which is known for the presence of RA while it is – with a few exceptions – missing in the other Lamiaceae subfamilies (see Petersen et al., 2009 for an overview). RA can, however, also be found in numerous other plant taxa throughout the plant kingdom from hornworts to monocots (Abdullah et al., 2008; Petersen et al., 2009).

Cell cultures of *C. blumei*, *Salvia officinalis* and several other species of the families Lamiaceae and Boraginaceae have the ability to accumulate high levels of RA (Zenk et al., 1977; Hippolyte et al., 1992). RA up to 21% of the cell dry wt. has been detected in suspension cultures of *C. blumei* after only 10 days of cultivation in a medium with high sucrose concentration (Ulbrich et al., 1985; Petersen et al., 1994). For this reason, suspension cultures of *C. blumei* have been a good system to study the biosynthetic enzymes. Since *C. blumei* is an amphidiploid hybrid of unknown origin (Lebowitz, 1985) it is not a good system for genetic and regulatory investigations. A more suitable species is *M. officinalis*, a diploid and important widely used medicinal plant. Murin (1997) reports about 32 chromosomes in the genome of diploid *M. officinalis*, but according to Strid (1983) in some cases also plants with 64 chromosomes or 16 chromosomes could be found. RA accumulation as well as enzyme activities in *M. officinalis* suspension cultures show time curves similar to those of *C. blumei* (Weitzel, 2009; Weitzel and Petersen, 2010) making *M. officinalis* a good *in vitro* system for studies on the regulation of RA biosynthesis and accumulation.

The biosynthesis of RA (as established for *C. blumei*; Fig. 1) has been thoroughly described by Petersen et al. (1993, 2009) and Petersen (1997). The intermediary precursors 4-coumaroyl-CoA formed from L-phenylalanine and 4-hydroxyphenyllactate (pHPL) derived from L-tyrosine are coupled in a transesterification

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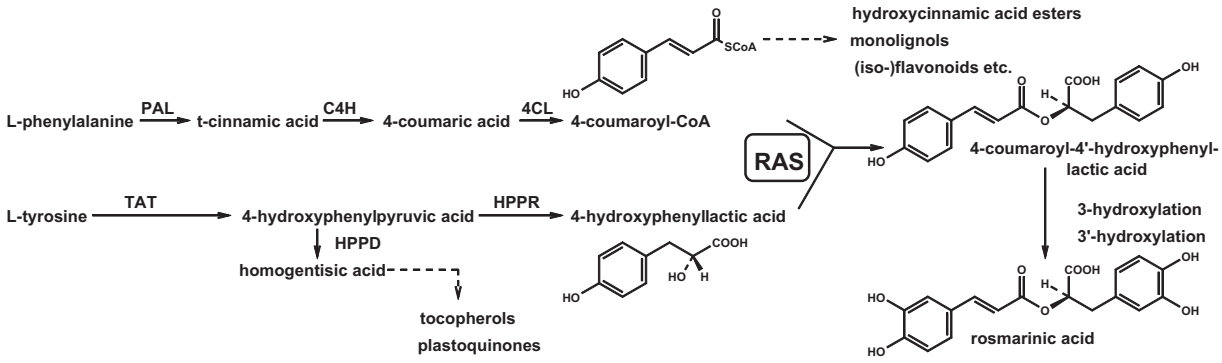

Fig. 1. Biosynthetic pathway for rosmarinic acid with some side reactions. PAL = phenylalanine ammonia-lyase, C4H = cinnamic acid 4-hydroxylase, 4CL = 4-coumarate:CoA-ligase, TAT = tyrosine aminotransferase, HPPR = hydroxyphenylpyruvate reductase, HPPD = hydroxyphenylpyruvate dioxygenase, RAS = rosmarinic acid synthase.

reaction catalysed by rosmarinic acid synthase (RAS; hydroxycinnamoyl-CoA:hydroxyphenyllactate hydroxycinnamoyltransferase; Petersen and Alfermann, 1988; Petersen, 1991). Finally this ester is hydroxylated by cytochrome P450 monooxygenase activities at the aromatic positions 3 and 3′ to RA (Petersen, 1997; Eberle et al., 2009). The activities of all the enzymes involved in the biosynthesis of RA in *C. blumei* could also be shown in protein extracts or microsomal preparations from suspension cultured *M. officinalis* cells (Weitzel, 2009; Weitzel and Petersen, 2010 and own unpublished results).

Investigations of the enzymes involved in this biosynthetic pathway have taken place in cell cultures of several plant species of the families Lamiaceae and Boraginaceae during the last years. Genes and cDNAs encoding enzymes of the general phenylpropanoid metabolism as well as RA biosynthesis have been isolated from, e.g. *Lithospermum erythrorhizon* (Yamamura et al., 2001; Matsuno et al., 2002), *Salvia miltiorrhiza* (Huang et al., 2008a,b), *Ocimum basilicum* (Gang et al., 2002), *C. blumei* (Kim et al., 2004; Berger et al., 2006; Eberle et al., 2009) and *M. officinalis* (Weitzel, 2009; Weitzel and Petersen, 2010 and other own unpublished results). Sequence similarities of the isolated genes have revealed their membership to prominent gene families, e.g. HPPR as member of the D-isomer specific 2-hydroxy acid dehydrogenases (Kim et al., 2004; Janiak et al., 2010), and the aromatic 3/3′-hydroxylase to the CYP98A subfamily (Eberle et al., 2009). The latter genes show high similarities to genes involved in the formation of caffeoylshikimate and chlorogenate. The latter, however, is not accumulated in *M. officinalis* according to our own investigations (Petersen et al., 2009).

Even though RAS probably is the most specific enzyme of the RA biosynthetic pathway, not much is known about this enzyme so far. In 2006, the first cDNA of RAS has been cloned from *C. blumei* (Berger et al., 2006). According to sequence similarities RAS belongs to the superfamily of BAHD acyltransferases (St. Pierre and De Luca, 2000; D'Auria, 2006). A second putative RAS sequence from *S. miltiorrhiza* has recently been deposited in the EMBL databank (FJ906696) but without biochemical proof of function.

We here report on the cloning and heterologous expression as well as biochemical characterisation of RAS from *M. officinalis* and its involvement in RA accumulation in suspension cultures of this plant.

2. Results and discussion

2.1. Characterisation of suspension cultures of M. officinalis

During a culture period of 11 days suspension-cultured cells of *M. officinalis* were monitored with regard to growth (dry wt.), RA accumulation and rosmarinic acid synthase activity (Fig. 2). The cultures were kept in CB-medium with 2% sucrose. Increasing the sucrose concentration of the medium to 4% resulted in an enhanced RA content (doubling from 3.5% to 6.7% in a previous experiment; data not shown), but the increase was not as significant as observed for cell cultures of *C. blumei* (Petersen et al., 1994). Additionally, the mRNA expression profile of RAS was examined.

The fr. wt. of the cells increased from 2.7 to 16.3 g per flask on day 7 (data not shown). The dry wt. of the cells increased continuously until a maximum of 0.63 g/flask was reached on day 5 of the culture period. Afterwards it decreased slowly. The RA content increased until day 6 (6.1% of the dry wt.) and then remained rather stable. The increase from day 10 to 11 is an artefact and has not been observed in all other culture characterisation experiments. The specific activity of RAS decreased from day 0 to 3 from 453 to 225 μkat/kg, increased until day 7 (510 μkat/kg) and then decreased until the end of the observation period.

Fig. 3 shows the mRNA expression level of RAS in comparison with the RAS activity in protein extracts from suspension cultures of *M. officinalis*. The comparatively equal amounts of mRNA used in the semi-quantitative PCR amplification can be seen in the bands of the control amplification, a 315 bp part of the 18S rRNA. The PCR-amplification of RAS-cDNA and RAS activity run in parallel, with the highest activity and expression level on day 3. The strongly decreasing RAS activity corresponds to the nearly undetectable mRNA levels from day 6 until the end of the cultivation period. The cellular RA content still increases until the end of the observation period due to residually present and active RAS protein.

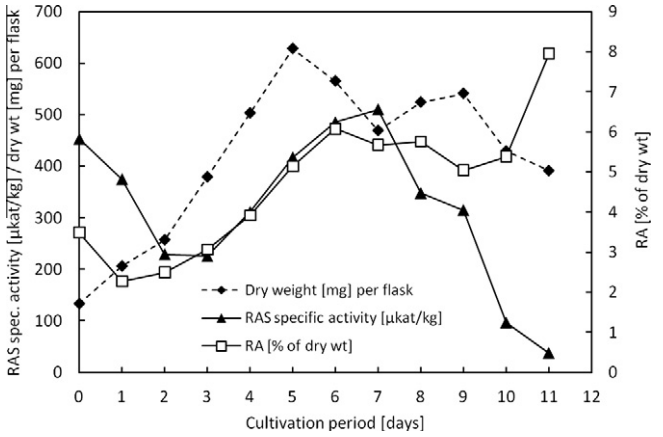

Fig. 2. Characterisation (dry wt., specific RAS activity, RA content) of a suspension culture of *Melissa officinalis* in CB-medium with 2% sucrose.

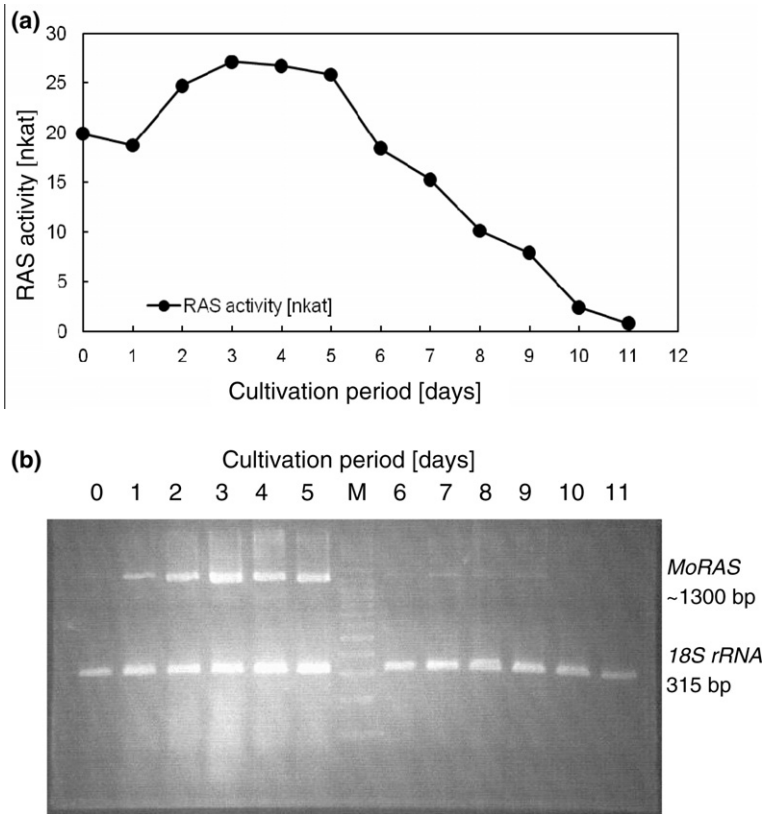

Fig. 3. RAS activity in protein extracts from suspension-cultured cells of *Melissa officinalis* (a) and (b) PCR amplification of the complete open reading frame of *MoRAS* as well as a 315 bp part of the 18S-rRNA from cDNA synthesised from RNA isolated on day 0–11 of the culture period.

Growth, RA accumulation and RAS activity in a suspension culture of *M. officinalis* essentially behaved like the corresponding data in *C. blumei* suspension cultures (Petersen et al., 1994), however, the level of RA accumulation remained lower and the effect of an increased sucrose concentration in the medium was less prominent (data not shown). In *C. blumei*, doubling the sucrose content of the medium (2 → 4%) resulted in a 10-fold increase in the RA content up to 19% of the cell dry wt. (Petersen and Alfermann, 1988) while only a doubling was observed in the *Melissa* culture. However, this behaviour is not observed in all suspension cell lines of *C. blumei* (own unpublished results). The RA content and the specific activity of RAS increase and decrease nearly parallel indicating a close correlation of these two parameters.

2.2. Isolation of a RAS cDNA and gene and determination of gene copy number

By using primers for amplification of the complete open reading frame of the C. *blumei* RAS-cDNA also the RAS-cDNA of *M. officinalis* could be amplified. To verify the sequences in the primer regions, RACE–PCR was performed. The obtained full-length cDNA of *MoRAS* was 1281 bp in length encoding a polypeptide of 427 amino acids (Supplementary Fig. 1). The cloned sequence showed high identity to the RAS-cDNA of *C. blumei* (84%) (Berger et al., 2006) and to a putative alcohol acyltransferase from *Lavandula angustifolia* (82%; EMBL Accession No. DQ886904). On the basis of the amino acid sequence, the identity between CbRAS and MoRAS ranged at 82%, the similarity at 91% (Supplementary Fig. 2). As RAS from *C. blumei*, MoRAS belongs to the superfamily of BAHD acyltransferases. The two conserved sequence motifs HxxxDG and DFGWG are also found in the sequence from *M. officinalis* (bold letters in Supplementary Fig. 1). To our knowledge this is only the second published RAS sequence that has been expressed and characterised with respect to the enzyme properties (see below). A recently submitted putative RAS sequence from *S. miltiorrhiza* (UniProt D6BR39) was only assigned according to sequence similarities. These sequences from *M. officinalis* and *S. miltiorrhiza* have identities of 89% on amino acid level and similarities of 94% (Supplementary Fig. 2).

Using genomic DNA isolated from *M. officinalis* suspension cells, PCR amplifications with primers amplifying the complete open reading frame of *MoRAS* gave an amplicon with exactly the same length and sequence as the previously isolated cDNA sequence. Thus, the RAS gene in *M. officinalis* is intron-free. This is somewhat surprising, since BAHD acyltransferase genes may be devoid of introns, but the hydroxycinnamoyltransferases of the subclade V according to D'Auria (2006) or subclade Ib as identified by Yu et al. (2009) mostly carry an intron. However, intron-free members of this subclade have been identified both in *Populus tremuloides* and *Arabidopsis thaliana* (Yu et al., 2009). The genomic *CbRAS* contained a phase 0 intron of 914 bp inserted after nucleotide 405; this intron is typically inserted after the codon for the amino acid glutamine (''Q-intron'').

The gene copy number of RAS in the genome of *M. officinalis* was determined by Southern-blot analysis. None of the restriction enzymes chosen for digestion of the genomic DNA did cut in the coding sequence of *MoRAS*. The result suggests the existence of only one *RAS* gene in the haploid genome of *M. officinalis* (Fig. 4). Two other genes of RA biosynthesis, phenylalanine ammonia-lyase and 4-coumarate:CoA-ligase, have been investigated as well for their gene copy numbers in *M. officinalis* and in both cases only two gene copies have been suggested (Weitzel and Petersen, 2010). *M. officinalis* as a diploid plant therefore is much better suited for investigations into the regulation of RA biosynthesis on

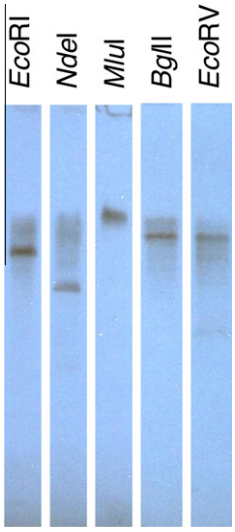

Fig. 4. Determination of RAS gene copy number in *Melissa officinalis* by Southern-blot analysis.

gene level than the amphidiploid *C. blumei*. A better understanding of regulatory processes involved in the formation and accumulation in this plant species is of high pharmaceutical interest.

It can be expected that further hydroxycinnamoyltransferases are present in *M. officinalis*. A hydroxycinnamoyl-CoA:shikimic acid hydroxycinnamoyltransferase (HST) should be present in all lignin-containing plants, since this HST has been assigned an important function in the biosynthesis of monolignols with higher substitution level at the aromatic ring (Hoffmann et al., 2004). Own recent investigations have shown a first proof of the presence of MoHST (unpublished). The formation of chlorogenic acid and/or other caffeoylquinic acids in lemon balm is reported controversely in the literature. While Litvinenko et al. (1975), Pedersen (2000) and Fecka and Turek (2007) did not find chlorogenic acid in *M. officinalis*, Marques and Farah (2009) have found several different caffeoylquinic acids. It is therefore worthwhile looking for enzymes/genes catalysing the formation of chlorogenic and other caffeoylquinic acids in this plant species.

2.3. Characterisation of RAS in crude extracts from M. officinalis suspension cells

Protein extracts were prepared from 4 to 5 day old suspension-cultured cells of *M. officinalis* in order to characterise RAS directly from plant cells. The pH-optimum of RAS is at pH 7.1 in 0.1 M K–Pi buffer at 30 °C. In the same buffer, the temperature optimum is at 45 °C. The RAS reaction in a standard assay was linear with protein concentrations up to 1.5 mg/ml of the crude extract in the assay and with time up to 10 min at 30 °C. Kinetic parameters were determined for 4-coumaroyl- and caffeoyl-CoA and for pHPL and 3,4-dihydroxyphenyllactic acid (DHPL) in all possible combinations (Table 1). 4-Coumaroyl-CoA and caffeoyl-CoA were accepted with apparent K_m-values of 4.8 and 5.8 μM with pHPL and of 7.3 and 5.2 μM with DHPL as acceptor substrate. Both hydroxycinnamoyl-CoA thioesters are thus accepted with similar affinities. pHPL is converted with higher affinity than DHPL. The apparent K_m-values for pHPL are at 16.9 μM with 4-coumaroyl-CoA and 74.0 μM for caffeoyl-CoA. The corresponding values for DHPL are at 152.8 and 233.6 μM. Saturation concentrations ranged at 50 μM for all CoA-activated acids. For the hydroxyphenyllactic acids saturation was achieved between 100 and 900 μM (see Table 1). The K_m-values of RAS in crude extracts from *M. officinalis* cells are similar to those determined for the enzyme isolated from *C. blumei* suspension cells (Petersen, 1991).

2.4. Characterisation of MoRAS heterologously expressed in Escherichia coli

E. coli BL21(DE3)pLysS harbouring the pET-15b plasmid with the open reading frame of *MoRAS* was used to heterologously synthesise MoRAS with *N*-terminally attached 6×His-tag. Crude bacterial protein extracts were used to determine the general enzyme characteristics of MoRAS. The temperature optimum was at 45 °C, the pH-optimum at pH 7.3 in K–Pi buffer. These two values are comparable in the crude enzyme preparation and the heterologously synthesised MoRAS. In order to test the range of acceptor substrates for MoRAS, we used pHPL, shikimate and quinate together with caffeoyl-CoA. Only pHPL was used for ester formation, thus the *MoRAS* sequence encodes a specific rosmarinic acid synthase as also described for *CbRAS* (Berger et al., 2006). Other hydroxyphenyllactic acids were accepted as well. Recently, however, it was shown for RAS from *C. blumei* that also substrates like D-phenylalanine, D-tyrosine and D-3,4-dihydroxyphenylalanine are accepted by the recombinantly synthesised enzyme putatively resulting in the formation of amides (Sander and Petersen, in press).

The apparent K_m-values were determined for pHPL and DHPL as acyl acceptor substrates together with both, 4-coumaroyl- and caffeoyl-CoA (Table 1). Severe substrate inhibition was observed for 4-coumaroyl-CoA with pHPL as acceptor and less with DHPL. This influenced the exact determination of the substrate saturation, V_{max} and app. K_m-value. The app. K_m-values for the donor substrates are not severely different and range between 2 and 9 μM (Table 1). However, the apparent K_m-values for pHPL or DHPL are highly dependent on the donor substrate and are considerably lower when 4-coumaroyl-CoA is the acyl donor (app. K_m for pHPL at

Table 1
Kinetic characterisation of RAS from *Melissa officinalis* tested either as crude enzyme in protein extracts from suspension-cultured plant cells or heterologously expressed as 6×His-fusion protein in *E. coli*. The substrate concentration ranges used for the variable substrate in assays with crude plant enzyme/heterologously synthesised enzyme are indicated. The constant substrate was used in saturating concentrations.

Variable substrate	Constant substrate	RAS in crude protein extracts from *M. officinalis* suspension cells		RAS expressed in *E. coli*		
		App. K_m (μM)	Saturation (μM)	App. K_m (μM)	V_{max} (μkat kg^{-1})	V_{max}/K_m (μkat kg^{-1}/mM)
pC-CoA (0–60/0–108 μM)	pHPL	4.8	~50	9.4	1076	114.5
Caf-CoA (0–180/0–48 μM)	pHPL	5.8	~50	2.3	283	123.0
pC-CoA (0–52/0–32 μM)	DHPL	7.3	~50	3.2	294	91.9
Caf-CoA (0–120/0–48 μM)	DHPL	5.2	~50	3.3	108	32.7
pHPL (0–80/0–50 μM)	pC-CoA	16.9	~100	18.9	1432	75.8
pHPL (0–0.8 mM/0–1.2 mM)	Caf-CoA	74.0	~600	156.5	153	0.9
DHPL (0–400/0–880 μM)	pC-CoA	152.8	~500	85.3	294	3.4
DHPL (0–880/0–600 μM)	Caf-CoA	233.6	~900	212.6	156	0.7

18.9 μM with 4-coumaroyl-CoA and at 156.5 μM with caffeoyl-CoA; app. K_m for DHPL at 85.3 μM with 4-coumaroyl-CoA and at 212.6 μM with caffeoyl-CoA). The determined K_m- and V_{max}-values as well as the value V_{max}/K_m indicate that 4-coumaroyl-CoA or caffeoyl-CoA together with pHPL are the best substrate combinations (from those substrates tested). Very similar apparent K_m-values have recently been determined for recombinantly synthesised RAS from *C. blumei* (Sander and Petersen, in press). Which donor substrate is used in the biosynthesis of RA might also be dependent on the availability of these two hydroxycinnamoyl-CoA esters in the cytoplasm. The preference for monohydroxylated substrates is consistent with the proposed biosynthetic pathway for RA in *C. blumei* (Petersen et al., 1993), where the 3- and 3′-hydroxyl groups of the aromatic rings are introduced by specific cytochrome P450 monooxygenases (Petersen, 1997; Eberle et al., 2009).

3. Conclusion

Rosmarinic acid synthase was characterised in crude enzyme preparations from *M. officinalis*. The gene encoding this enzyme shows high similarities to the corresponding gene from *C. blumei* although it does not contain an intron. The genome of *M. officinalis* only contains one copy number. The expression of *MoRAS* during a cultivation period of *M. officinalis* suspension cells parallels RAS activity. The heterologously expressed MoRAS shows high affinities to 4-coumaroyl- and caffeoyl-CoA as acyl donor and pHPL and DHPL as acceptor substrates; shikimate and quinate were not accepted as acceptors. Overall, MoRAS shows similar characteristics as the corresponding gene and enzyme from *C. blumei*. Therefore, the diploid *M. officinalis* is a suitable medicinally important plant/cell culture system for studies of the regulation of RA biosynthesis.

4. Experimental

4.1. Establishment of suspension cultures

To establish a suspension culture, plant material of *M. officinalis* L. was harvested from the medicinal plant garden of the Institut für Pharmazeutische Biologie und Biotechnologie (Philipps-Universität Marburg, Germany). After young stems and leaves had been washed in running tap water they were blotted dry on tissue paper and pre-sterilised with EtOH (96%) for 1 min. Sterilisation was performed in a 10% commercial chlorine disinfectant solution for 10–15 min. Before placing the plant parts on CB2 agar medium (Petersen and Alfermann, 1988) the green plant tissues were washed three times with sterile distilled water and cut into segments. Callus was further sub-cultivated on the same medium with transfer to fresh medium in intervals of 3–4 weeks. Suspension cultures were established by transferring 5 g callus cells to 50 ml liquid CB2-medium in a 250 ml Erlenmeyer flask and shaking at 110 rpm. Sub-cultivation of the suspension cultures took place weekly with 5 g wet cells/50 ml medium. Callus as well as suspension cultures were cultivated at 25 °C in the dark.

4.2. Characterisation of a M. officinalis suspension culture

For characterisation during a culture period of 11 days, a sufficient number of Erlenmeyer flasks with 5 g suspension cells/50 ml medium were inoculated, one or two of which were harvested daily at the same time of the day. Cells and medium were separated by suction filtration, then the cells were weighed (fr. wt.). To obtain the dry wt. 0.5 g cells were frozen at −20 °C and lyophilised. To extract RA, 50 mg lyophilised cells were suspended in 2 ml 70% EtOH and mixed vigorously. Extraction was performed by sonicating the samples in an ultrasonic bath at 70 °C for 10 min twice with intermediate vigorous mixing. After the samples had been centrifuged for 10 min at 3000*g* the supernatant was removed and diluted 1:10 with 50% MeOH acidified with 0.01% H_3PO_4 followed by centrifugation (5 min, 16,000*g*) before HPLC analysis.

4.3. Protein extraction

Freshly harvested cells were homogenised together with 20% of the cell fr. wt. Polyclar 10 and 1 ml per 1 g fr. wt. 0.1 M K–Pi buffer pH 7.0 containing 1 mM dithiothreitol (DTT) with help of an Ultra-Turrax (Janke and Kunkel, Staufen i. Br., Germany) for 3 × 30 s with intermediate cooling on ice. To obtain the protein extract used for enzyme assays the preparation was centrifuged at 8000*g* for 20 min at 4 °C. The protein extract was kept on ice or stored at −20 °C.

To harvest bacterial cells the bacterial suspension was centrifuged for 5 min at 5000*g* at 4 °C. The cell-free medium was discarded and the bacterial pellets frozen over-night at −70 °C. After thawing on ice the cells were resuspended in 2–5 ml buffer (0.1 M K–Pi buffer pH 7.0) per 1 g bacterial cells. Cells were disrupted by ultrasonication (3 × 30 s) and cell residues sedimented by centrifugation at 4 °C for 10 min at 10,000*g*. The supernatant was used for enzyme assays or stored at −20 °C until use.

Protein concentrations were determined photometrically according to the method of Bradford (1976) with bovine serum albumin as standard.

4.4. Determination of rosmarinic acid synthase activity

Assays for the determination of RAS activity contained in a total volume of 125 μl, 67.5 μl 0.1 M K–Pi buffer pH 7.0, 12.5 μl 0.1 M DTT, 10 μl 12.5 mM ascorbic acid, 10 μl 2.5 mM hydroxycinnamoyl-CoA, 5 μl 20 mM D/L-pHPL (alternatively DHPL, shikimate, quinate) and 20 μl of the protein preparation. They were incubated at 30 °C for 10 min and then stopped by addition of 20 μl 6 N HCl. After extraction with 500 μl EtOAc twice and evaporation of the solvent, the residue was dissolved in 50% MeOH/0.01% H_3PO_4.

For the determination of kinetic parameters, standard assays were used with the following alterations: the incubation time was reduced to 30 or 60 s. The concentration ranges for the varied substrates are indicated in Table 1.

4.5. HPLC analysis

For isocratic HPLC analysis an Equisil ODS column (particle size 5 μm; 250 mm length, 4 mm diameter, 20 mm precolumn; Dr. Maisch HPLC GmbH, Ammerbuch, Germany) was used with 50% MeOH/0.01% H_3PO_4 as eluent at flow rates of 0.8–1.0 ml/min. Hydroxycinnamic acid esters were detected at 333 nm and quantified using authentic standards (25 μM).

4.6. Isolation of RNA and DNA and cDNA synthesis

The method of Chomczynski and Sacchi (1987) was used to isolate total RNA from suspension cultures of *M. officinalis*. cDNA was synthesised with the RevertAid® First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol. Total DNA was isolated by the slightly modified method of Rogers and Bendich (1985).

4.7. Isolation of a RAS cDNA

A first amplification of a RAS-cDNA from *M. officinalis* was achieved with primers designed for RAS from *C. blumei* (RAS-VL5-CB, RAS-VL3-CB; Table 2). The original sequence of the *Melissa* RAS

Table 2
Sequences of primers used in this study.

Primer name	Primer sequence
RAS-VL5-CB	5′-ATTACATATGAAGATAGAAGTCAAAGACTC-3′
RAS-VL3-CB	5′-TAGGATCCTCATCAAATCTCATAAAACAACTTCTC-3′
MoRAS5-pET	5′-TTGGATCCATGAGGATCGATATCAAGGACTC-3′
MoRAS3-pET	5′-TAGGATCCTCATCAAATCTCATAAAACAACTTCTC-3′
MoRAS-SBf	5′-GCAAACGCGCACCACTTATCCGACGGCGTCGCCGCC-3′
MoRAS-SBr	5′-CACCGCCTTCCCCCACCC(AG)AA(AG)TCCGGCTC(AG)TA-3′
18Sf	5′-AGGAATTGACGGCCAAACAC-3′
18Sr	5′-GCACATCTAAGGGCATCACA-3′)

was verified by RACE-PCR and finally the primers MoRAS5-pET and MoRAS3-pET (Table 2) were used for full-length amplification with introduction of *Bam*HI restriction sites for ligation into the expression vector pET-15b (Novagen, Darmstadt, Germany). The plasmid was transferred into *E. coli* BL21(DE3)pLysS. Expression of MoRAS was performed in LB-medium containing 100 μg ampicillin. Over-night culture (1.5 ml) were used to inoculate 100 ml medium and cultures were incubated at 37 °C with shaking at 220 rpm until an OD_{600} of ca. 0.6 was reached. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactoside and the cultures cultivated for another 5 h before they were harvested as described above.

Sequencing and primer synthesis were done by Eurofins MWG Operon, Ebersberg, Germany.

4.8. Southern-blot analysis

Thirty-five micrograms of total DNA of *M. officinalis* were digested over-night with 100 U of suitable restriction enzymes (see Fig. 4) in the buffer proposed by the manufacturer (Fermentas). At the following morning additional 10 U of each restriction enzyme were added. DNA was precipitated by addition of 0.15 M sodium acetate and an equal volume of isopropanol at −20 °C and collected by centrifugation for 10 min at 16,000*g*. The precipitated DNA was washed with 70% EtOH and redissolved in 30 μl TE buffer at 56 °C for 10 min. The DNA was electropherographed on a 0.7% agarose gel and blotted onto a positively charged nylon membrane according to standard procedures. 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 to synthesise a MoRAS probe. The primers MoRAS-SBf and MoRAS-SBr (Table 2) yielded a 700 bp amplicon that was used for hybridisation. Washes were done at 58 °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 disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3.3.1.1^{3,7}]-decan}-4-yl)phenyl phosphate (CSPD).

4.9. Semi-quantitative PCR

During the characterisation of a *M. officinalis* suspension culture a small amount of the harvested cells was frozen at −70 °C and used to isolate total RNA according to the method of Chomczynski and Sacchi (1987). The concentration of RNA was measured photometrically at 260 nm. With help of the RevertAid® First Strand cDNA Synthesis Kit (Fermentas) exactly the same concentration of each RNA preparation was reverse-transcribed into cDNA according to the manufacturer's protocol. PCR was performed using 2 μl cDNA (equivalent to 5.4 μg RNA) and primers both for a 315 bp-part of the 18S subunit of rRNA (18S-f, 18S-r; Table 2) and *MoRAS* (MoRAS5-pET and MoRAS3-pET, see above). GoTaq polymerase (Promega, Mannheim, Germany) was used according to the manufacturer's protocol with the following program: 2 min 94 °C (one cycle); 30 s 94 °C, 1 min 54 °C, 2 min 70 °C (30 cycles); 10 min 70 °C (one cycle). The DNA was separated by electrophoresis on a 2% agarose gel and stained by incubating the gel for 15 min in 0.005% aqueous ethidiumbromide and subsequent washing in distilled water.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.01.039.

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