Differential Gene Expression in *Papaver*-Species in Comparison with Alkaloid Profiles

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Abstract

Papaver species are known to produce a large variety of benzylisoquinoline alkaloids, with each species exhibiting a specific alkaloid profile, but only a few genes involved in the biosynthesis or regulation of these complex pathways are known so far. Here we used a genomic approach to discover genes responsible for the determination of a specific alkaloid profile. A stem expressed sequence tag database of ~1100 unique genes from *Papaver somniferum* was created. Gene expression analysis of these sequences in *P. bracteatum, P. somniferum* and *P. somniferum* 'Noscapine' exhibited 39 cDNAs showing differential expression coincident with morphine accumulation.

INTRODUCTION

The tetrahydrobenzylisoquinoline class of alkaloids contains many physiologically active compounds, such as the narcotic analgesic, morphine, the antitussive, codeine, and noscapine, or the vasodilator papaverine. The tetrahydrobenzylisoquinolines can be divided into several classes such as pavines (isopavines, benzophenanthridines, rhoeadines) papaverrubines, protopines, phthalideisoquinolines, protoberberines, aporphines and morphinans (Preininger, 1986). Common to all these classes are the first steps in the biosynthesis, which includes the condensation of two tyrosine derived molecules to norcoclaurine and subsequent *N*- and *O*-methylations and hydroxylation steps leading to (*S*)-reticuline (Kutchan et al., 1998). This central intermediate enters different pathways for the generation of the individual tetrahydrobenzylisoquinoline classes. In contrast to the biosynthetic enzymes leading to (*S*)-reticuline, little is known about the following steps on the enzymatic and molecular level, but from the individual reactions one can assume that *O*-methyltransferases and P450-monooxygenases predominate. Similarly, knowledge about the regulation of the whole pathway and transport processes of intermediates is scarce.

With a few exceptions, tetrahydrobenzylisoquinolines occur in the Ranunculales, specifically in the family of the Papaveraceae (Jensen, 1995). Among them, only species belonging to the genus *Papaver* are known to contain all classes of tetrahydrobenzylisoquinoline alkaloids, whereby strong variations in the alkaloid profiles are species specific (Waterman, 1998). We use the close genetic relationship but the diversity in the alkaloid profiles of *Papaver* species to correlate the gene expression with the alkaloid profiles in order to access cDNAs responsible for a distinct alkaloid profile.

MATERIALS AND METHODS

Plants were either grown in the field or in the greenhouse as indicated. Upper stems and latex of *Papaver* plants were harvested 4-6 days after petal fall.

For alkaloid analysis, stems and latex were extracted with 80% ethanol and analysed by HPLC at 210 nm on LiChrospher 60 RP-select B columns (5 µm, 250 mm x 4 mm, Merck Eurolab, Darmstadt, Germany) using an acetonitrile gradient from 2% to 46% in 25 min, 46% to 60% in 5 min, constant 60% for 5 min and finally an increase up to 100% in 5 min in 0.01% phosphoric acid.

RNA was extracted according to standard procedures (Sambrook et al., 1989) and the mRNA purified using an Oligotex mRNA purification kit (Qiagen, Hilden, Germany). The mRNA from stems of field grown *P. somniferum* plants was used to construct a directional SuperScript II RT cDNA library cloned in plasmid pZL1 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The plasmid preparations of randomly chosen individual clones were sequenced from the 5`-end using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit and run on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). Database searches were performed using the blastX algorithm (Altschul et al., 1997) on the NCBI server (http://www.ncbi.nlm.nih.gov/).

cDNAs chosen for arraying were PCR amplified using vector specific primers. The amplicons were purified using NucleoFast 96 PCR plates (Macherey-Nagel, Düren, Germany), adjusted to a concentration of 100 to 400 ng μl^{-1} and spotted in duplicates on Biodyne B transfer membrane (PALL, Portsmouth, England) using a *Micro*Grid II (*Bio*Robotics Ltd). The arrayed PCR amplicons were denatured by incubating the membrane on 0.5 N NaOH/1.5 M NaCl for 5 min, followed by neutralization with 1 M Tris-Cl pH 7.5/1.5 M NaCl and finally immobilized by UV crosslinking at 120 mjoules cm^{-2} using a Stratalinker 1800 (Stratagene, Amsterdam, The Netherlands). Membranes were prehybridized in 5x SSC, 5 x Dehnhardt solution, 0.1 % (w/v) SDS, 125 µg ml^{-1} denatured salmon sperm DNA for 3 hrs at 65°C, and hybridised overnight with labelled cDNA in the prehybridisation solution at 65°C. Filters were washed three times with 2 x SSC, 0,1% SDS for 15 min each at 65°C. Data were acquired with a Storm 860 Imaging System (Amersham Phamarcia Biotech, Freiburg, Germany) after the membranes were exposed to Storage Phosphor Screens. Data analyses was performed using the AIDA Image Analyzer Software (Raytest, Straubenhardt, Germany).

About 40 µg of total RNA was reversed transcribed using M-MLV reverse transcriptase RNase H minus (Promega, Mannheim, Germany) and the resulting cDNA labelled with [alpha-^{33}P]dATP using the Megaprime DNA Labelling Kit as described by the manufacturer (Amersham Pharmacia Biotech, Freiburg, Germany).

RESULTS AND DISCUSSION

Expressed Sequence Tag Sequencing

As a first step toward gene expression analysis, an expressed sequence tag (EST) project was initiated. Since *P. somniferum* is known to produce most of the tetrahydrobenzylisoquinoline alkaloids and is the only plant containing large amounts of morphine, on which compound our interest is mainly focussed, we decided to perform the EST project with this species. The stem-sections 2-4 cm below the capsule has been shown to possess high biosynthetic activity for alkaloids (Unterlinner et al., 1999; Huang and Kutchan, 2000; Grothe et al., 2001) and were therefore taken as a source for the RNA to construct a stem specific cDNA library. Sequencing of about 2000 randomly chosen cDNA-clones yielded more than 1000 unique sequences. More than half of the sequences showed homology to proteins with known function (Fig. 1). The largest groups of genes are represented by proteins involved in cell cycle, gene regulation, protein synthesis and turnover, as well as stress response proteins and proteins responsible for redox control. Another group of genes highly represented in our database codes for proteins participating in metabolism, mainly primary metabolism. To twenty sequences, a role in secondary metabolism could be ascribed, such as flavanol 3-hydroxylase and caffeic acid 3-*O*-methyltransferase of the phenylpropanoid pathway, farnesyldiphosphate synthase of the isoprenoid pathway or tyrosine/DOPA decarboxylase and codeinone reductase of alkaloid biosynthesis. The high percentage (40%) of sequences either coding for predicted proteins with unknown function or having no homologs in the databases suggests that *P. somniferum* stems might be an intriguing source for novel genes.

Alkaloid Profiles

The selection of plants used for gene expression analysis is based on their alkaloid profile and their phylogenetic relationship. Therefore, we examined the latex or stems of different *Papaver* species and recorded the major tetrahydrobenzylisoquinolines by HPLC. *P. somniferum* grown in the field produced a large amount of morphine, followed by a less strong but still high accumulation of codeine and thebaine, the biosynthetic precursors of morphine (Fig. 2). Greenhouse plants show the same alkaloid profile with a decreased proportion of morphine. This profile changes also qualitatively when a mutated plant, *P. somniferum* 'Noscapine', is investigated. This plant accumulates large amounts of noscapine, which belongs to the phthalideisoquinolines, but still shows substantial amounts of the three morphinan alkaloids morphine, codeine and thebaine. On the other hand, *P. bracteatum* produces large amounts of thebaine, but neither codeine nor morphine could be detected. This indicates that this species is impaired in the biosynthesis of morphine downstream of thebaine.

Gene Expression Analysis

For the analysis of gene expression in different *Papaver* species, we started with the comparison of the above mentioned plants, since *P. bracteatum* seems to lack only the three last steps to the production of morphine. Furthermore, the comparison of field grown and greenhouse *P. somniferum* should help us to exclude gene expression differences based on different growth conditions. As an initial experiment, we spotted a macroarray with 1152 PCR fragments derived from the EST sequences. As source for the RNA used as the probe for hybridisation, we used 1-3 cm long stems cut about 1 cm below the capsule 2-4 days after petal fall. Based on a two-fold difference in expression as the threshold, 117 differentially expressed cDNA fragments were detected in the comparison of *P. bracteatum* and *P. somniferum*, both grown in the field. A similar comparison but using *P. somniferum* grown in the greenhouse yielded 153 differentially expressed cDNA fragments. The number of cDNA fragments could be reduced to 77 under the assumption that cDNA fragments possibly responsible for morphine biosynthesis should show differential expression in both experiments (Fig. 3). The gene expression analysis of *P. bracteatum* compared to *P. somniferum* 'Noscapine' yielded 170 differentially expressed cDNA fragments. The combinations of all three datasets yielded 44 cDNA fragments. Of these, five showed differential expression between field grown and greenhouse grown *P. somniferum* plants. Since both plants contain high levels of morphine, one can assume that these fragments do not contribute to morphine biosynthesis. Of the remaining 39 cDNA fragments, 27 are more highly expressed in morphine containing plants. Among all 39 sequences, 25 showed no homology or homology to proteins with unknown function. Based on sequence homology to database entries, the other cDNAs code for proteins with diverse functions. Some play a role in primary and secondary metabolism such as catechol *O*-methyltransferase and *S*-adenosylhomocysteine hydrolase or could be involved in gene regulation such as a G-binding protein or RING zinc finger protein (Table 1). These genes might represent candidates for species specific differences in metabolite profiles. Others, such as ribosomal proteins or the protein psbW from photosystem II are most likely the result of different species specific expression levels of housekeeping genes.

In order to further reduce the number of cDNAs possibly involved in morphine biosynthesis, more gene expression studies compared to alkaloid profiles in several *Papaver* species will be performed. This procedure should enable us to decrease the number of cDNAs such that a functional characterisation of clones with unknown function is becomes feasible.

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Tables

Table 1. Differences in expression of selected genes between *P. bracteatum* and the plants indicated.

P. somniferum field grown (-fold expression)	*P. somniferum* greenhouse (-fold expression)	*P. somniferum* Noscapine (-fold expression)	
11.6	11.5	5.5	catechol *O*-methyltransferase
3.3	3.1	2.5	mutT domain like
3.2	2.7	2.3	stress induced cysteine proteinase
3.0	3.0	2.4	dTDP-glucose 4-6-dehydratase
2.9	3.9	2.8	triosephosphate isomerase
2.5	2.6	2.4	glutamine synthetase
3.1	2.3	2.8	S-adenosylhomocysteine hydrolase
-2.8	-3.3	-2.2	G-box-binding protein
-3.5	-2.8	-2.6	RING zinc finger protein
-4.5	-3.3	-2.4	40S ribosomal protein S6
-4.9	-4.2	-2.9	60S ribosomal protein L144
-4.9	-3.4	-3.1	ferredoxin-$NADP^+$ reductase
-8.9	-7.0	-5.3	PSII protein psbW
-16.0	-8.3	-8.9	metallothionein

Figures

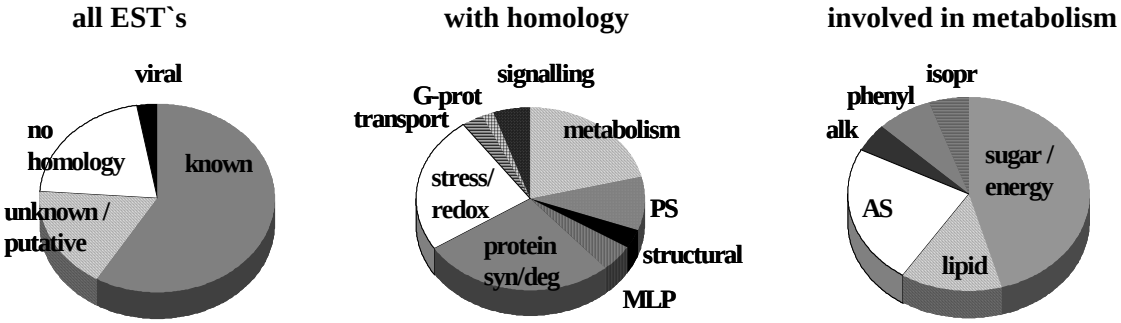

Fig. 1. Functional classification of *P. somniferum* stem ESTs. Abbreviations: PS: photosynthesis, MLP: major latex proteins, protein syn/deg: protein synthesis and degradation, G-prot: G-proteins, AS: amino acids, alk: alkaloids, phenyl: phenylpropanoids, isopr: isoprenoids.

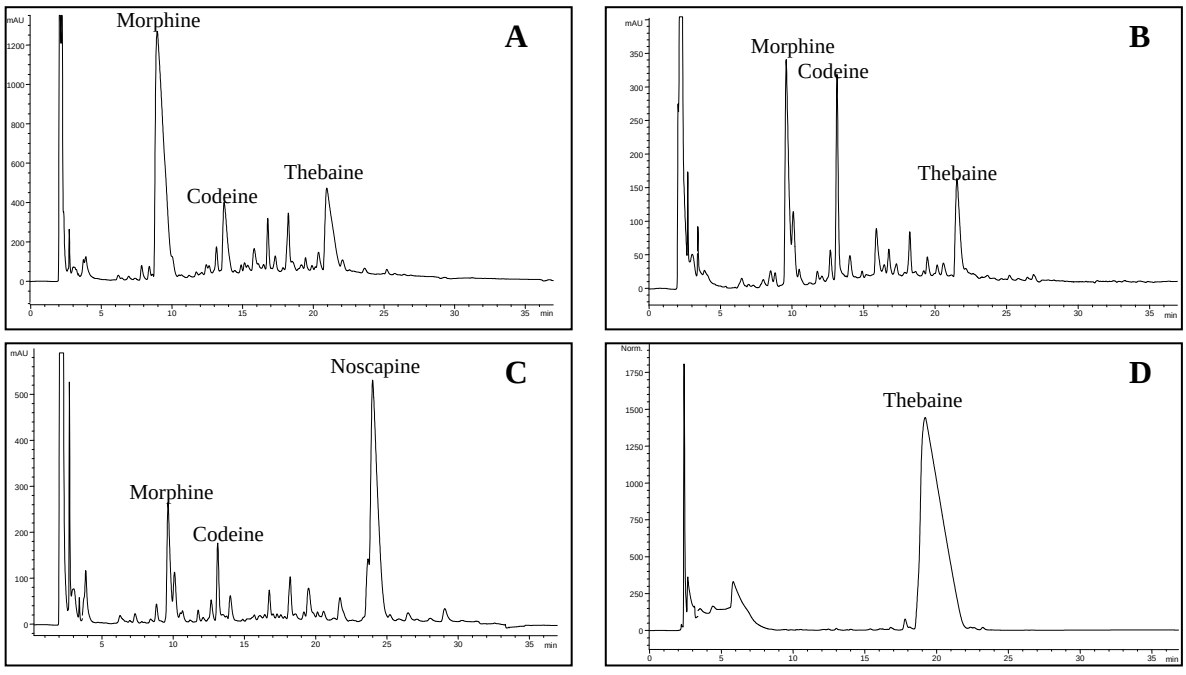

Fig. 2. HPLC chromatograms of latex samples from A: *P. somniferum* grown in the field, B: *P. somniferum* grown in the greenhouse, C: *P. somniferum* Noscapine, D: *P. bracteatum* grown in the field.

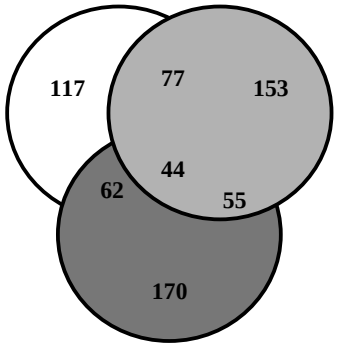

Fig. 3. Interloping diagram of the number of genes differentially expressed between *P. bracteatum* and *P. somniferum* grown in the field (white circle), *P. bracteatum* and *P. somniferum* grown in the greenhouse (light grey circle) and *P. bracteatum* and *P. somniferum* Noscapine (dark grey circle). The number in the overlapping areas indicates the number of genes that show differential expression in the combination of the respective comparisons.