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Short communication

Transformation of opium poppy (*Papaver somniferum* L.) with antisense berberine bridge enzyme gene (*anti-bbe*) via somatic embryogenesis results in an altered ratio of alkaloids in latex but not in roots

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Abstract

The berberine bridge enzyme cDNA *bbe* from *Papaver somniferum* L. was transformed in antisense orientation into seedling explants of the industrial elite line C048-6-14-64. In this way, 84 phenotypically normal T_0 plants derived from embryogenic callus cultures were produced. The selfed progeny of these 84 plants yielded several T_1 plants with an altered alkaloid profile. One of these plants T_1-47, and its siblings T_2-1.2 and T_2-1.5 are the subject of the present work. The transformation of these plants was evaluated by PCR, and northern and Southern hybridisation. The transgenic plants contained one additional copy of the transgene. The alkaloid content in latex and roots was determined with HPLC and LC-MS. We observed an increased concentration of several pathway intermediates from all biosynthetic branches, e.g., reticuline, laudanine, laudanosine, dehydroreticuline, salutaridine and (*S*)-scoulerine. The transformation altered the ratio of morphinan and tetrahydrobenzylisoquinoline alkaloids in latex but not the benzophenanthridine alkaloids in roots. The altered alkaloid profile is heritable at least to the T_2 generation. These results are the first example of metabolic engineering of the alkaloid pathways in opium poppy and, to our knowledge, the first time that an alkaloid biosynthetic gene has been transformed into the native species, followed by regeneration into a mature plant to enable analyses of the effect of the transgene on metabolism over several generations.

Introduction

Opium poppy contains more than eighty tetrahydrobenzylisoquinoline-derived alkaloids. These include the pharmaceutically important analgesic and narcotic drug morphine, the cough suppressant codeine, as well as the muscle relaxant papaverine, the antitumoric agent noscapine and the antimicrobial sanguinarine. Several genes from the biosynthetic pathways for reticuline, sanguinarine and morphine have been cloned (Kutchan et al., in press; ref. cited therein), including berberine bridge enzyme (BBE) (Dittrich & Kutchan, 1991; Facchini et al., 1996; Huang & Kutchan, 2000). Although morphine

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biosynthesis is well understood at the enzymatic level (for a review see Kutchan, 1998), the regulation and ecological function of the morphinan alkaloids *in planta* is still unknown. With a genetic transformation it would be possible to alter the alkaloid metabolism in commercial poppy cultivars in order to obtain varieties lacking alkaloids or to produce plants with tailored alkaloid profiles for industrial and pharmaceutical use.

In the present work, we transformed seedling explants of *P. somniferum* with antisense *bbe* cDNA. The aim of the experiment was to determine whether the transformation reduced or blocked benzophenanthridine alkaloid biosynthesis in roots of transgenic plants. Moreover, we are interested as to whether the transgene influences other pathway branches in opium poppy.

Materials, results and discussion

Construction of binary vector, transformation, selection and regeneration of transgenic plants

Hypocotyls from 8-day-old seedlings of the industrial elite line C048-6-14-64 (Tasmanian Alkaloids Pty Ltd, Westbury) were transformed with the binary vector *anti-bbe* pPLEX X002, named pPOP19. The blunt-ended full-length *P. somniferum* L. *bbe* coding region (Huang & Kutchan, 2000) was ligated into the *SnaB*I digested vector pPLEX X002.

pPLEX X002 was constructed at CSIRO Plant Industry, Canberra, by ligating the *Not*I cassette from the vector pPLEX 3002 (Schünmann et al., 2003) into the vector pBS435. The expression cassette of pPLEX X002 contains a subterranean clover stunt virus (SCSV) double promoter element (S4S4), followed by a multiple cloning site and the Me1′ terminator. The selection cassette harbours the *npt*II (neomycin phosphotransferase) gene driven by the cauliflower mosaic virus 35S promoter (Allen and Larkin, unpublished).

The direction of the inserted *bbe* gene was confirmed by restriction enzyme digest prior to nucleotide sequence determination with ABI PRISM^{TM} BigDye Terminator Cycle Sequencing according to the manufacturer's instructions (Primer sequences: 5′-TGA ACG TTT GGT TAA GGC-3′ and 5′-TGC ATG GAT GCA TGC AGC-3′). pPOP19 was transformed by electroporation in the disarmed *Agrobacterium tumefaciens* strain AGL1 (Lazo et al., 1991). Seed sterilisation, inoculation with *Agrobacterium*, cocultivation, selection of paromomycin-resistant calli and regeneration of transgenic plants were made following an optimised protocol (Chitty et al., 2003). In this way, 84 phenotypically normal T_0 plants were produced. The selfed progeny of these 84 plants yielded several plants with an altered alkaloid profile. One of these plants T_1-47, and its siblings T_2-1.2 and T_2-1.5 are the subject of the present work.

Analysis of transformation

Stable transformation of *P. somniferum* plants was evaluated by determining: (1) the integration of *bbe* and *npt*II into the plant genome by PCR; (2) the presence of *bbe* and *npt*II mRNAs by northern blotting; (3) the copy numbers of *bbe* and *npt*II genes with Southern blots; and (4) the concentration of morphinan-, tetrahydrobenzylisoquinoline- and benzophenanthridine alkaloids in latex and roots in comparison to wild type plants.

Plant genomic DNA and total RNA were extracted from leaves according to standard protocols (Maniatis et al., 1982). PCR was performed under the following conditions: a) for *npt*II: Primer sequences, 5′-GAG GCT ATT CGG CTA TGA CTG-3′ and 5′-ATC GGG AGC GGC GAT ACC GTA-3′ (Bonhomme et al., 2000); cycle 94 °C, 3 min, 35 cycles of 94 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min, cycle 72 °C, 5 min; (b) for S4S4 promoter and Me1′ terminator: Primer sequences, 5′-TAA GCG TAC TCA GTA CGC TTC-3′ and 5′-GCA TTA CAA CAT GCA TCT GAC-3′; cycle 94 °C, 3 min, 35 cycles of 94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min, cycle 72 °C, 5 min.

PCR performed with S4S4 and Me1′ primers resulted in the amplification of a single band with the expected size of 1870 bp. With primers specific for the *npt*II gene, a single product of 892 bp was amplified. These two bands could be detected in all regenerated paromomycin- resistant plants and their siblings (data not shown). No amplicons were obtained using genomic DNA isolated from wild type plants. Nucleotide sequence determination of the cloned PCR

product confirmed the direction and sequence of the inserted *bbe* gene.

The transgenic plants were next analysed by northern and Southern blot hybridisation. 10 μg of total RNA was fractionated on formaldehyde agarose gel. 10 μg genomic DNA were digested with *Sal*I and separated by agarose gel electrophoresis. Blotting onto nylon membranes (Biodyne B Membrane 0.45 μm, Pall) was performed as described by the manufacturer. The blots were hybridised with ^{32}P-labelled full-length *bbe* probes or with a ^{32}P-labelled *npt*II fragment, which were labelled with a Megaprime DNA Labelling System (Amersham) according to the manufacturer's instructions. Hybridisation was performed at 60°C (DNA) or 68°C (RNA).

Northern blot analysis (Figure 1(A)–(B)) showed a weak signal of *bbe* transcripts in wild type plants (Figure 1(A), lane 1). This signal represents the transcription of the endogenous *bbe* gene in opium poppy. In contrast, the additional expression of the transgene in the plant T_1-47 (Figure 1(A), lane 2) leads to a stronger signal in comparison to wild type plants. The labelling of the blot with a *npt*II probe (Figure 1(B)) confirmed the transformation. The expression of *npt*II was observed in transgenic (Figure 1(B), lane 2) but not in wild type plants (Figure 1(B), lane 1). An ethidium bromide-stained gel prior to blotting demonstrated equal loading of RNA samples (Figure 1(C))).

Southern blot analysis (Figure 1(D)–(E)) revealed a hybridisation pattern that is in agreement with one additional copy of *bbe* in the opium poppy genome. *Sal*I recognises one hydrolysis site within the *bbe* open reading frame yielding a hybridising band with an expected size of 3172 bp. This fragment could be observed in plant T_1-47 (Figure 1(D), lane 2), but not in the genomic DNA of wild type plants (Figure 1(D), lane 1). Additionally, a hybridising band width of about 1100 bp could be observed in all plants examined (Figure 1(D)). By digesting the *bbe* open reading frame with *Sal*I, two fragments of 474 and 1134 bp are expected from the endogenous *bbe* gene in opium poppy. The labelled band could represent the 1134 bp band observed. In the genome of plant T_1-47, one copy of the *npt*II resistance gene is present (Figure 1(E), lane 2), which could not be detected in wild type plants (Figure 1(E), lane 1).

Transformation of opium poppy with antisense bbe leads to an altered alkaloid profile in latex but not in roots

The concentration of morphinan-, tetrahydrobenzylisoquinoline- and benzophenanthridine-alkaloids in latex and roots were analysed by HPLC and LC-MS. The harvest of latex, the HPLC analysis and the LC-MS analysis of alkaloids of latex were described recently (Frick et al., in press).

After ripening of the capsule, the root weight was determined. Root samples were homogenised with ethanol containing dihydrocodeine as internal standard. After extraction with ethyl acetate, the concentrated samples were dissolved in 70% ethanol and analysed by HPLC with a modified gradient as follows: 0–25 min 0–60% acetonitrile, 25–26 min 100% acetonitrile, 26–33 min 100% acetonitrile and UV detection at 282 nm. Retention times and $(M+H)^+$ ions were as follows: morphine (7.8 min; m/z 286.1); dihydrocodeine (10.7 min; m/z 302.1); codeine (11.0 min; m/z 300.1); oripavine (12.4 min; m/z 298.1); reticuline (14.1 min; m/z 330.1); scoulerine, 1,2-dehydroreticuline, salutaridine (14.8 min; m/z 328.1); laudanine (15.7 min; m/z 344.1); thebaine (17.0 min; m/z 312.1) laudanosine (17.5 min; m/z 358.1). The molecular ion peak at m/z 328.1 characterised the MS-spectra of the alkaloids salutaridine, 1,2-dehydroreticuline and scoulerine. To distinguish between these three compounds selected reaction monitoring (SRM) mode was applied (Niessen, 1999). CIDMS were used both for identification and quantification. Raith et al. (2003) described full scan CIDMS data of the alkaloids.

In wild type latex (Figure 1(F), and 2(A)), the four major alkaloids were morphine, codeine, thebaine and oripavine. Other biosynthetic intermediates, such as reticuline, scoulerine, dehydroreticuline, salutaridine, laudanine and laudanosine, were present in small amounts only (Figure 1(F), and 2(A)). In root extracts, the major alkaloids in wild type plants were sanguinarine/10-hydroxysanguinarine and dihydrosanguinarine/10-hydroxydihydrosanguinarine (Figure 2(B)). The hydroxylated compounds could not be separated from their reduced counterparts under the HPLC conditions used. All other alkaloids detected in the latex could also be observed in traces in roots (Figure 2(C)). In Figure 2(D),

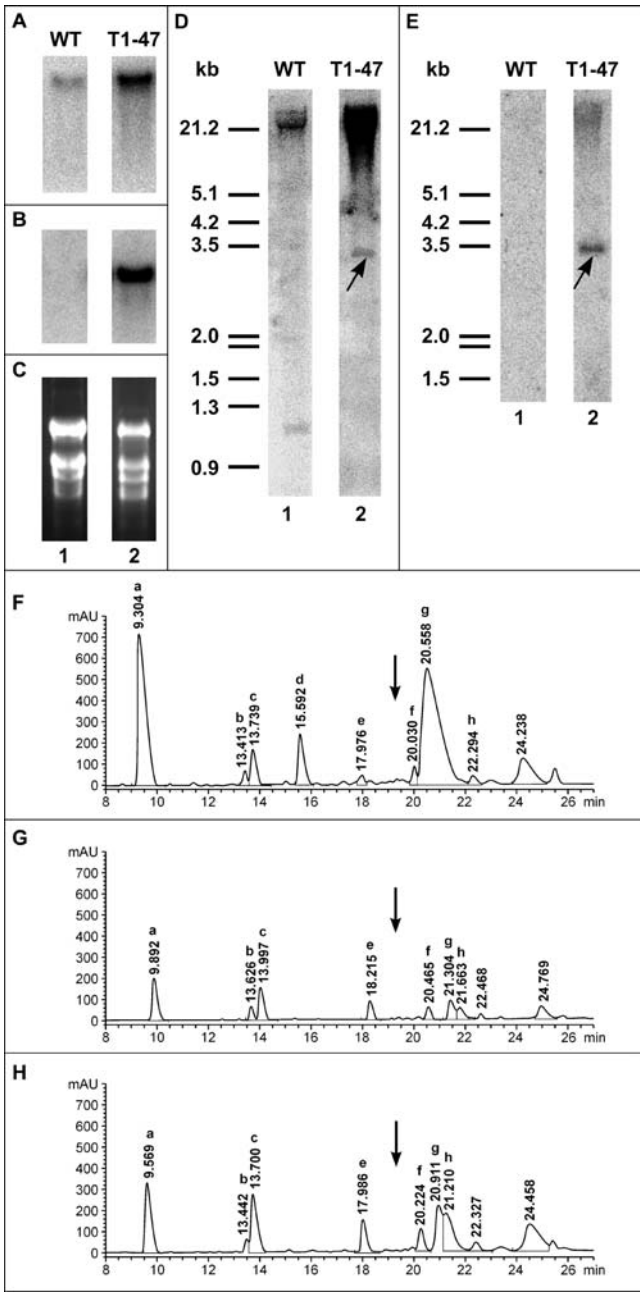

Figure 1. Northern and Southern blot hybridisation and HPLC analysis of latex from *Papaver somniferum* wild type (WT) and transgenic plants transformed with S4S4:*anti-bbe*. T_1 plant T_1-47 is the parental plant of T_2 plants T_2-1.2 and T_2-1.5. (A–C) Northern blot analysis of the expression of *bbe* and *npt*II genes. 10 μg total RNA (A) from mature leaves was fractionated on a 1.2% formaldehyde agarose gel and hybridised with a double stranded ^{32}P-labelled *bbe* full-length probe or (B) a ^{32}P-labelled *npt*II probe. (C) Ethidium bromide-stained gel prior to blotting shows equal loading of RNA samples. (D–E) Southern blot analysis of the copy number of the *bbe* and *npt*II genes. 10 μg plant genomic DNA was extracted from mature leaves, digested with *Sal*I, resolved on a 0.8% agarose gel and hybridised to (D) the double stranded ^{32}P-labelled *bbe* full-length probe or (E) the ^{32}P-labelled *npt* II probe. (F–H) HPLC elution profiles of latex extracts from (F) wildtype, (G) T_1 plant T_1-47 and (H) T_2 plant T_2-1.2. Peaks correspond to: a, morphine; b, dihydrocodeine (internal standard); c, codeine; d, oripavine; e, reticuline; f, laudanine; g, thebaine; h, laudanosine. Arrow marks 1,2-dehydroreticuline, salutaridine, and scoulerine at retention time 19.3 min.

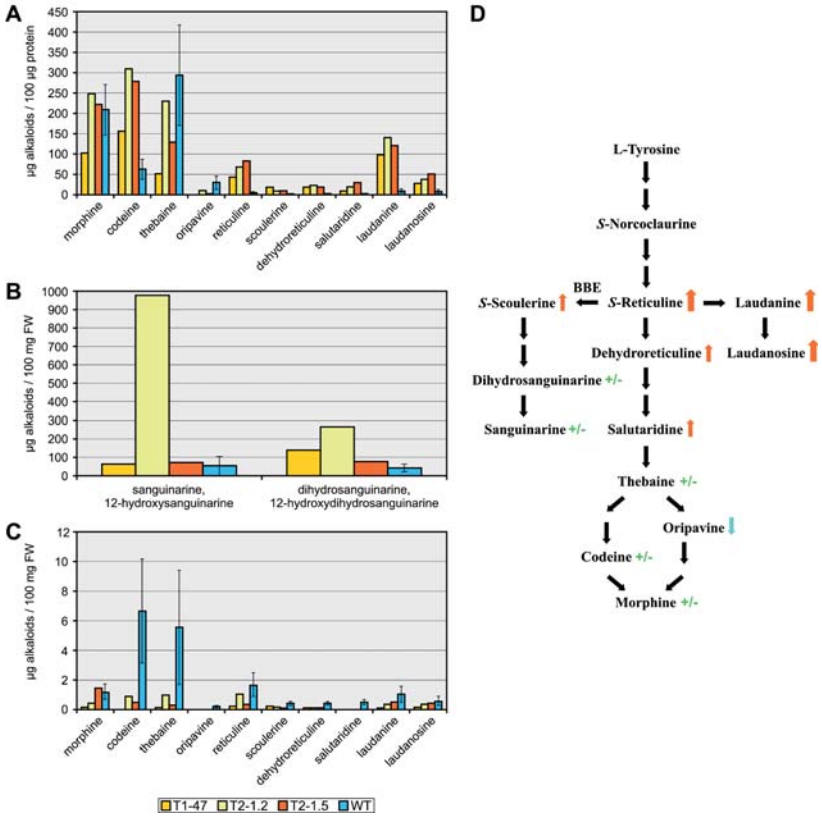

Figure 2. Determination of (A) benzylisoquinoline alkaloids in latex, (B) benzophenanthridine alkaloids in roots and (C) benzylisoquinoline alkaloids in roots. Alkaloid concentrations in *Papaver somniferum* wild type (WT), transgenic T_1 plants T_1-47 and transgenic T_2 plants T_2-1.2 and T_2-1.5. T_1 plant T_1-47 is the parental plant of T_2 plants T_2-1.2 and T_2-1.5. The data are presented as μg alkaloid/100 μg of soluble protein in latex or μg alkaloid / 100 mg root fresh weight. Panels (A) and (C) represent the average alkaloid content of 29 wild type plants and panel (B) shows the average benzophenanthridine content measured in 26 wild type plant roots with the corresponding standard deviations. Alkaloid concentrations of the transgenic plants are compared to those of wild type. (D) Schematic biosynthetic pathway leading from L-tyrosine to sanguinarine, laudanosine and morphine in opium poppy. Selected intermediates are shown; the position of BBE is indicated. Double arrows indicate two or multiple enzyme steps. +/−: concentration not changed, ↑: concentration increased, ↓: concentration decreased.

the most important intermediates and end products of all three biosynthetic branches present in opium poppy, as well as the site of action of the BBE, is shown. The alkaloid analysis of three transgenic plants T_1-47, T_2-1.2, T_2-1.5 is presented in Figure 1(G)–(H) and Figure 2(A)–(C). T_1-47 is the parental plant of T_2-1.2 and T_2-1.5. The major alkaloids in latex of these three transgenic plants (Figure 1(G)–(H), Figure 2(A)) were also morphine, codeine, and with the exception of plant T_1-47, thebaine. Oripavine in comparison to wild type plants was drastically reduced, or as in plant T_1-47, undetectable. The other biosynthetic intermediates were abundant in latex in significantly higher concentrations compared to wild type plants. The highest concentrations were displayed by laudanine, reticuline, and laudanosine, followed by salutaridine, dehydroreticuline and scoulerine (Figure 2(A)). The major alkaloids in roots were sanguinarine/10-hydroxysanguinarine and dehydrosanguinarine/10-hydroxydehydrosanguinarine (Figure 2B). Interestingly, the transgenic plants showed no reduction in the concentration of benzophenanthridine alkaloids in roots. Other alkaloids detectable in the roots were morphine, codeine, thebaine, reticuline, scoulerine, dehydro-

reticuline, laudanine, and laudanosine. Oripavine and salutaridine were not detectable in the roots of any of the three transgenic plants (Figure 2(C)). The results of the alkaloid determination are summarised schematically in Figure 2(D).

The transformation of opium poppy hypocotyls with S4S4:*anti-bbe* demonstrated herein is the first attempt to metabolically engineer whole plants of *P. somniferum*. Other attempts at metabolic engineering of isoquinoline alkaloids pathways have been published recently. California poppy (*Eschscholzia californica* Cham.) cell suspension and root cultures have been transformed with an antisense *bbe* construct (Park et al., 2002, 2003). In both cases, the authors selected transgenic cell lines that expressed antisense *bbe*, displayed low levels of *bbe* mRNA and showed a reduced accumulation of benzophenanthridine alkaloids. Pathway intermediates were unfortunately not detected (Park et al., 2002, 2003).

Morphine biosynthesis occurs in the aerial parts of the plant as well as in the roots. The localisation of the enzymes leading specifically to the benzophenanthridine alkaloids is not yet known. Transcript *bbe*, involved in the first step of the biosynthesis to the benzophenanthridines, has been found in root, stem and leaf (Huang & Kutchan, 2000). The detection of *bbe* transcripts in some aerial organs of poppy (Huang & Kutchan, 2000; Facchini & Park, 2003) was unexpected, since sanguinarine was reported to accumulate only in roots (Facchini & De Luca, 1995). The possible root-specific expression of genes encoding enzymes downstream of BBE could explain the lack of sanguinarine in the aerial parts of opium poppy. Furthermore, in developing seedlings, sanguinarine starts to increase five days after germination, although *bbe* mRNAs could be observed earlier in development (Facchini et al., 1996; Huang & Kutchan, 2000). These data suggest the role of enzymes other than BBE in the control of sanguinarine biosynthesis in opium poppy. The enzymes downstream of BBE could be restricted to the roots. If enzymes downstream of BBE were involved in the regulation of sanguinarine biosynthesis in opium poppy root, this would be a possible explanation for the unchanged sanguinarine levels in antisense *bbe* transgenic plants. The fact that two related plant species may display different regulation in alkaloid metabolism has been shown for *Datura metel* and *Hyoscyamus muticus* (Moyano et al., 2003).

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