Cations modulate the substrate specificity of bifunctional class I *O*-methyltransferase from *Ammi majus*

Richard Lukačin[a], Ulrich Matern[a], Silvia Specker[a], Thomas Vogt[b,*]

[a] *Institut für Pharmazeutische Biologie der Philipps-Universität Marburg, Deutschhausstr. 17A, D- 35037 Marburg/Lahn, Germany*
[b] *Leibniz-Institute of Plant Biochemistry, Department of Secondary Metabolism, Weinberg 3, D-06120 Halle/Saale, Germany*

Received 2 August 2004; revised 23 September 2004; accepted 11 October 2004

Available online 22 October 2004

Edited by Judit Ovádi

Abstract Caffeoyl-coenzyme A *O*-methyltransferase cDNA was cloned from dark-grown *Ammi majus* L. (Apiaceae) cells treated with a crude fungal elicitor and the open reading frame was expressed in *Escherichia coli*. The translated polypeptide of 27.1-kDa shared significant identity to other members of this highly conserved class of proteins and was 98.8% identical to the corresponding *O*-methyltransferase from parsley. For biochemical characterization, the recombinant enzyme could be purified to apparent homogeneity by metal-affinity chromatography, although the recombinant enzyme did not contain any affinity tag. Based on sequence analysis and substrate specificity, the enzyme classifies as a cation-dependent *O*-methyltransferase with pronounced preference for caffeoyl coenzyme A, when assayed in the presence of Mg^{2+}-ions. Surprisingly, however, the substrate specificity changed dramatically, when Mg^{2+} was replaced by Mn^{2+} or Co^{2+} in the assays. This effect could point to yet unknown functions and substrate specificities in situ and suggests promiscuous roles for the lignin specific cluster of plant *O*-methyltransferases.

Keywords: Lignin biosynthesis; Caffeoyl coenzyme-A dependent *O*-methyltransferase; Substrate specificity; Bivalent cation; *Ammi majus* L., Apiaceae

1. Introduction

Methylation by *S*-adenosyl-L-methionine (AdoMet)-dependent *O*-methyltransferases (OMTs) (EC 2.1.1) is a common alkylation reaction observed in natural product biosynthesis, and site-specific *O*-methylations of phenolic hydroxyl groups modulate the chemical reactivity and the physiological properties of aromatic compounds [1]. Based on the classification by Joshi and Chiang [2], plant class I OMTs designate a group of low molecular weight (23–27 kDa) and Mg^{2+}-dependent enzymes which were initially characterized as caffeoyl CoA OMTs (CCoAOMTs) and shown to play an important role in the methylation of guaiacyl residue precursors of lignin, like caffeoyl or 5-hydroxyferuloyl CoA [3]. These enzymes, which do not accept caffeic acid as a substrate, appear to be crucial for the composition of lignin, and their function has been considered to be confined to lignin biosynthesis [4]. However, the methylation profile in angiosperms may also be generated by class II OMTs, referred to as COMTs, which methylate caffeic acid, caffeoyl aldehyde or caffeoyl alcohol independently of Mg^{2+} [5–7]. The apparent redundancy seemingly results in a cell- and tissue-specific metabolic grid, which is essential for plants to regulate their lignin composition and structure.

Class II enzymes display a rather promiscuous substrate specificity. Aside from lignin biosynthesis, additional natural products have been suggested recently as possible substrates for class I OMTs. This proposal was founded on the identification of a novel subclass of substrate-tolerant CCoAOMTs from the ice plant and from *Arabidopsis thaliana*, which in addition to caffeoyl CoA methylate a variety of plant metabolites with catechol-type functionality, including quercetin, caffeic acid or caffeoylglucose (Fig. 1) [8]. This subclass of enzymes can be distinguished from designated lignin-specific CCoAOMTs not only because of their differential substrate specificities, but also by clear differences in the polypeptide sequences [6]. Both types of these class I OMTs were reported to be inducible by biotic or abiotic stressors [9–11].

In this report, we describe the substrate specificities of a stress-inducible class I OMT, cloned from *Ammi majus* L. (bishop's weed). The enzyme classifies with the lignin specific CCoAOMTs from a variety of plant species based on high sequence identities. *Ammi majus* is a member of the Apiaceae and is well known for the accumulation of psoralene derivatives, used in the treatment of vitiligo, a patchy depigmentation of the skin [9,12]. Detailed kinetic studies performed with the heterologously expressed enzyme and with different cations revealed considerable variations in the substrate preference depending on the metal cofactor used in the assays. Thus, also the enzymes of the lignin specific cluster of CCoAOMTs may serve multiple functions in natural product biosynthesis.

2. Materials and methods

2.1. Materials

Quercetin and quercetagetin were purchased from Roth (Karlsruhe, Germany) and from Extrasynthese (Genay, France), respectively. 3,4-Dihydroxy benzoic acid was obtained from Sigma (Deisenhofen, Germany). 5-Hydroxyferulic acid was a kind gift from Vincent Chiang (North Carolina State University, USA). Caffeoylglucose was prepared and quantified from caffeic acid and UDP–glucose with the purified

* Corresponding author. Fax: +49-345-5582-1009.
E-mail address: tvogt@ipb-halle.de (T. Vogt).

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; CCoAOMT, caffeoyl-coenzyme A dependent *O*-methyltransferase

0014-5793/$22.00
doi:10.1016/j.febslet.2004.10.032

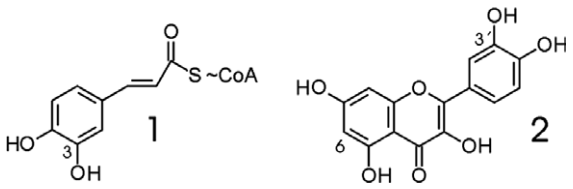

Fig. 1. Substrates of *A. majus* CCoAOMT. The enzyme methylates the 3- or 3′-phenolic group of caffeoyl CoA (1) and quercetin (2), respectively. The structure of quercetagetin differs from quercetin by an additional hydroxyl group at carbon C-6.

recombinant sinapic acid glucosyltransferase according to Ibdah et al. [8]. Concentrations of caffeoylglucose were calculated based on a standard of sinapoyl glucose (provided by A. Baumert, IPB, Halle) and calculated with an extinction coefficient of $\varepsilon_{330\,nm}$ of 23 000. Caffeoyl-CoA was prepared according to Strack et al. [13] based on a method by Stöckigt and Zenk [14].

2.2. Cloning, expression, and purification

Cell suspension cultures of *A. majus* were grown continuously in the dark and induction of the cells with the crude cell wall elicitor from *Phytophthora sojae* (*Pmg*) was performed as described elsewhere [11]. Total RNA was isolated from *Pmg* elicitor-induced cells following the protocol of Giuliano et al. [15]. Specific CCoAOMT-cDNA fragments of *A. majus* were amplified by RT-PCR using oligonucleotide primers which had been designed according to the sequence of parsley CCoAOMT (GenBank Accession No. M69184). Full length clones were generated by 5′- and 3′-RACE. Two *Nco*I sites contained in the open reading frame (ORF) of the CCoAOMT were deleted by using QuikChange® Multi Site-Directed Mutagenesis Kit as described by the manufacturer (Stratagene, Heidelberg, Germany) without altering the amino acid sequence, and 5′- and 3′-flanking *Nco*I and *Bam*HI sites, respectively, were introduced at the start codon and proximal downstream of the stop codon. The mutations were verified by DNA sequencing [16], and the CCoAOMT-coding region was isolated by digestion with *Nco*I and *Bam*HI and ligated in-frame into pQE60 vector (Qiagen, Hilden, Germany) for functional expression in *E. coli* strain M15 (Qiagen) harboring the plasmid pRep4. The expression was induced by the addition of 1.0 mM isopropyl-thio-β-D-galactoside (IPTG) [11], and the bacteria from two liters of culture were harvested as described [17]. Crude bacterial extract was prepared by ultrasonication and centrifugation (30 000 × g, 4 °C, 10 min). The supernatant was treated with 0.05% protamine sulfate for 5 min, cleared by centrifugation, and the enzyme was purified from the crude extract by affinity chromatography on 1 ml HiTrap HP (Amersham, Freiburg, Germany). Finally, the enzyme fraction was desalted through PD10 columns (Amersham) into 20 mM KPi, pH 7.5, 10% glycerol, and 150 mM NaCl and adjusted to 1 mg/ml protein based on a calculated extinction coefficient of 28 000 ± 5%. Total yield was 20 mg of highly purified CCoAOMT. The purification was monitored by SDS–PAGE [18] and enzyme activity assays of individual fractions. Protein amounts were quantified by the absorbance at 280 nm. Purified protein was stable for several weeks when stored at −20 °C.

2.3. Enzyme assays

Enzyme assays and kinetic measurements were performed in 100 mM KPi buffer, pH 7.5 (total volume 60 μl), containing 10% glycerol, 5 mM sodium ascorbate, 1 mM 2-mercaptoethanol and 150 μM $MgCl_2$, in the presence of 2–40 μM substrate (dissolved in 50% DMSO in case of flavonols and 3,4-dihydroxy benzoic acid), 100 μM AdoMet and 1.5 μg of purified CCoAOMT. The assays were conducted at 37 °C for 1–10 min, depending on the substrate used, after which the incubation was terminated by addition of 20 μl 3.5% TCA in 50% acetonitrile. The reaction products were analyzed by reverse phase liquid chromatography on a Nucleosil 5 μm C18 column (5 cm × 4 mm i.d.; Macherey & Nagel, Düren, Germany), as described previously [8,19]. Compounds were analyzed with a linear gradient from 5% to 50% acetonitrile (solvent B) in 1.5% aqueous phosphoric acid (solvent A) for free acids and CoA esters, from 5% to 30% B (glucose esters), and from 20% to 80% B in A (flavonoids) within 4 min at a flow rate of 1 ml min^{-1}. Detection was performed at 364 nm (flavonols), 340 nm (CoA esters), 330 nm (hydroxycinnamic acids and caffeoylglucose), and 276 nm (3,4-dihydroxybenzoic acid), respectively. The K_m and V_{max} values were visualized by Lineweaver–Burk plots and quantified by linear regression analysis. All enzyme activities and kinetic data were recorded in triplicates, based on two independently performed experiments.

3. Results and discussion

3.1. cDNA cloning, functional expression, and purification

Oligonucleotide primers designed for the cloning of CCoAOMT from parsley [10] were used for RT-PCR amplifications with total RNA template from *A. majus* cells which had been elicited for 4 h. Extension of the amplified fragment by 3′- and 5′-RACE generated one full size cDNA clone spanning an ORF of 723 bp (GenBank Accession No. AY620245) and encoding a polypeptide of 241 amino acids. Functional expression of the *A. majus* coding region in *E. coli* provided the final proof of identity, because the IPTG-induced transformants expressed CCoAOMT activity (about 1 μkat/kg) which was absent in extracts from non-transformed controls. The sequence identity of the translated polypeptide with heterologous CCoAOMTs of about 80–90% was striking and is consistent with the high conservation to be expected among the class I OMTs. These enzymes have likely played an important role during evolution with a strong impact on lignin monomer composition, because a similarity close to 80% was observed even with the corresponding enzymes from monocots, i.e., maize, or from gymnosperms like pine [20,21]. Not surprisingly, the closest sequence identity of the enzyme from *A. majus* was observed to the parsley CCoAOMT, which also is a member of the Apiaceae, sharing 238 identical amino acids (98.8%) differing by only three residues (Glu-140, Thr-141 and Val-176). In contrast, there is no apparent sequence identity to the class II caffeic acid OMTs, which in general display a broad substrate specificity [8]. This might suggest that essentially the distinct substrate specificity was the driving force for sequence conservation during evolution. Other factors such as an hypothetical interaction with other proteins or the conditions of subcellular localization remain to be investigated.

The recombinant CCoAOMT, expressed as an untagged polypeptide, was purified from the crude extracts by a one step chromatography procedure. Since a distinct metal-affinity of the enzyme had been observed in preliminary assays, the eventual purification was accomplished by affinity chromatography on a high affinity HisTrap-column. At pH 7.5, the enzyme bound to the metal-affinity matrix and could be eluted in the presence of 30 mM imidazole, yielding 20 mg total of apparently homogenous CCoAOMT protein from two liters of *E. coli* culture (Fig. 2). Examination of the fractionated enzyme by SDS–PAGE revealed a single band of 27 kDa (lane 3, Fig. 2), which was absent in protein extracts of non-transformed *E. coli* control cells. The purified recombinant *A. majus* enzyme was used for kinetic investigations.

3.2. Metal dependency and substrate specificity

CCoAOMTs are generally referred to as Mg^{2+}-dependent enzymes showing the highest catalytic activity with caffeoyl-CoA substrate. Accordingly, other bivalent cations have rarely been tested, and most of the assays were confined to the preferred substrate. However, the binding of the *Ammi* CCoAOMT to the Ni^{3+}-loaded HisTrap-column on purification already suggested a wider metal affinity for the enzyme.

Therefore, various metals as well as a number of potential catechol-type substrates were employed in an attempt to accurately delineate substrate and cofactor requirements for CCoAOMT from *A. majus* (Table 1). The substitution of Mg^{2+} by Ca^{2+} or Zn^{2+} in the CCoAOMT assays marginally affected the turnover rate of substrate and the substrate preference, whereas 150 μM Cu^{2+} completely abolished the enzyme activity (data not shown). However, when the recombinant homogeneous enzyme was incubated in the presence of Mn^{2+} or Co^{2+}, a dramatic increase in enzyme activity was observed, because at 150 μM the rate of methylation of caffeoyl-CoA increased about twofold. Comparison of the kinetic data recorded with Mg^{2+} vs. Co^{2+} still suggested caffeoyl-CoA as the preferred substrate (Table 1), which is consistent with a classification as a "lignin specific" CCoAOMT. Nevertheless, a bias towards catechol-type flavonols, like quercetin (Fig. 3), was observed in the presence of Co^{2+} (Fig. 3a, Table 1). Obviously, the substitution of Mg^{2+} by Co^{2+} in the in vitro assays decreased the affinity of the enzyme to caffeoyl-CoA (and also to quercetin), but in both instances this effect was overcompensated by the considerably enhanced K_{cat}-values. Moreover, replacing Mn^{2+} or Co^{2+} for Mg^{2+} drastically shifted the substrate preferences towards caffeic, 5-hydroxyferulic and 3,4-dihydroxybenzoic acids (Table 1), which were not at all accepted in assays with Mg^{2+} as cofactor. Furthermore, 5-hydroxyferulic acid or caffeoylglucose was methylated in the presence of Mn^{2+} or Co^{2+} at rates equivalent to quercetin (Table 1, Fig. 3b). In fact, in the presence of Co^{2+} all of these compounds were methylated with affinities similar to that observed with caffeoyl-CoA, although at considerably lower K_{cat} values (Table 1), while the methylation of caffeoylglucose was 50-fold more efficient (or 20-fold in the presence of Mn^{2+}; data not shown) as compared to the standard assay conditions (at 150 μM Mg^{2+}).

Incubation of the *A. majus* CCoAOMT with quercetagetin under standard conditions (150 μM Mg^{2+}) resulted in one product, identified as 3′-hydroxylated quercetagetin, based on co-chromatography with authentic standards (Fig. 1), and is consistent with previous data [8]. In the presence of Co^{2+}, the assay yielded up to six products (data not shown) which cor-

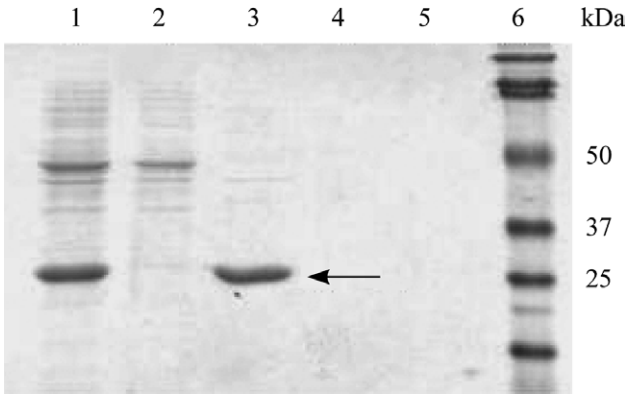

Fig. 2. Separation of recombinant *A. majus* CCoAOMT (1.5 μg) by SDS–PAGE using 12% separation and 5% stacking gels and stained with Coomassie brilliant blue R 250. The enzyme protein band is marked with an arrow and marker proteins in the range of 17–95 kDa (Biorad, Munich, Germany) were used for calibration. Lane 1, crude protein extract, lane 2, His Trap wash; lane 3, 30 mM imidazole washing with 5 μg *A. majus* OMT (arrow); lanes 4 and 5, subsequent 300 mM imidazole washings; lane 6, molecular weight marker.

Table 1
Kinetic properties of recombinant CCoAOMT from *A. majus* in the presence of bivalent cations (150 μM)

Substrate	Cation	K_m [μM]	K_{cat} [s^{-1}]	K_{cat}/K_m (s^{-1} M^{-1})
Caffeoyl CoA	Co^{2+}	20	1.043	52100
	Mg^{2+}	4.1	0.149	36300
Quercetin	Co^{2+}	73	0.938	12800
	Mg^{2+}	31	0.268	8600
Quercetagetin	Co^{2+}	n.d.	n.d.	n.d.
	Mg^{2+}	9	0.071	7900
Caffeoylglucose	Co^{2+}	61	0.631	10300
	Mg^{2+}	930	0.206	220
Caffeic acid	Co^{2+}	88	0.068	770
	Mg^{2+}	**	**	**
5-hydroxy- ferulic acid	Co^{2+}	68	0.143	2100
	Mg^{2+}	**	**	**
3,4-dihydroxy- benzoic acid	Co^{2+}	39	0.013	330
	Mg^{2+}	**.	**	**

n.d., not determined; **, no product formation detected.
The assays were performed at 37 °C for 1–10 min using from 1 μg (caffeoyl CoA or flavonols as substrate) or 10 μg (caffeic acid and 3,4-dihydroxy benzoic acid as substrate) of the affinity purified enzyme.

responded chromatographically to the products generated previously by the promiscuous CCoAOMTs from *Mesembryanthemum crystallinum* or *A. thaliana*, including the fluorescent 5-*O*-methylquercetagetin [8]. However, kinetic data were not determined because of very low recovery of quercetagetin and its products, even after short times of incubation (1 min). The reasons have remained unclear.

The enzyme was also tested at very low Mg^{2+}, Mn^{2+} and Co^{2+} concentrations (15 and 1.5 μM), which might reflect more accurately the in vivo concentrations of any of these cations although to match in vivo concentration of these cations on a subcellular level is a very challenging task. With the lower cation concentrations, except a reduced turnover, no significant differences in substrate and position specificities as compared to the 150 μM concentrations were observed (data not shown). The whole set of incubations was also repeated with recombinant highly homologous CCoAOMT from tobacco [22] in order to examine the generality of the specificity effects of metal cofactors in vitro. This enzyme also showed stringent specificity in the presence of Mg^{2+} or Ca^{2+} and promiscuous specificity with Mn^{2+} or Co^{2+} as a cofactor (data not shown). The mechanistic basis of this effect remains to be investigated.

In previous reports, it was demonstrated that plants have two subsets of class I OMTs for apparently promiscuous and for substrate specific tasks [8,19]. The novel data might now suggest that plants are capable of adjusting the substrate preference of the previously considered "specific subset" of CCoAOMTs by regulating the intracellular concentration of divalent cations. Alternatively, the promiscuous specificity of this class observed in vitro could be considered as a relic feature preserved from an ancient enzyme precursor. In case the methylation of caffeoyl-CoA is required exclusively for the biosynthesis of guaiacyl monomers, the participation of Mg^{2+} as cofactor is sufficient to support the high activity of CCoAOMT and ensure specificity. CCoAOMTs have commonly been characterized as elicitor inducible enzymes [10]. The rapid transcript accumulation of *A. majus* cells extracted

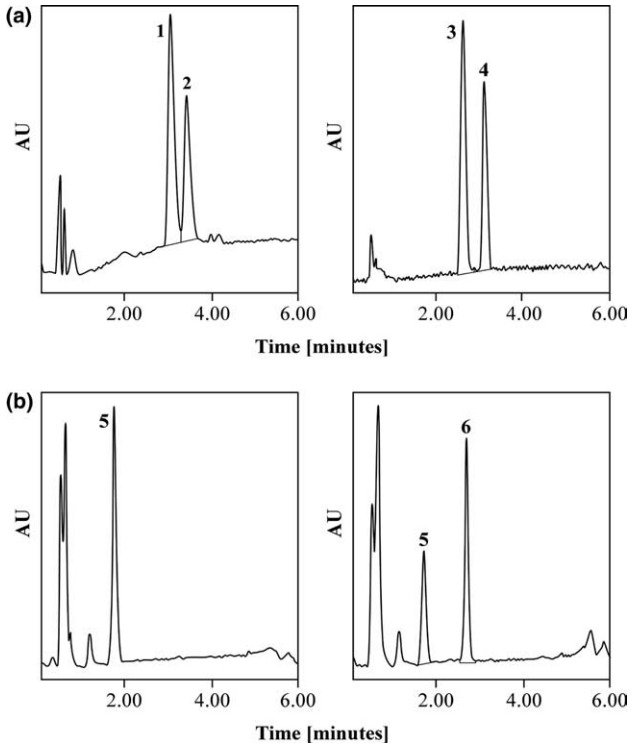

Fig. 3. (a) HPLC-analysis of product formation from caffeoyl-CoA (left) or quercetin (right) by apparently homogenous *A. majus* CCoAOMT. The separation was monitored by absorption at λ_{max} 340 and 364 nm, respectively, and the compounds were identified as reported previously [6,16]. Peak identification: 1, caffeoyl CoA; 2, feruloyl CoA; 3, quercetin; 4, quercetin-3′-*O*-Me (isorhamnetin). Product identification was based on co-chromatography with authentic standards [6,16]. (b) HPLC-analysis of product formation from 3,4-dihydroxybenzoic acid by apparently homogenous *A. majus* CCoAOMT. The incubations were carried out in the presence of 150 μM Mg^{2+} (left) or 150 μM Co^{2+}, and the separation was monitored at λ_{max} 276 nm. Peak identification: 5, substrate (3,4-dihydroxybenzoic acid); 6,4-hydroxy-3-methoxybenzoic acid.

at various time points following the addition of the *Pmg* elicitor (data not shown) could point to an essential function of the enzyme in the overall defense response, although the cell cultures may not necessarily reflect the in planta situation.

Through the course of evolution, plants have developed two very different classes of enzymes, at least, for the methylation of the wide variety of polyhydroxylated compounds. The metal-independent caffeic acid class II OMT (COMT) already known for its promiscuous substrate specificity towards a variety of potential lignin monomer ''precursors'' [8,23] has recently also been shown also methylate even simple phenolics, like syringic acid [24]. Overall, the modulation of substrate specificity of class I OMTs by bivalent cations adds a further degree of complexity to the metabolic grid controlling the methylation pattern of polyhydroxylated phenolics in plants.

In addition to the modeling data already available for the CCoAOMT from tobacco [22], the crystal structure of the corresponding enzymes in combination with a yet to be developed sensitive in vivo ICPMS (inductively coupled plasma mass spectrometry)-analyses might provide a detailed look at the active sites of the class I OMTs in vitro and in vivo in due course.

Acknowledgements: Financial support by the Deutsche Forschungsgemeinschaft and Fonds der chemischen Industrie is gratefully acknowledged. We are indebted to D. Knöfel for skilled technical assistance.

References

[1] Zhu, B.T., Ezell, E.L. and Liehr, J.G. (1994) J. Biol. Chem. 269, 292–299.
[2] Joshi, L. and Chiang, V.L. (1998) Plant Mol. Biol. 37, 663–674.
[3] Ye, Z.H., Kneusel, R.E., Matern, U. and Varner, J.E. (1994) Plant Cell 6, 1427–1439.
[4] Humphreys, J. and Chapple, C. (2002) Curr. Opin. Plant Biol. 5, 224–229.
[5] Osakabe, K., Tsao, C.C., Li, L., Popko, J.L., Umezawa, T., Carraway, D.T., Smeltzer, R.H., Joshi, C.P. and Chiang, V.L. (1999) Proc. Natl. Acad. Sci. USA 96, 8955–8960.
[6] Maury, S., Geoffroy, P. and Legrand, M. (1999) Plant Physiol. 121, 215–224.
[7] Parvathi, K., Chen, F., Guo, D., Blount, J.W. and Dixon, R.A. (2001) Plant J. 25, 193–202.
[8] Ibdah, M., Zhang, X.-H., Schmidt, J. and Vogt, T. (2003) J. Biol. Chem. 278, 43961–43972.
[9] Pakusch, A.E., Kneusel, R.E. and Matern, U. (1989) Arch. Biochem. Biophys. 27, 488–494.
[10] Schmitt, D., Pakusch, A.-E. and Matern, U. (1991) J. Biol. Chem. 266, 17416–17423.
[11] Hehmann, M., Lukaèin, R., Ekiert, H. and Matern, U. (2004) Eur. J. Biochem. 271, 932–940.
[12] Bethea, D., Fullmer, B., Syed, S., Seltzer, G., Tiano, J., Rischko, C., Gillespie, L., Brown, D. and Gasparro, F.P. (1999) J. Dermatol. Sci. 19, 78–88.
[13] Strack, D., Keller, H. and Weissenböck, G. (1987) J. Plant Physiol. 131, 61–73.
[14] Stöckigt, J. and Zenk, M.H. (1975) Z. Naturforsch. C 30, 352–358.
[15] Giuliano, G., Bartley, G.E. and Scolino, P.A. (1993) Plant Cell 5, 379–387.
[16] Sanger, F., Nicklen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[17] Lukaèin, R. and Britsch, L. (1997) Eur. J. Biochem. 249, 748–757.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Vogt, T. (2004) FEBS Lett. 561, 159–162.
[20] Civardi, L., Rigau, J. and Puigdomenech, P. (1999) Plant Physiol. 120, 1206.
[21] Li, L., Osakabe, Y., Joshi, C.P. and Chiang, V.L. (1999) Plant Mol. Biol. 40, 555–565.
[22] Hoffmann, L., Maury, S., Bergdoll, M., Thion, L., Erard, M. and Legrand, M. (2001) J. Biol. Chem. 276, 36831–36838.
[23] Edwards, R. and Dixon, R.A. (2000) Arch. Biochem. Biophys. 287, 372–379.
[24] Kota, P., Guo, D., Zubieta, C., Noel, J. and Dixon, R.A. (2004) Phytochemistry 65, 837–846.