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Molecular Characterization of Berberine Bridge Enzyme Genes from Opium Poppy[1]

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In *Papaver somniferum* (opium poppy) and related species, (*S*)-reticuline serves as a branch-point intermediate in the biosynthesis of numerous isoquinoline alkaloids. The berberine bridge enzyme (BBE) ([*S*]-reticuline:oxygen oxidoreductase [methylene bridge forming], EC 1.5.3.9) catalyzes the stereospecific conversion of the *N*-methyl moiety of (*S*)-reticuline into the berberine bridge carbon of (*S*)-scoulerine and represents the first committed step in the pathway leading to the antimicrobial alkaloid sanguinarine. Three unique genomic clones (*bbe1*, *bbe2*, and *bbe3*) similar to a BBE cDNA from *Eschscholtzia californica* (California poppy) were isolated from opium poppy. Two clones (*bbe2* and *bbe3*) contained frame-shift mutations of which *bbe2* was identified as a putative, nonexpressed pseudogene by RNA blot hybridization using a gene-specific probe and by the lack of transient expression of a chimeric gene fusion between the *bbe2* 5′ flanking region and a β-glucuronidase reporter gene. Similarly, *bbe1* was shown to be expressed in opium poppy plants and cultured cells. Genomic DNA blot-hybridization data were consistent with a limited number of *bbe* homologs. RNA blot hybridization showed that *bbe* genes are expressed in roots and stems of mature plants and in seedlings within 3 d after germination. Rapid and transient BBE mRNA accumulation also occurred after treatment with a fungal elicitor or with methyl jasmonate. However, sanguinarine was found only in roots, seedlings, and fungal elicitor-treated cell cultures.

The BBE ([*S*]-reticuline:oxygen oxidoreductase [methylene bridge forming], EC 1.5.3.9) catalyzes the stereospecific transformation of the *N*-methyl group of (*S*)-reticuline into the C-8 berberine bridge carbon of (*S*)-scoulerine (Rink and Böhm, 1975; Steffens et al., 1985; Frenzel et al., 1988) (Fig. 1). (*S*)-Scoulerine then serves as precursor in the species-specific biosynthesis of numerous benzophenanthridine, protopine, and protoberberine alkaloids that are widely distributed in the Papaveraceae, Fumariaceae, and Berberidaceae plant families. Some benzophenanthridine alkaloids, such as the antimicrobial alkaloid sanguinarine (Dzink and Socransky, 1985; Cline and Coscia, 1988), are involved in the plant defense response to a pathogen challenge. The accumulation of sanguinarine in response to the addition of various elicitors has been demonstrated for cell-suspension cultures of *Eschscholtzia californica*, the California poppy (Schumacher et al., 1987), and *Papaver somniferum*, the opium poppy (Eilert et al., 1985).

(*S*)-Reticuline also serves as the universal biosynthetic precursor to a variety of other isoquinoline alkaloids in plants. In some members of the genus *Papaver*, (*S*)-reticuline is converted to its (*R*)-epimer to initiate the biosynthetic pathway to morphine (Weiczorek et al., 1986) (Fig. 1). In aerial organs of opium poppy, morphine is the major alkaloid, whereas in poppy roots sanguinarine is most abundant, although some morphine is also present (Facchini and De Luca, 1995). The differential accumulation of specific alkaloids in opium poppy shoots and roots is reflected by the differential and tissue-specific expression of genes for TYDC (EC 4.1.1.25) (Facchini and De Luca, 1994, 1995), which catalyzes the first steps in the biosynthesis of isoquinoline alkaloids in plants (Stadler et al., 1987). However, little is known about the regulatory mechanisms in opium poppy and related species whereby the central intermediate (*S*)-reticuline is partitioned to serve as precursor for morphine accumulation in aerial organs and predominantly as precursor for the constitutive accumulation of sanguinarine in roots. The role of (*S*)-reticuline as a central branch-point intermediate in alkaloid biosynthesis suggests that the regulation of branch-point enzymes such as BBE may play a key role in the control of sanguinarine and morphine accumulation in opium poppy.

A cDNA-encoding BBE has previously been isolated from *E. californica* using degenerate oligonucleotides based on amino acid sequences from the purified native enzyme (Dittrich and Kutchan, 1991). Heterologous expression of the cDNA in insect cell culture using a baculovirus expression vector (Kutchan et al., 1993) provided enough pure enzyme to demonstrate that BBE is covalently flavinylated and that closure of the berberine bridge ring system occurs by the formation of a methylene iminium ion as a reaction intermediate, followed by an ionic mechanism (Kutchan and Dittrich, 1995). The oxidative cyclization catalyzed by BBE consumes 1 mol of O_2 and produces 1 mol each of (*S*)-scoulerine and H_2O_2 per mol of (*S*)-reticuline (Steffens

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Abbreviations: BBE, berberine bridge enzyme; CaMV, cauliflower mosaic virus; GGLO, L-gulono-γ-lactone oxidase; 6-HDNO, 6-hydroxy-D-nicodine oxidase; Luc, luciferase; MCRA, mitomycin C radical oxidase; MeJA, methyl jasmonate; ORF, open reading frame; TYDC, Tyr/dopa decarboxylase.

et al., 1985). Induction of BBE mRNAs prior to sanguinarine accumulation has been demonstrated to occur in *E. californica* cell-suspension cultures in response to treatment with yeast elicitor (Dittrich and Kutchan, 1991) and MeJA (Kutchan, 1993; Kutchan and Zenk, 1993). In addition to BBE, four Cyt P450-dependent enzymes involved in the pathway from (*S*)-reticuline to sanguinarine are induced in response to elicitor treatment (Blechert et al., 1995), and all five enzymes are membrane-associated. BBE, in particular, is localized within a subcellular particle of a density ρ = 1.14 g mL^{-1} (Amann et al., 1986).

In this paper we report the cloning and characterization of functional and nonfunctional members of a gene family for BBE in opium poppy. We show that one member (*bbe1*) is expressed, whereas another (*bbe2*) is not, using gene-specific probes in the region 3′ to the putative stop codons and by transient GUS expression in cultured opium poppy cells harboring *bbe1* promoter-GUS and *bbe2* promoter-GUS fusion constructs. In addition, the tissue-specific, developmental-specific, and elicitor-inducible expression of *bbe* genes is discussed in the context of the regulation of isoquinoline alkaloid biosynthesis in opium poppy.

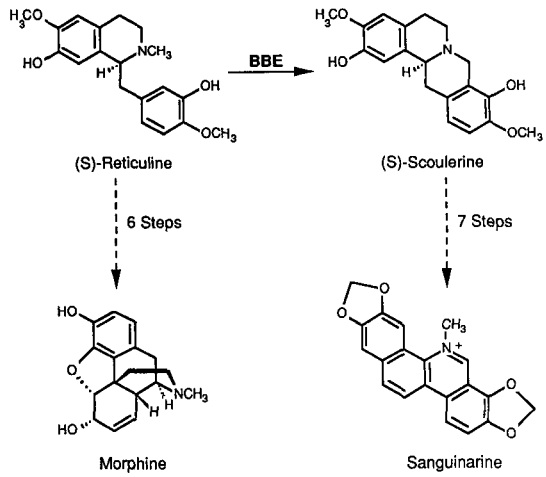

Figure 1. The BBE catalyzes the formation of (*S*)-scoulerine via the oxidative cyclization of the *N*-methyl moiety of (*S*)-reticuline. In opium poppy, (*S*)-scoulerine is the precursor to the antibiotic benzophenanthridine alkaloid sanguinarine, whereas (*S*)-reticuline, following its epimerization to (*R*)-reticuline, also serves as the precursor to morphine.

MATERIALS AND METHODS

Plant Material and Cell-Suspension Cultures

Opium poppy (*Papaver somniferum* cv Marianne) plants were grown in greenhouse conditions at a day/night temperature regime of 20/18°C. Tissue samples were collected from mature flowering plants 1 d after anthesis. Seedlings were grown at 23°C in Petri plates containing moist filter paper. Seeds were surface-sterilized with 20% (v/v) sodium hypochlorite for 15 min, washed thoroughly with sterile distilled water, and allowed to imbibe water for 24 h (d 0). Seeds were kept in the dark for 3 d following imbibition and then transferred to a photoperiod of 16 h of light/8 h of dark.

Cell-suspension cultures of opium poppy (cell line 2009 SPF) were maintained in diffuse light at 23°C on Gamborg 1B5C medium consisting of B5 salts and vitamins plus 100 mg L^{-1} *myo*-inositol, 1 g L^{-1} hydrolyzed casein, 20 g L^{-1} Suc, and 1 mg L^{-1} 2,4-D. Cells were subcultured every 6 d using a 1:3 dilution of inoculum to fresh medium.

Elicitor Preparation and Treatment

Fungal elicitors were prepared according to the method of Eilert et al. (1985). Sections (1 cm^2) of mycelium grown on potato dextrose agar were used to inoculate 50 mL of 1B5C plant cell-suspension culture medium including supplements but excluding 2,4-D. Fungal mycelium cultures of *Botrytis* sp. and *Pythium aphanidermatum* were grown on a gyratory shaker (120 rpm) at 22°C in the dark for 6 d. Mycelia and medium were homogenized with a Polytron (Brinkmann), autoclaved (121°C) for 20 min, and subsequently centrifuged under sterile conditions with the supernatant serving as elicitor. Opium poppy cell-suspension cultures in rapid growth phase, corresponding to 4 d after subculture, were used for all experiments. Elicitor treatments were initiated by the addition of 1 mL of fungal homogenate 50 mL^{-1} cell culture, unless otherwise indicated, or by the addition of (±)-MeJA to a final concentration of 100 μM. Samples were collected by vacuum filtration, frozen in liquid nitrogen, and stored at −80°C until used.

Isolation and Analysis of Nucleic Acids

Poppy leaf genomic DNA was isolated (Murray and Thompson, 1980), digested with *Eco*RI and *Hin*dIII, electrophoresed on a 1.0% agarose gel, and transferred to a nylon membrane (Sambrook et al., 1989). Total RNA for gel-blot analysis was isolated according to the method of Logemann et al. (1987), and 15 μg was fractionated on 1.0% formaldehyde agarose gels before transfer to nylon membranes (Sambrook et al., 1989). RNA and DNA blots were hybridized, unless otherwise indicated, with a random primer ^{32}P-labeled (Feinberg and Vogelstein, 1984) 2.0-kb *Sal*I fragment from the *bbe1* genomic clone that contained approximately 1.3 kb of the ORF. Alternatively, a 0.6-kb fragment amplified from within the ORF by PCR (sense primer = 5′-AAAGAATGTAGGAATCGA-3′; antisense primer = 5′-TATTTCTCCAATCTATCC-3′) was used. Random primer ^{32}P-labeled gene-specific probes for *bbe1* and *bbe2* were synthesized from DNA fragments amplified by PCR (*bbe1* sense primer = 5′-TAACCATCCACAGAGTAT-3′; *bbe1* antisense primer = 5′-AACGAGGTCTATCATTTC-3′; *bbe2* sense primer = 5′-CTATGATCATTTAGTACA-3′; *bbe2* antisense primer = 5′-CATAGTAGGTTCTCATGG-3′). Hybridizations were performed at 65°C in 0.25 M sodium phosphate buffer, pH 8.0, 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA. Blots were washed at 65°C, twice with 2× SSC, 0.1% (w/v) SDS, and twice with 0.2 × SSC,

0.1% (w/v) SDS (Sambrook et al., 1989) (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). RNA and DNA blots were autoradiographed with an intensifying screen at −80°C.

Library Construction and Screening

A λEMBL3 (Stratagene) genomic library was constructed from poppy leaf DNA partially digested with *Mbo*I (Sambrook et al., 1989). A primary library of 1.1×10^7 plaques was obtained (Facchini and De Luca, 1994) and 2.5×10^9 plaques of the amplified library were screened at high stringency, as described above, with a ^{32}P-labeled probe synthesized from the full-length coding region of BBE from *Eschscholtzia californica* (Dittrich and Kutchan, 1991). The coding region of BBE was amplified by 35 cycles of PCR from 100 ng of *E. californica* genomic DNA using *Taq* DNA polymerase and primers designed to the published sequence (sense primer = 5′-AAACTCCCATCTTCTTCT-3′; antisense primer = 5′-CCACCATCACTCCCCAAA-3′) at an annealing temperature of 50°C. The 1.6-kb PCR product was blunt-end-cloned into the *Sma*I site of pBluescript SK$^+$ (Stratagene) and partially sequenced to verify its identity. The 1.6-kb insert was then purified on an agarose gel, random primer-labeled, and used to screen the poppy genomic library. Isolated genomic DNA fragments that cross-hybridized to the *E. californica* BBE probe were subcloned into pBluescript SK$^+$ for further characterization.

Double-stranded DNA was sequenced using the dideoxynucleotide chain-termination method (Sanger et al., 1977) and a recombinant T7 DNA polymerase (United States Biochemical). Insert DNA fragments were sequenced from their ends using T7 and T3 primers. In addition, 18-mer oligonucleotides synthesized according to obtained sequence information were used directly as primers for further sequencing. Comparative sequences were obtained from published reports or from the GenBank and Swiss-Prot sequence databases. Sequence compilation and analysis were performed using the FASTA program package (Pearson and Lipman, 1988).

Construction of Transient Expression Vectors

A cassette containing the GUS ORF, followed by the nopaline synthase polyadenylation signal from a modified version of the promoterless binary vector pBI 101 (Jefferson et al., 1987), designated pBI 102, was transferred to pUC19 between the *Hin*dIII and *Eco*RI sites and used for the construction of promoter-GUS fusions. The modified vector in pUC19, designated pUC 202, contains the additional restriction sites *Apa*I, *Xho*I, *and Kpn*I as the result of the blunt-end insertion of an adapter fragment into the *Sma*I site of pBI 101. The CaMV 35S promoter from pBI 121 (Jefferson et al., 1987) was introduced into pUC 202 between the *Hin*dIII and *Bam*HI sites to yield the pUC 35S::GUS construct. Promoter regions of *bbe1* and *bbe2* were amplified by PCR using primers designed to include *Hin*dIII and *Bam*HI restriction sites at the 5′ and 3′ ends of the promoter fragment, respectively (*bbe1* sense primer = 5′-CCCCCCGGATCCGTTGGAGAAGTAC GTCAA-3′; *bbe1* antisense primer = 5′-CCCCCCAAGCTTGAATTCAGAATGGGTTAGTC-3′; *bbe2* sense primer = 5′-CCCCCCAAGCTTATATTGACTCCTTGAGCTAC-3′; *bbe2* antisense primer = 5′-CCCCCCGGATCCTTAACAGTGACTAGTGAC-3′). The *bbe1* and *bbe2* promoters extended 2.5 and 2.0 kb, respectively, upstream of the putative translational start codon in each gene. The PCR-generated *bbe1* and *bbe2* promoter fragments were inserted into pUC 202 between the *Hin*dIII and *Bam*HI sites to yield the pUC BBE1::GUS and pUC BBE2::GUS constructs, respectively. The assembly of all constructs was verified by sequencing through the promoter-GUS junction.

A construct (pCaLucNOS) harboring a CaMV 35S-Luc ORF fusion, followed by the nopaline synthase polyadenylation signal, was a kind gift from Dr. Larry Holbrook (SemBioSys Genetics, Calgary). All plasmids were purified by PEG precipitation, phenol/chloroform extraction, LiCl precipitation, and RNase digestion, followed by an additional phenol/chloroform extraction and ethanol precipitation before use.

Microprojectile Bombardment of Cultured Opium Poppy Cells

Gold particles (60 mg; 1.6-μm diameter; Bio-Rad) were sterilized by vortexing in 1 mL of 100% ethanol for 5 min, washed twice with sterile, distilled water, and resuspended in 1 mL of sterile, distilled water. A 50-μL aliquot of the suspension was removed and 15 μg of each plasmid DNA, 50 μL of 2.5 M $CaCl_2$, and 20 μL of 0.1 M spermidine were successively added. The gold particles were incubated on ice for 5 min after each addition. The mixture was then vortexed at room temperature for 4 min, washed twice with ethanol, and resuspended in 45 μL of 100% ethanol. For each bombardment, 15 μL of the particle suspension (1 mg of particles per shot) was pipetted onto the center of microcarriers that were sterilized with 100% ethanol and used after all ethanol had evaporated.

Opium poppy cell-suspension cultures in logarithmic growth phase (d 2) were collected on Whatman GF/A microfiber filters by gentle vacuum filtration to form a thin cell layer within a circular area of approximately 2.0 cm in diameter. Filters containing the plant cells were placed in sterile Petri plates and positioned below a microprojectile stopping screen. Bombardments were performed using a Biolistic particle acceleration device (PDS 1000/He, Bio-Rad) under a chamber pressure of 26 mm of Hg, at a distance of 1.5, 2.0, and 6.5 cm from the rupture disc to the microcarriers to the stopping screen to the target, respectively, and at a He pressure of 1,100 p.s.i. After bombardment, 600 μL of sterile 1B5C medium and 60 μL of the *Botrytis* elicitor preparation were added to the cultured cell layers, which were then incubated at 23°C in the same Petri plates.

GUS and Luc Assays

Forty-eight hours after microprojectile bombardment, cultured cells were collected by vacuum filtration and ground in a microtissue grinder with extraction buffer consisting of 50 mM KPO_4 buffer (pH 7.0), 1 mM EDTA, and

10 mM β-mercaptoethanol. The GUS fluorometric assay buffer consisted of 50 mM $NaPO_4$ buffer (pH 7.0), 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% (w/v) sodium lauryl sarcosine, and 0.1% (w/v) Triton X-100. 4-Methylumbelliferyl-β-D-glucuronide was added at a final concentration of 0.44 mg mL^{-1}. Assays were performed on 80 μL of bombarded cell culture extract for 3 h at 37°C and stopped with a 10× volume of 0.2 M Na_2CO_3. A fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan) was used to quantify the amount of methylumbelliferone cleaved from 4-methylumbelliferyl-β-D-glucuronide.

The Luc assay buffer consisted of 25 mM Tricine, pH 7.8, 15 mM $MgCl_2$, 5 mM ATP, 0.5 mg mL^{-1} BSA, and 7 mM β-mercaptoethanol. Bombarded cell culture extract (20 μL) was mixed with 200 μL of assay buffer and incubated at room temperature for 15 min (de Wet et al., 1987). Luciferin (100 μL of 0.5 mM diluted with 1 mM Tricine, pH 7.8, from 10 mM stock; Boehringer Mannheim) was injected into the reaction mixture, and the light emitted within the first 10 s after luciferin injection was quantified using a luminometer (Monolight 2010, Analytical Luminescence Laboratories, San Diego, CA). Protein concentration was determined by the method of Bradford (1976) using BSA as the standard.

Sanguinarine Extraction, Identification, and Quantification

Plant tissues were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The powdered tissue was extracted in methanol for 10 min at 100°C and insoluble debris was removed by filtration. Extracts were reduced to dryness under vacuum, redissolved in 1.0 M sodium carbonate/bicarbonate (3:2) buffer, pH 10.0, and extracted three times with ethyl acetate. The pooled ethyl acetate fractions were reduced to dryness and the residue was taken up in 1.0 mL of methanol. Sanguinarine was quantified by HPLC on a Waters 600E HPLC system and Waters 991 photodiode array detector. Alkaloid extracts were separated and monitored at 280 nm on a Waters Nova Pak C_{18} reverse-phase column (3.9 × 300 mm) at 1200 p.s.i. with an isocratic gradient of methanol:water (6:4) containing 0.1% triethylamine and a flow rate of 0.5 mL min^{-1}. The sanguinarine peak was identified from its UV spectrum and by comparison of its retention time with that of the authentic standard. The identity of sanguinarine was further confirmed by low-resolution, direct probe MS (VG 7070F GC/MS System; VG Analytical, Manchester, UK) in comparison with the spectrum of an authentic standard, as described previously (Facchini et al., 1996).

RESULTS

Isolation of Opium Poppy Genes Similar to BBE

Screening of an opium poppy genomic library in λEMBL3 with the 1.6-kb full-length coding region of *E. californica* BBE resulted in the isolation of eight different genomic clones with an average insert length of approximately 12 kb. Restriction mapping and nucleotide sequence analysis showed that each clone contained one of three unique sequences with similarity to BBE, designated *bbe1*, *bbe2*, and *bbe3* (Fig. 2). An uninterrupted ORF predicting an amino acid sequence with strong similarity to *E. californica* BBE was found in *bbe1* (Fig. 3). In contrast, *bbe2* contained an extra thymidine between codons encoding V-226 and F-227, which introduced a frame-shift mutation in the ORF (Fig. 2). With the exception of this frame shift, the ORFs on either side of the mutation predicted polypeptides with extensive identity to BBE1 from opium poppy and BBE from *E. californica* (Fig. 3). Finally, *bbe3* was found to possess homology with only about 1.0 kb at the 3′ end of the opium poppy *bbe1*- and *bbe2*-coding regions and the *E. californica* BBE cDNA (Fig. 2). In addition, this truncated *bbe3* clone had numerous frame-shift mutations within the remnants of the region corresponding to the ORF of other *bbe* clones.

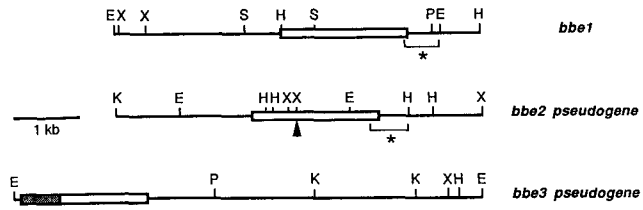

Figure 2. Restriction maps of isolated opium poppy genomic clones similar to the BBE cDNA from *E. californica* (Dittrich and Kutchan, 1991). The open boxes correspond to coding regions. The shaded box represents a region of the *bbe3* clone with no apparent homology to the corresponding region of other clones. The arrowhead shows the location of an extra nucleotide that results in a frame-shift mutation in the ORF of *bbe2*. The brackets and asterisks identify the location of DNA fragments used as gene-specific probes in experiments illustrated in Figure 7. Restriction sites are as follows: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; X, *Xba*I.

Not All BBE Homologs in Opium Poppy Are Functional

Our data suggested that *bbe2* and *bbe3* were pseudogenes and that only *bbe1* was expressed and encoded a functional BBE protein in opium poppy. However, the possibility remained that the single frame-shift mutation in *bbe2* might represent a cloning artifact; thus, the expressions of *bbe1* and *bbe2* were tested using two independent approaches (Fig. 4). First, 2.5 and 2.0 kb of the 5′ regions flanking *bbe1* and *bbe2*, respectively, were amplified by PCR using primers designed to introduce *Hin*dIII and *Bam*HI sites at the 5′ and 3′ ends, respectively, of each DNA fragment. Chimeric gene fusions were constructed between the *bbe1* and *bbe2* promoter regions and the GUS reporter gene and transferred into cultured opium poppy cells by microprojectile bombardment. As shown in Figure 4A, GUS activity was detected only in cells bombarded with the *bbe1* promoter-GUS and the CaMV 35S-GUS constructs, whereas no GUS activity was detected in cells bombarded with the *bbe2* promoter-GUS or the promoterless-GUS constructs. Cells were co-bombarded with pCaLucNOS to allow GUS activity to be normalized against Luc activity to account for differences in expression efficiency between bombardments. The specific normalized GUS activities in poppy cultures harboring the pUC BBE1::GUS and pUC 35S::GUS constructs were 942 ± 95 and 867 ± 260 pmol 4-methylumbelliferone min^{-1} mg^{-1} protein/relative lucif-

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                                                        ΔΔΔ
EcBBE     M-ENKTPIFFSLSIFLSLL-NCALGG----NDLLSCLTFNGVRNHTVF-   42
PsBBE1    MMCRSLTLRFF-L--FIVLLQTCVRGGDVNDNLLSSCLNSHGVHNFTTL-   46
PsBBE2    MVMKFMLNHYSLFIF-LLMSLVVPFLSEPTNIISSCLTQNQVKNFTLNA    48

EcBBE     -SADSDSDFNRFLHLSIQNPLFQNSLISKPSAIILPGSKEELSNTIRC-I   90
PsBBE1    -STDTNSDYFKLLHASMQNPLFAKPTVSKPSFIVMPGGKEELSSTVHCCT   95
PsBBE2    NSGNDTSEFHKLLSFSIQNLRYAESTYTKPIVIVLPDTREELANTVLC-S   97

                   ••••••••••
EcBBE     RKGSWTIRLRSGGHSYEGLS----YTSDTPFILIDLMNLNRVSIDLESET  136
PsBBE1    RE-SWTIRLRSGGHSYEGLS----YTADTPFVIVDMMNLNRISIDVLSET  140
PsBBE2    KQASLAIRVRCGGHSYEGLSSVSTYTGEL-FMIIDVMNLNNVSVDLESET  146

EcBBE     AWVESGSTLGELYYAITESSSKLGFTAGWCPTVGTGGHISGGGFGMMSRK  186
PsBBE1    AWVESGATLGELYYAIAQSTDTLGFTAGWCPTVGSGGHISGGGFGMMSRK  190
PsBBE2    AWVEGGATLGETCQAIAESSHLHGFSAGSCPTVGSGGHISGGGFGLLSRK  196

EcBBE     YGLAADNVVDAILIDANGAILDRQAMGEDVFWAIRGGGGGVWGAIYAWKI  236
PsBBE1    YGLAADNVVDAILIDSNGAILDREKMGDDVFWAIRGGGGGVWGAIYAWKI  240
PsBBE2    YGLAADNVVDAILIDANGRFLDRKAMGEDVFWAIRGGGGGVWGIIYAWKV  246

EcBBE     KLLPVPEKVTVFRVTKNVAIDEATSLLHKWQFVAEELEEDFTLSVLGGAD  286
PsBBE1    KLLPVPEKLTVFRVTKNVGIEDASSLLHKWQYVADELDEDFTVSVLGGVN  290
PsBBE2    QLVPVPETVTSFILSRPGTKNSVAKLVHKWKFVAPHLEDEFYLSVFVGAG  296

EcBBE     EKQVWL-----TMLGFHFGLKTVAKSTFDLLFPELGLVEEDYLEMSWGES  331
PsBBE1    GNDAWL-----MFLGLHLGRKDAAKTIYDEKFPELGLVDKEFQEMSWGES  335
PsBBE2    LPETRTIGISATFKGFYLGSTAEAISTLNRKFPELGVAEDDCKEMSWIES  346

EcBBE     FAYLAGLETVSQ---LNNRFLKFDERAFKTKVDLTKEPLPSKAFYGLLER  378
PsBBE1    MAFLSGLDTISE---LNNRFLKFDERAFKTKVDFTKVSVPLNVFRHALEM  382
PsBBE2    VVFFSGLRNGSTVLDLKNRYLDDKG-YFKAKSDYVKEPISMRGIKTALRI  395

                                                        ΔΔΔ
EcBBE     LSKEPNGFIALNGFGGQMSKISSDFTPFPHRSGTRLMVEYIVAWNQSEQK  428
PsBBE1    LSEQPGGFIALNGFGGKMSEISTDFTPFPHRKGTKLMFEYIIAWNQDEES  432
PsBBE2    LENEPKGYVILDPYGGLMSKISSDSIPYPHRAGNIYAIQYLVAWNSTDNS  445

                                                        ΔΔΔ
EcBBE     KKTEFLDWLEKVYEFMKPFVSKNPRLGYVNHIDLDLGGIDWGNKT-VVNN  477
PsBBE1    KIGEFSEWLAKFYDYLEPFVSKEPRVGYVNHIDLDIGGIDWRNK-SSTTN  481
PsBBE2    KNDVFISWIRNFYNSMTNYVAKGPRAAYVNYLDLDLGEMNLLSLTSSTTM  495

EcBBE     A------IEISRSWGESYFLSNYERLIRAKTLIDPNNVFNHPQSIPPMAN  521
PsBBE1    A------VEIARNWGERYFSSNYERLVKAKTLIDPNNVFNHPQSIPPMMK  525
PSBBE2    ARSQSDAVKIAKVWGEKYFLKNYDHLVQAKTLIDPDNVFYNQQGIPPLPT  545

EcBBE     FD--YLEKTLGSDGGEVVI*                                538
PsBBE1    FEEIYMLKEL*                                         535
PsBBE2    SR---LHSNI*                                         552
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Figure 3. Alignment of predicted amino acid sequences of the BBEs from *E. californica* (EcBBE) and *P. somniferum* (PsBBE1 and PsBBE2). Double dots refer to identical amino acid residues and single dots indicate chemically conserved positions. A single bp insertional mutation between V-226 and F-227 in the *bbe2* sequence was removed for the purposes of aligning the encoded amino acid sequences on either side of the frame shift. The closed circles indicate the putative flavin-binding domain. The open triangles mark the location of putative consensus sequences for *N*-linked glycosylation sites.

erase unit $\times 10^{-5}$, respectively. Specific normalized GUS activities in cultures harboring the pUC 102 and pUC BBE2::GUS constructs were 38 ± 37 and 40 ± 23 pmol 4-methylumbelliferone min^{-1} mg^{-1} protein/ relative luciferase unit $\times 10^{-5}$, respectively. All activities represent values from which the background measured in control cultures (bombarded with gold particles with no absorbed DNA) was removed. The specific Luc activity was similar in each case. The expression of *bbe* genes in opium poppy plants was confirmed using probes designed to unique sequences 3′ to the putative stop codon of each gene. Hybridization of radiolabeled gene-specific probes to RNA gel blots containing samples from tissues known to accumulate abundant BBE mRNAs showed that only *bbe1*-specific, not *bbe2*-specific mRNAs were present (Fig. 4B).

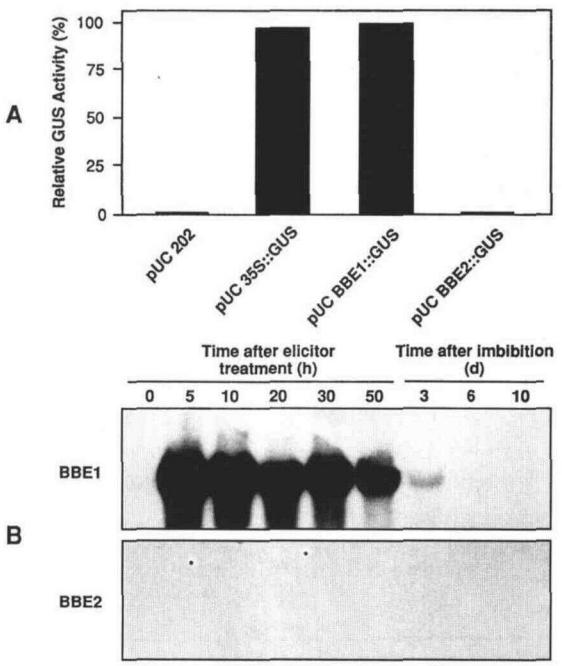

Figure 4. Expression of *bbe1* and *bbe2* determined by transient expression of promoter-GUS fusions in opium poppy cell cultures (A) and detection of BBE mRNAs using gene-specific probes for *bbe1* and *bbe2* (B). A, Relative normalized GUS activity in cultured poppy cells following microprojectile bombardment with constructs containing different promoters: pUC 102 (promoterless), pUC 35S::GUS (CaMV 35S promoter), pUC BBE1::GUS (*bbe1* promoter), and pUC BBE2::GUS (*bbe2* promoter). Values represent the means of three independent experiments whereby poppy cells were co-bombarded with promoter-GUS and CaMV 35S-Luc (Luc) constructs, and GUS activity was normalized against Luc activity, which was generally similar in each case. B, Levels of BBE1 and BBE2 mRNAs in cultured poppy cells at different times following treatment with 1 mL *Botrytis* elicitor 50 mL^{-1} culture (Fig. 7) and in poppy seedlings at different times following imbibition (Fig. 6). Total RNA was extracted and 15 μg was fractionated on 1.0% formaldehyde agarose gels, transferred to nylon membranes, and hybridized at high stringency with ^{32}P-labeled PCR products corresponding to regions 3′ of the putative stop codons of *bbe1* or *bbe2*. Gels were stained with ethidium bromide prior to blotting to ensure equal loading.

Characterization of Opium Poppy BBE Genes

The nucleotide sequence of *bbe1* consisted of an ORF of 1605 bp containing no intervening sequences, which is similar to the absence of introns in the numerous *tydc* genes reported from opium poppy (Facchini and De Luca, 1994; Maldonado-Mendoza et al., 1996) that are presumed to be involved in alkaloid biosynthesis. The predicted translation product initiated at the first in-frame ATG contained 535 amino acids with a molecular mass of 59.9 kD. In *E. californica*, the mature protein excludes 22 amino acids that constitute the putative hydrophobic signal peptide that directs the preprotein into the specific alkaloid biosynthetic vesicle in which it accumulates (Amann et al., 1986; Dittrich and Kutchan, 1991). A similar hydrophobic domain is found at the N terminus of opium poppy *bbe1* (Fig. 3). As in *E. californica* BBE (Dittrich and Kutchan, 1991), opium poppy BBE1 possesses consensus sequences corresponding to the motif NX(S/T) for putative *N*-linked glycosylation sites.

Comparison of the predicted amino acid sequence for opium poppy BBE1 with sequences present in the GenBank and Swiss-Prot databases revealed similarity to other plant, bacterial, and animal oxygen oxidoreductases (August et al., 1994; Kutchan and Dittrich, 1995). BBE1 was found to share 30% overall homology to 6-HDNO from *Arthrobacter oxidans* (Brandsch and Bichler, 1985; Brandsch et al., 1987), 24% overall homology to MCRA encoded by the mitomycin C resistance gene *mcrA* from *Streptomyces lavendulae* (August et al., 1994), and 23% overall homology to GGLO from rat liver (Koshizaka et al., 1988). Extensive sequence similarity (50% identity; 60% homology) was also detected between the central region of BBE1 and the translated ORF (110 amino acids) of an *Arabidopsis thaliana* expressed sequence tag (clone TAP0059; GenBank accession no. F14356). Direct comparison shows that *E. californica* BBE and opium poppy BBE1 are 88% identical within the 110 amino acids corresponding to the available sequence of TAP0059.

The putative flavin-binding consensus sequence for a covalently flavinylated His is found in opium poppy BBE near the N terminus (Fig. 3), as it is in *E. californica* BBE (Dittrich and Kutchan, 1991; Kutchan and Dittrich, 1995), 6-HDNO from *A. oxidans* (Brandsch and Bichler, 1985; Brandsch et al., 1987), MCRA from *S. lavendulae* (August et al., 1994), and GGLO from rat liver (Koshizaka et al., 1988). The amino acid motif SGGH occurs at a unique location in *A. oxidans* 6-HDNO, *E. californica* BBE, and opium poppy BBE1, whereas this motif is replaced by the amino acid sequences ATGH and GGGH in MCRA and GGLO, respectively. August et al. (1994) provided a complete amino acid sequence alignment of BBE, 6-HDNO, MCRA, and GGLO.

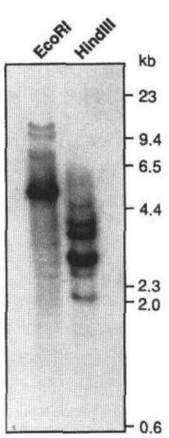

Figure 5. DNA gel blot of opium poppy genomic DNA hybridized to the *SalI/EcoRI* fragment of *bbe1*. Opium poppy genomic DNA (20 μg) was digested with *EcoRI* and *HindIII*, fractionated in a 1.0% agarose gel, transferred to a nylon membrane, and hybridized at high stringency with a ^{32}P-labeled DNA probe. Numbers on the right refer to the sizes of DNA size markers.

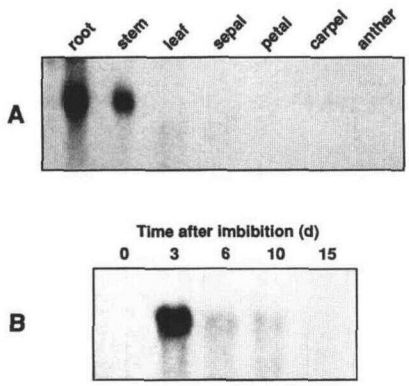

Figure 6. Levels of BBE mRNAs in different organs of mature opium poppy plants (A) and in opium poppy seedlings at different times following imbibition (B). Total RNA was extracted and 15 μg was fractionated on 1.0% formaldehyde agarose gels, transferred to nylon membranes, and hybridized at high stringency with a ^{32}P-labeled *SalI/EcoRI* fragment of *bbe1*. Gels were stained with ethidium bromide prior to blotting to ensure equal loading.

The nucleotide sequence of *bbe1* shares 52 and 65% identity with corresponding regions of *bbe2* and *bbe3*, respectively, and 72% identity with the ORF of the BBE cDNA from *E. californica*. The predicted amino acid sequence of opium poppy BBE1 is 67% identical and 80% homologous to the amino acid sequence of *E. californica* BBE (Dittrich and Kutchan, 1991) and 47% identical and 66% homologous to the frame-shift-corrected amino acid sequence of opium poppy BBE2 (Fig. 3). DNA gel-blot analysis data are consistent with the existence of a limited number of genes with homology to *bbe1* in the opium poppy genome (Fig. 5). Under high-stringency hybridization conditions, a probe comprising the 2.0-kb *SalI/EcoRI* fragment of *bbe1* hybridized strongly to 5-kb *EcoRI* and 3-kb *HindIII* restriction fragments that correspond to *bbe1* (Fig. 1) and less strongly to a limited number of other bands. Some of these other bands might be accounted for by restriction fragments corresponding to *bbe2* and *bbe3*; however, the possibility that additional *bbe*-like genes may also be present cannot be completely ruled out by these experiments.

Developmental, Tissue-Specific, and Elicitor-Induced Expression of BBE in Opium Poppy Plants and Cell Cultures

RNA gel-blot analysis revealed that *bbe* genes are differentially expressed in mature poppy tissues (Fig. 6A). BBE mRNAs were detected most abundantly in roots and, to a

slightly lesser extent, in stems. BBE transcripts were not detected in leaves or reproductive organs. A transient developmental induction of BBE mRNAs was also detected in opium poppy seedlings immediately following germination (Fig. 6B). BBE transcript levels increased rapidly to a maximum 3 d following imbibition and subsequently declined rapidly. Low levels of BBE mRNAs were detectable in total RNA samples up to 14 d following imbibition.

The level of BBE mRNAs also increased in opium poppy cell-suspension cultures in response to treatment with a fungal elicitor (Fig. 7). BBE transcripts were not detected in control cultures, but message levels began to increase about 2 h after the addition of elicitor prepared from the opium poppy pathogenic fungus of the genus *Botrytis* (Fig. 7A). BBE mRNAs reached maximum levels 10 h following the addition of elicitor and declined to near baseline levels 80 h after elicitation. The induction of BBE mRNA levels was shown to be dependent on the amount of *Botrytis* elicitor added to the cell cultures (Fig. 7B). The possible involvement of a specific elicitor component(s) in the crude *Botrytis* elicitor preparation was suggested by the ineffectiveness of a crude *Pythium* elicitor preparation in the induction of BBE mRNA levels (Fig. 7C). BBE mRNA levels were also induced after treatment of cell cultures with MeJA (Fig. 8) following a time course similar to the induction of BBE mRNAs in response to the *Botrytis* elicitor (Fig. 7A). However, the induction was weaker than that observed using the fungal elicitor.

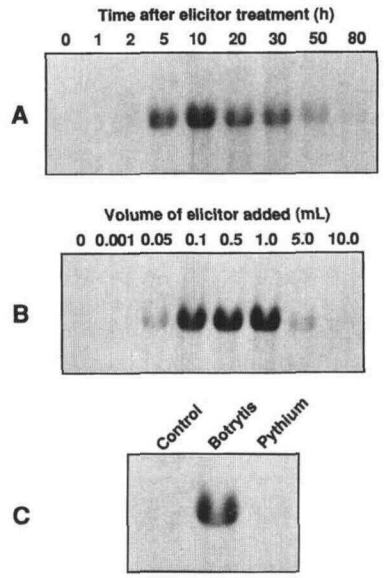

Figure 7. Levels of BBE mRNAs in cell-suspension cultures of opium poppy treated with a fungal elicitor. A, Time course of changes in BBE mRNA levels following treatment of poppy cells with 1 mL *Botrytis* elicitor 50 mL^{-1} culture. B, Dose-response changes in BBE mRNA levels following treatment with different volumes of *Botrytis* elicitor 50 mL^{-1} of culture. C, Differential accumulation of BBE mRNAs following treatment with 1 mL *Botrytis* or *Pythium* elicitor 50 mL^{-1} culture. Total RNA was extracted and 15 μg was fractionated on 1.0% formaldehyde agarose gels, transferred to nylon membranes, and hybridized at high stringency with a ^{32}P-labeled *Sall/EcoRI* fragment of *bbe1*. Gels were stained with ethidium bromide prior to blotting to ensure equal loading.

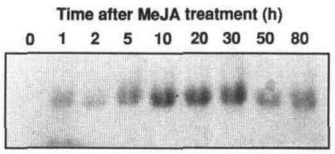

Figure 8. Time-course changes in BBE mRNA levels following treatment of opium poppy cell-suspension cultures with 100 μM MeJA. Total RNA was extracted and 15 μg was fractionated on 1.0% formaldehyde agarose gels, transferred to nylon membranes, and hybridized at high stringency with a ^{32}P-labeled 0.6-kb internal fragment of *bbe1*. Gels were stained with ethidium bromide prior to blotting to ensure equal loading.

Sanguinarine Accumulates in Most Tissues Expressing BBE

Analysis of alkaloid extracts from various opium poppy tissues by HPLC showed that sanguinarine accumulates most abundantly in the roots of mature plants, in developing opium poppy seedlings between 4 and 12 d after imbibition, and in cell cultures treated with the *Botrytis* elicitor (Table I). Much lower levels were found in cell cultures treated with the *Pythium* elicitor. Sanguinarine was not detected in any mature stem or leaf tissues, including the isolated latex, or in cell cultures treated with MeJA (Table I).

DISCUSSION

The biosynthesis of morphine and sanguinarine in opium poppy is thought to proceed in three stages. The first stage begins with the decarboxylation of L-Tyr and leads to the biosynthesis of (*S*)-reticuline (Frenzel and Zenk, 1990) and is a general pathway found in many plant families that produce isoquinoline alkaloids. The second and third stages diverge from (*S*)-reticuline and lead to morphine and sanguinarine biosynthesis, respectively (Fig. 1). The role of (*S*)-reticuline as a common branch-point intermediate in the biosynthesis of many different plant

Table I. *Accumulation of sanguinarine in various opium poppy tissues*

Tissue	Sanguinarine Content
	μg/g dry wt
Mature plant	
Stem excluding latex[a]	nd[c]
Leaf excluding latex[a]	nd
Latex[b]	nd
Root	1650 ± 150
Seedling	
4 d after imbibition	Trace
12 d after imbibition	1445 ± 55
Cell culture	
Control	Trace
Treated with *Botrytis* elicitor[d]	2050 ± 150
Treated with *Pythium* elicitor[d]	160 ± 50
Treated with MeJA[d]	Trace

[a] Stem and leaf tissue was cut into 1-cm^2 sections, latex was allowed to drain completely, and tissues were rinsed with distilled water before extraction. [b] Latex was collected from fresh tissue using a glass capillary and 25 μL was extracted. [c] nd, Not detected. [d] Treatment was for 80 h.

isoquinoline alkaloid classes suggests that branch-point enzymes that direct (*S*)-reticuline into specific branch pathways might regulate the carbon and nitrogen flux into specific end products. BBE catalyzes the conversion of (*S*)-reticuline to (*S*)-scoulerine, the first committed step in sanguinarine biosynthesis. The unique vesicular localization of BBE among sanguinarine biosynthetic enzymes (Amann et al., 1986; Blechert et al., 1995) suggests that transport into these vesicles precludes the entry of (*S*)-reticuline into any other pathway, even if morphine biosynthetic enzymes are present in the cytosol or in other compartments (Amann et al., 1986). The possible regulatory function of BBE was further suggested by the induction of BBE mRNAs (Dittrich and Kutchan, 1991; Kutchan, 1993) and enzyme activity (Blechert et al., 1995) in response to yeast elicitor and MeJA treatments in *E. californica* cell cultures before the accumulation of sanguinarine.

In the opium poppy cultivar Marianne, BBE is encoded by a limited number of genes of which *bbe1* is expressed and *bbe2* and *bbe3* are not (Fig. 2). Our data do not preclude the possibility of another expressed, but as yet unidentified, *bbe* gene in opium poppy. The predicted ORF from *bbe1* results in an amino acid sequence that shares extensive identity (>67%) and homology (>80%) with the predicted translation product of the BBE cDNA isolated from *E. californica* (Dittrich and Kutchan, 1991). The sequences include a colinear alignment of the putative FAD-binding site (Kutchan and Dittrich, 1995) centered at H-108 and H-104 of opium poppy BBE1 and *E. californica* BBE, respectively (Fig. 3). Opium poppy BBE1 also exhibits a putative hydrophobic N-terminal signal peptide with 42% identity and 50% homology to the known *E. californica* BBE signal peptide (Dittrich and Kutchan, 1991). Both preproteins share an identical molecular mass of 59.9 kD and, assuming an identical signal peptide cleavage site, comparable mature protein molecular masses of 57.1 and 57.4 for opium poppy BBE1 and *E. californica* BBE (Dittrich and Kutchan, 1991), respectively. The extensive similarity between opium poppy BBE1 and *E. californica* BBE strongly suggests that these proteins are functionally identical.

Significant alignments to BBE proteins were found with a bacterial enzyme (6-HDNO) involved in nicotine catabolism that catalyzes the two-electron oxidation of the D-6-hydroxynicotine pyrrolidine ring (Brandsch et al., 1987), the predicted translation product (MCRA) of a bacterial mitomycin C resistance gene *mcrA* (August et al., 1994), and an FAD-dependent microsomal GGLO involved in ascorbate biosynthesis in rat liver (Koshizaka et al., 1988). All of these proteins have been characterized as flavinylated oxygen oxidoreductases. The function of the gene product encoded by the *A. thaliana* TAP0059-expressed sequence tag is not known, but it could be an oxygen oxidoreductase due to its homology to this class of enzymes. The extensive identity among opium poppy BBE1, *E. californica* BBE, and the *A. thaliana* TAP0059 clone suggests that at least some plant oxygen oxidoreductases might share a common evolutionary origin.

In an effort to understand the overall regulation of isoquinoline alkaloid biosynthesis in opium poppy and related species, the tissue-specific, developmental-specific, and elicitor-induced expression of *bbe* genes was studied in opium poppy plants and cultured cells in relation to the accumulation of sanguinarine (Table I). In the plant, sanguinarine accumulation is essentially restricted to roots (Facchini and De Luca, 1995) and seedlings. Sanguinarine accumulation is inducible in cell cultures after treatment with specific fungal elicitors (Facchini et al., 1996). In the mature plant, BBE mRNA levels are highest in the root (Fig. 6A), as expected. However, the high level of BBE mRNAs in the stem (Fig. 6A) was not anticipated because of the absence of detectable levels of sanguinarine in this organ (Table I). Although symptoms of microbial infection were not observed, the plants were not grown under axenic conditions; thus, it is possible that *bbe* expression in the stems was induced in response to some unidentified environmental factor. However, RNA was extracted from three independent plants and identical results were obtained in each case; thus, it also becomes possible that under normal growth conditions, sanguinarine biosynthesis occurs not only in the root, where it accumulates, but also in the stem. Similarly, morphine accumulates mainly in the latex of aerial organs (Facchini and De Luca, 1995), but salutaridine synthase, a late enzyme of morphinan alkaloid biosynthesis, is abundant in stems and roots (Gerardy and Zenk, 1993). The antiparallel translocation of sanguinarine and morphine from stem to root and root to stem, respectively, remains to be verified. However, nicotine and hyoscyamine are classic examples of alkaloids that are synthesized in one organ (i.e. roots) and predominantly accumulate in another (i.e. leaves) as the result of their translocation through the xylem (Hashimoto and Yamada, 1992). Similar mechanisms could exist in members of the Papaveraceae family.

The transient expression of *bbe* genes in germinating poppy seeds (Fig. 6B) is identical to that of *tydc5* (Maldonado-Mendoza et al., 1996) and *tydc2* (P.J. Facchini, unpublished results) and further supports the notion that sanguinarine accumulates (Table I) to protect the seedling during this vulnerable developmental stage. The transient increase in BBE mRNA in cultured poppy cells in response to a species-specific fungal elicitor (Fig. 7) is consistent with the induction of BBE mRNA in *E. californica* cultures treated with a yeast elicitor (Dittrich and Kutchan, 1991). The time course for *bbe* gene expression in poppy cultures is also similar to that for *tydc* genes (Facchini et al., 1996). These data further support the suggestion of a defensive role for sanguinarine and agree with the proposed regulatory function of BBE in its biosynthesis (Dittrich and Kutchan, 1991; Kutchan, 1993; Blechert et al., 1995). However, the induction of BBE mRNA levels in poppy cultures without a concomitant increase in sanguinarine accumulation in response to MeJA treatment suggests that the regulation of at least one of four other inducible biosynthetic enzymes (Blechert et al., 1995) is not mediated by jasmonic acid. Although these data do not dispute the putative key role of BBE in the control of alkaloid biosynthesis in opium poppy and related species, the regulation of other biosynthetic enzymes must participate in the complex control architecture of these pathways.

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The GenBank accession numbers for sequences reported in this paper are U59232 and U59233.

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