The Tzs protein from *Agrobacterium tumefaciens* C58 produces zeatin riboside 5′-phosphate from 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate and AMP

Lilian Krall[a], Maja Raschke[b], Meinhart H. Zenk[b], Christian Baron[a,c,*]

[a] *Department Biologie I, Ludwig-Maximilians-Universität, Bereich Mikrobiologie, Maria-Ward-Str. 1a, D-80638 München, Germany*
[b] *Biozentrum der Martin-Luther-Universität Halle-Wittenberg, Weinbergweg 22, D-06120 Halle/Saale, Germany*
[c] *Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON, Canada LS8 4K1*

Received 19 June 2002; revised 2 August 2002; accepted 8 August 2002

First published online 22 August 2002

Edited by Marc Van Montagu

Abstract The plant pathogen *Agrobacterium tumefaciens* produces cytokinins upon induction of the virulence genes by secondary metabolites from wounded plants, and these hormones are believed to stimulate the infection process. To study the biosynthetic pathway, the *tzs* gene, encoding the Tzs (*trans*-zeatin-synthesizing) protein from *A. tumefaciens*, was cloned and the protein was overproduced and purified. Analysis of its reactivity with radioactively labeled substrate demonstrated conversion of 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate, a product of the deoxyxylulose phosphate pathway, with AMP to zeatin riboside 5′-phosphate. This suggests that *A. tumefaciens* uses an alternative pathway of cytokinin biosynthesis, which had previously been hypothesized to operate in plants. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cytokinin; Gene transfer; Isoprenoid biosynthesis; Non-mevalonate pathway; Plant hormone; Terpene

1. Introduction

Agrobacterium tumefaciens is a natural genetic engineer, which is able to transfer genetic information from its tumor-inducing (Ti-) plasmid into the nuclear genome of plant cells [1,2]. Under natural conditions, the infection occurs at plant wound sites and plant-derived phenylpropanoids, such as acetosyringone, are required for the induction of the bacterial virulence system [3]. The virulence proteins mediate the excision of the transferred DNA (T-DNA) from the Ti-plasmid and its translocation into the plant cells, and they also contribute to its nuclear uptake and integration into the genome [4]. Expression of the translocated genes leads to synthesis of plant hormones (cytokinins and auxins), causing the formation of tumors, and to the production of opines, which can be metabolized by *A. tumefaciens*.

A subgroup of *A. tumefaciens* strains encodes biosynthetic enzymes for the synthesis of nopaline-type opines on their T-DNA, and the genomic sequence of model nopaline strain C58 was reported recently [5,6]. Cytokinin biosynthesis is catalyzed by T-DNA-encoded proteins, which share significant sequence similarity with plant-encoded biosynthetic enzymes [7]. C58 encodes the Tmr (Ipt) protein and it has enzymatic activity as isopentenyltransferase converting dimethylallyl diphosphate (DMAPP) and AMP to the cytokinin iPMP (isopentenyladenosine 5′-monophosphate) [8,9]. In addition to the T-DNA-coded cytokinin biosynthetic enzymes, nopaline strains of *A. tumefaciens* encode enzymes for *trans*-zeatin synthesis (Tzs) in the virulence region, which is not translocated to the plant cells. Expression of the *tzs* gene of strain C58 is subject to virulence gene regulation, and zeatin secretion is believed to stimulate gene transfer efficiency [10,11].

Tmr as well as Tzs and some of their plant homologs were shown to have isopentenyltransferase activity, leading to the production of the cytokinin iPMP [7–9,11]. However, *Escherichia coli* as well as *A. tumefaciens* producing these proteins were shown to secrete zeatin riboside 5′-phosphate (ZMP) in addition to iPMP. The factors required for hydroxylation of iPMP to ZMP and further conversion to zeatin have neither been characterized in microorganisms nor in plants. Åstot et al. (2000) recently proposed an alternative pathway for the production of ZMP in *Arabidopsis thaliana*, implying the direct incorporation of a hydroxylated side chain precursor [12]. A putative precursor is 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate (HMBPP), which was identified as an intermediate of the non-mevalonate (1-deoxyxylulose 5-phosphate, DXP) pathway of isoprenoid biosynthesis in *E. coli* [13–15]. HMBPP likely is a biosynthetic precursor of isopentenyl diphosphate (IPP) and DMAPP in this pathway, which occurs in most microorganisms and in the chloroplasts of plants [16,17]. In addition, HMBPP may serve as substrate of the alternative cytokinin biosynthetic pathway [12] and the synthesis of the radioactively labeled compound enabled us to directly address this question [18]. Using purified Tzs protein and radioactively labeled substrate, we showed for the first time that Tzs also metabolizes HMBPP, thus directly producing ZMP.

2. Materials and methods

2.1. DNA manipulations

Since *tzs* contains an internal *Nde*I site, pT7-7Nco was generated by

*Corresponding author. Fax: (49)-89-2180 996138.
E-mail address: cbaron@lrz.uni-muenchen.de (C. Baron).

Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxyxylulose 5-phosphate; HMBPP, 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate; iPMP, isopentenyladenosine 5′-monophosphate; ZMP, zeatin riboside 5′-phosphate; ZR, zeatin riboside

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)03258-1

inverse PCR from pT7-7 [19], replacing the *Nde*I site adjacent to promoter and Shine-Dalgarno sequence with an *Nco*I site. The *tzs* gene was PCR-amplified from *A. tumefaciens* C58 cells with oligonucleotides Tzs5 (5′-GGCTCATGATACTCCATCTCATCTACGGAC-C-3′) and Tzs3 (5′-CGGAAGCTTCACCGAATTCGAGTCAGCG-TG-3′) using cycling parameters of 95°C for 1 min (one cycle), 50°C for 1 min, 72°C for 1 min and 95°C for 30 s (30 cycles), and termination at 4°C. The 747 bp PCR product was cleaved with *Bsp*HI and *Hin*dIII (underlined above), ligated with *Nco*I/*Hin*dIII-cleaved pT7-7Nco, and the sequence of the resulting pT7-7tzs was confirmed.

2.2. Purification of Tzs and protein analysis

E. coli strain GJ1158 pT7-7tzs (8×200 ml) was grown in LBON medium (1% trypton, 0.5% yeast extract) with carbenicillin (100 μg/ml) at 37°C to the late exponential growth phase (OD_{600} = 0.8), followed by addition of NaCl at 0.3 M for induction of protein production [20], and shift to 26°C to ensure accumulation of soluble protein. After growth for 18 h, cells were sedimented and washed with phosphate-buffered saline (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4, pH 7.0), followed by addition of 20 ml of the same buffer with 0.5 mM phenylmethylsulfonyl fluoride and lysis in a French press cell (3 times at 18 000 psi). The following steps were conducted on ice or at 4°C. The lysate was cleared by successive centrifugation in an SS-34 rotor at 12 000 rpm (1 h) and at 40 000 rpm in a 70.1 Ti rotor in an OTD 50B centrifuge (2 h). Proteins in the supernatant were fractionated by $(NH_4)_2SO_4$ precipitation, and the highest amounts of Tzs were detected in the 30–40% fraction. The protein was suspended in buffer A (50 mM HEPES, 50 mM NaCl, pH 7) and chromatographed over a Superdex 75 gel filtration column (Amersham) with a flow rate of 0.5 ml/min. Tzs eluted from the column in a broad molecular mass range and fractions with relatively minor amounts of other proteins were applied to a Q-Sepharose anion exchange column (Amersham) in buffer A. The protein was eluted with a gradient of 10 column volumes from buffer A to buffer B (50 mM HEPES, 1 M NaCl, pH 7). Fractions containing the lowest amounts of impurities were dialyzed for 24 h in buffer A, followed by 24 h in glycerol buffer (buffer A, 50% glycerol, 0.5 mM dithiothreitol) and storage at −20°C. The amount of protein was determined by Bradford assay as 0.55 μg/ml.

2.3. 4-Hydroxy-3-methyl-2-(E)-butenyl (HMB) transferase assay and identification of reaction products

The reaction mixture (70 μl) contained 100 mM Tris/HCl pH 7.6, 0.7 mM Na-AMP, 8.5 mM $MgCl_2$, 5 mM NaF and 5.2 μM [4-^{3}H]HMBPP (specific activity: 5.77×10^4 Bq/nmol) synthesized as described [18]. The reaction was started by the addition of 14 μg Tzs and incubation was for 90 min at 30°C.

The reaction products were adsorbed onto a small (200 mg) octadecylsilica column (Sep-Pak, Waters), washed with water (100 μl), eluted with methanol (2 ml) and dried. The sample was dissolved in 50% aqueous methanol and subjected to high-performance liquid chromatography (HPLC) using an ion pair reversed phase system (radioactivity detector: Raytest Ramona 2000, column: Phenomenex Luna 5u C8(2); solvent system: 10 mM tetra-*n*-*butyl*-ammonium hydrogen sulfate (TBAS) (solvent A), 10 mM TBAS in 70% methanol (solvent B); flow rate: 0.75 ml/min; gradient: 0 min 100% solvent A, 20 min 100% solvent A, 80 min 40% solvent A/60% solvent B, 85 min 100% solvent A, 100 min 100% solvent A) [21].

An aliquot of the reaction product, enriched by Sep-Pak adsorption, was suspended in 50 μl 10 mM Tris/HCl pH 7.6. We added 10 units of bovine intestinal mucose alkaline phosphatase (Sigma), followed by hydrolysis of the phosphate ester for 90 min at 30°C. The reaction product was separated via Sep-Pak adsorption, and an aliquot of the eluent was subjected to HPLC as above. Another aliquot of the dephosphorylated sample in the methanol eluate of the Sep-Pak column was chromatographed on silicagel plates (Macherey Nagel) with n-butanol:0.14 N NH_4OH:H_2O (6:1:2) [11].

In a separate experiment, using 10.4 μM [4-^{3}H]HMBPP, the methanol eluent of the Sep-Pak column was taken to dryness, dephosphorylated, and 1 μM of unlabeled zeatin riboside (ZR; Sigma) in methanol was added. The specific activity was determined and the sample was again taken to dryness. We added 200 μl dry pyridine and 10 μl acetic anhydride followed by incubation for 48 h at room temperature. The reaction mixture was taken to dryness, dissolved in 100 μl methanol, subjected to thin layer chromatography (TLC) and scanned for radioactivity. The compound with the R_f value of 0.68 was isolated and the specific activity was determined. An unlabeled sample of ZR was acetylated the same way and subjected to electron spray ionization mass spectroscopy using a Q-TOF system (Micromass, UK). The concentrations of zeatin and derivatives were determined by UV spectroscopy with synthetic standards in methanol.

3. Results

3.1. Cloning of the tzs gene, overproduction and purification of Tzs

The *tzs* gene from the Ti-plasmid was PCR-amplified from cells of *A. tumefaciens* C58 and cloned into expression vector pT7-7Nco. Expression of the gene in strain GJ1158 at 37°C by addition of NaCl resulted in accumulation of large amounts of a protein of the expected molecular mass, but predominantly as insoluble inclusion bodies. Cell cultivation and expression at 26°C resulted in lower amounts of the protein, but about 50% was soluble, so that these conditions were applied for large-scale growth. The protein was purified by successive steps of $(NH_4)_2SO_4$ precipitation, Superdex 75 gel filtration and Q-Sepharose anion exchange chromatography, resulting in fractions with only minor impurities (Fig. 1), which were used for assays of its enzyme activity. The protein was also used to raise antisera, which detected the authentic gene product in virulence gene-induced *A. tumefaciens* C58, thus confirming its identity (not shown). A protein of slightly larger molecular mass, which may be an *E. coli* protein, constantly co-purified with Tzs. Whereas we can not formally exclude that it carries HMBPP-metabolizing activity, this possibility is unlikely, because isopentenyltransferase activity in *E. coli* was shown to be dependent on the production of heterologous enzymes [7–11].

3.2. Production of ZMP shows HMB transferase activity of Tzs

The availability of radioactively labeled HMBPP [18] and of purified Tzs allowed direct assessment of the question, whether the enzyme catalyzes the formation of ZMP. Incubation of AMP and labeled [4-^{3}H]HMBPP in the presence of Tzs led to quantitative conversion to a labeled product, de-

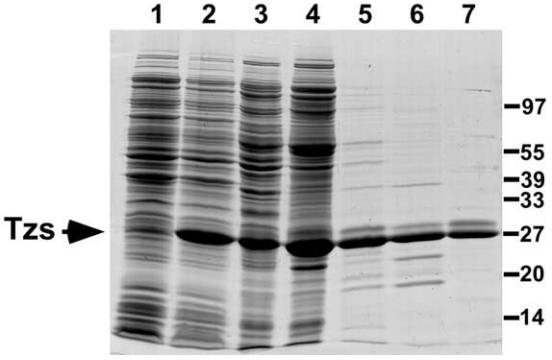

Fig. 1. Purification of Tzs from *E. coli* GJ1158 pT7tzs. Coomassie-stained sodium dodecylsulfate–polyacrylamide gel (12%) showing steps leading to purification of Tzs. Lanes as follows: pT7tzs-carrying strain GJ1158 (1) before and (2) after induction with NaCl for 18 h, (3) supernatant after cell lysis and ultracentrifugation, (4) 30–40% ammonium sulfate precipitate, (5) Superdex 75 gel filtration chromatography and (6/7) pooled fractions from Q-Sepharose anion exchange chromatography. Protein from fractions shown in lane 7 was used for enzyme assays.

tected as a single peak with an R_t of 71 min after HPLC analysis (Fig. 2A). This compound separated clearly from HMBPP ($R_t = 52$ min) (Fig. 2B) and subsequent dephosphorylation led to a compound with a chromatographic behavior identical to synthetic ZR ($R_t = 63$ min). Analysis of the dephosphorylated labeled reaction product by TLC revealed that the R_f was identical to ZR ($R_f = 0.55$), showing that the Tzs-mediated reaction likely produced ZMP.

To obtain unequivocal evidence for its identity, the dephosphorylated radioactively labeled reaction product was mixed with unlabeled ZR, giving a specific activity of 98 Bq/nmol. The sample was subjected to acetylation with acetic anhydride, separated by TLC, followed by determination of the specific activity as 94 Bq/nmol. Unlabeled ZR was acetylated in parallel and mass spectrometric analysis revealed that the singly-charged ion of the acetylated reference compound was [M+H] *m/z* 520, which is identical with the calculated molecular mass of ZR tetraacetate. The observed fragment ions were *m/z* 262, 202 and 185, which is in accordance with the tetra-acetylated ZR derivative. Separation of radioactively labeled acetylation products and the unlabeled reference compound by TLC revealed an identical R_f value of 0.68 (Fig. 3), thus confirming the identity of the labeled reaction product as tetraacetyl ZR. The specific activity of the acetylated compound retrieved from the TLC plate deviated from that of the original sample by only 3.6%, which is within experimental error. Taken together, our results demonstrate that ZMP is the product of the Tzs-mediated conversion of HMBPP with AMP.

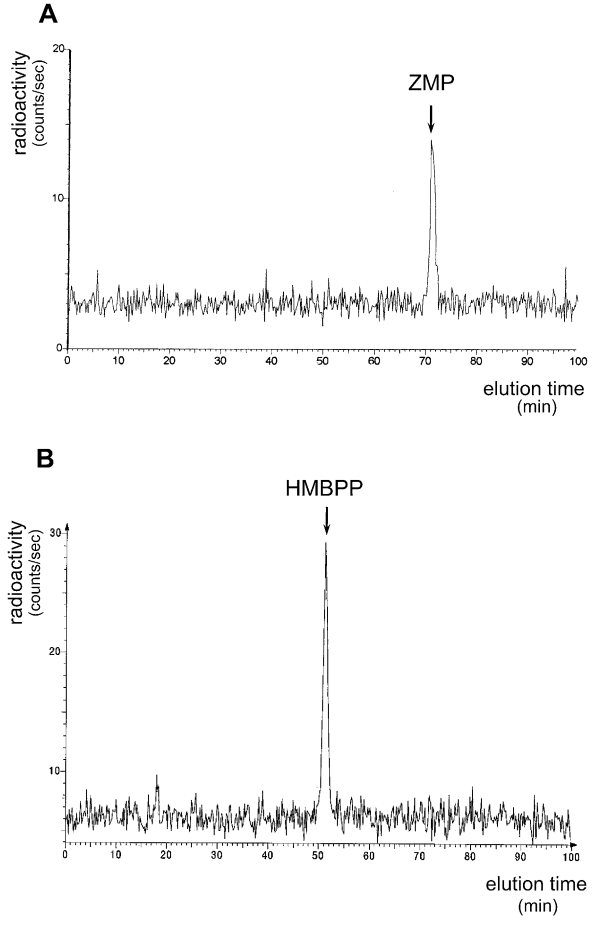

Fig. 2. HPLC analysis shows Tzs-mediated incorporation of radioactively labeled HMB into ZMP. Products with (A) and without (B) added Tzs.

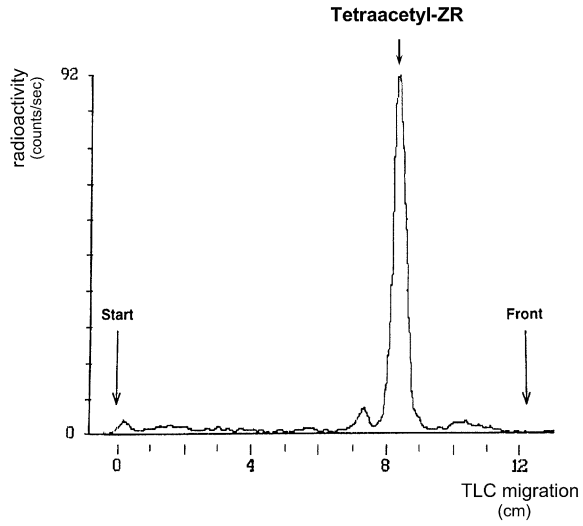

Fig. 3. Tzs-mediated incorporation of radioactively labeled HMB into ZMP demonstrated by TLC. Reaction product ZMP was dephosphorylated to ZR, diluted with unlabeled ZR and subjected to acetylation and separation by TLC.

4. Discussion

It is well established that many plant-associated bacteria produce cytokinins and they contribute to their virulence in some cases [22,23]. In spite of this knowledge, the biosynthetic origin of those plant hormones remained elusive until recently. Analysis of microbial genomes combined with biochemical experiments established the existence of the DXP pathway of isoprenoid biosynthesis in some bacteria and in the chloroplasts of plants [16,17]. Analysis of clusters of orthologous groups revealed that the genes encoding enzymes of

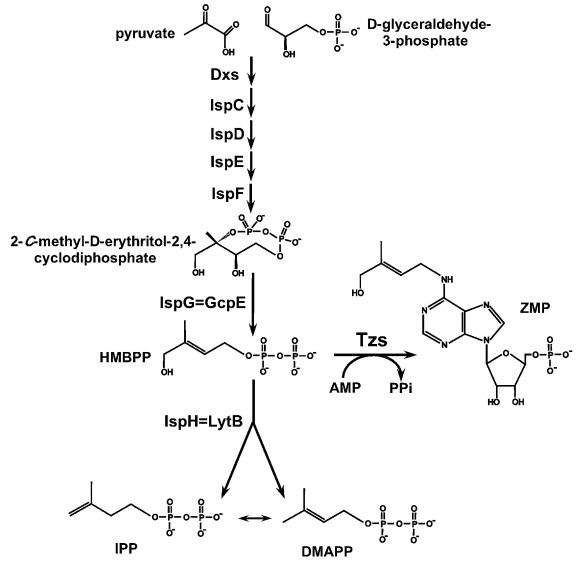

Fig. 4. Proposed DXP pathway for the production of ZMP in *A. tumefaciens* C58. Genes encoding the enzymes of the putative DXP pathway (Dxs, IspC, IspD, IspE, IspF, IspG, IspH) were detected in the genomic sequence [5,6].

this pathway are present in most bacteria analyzed so far. In contrast, the genes encoding enzymes of the mevalonate pathway are present mostly in Gram-positive bacteria, leading to the prediction that the majority of bacteria uses the DXP pathway [16,17]. In contrast, genes for DXP biosynthetic enzymes are absent from Archaea, and similar to Eukarya, they are predicted to use the mevalonate pathway. Analysis of the genomic sequence of *A. tumefaciens* C58 showed that it encodes the enzymes of the DXP pathway, and those of the mevalonate pathway are absent [5,6]. Based on these results we postulate that *A. tumefaciens* uses the DXP pathway for synthesis of precursors of the Tzs-mediated production of zeatin derivatives (Fig. 4). In addition to HMBPP, previous studies [11] and our own work (not shown) demonstrated that Tzs also accepts DMAPP. However, most of the analyzed bacteria secrete primarily hydroxylated cytokinins, suggesting that HMPBB may be the main precursor [24]. Future in vivo and in vitro studies have to show how the substrate flux of the two side chain precursors towards the biosynthesis of cytokinins is regulated.

The T-DNA-encoded Tmr (Ipt) protein also has DMAPP:AMP isopentenyltransferase activity in vitro [8,9]. Due to the lack of knowledge on its plant homologs, this protein was employed as a model biosynthetic enzyme for analysis and modulation of plant cytokinin production [12,25]. The analysis of Ipt-overproducing *A. thaliana* provided conclusive evidence for the existence of an iPMP-independent pathway of cytokinin biosynthesis [12]. Similar to the Tzs protein, Ipt may accept such a precursor leading to direct production of ZMP. This notion is further supported by the investigation of Ipt and eight recently identified homologs from *A. thaliana*. *E. coli* strains overproducing the recombinant proteins produced isopentenyladenine as well as *trans*-zeatin, suggesting that the plant enzymes may contribute to the alternative iPMP-independent pathway [7]. In silico analysis of their cellular location suggests that AtIPT1 and AtIPT8 localize to the chloroplast, whereas the other enzymes are predicted to reside in the cytoplasm (Software TargetP v1.01, http://www.cbs.dtu.dk/services/TargetP, not shown). This opens the possibility that cellular localization determines the biosynthetic origin of zeatin-type cytokinins from the cytoplasmic mevalonate or the chloroplasmic DXP pathway, using precursors DMAPP or HMBPP, respectively.

Analysis of the bacterial Tzs protein does not allow direct conclusions on the metabolic activities of plant enzymes. Nevertheless, we showed for the first time the direct production of ZMP-type cytokinins from the precursor HMBPP, and thereby confirmed previous hypotheses [12,18,26]. Since the radioactively labeled substrate for such analyses is now available, future studies will address the biosynthetic origin of zeatin-type cytokinins in plants.

Acknowledgements: We thank August Böck and Patricia C. Zambryski for continued support and discussions, and Heike Schmidt-Eisenlohr for construction of pT7-7Nco. We are indebted to Klaus Raith for measurement and interpretation of the mass spectra. This work was supported by the Deutsche Forschungsgemeinschaft (DFG) via Sonderforschungsbereich 369 (projects A1 and C9), and the Fonds der Chemischen Industrie.

References

[1] Zupan, J., Muth, T.R., Draper, O. and Zambryski, P.C. (2000) Plant J. 23, 11–28.
[2] Zhu, J., Oger, P.M., Schrammeijer, B., Hooykaas, P.J., Farrand, S.K. and Winans, S.C. (2000) J. Bacteriol. 182, 3885–3895.
[3] Stachel, S.E., Messens, E., van Montagu, M. and Zambryski, P.C. (1985) Nature 318, 624–629.
[4] Ziemienowicz, A., Merkle, T., Schoumacher, F., Hohn, B. and Rossi, L. (2001) Plant Cell 13, 369–383.
[5] Wood, D.W. et al. (2001) Science 294, 2317–2323.
[6] Goodner, B. et al. (2001) Science 294, 2323–2328.
[7] Takei, K., Sakakibara, H. and Sugiyama, T. (2001) J. Biol. Chem. 276, 26405–26410.
[8] Blackwell, J.R. and Horgan, R. (1993) Phytochemistry 34, 1477–1481.
[9] Akiyoshi, D.E., Klee, H., Amasino, R.M., Nester, E.W. and Gordon, M.P. (1984) Proc. Natl. Acad. Sci. USA 81, 5994–5998.
[10] John, M.C. and Amasino, R.M. (1988) J. Bacteriol. 170, 790–795.
[11] Powell, G.K., Hommes, N.G., Kuo, J., Castle, L.A. and Morris, R.O. (1988) Mol. Plant-Microbe Interact. 1, 235–242.
[12] Åstot, C., Dolezal, K., Nordstrom, A., Wang, Q., Kunkel, T., Moritz, T., Chua, N.H. and Sandberg, G. (2000) Proc. Natl. Acad. Sci. USA 97, 14778–14783.
[13] Hecht, S., Eisenreich, W., Adam, P., Amslinger, S., Kis, K., Bacher, A., Arigoni, D. and Rohdich, F. (2001) Proc. Natl. Acad. Sci. USA 98, 14837–14842.
[14] Hintz, M. et al. (2001) FEBS Lett. 509, 317–322.
[15] Seemann, M., Campos, N., Rodriguez-Concepción, M., Ibañez, E., Duvold, T., Tritsch, D., Boronat, A. and Rohmer, M. (2002) Tetrahedron Lett. 43, 1413–1415.
[16] Rohdich, F., Kis, K., Bacher, A. and Eisenreich, W. (2001) Curr. Opin. Chem. Biol. 5, 535–540.
[17] Rohmer, M. (1999) Nat. Prod. Rep. 16, 565–574.
[18] Gao, W., Loeser, R., Raschke, M., Dessoy, M.A., Fulhorst, M., Alpermann, H., Wessjohann, L.A. and Zenk, M.H. (2002) Angew. Chem. Int. Ed. 41, 2604–2607.
[19] Studier, F.W. and Moffatt, B.A. (1986) J. Mol. Biol. 189, 113–130.
[20] Bhandari, P. and Gowrishankar, J. (1997) J. Bacteriol. 179, 4403–4406.
[21] McCaskill, D. and Croteau, R. (1993) Anal. Biochem. 215, 142–149.
[22] Costacurta, A. and Vanderleyden, J. (1995) Crit. Rev. Microbiol. 21, 1–18.
[23] Goethals, K., Vereecke, D., Jaziri, M., Van Montagu, M. and Holsters, M. (2001) Annu. Rev. Phytopathol. 39, 27–52.
[24] Akiyoshi, D.E., Regier, D.A. and Gordon, M.P. (1987) J. Bacteriol. 169, 4242–4248.
[25] Gan, S. and Amasino, R.M. (1995) Science 270, 1986–1988.
[26] Ward, J.L. and Bearle, M.H. (2002) J. Chem. Soc. Perkin Trans. 1, 710–712.