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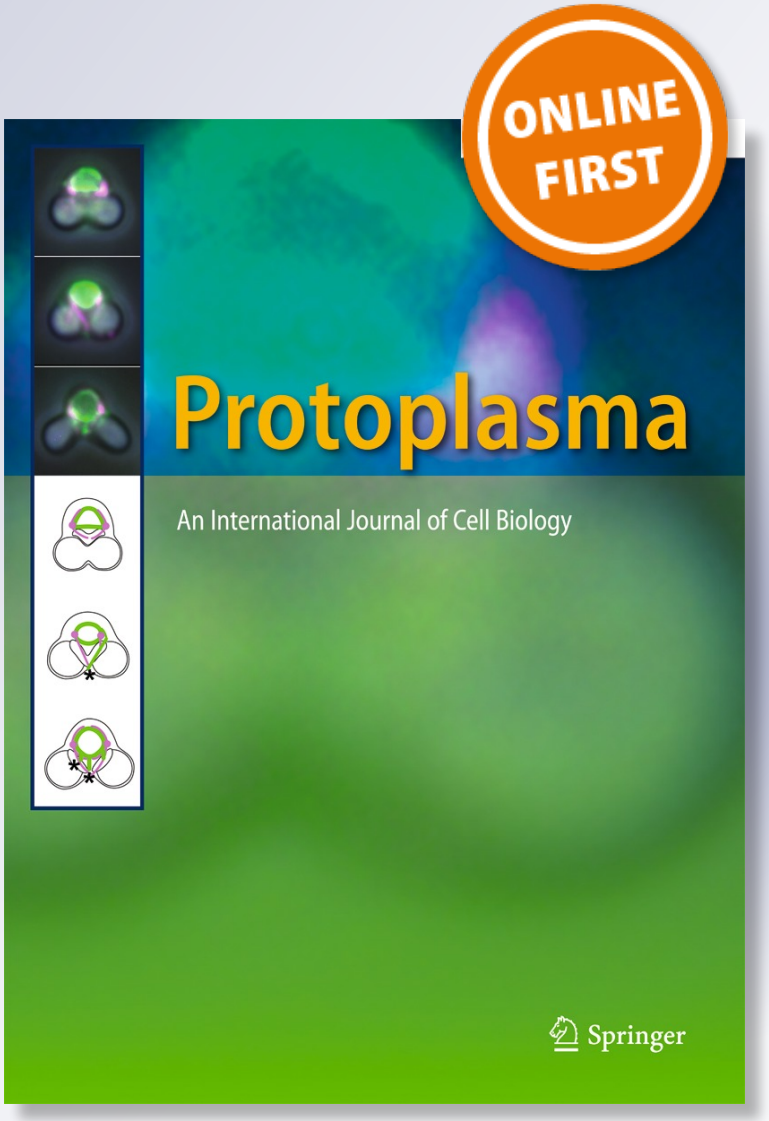


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ORIGINAL ARTICLE

# Comparative analysis of *Papaver somniferum* genotypes having contrasting latex and alkaloid profiles

**Nidarshana Chaturvedi · Mridula Singh · Ashutosh K. Shukla · Ajit K. Shasany · Karuna Shanker · Raj K. Lal · Suman P. S. Khanuja**

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**Abstract** *Papaver somniferum* produces therapeutically useful benzylisoquinoline alkaloids (BIAs) like papaverine, thebaine, codeine, and morphine that accumulate in its capsular latex. Morphine is a potent analgesic but is also abused as a narcotic, which has increased the demand for non-narcotic thebaine that can be converted into various analgesics. To curtail the narcotic menace, many distinct genotypes of the plant have been developed that are deficient in morphine and/or latex. *Sujata* is one such latex-less low alkaloid-producing variety developed from the alkaloid-rich gum harvest variety *Sampada*. Its utility for gene prospecting and studying differential gene regulation responsible for its low alkaloid, nutritive seed oil, and latex-less phenotype has been exploited in this study. BIA profiling of *Sujata* and *Sampada* capsules at the early and late stages indicated that except for thebaine, *Sujata* had a depressed alkaloid phenotype as compared to *Sampada*. Comparative transcript-based analysis of the two genotypes was carried out in the early stage capsule (higher thebaine) using subtractive hybridization and microarray. Interrogation of a *P. somniferum* array yielded many differentially expressing transcripts. Their homology-based annotation classified them into categories—latex related, oil/lipid related, alkaloid related, cell wall related, and others. These leads will be useful to characterize the highly sought after *Sujata* phenotype.

**Keywords** Genotype · Latex · Microarray · Morphine · Subtractive hybridization · Thebaine

## Introduction

*Papaver somniferum* (Papaveraceae) is well known for its economically important therapeutic alkaloids—papaverine, thebaine, codeine, and morphine. Papaverine has use as a vasodilator, thebaine is the starting source for medically useful derivatives including the analgesics oxycodone and buprenorphine, codeine is an antitussive, and morphine is a potent analgesic. Chemical synthesis of most of these alkaloids is possible, but not commercially viable and the alternative of bioreactor production is not possible in many cases, which leaves the plant as the most important source of the medicinal alkaloids. In spite of its notoriety (narcotic nature and easy conversion into the more narcotic heroin), morphine has an evergreen demand as an analgesic. However, recent global trends indicate that it is likely that there will also be a major demand for thebaine-derived drugs.

Dried latex or gum (opium) from the poppy (*P. somniferum*) capsule is the major source of alkaloids. India is the only country where gum harvest (GH) varieties of *P. somniferum* are cultivated for producing licit opium whereas other poppy-growing countries (where opium collection is not prevalent) cultivate concentrate of poppy straw (CPS) varieties for direct extraction of alkaloids (mainly morphine) from the straw (capsule hulls) after the removal of seeds (Sharma et al. 2002a). The CPS cultivars also possess opium but they are not lanced for opium collection. Illicit cultivation of poppy (mainly GH varieties for latex collection) is a problem in some regions of the world and in order to curtail it, ideally there should be poppy genotypes that are either deficient in latex morphine (designer

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plants blocked to produce only morphine precursors) or latex-less varieties that would not produce latex upon lancing. Both these approaches have been attempted in the past. In thebaine oripavine poppy (*top1*) the morphinan alkaloid biosynthetic pathway is blocked at the precursors, thebaine and oripavine, whereby the plant latex is devoid of morphine and codeine (Fist et al. 2000; Millgate et al. 2004). On the other hand, the Indian poppy variety *Sujata* is latex-less, which provides a deterrent for lancing of the plant capsule (Sharma et al. 1999, 2002b, 2004).

*Sujata* genotype was developed through mutation breeding from the parental alkaloid-rich GH variety *Sampada* (initially known as *Mass-2B*) (Sharma et al. 2002a, b). It is difficult to differentiate *Sujata* and *Sampada* based on their morphological characters. However, the major difference between the two is evident only upon lancing their capsules, whereby *Sujata* is latex-less and *Sampada* oozes out latex profusely (Sharma et al. 2002b). The seeds of *Sujata* have high nutritive value and contain around 24 % protein and >58 % oil with a high proportion (>82 %) of unsaturated fatty acids, which makes it an attractive seed poppy for edible purposes (Sharma et al. 2002b). Although initially *Sujata* was considered to be alkaloid-free (based on thin-layer densitometry), later analyses (more sensitive HPLC in the present study) showed that it may not be entirely devoid of alkaloids. However, it does have a depressed alkaloid phenotype (except for thebaine) as compared to its parent, *Sampada*.

Although the molecular mechanisms underlying the phenotype of the altered plants of *Sujata* are not exactly known, it can be used as an excellent tool for gene prospecting and studying differential gene regulation responsible for the low alkaloid, nutritive seed oil, and latex-less phenotype. In the present study, a comparative analysis of the familial poppy genotypes *Sujata* and *Sampada* was carried out at the metabolite level in conjunction with related gene expression profiling. For gene expression profiling, a high-utility small scale (750 target genes) microarray chip of *P. somniferum* containing the significant alkaloid biosynthesis-related genes was designed and used, whereby the target genes were selected from over 20,000 *P. somniferum* expressed sequence tags (ESTs) in database of expressed sequence tags (dbEST) (National Center for Biotechnology Information [NCBI]). The microarray data was validated and many differentially expressing candidates were identified in both the genotypes that will provide leads for further research on the plant.

## Material and methods

### Plant material

Selfed seeds of *P. somniferum* genotypes, *Sampada* and *Sujata*, were obtained from the National Gene Bank for Medicinal and Aromatic Plants, Council of Scientific and Industrial Research–Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India. Alkaloid content was analyzed in early (75 day old) and late stage (115 day old) plants. Capsules of early stage plants were used for RNA isolation for microarray and subtractive hybridization analyses.

### RNA isolation

Total RNA was isolated from early stage capsules of the plants using the RNeasy Mini Kit (QIAGEN). RNA concentration and purity was evaluated by measuring absorbance at 260 and 280 nm using a NanoDrop (NanoDrop, USA). Its quality was evaluated by electrophoretic analysis with Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). Total RNA that met the quality standards was released for probe generation.

A previously described protocol, which yields high quality poly $(A)^+$ mRNA from medicinal and aromatic plants (Shukla et al. 2005), was used to isolate mRNA for preparing cDNA for subtractive hybridization.

### Microarray preparation and design

A small scale microarray chip (spotted oligonucleotides for 750 target genes) was designed for *P. somniferum*. The target genes were selected from the over 20,000 *P. somniferum* ESTs in dbEST (NCBI), including the in-house generated 300 ESTs (GR979624-GR979913, GT735891-GT735900) of *Sampada* genotype and the known genes of *P. somniferum* in GenBank (NCBI).

The in-house-generated ESTs (capsule and leaf combined) were clustered using CAP3 into unique sequences (contigs and singletons) and annotated using BLAST and two RefSeq-derived databases—*Arabidopsis* and non-*Arabidopsis* flowering plants. Care was taken to avoid inclusion of any sequence showing homology to ribosomal genes. The other *P. somniferum* EST sequences from the dbEST were retrieved from NCBI and clustered using TIGR gene indices clustering tools (Pertea et al. 2003). A TBLASTx process was used to annotate the created cluster set and the remaining singleton EST sequences by comparing with the *Arabidopsis* RefSeq databases. The annotated sequences were then classified into various categories like decarboxylases, reductases, synthases, synthetases, regulators, transferases, transporters, transcription factors, transcription-related, etc. A CAP3-based clustering was done again to remove any further redundancy among the dbEST-derived sequences and the in-house derived ESTs. The known genes of *P. somniferum* were also taken up from the nucleotide database of GenBank (NCBI) [Out of the 45 sequences available (24 genes, some represented by more than one allelic form) probes could not be designed for two—*cor* 1-6

(AF108437) and *cor*1-5 (AF108436). The remaining 43 known genes were represented by the 36 oligo probes]. Further removal of redundancy was done using BLAST and a total of 750 target genes were shortlisted based on the basis of their possible/putative involvement in alkaloid biosynthesis/metabolism/diversion pathways/regulation. Bioinformatics-based feasibility study showed that oligonucleotide probes could be designed for the selected target 750 genes. Cross hybridization of the designed 50 mer oligos was checked. The custom *P. somniferum* array was prepared by Ocimum Biosolutions (Hyderabad, India). The Platform data for the *P. somniferum* custom array has been submitted to Gene Expression Omnibus (GEO) (NCBI) under accession number GPL14625.

## Probe generation, hybridization, and analysis

A dual channel procedure with dye-swap arrangement was adopted in the study on the two samples—*Sampada* and *Sujata*. The interest was to compare the expression levels of target genes in *Sujata* against *Sampada*. The expression data was generated on 1,569 probes, in which each probe was replicated twice.

Five micrograms of total RNA was used for amplification using Amino Allyl MessageAmp™ II aRNA Amplification Kit from Ambion by linear transcription based RNA amplification system to produce cRNA. Briefly, mRNA was reverse transcribed with an oligo (dT) primer bearing T7 promoter at 42 °C for 2 h and second-strand cDNA synthesis was carried out at 16 °C for 2 h. The resulting cDNA was then purified and was then transcribed with T7 RNA polymerase to generate multiple copies of aminoallyl antisense RNA (aRNA) at 37 °C for 16 h. aRNA was then labeled with Cy3™/Cy5™ post-labeling reactive dye pack (GE Healthcare, UK) at room temperature and unincorporated Cy3/Cy5 molecules were removed by purification process. Samples were purified using QIAGEN PCR purification kit before hybridization.

Ten micrograms of the labeled aRNA in 75 μl of Ocimum's Hyb buffer was used for hybridization with the *P. somniferum* custom array chip. Hybridized chips were scanned using Affymetrix 428™ Array Scanner at three different photomultiplier (PMT) gains (40, 50, 60) and the data was analyzed using Genowiz software (Ocimum Biosolutions, Hyderabad, India).

Image analysis was carried out using Imagene, version 5.6.1. The signal values obtained at the three PMT settings were averaged to get the signal mean for further analysis. Replicate genes were also averaged before normalization. Thus, obtained signal values from each channel were log2 transformed and normalized using LOWESS algorithm and median absolute deviation (MAD) scaling. For each sample, Cy3 and Cy5 intensities related to that sample were averaged, and were further used to compare the samples. During normalization, paired slide dye-swap method was followed to overcome the dye-bias during the comparison and then MAD was performed to adjust data into same scale (Yang et al. 2002). The normalized (adjusted) data was subsequently used in differential expression analysis, which was performed using the fold–change technique.

The data from this microarray experiment has been submitted to GEO (NCBI) under series accession number GSE37983 [associated sample data GSM931298 (*Sujata*) and GSM931299 (*Sampada*)] (The OBSca1001_ prefix in the gene/probe IDs in the submitted data has been abbreviated here as Psom for convenience).

## Subtractive hybridization

The Clontech PCR-Select™ cDNA subtraction kit (Diatchenko et al. 1996) was used for suppression subtractive hybridization to identify genotype-specific/abundant transcripts in *P. somniferum*. All the steps were performed as per the manufacturer's guidelines. The primary and secondary PCRs to amplify the subtracted messages (PsSamTSujD = Sampada-tester Sujata-driver; PsSujTSamD = Sujata-tester Sampada-driver) were carried out for 30 and 17 cycles, respectively. The secondary PCR products were cloned on pGEM®-T Easy vector using T/A cloning. The ligation products were used to transform *E. coli* strain DH5α-competent cells and the recombinants were selected by blue/white screening. The inserts contained in the selected recombinant plasmids were subjected to end sequencing to establish ESTs. The edited sequences were analyzed as described previously (Shukla et al. 2006).

## RT-PCR analysis for validation

The gene expression profiles obtained in the microarray analysis were validated through semi-quantitative RT-PCR. The total RNA used was derived from the capsule wall of *Sujata* and *Sampada* plants using Trizol® reagent (Invitrogen) and treated with DNaseI. Exactly equal amount (~1.6 μg) of total RNA (as determined by ethidium bromide staining as well as spectrophotometrically through Nanodrop ND1000) isolated from both the samples to be compared was used for first-strand cDNA synthesis with oligo (dT) $_{20}$ primer in a 20 μl reaction volume using the ThermoScript™ RT-PCR System (Invitrogen) employing the manufacturer's protocol. For the PCR step, 1 μl of the cDNA synthesis reaction, 2.5 μl of 10X Taq polymerase buffer, 1 μl of 10 mM (each) dNTP mix, 10 pmol of forward primer, 10 pmol of reverse primer, 0.6 units of Taq DNA polymerase (3 U/μl) and sterile water (to make up the final volume to 25 μl) were added in a PCR tube. The thermal cycler was programmed as 94 °C for 5 min; 28 to 30 cycles of 94 °C for 1 min, × °C (depending on primer Tm) for 1 min, 72 °C for 2 min; 72 °C for 5 min; and 4 °C for

infinite period. The RT-PCR product was analyzed on a 1.2 % agarose gel. The *P. somniferum* actin gene was used as the housekeeping gene control. Primers for the poppy actin gene were based on poppy EST (Accession number EB740770) and *Arabidopsis thaliana* Actin 8 (Accession number NM_103814). The primer sequences used for the semi-quantitative RT-PCR-based validation of selected target genes are listed in Supplementary Table 1. The gene expression profiles of some of the known genes of *P. somniferum*, mainly belonging to the morphinan alkaloid biosynthetic pathway were validated through TaqMan chemistry-based real-time PCR (Supplementary Table 2). For real-time PCR, ~4 μg of DNaseI-treated total RNA was used for first-strand cDNA synthesis with random hexamer primer in a 20 μl reaction volume using the ThermoScript™ RT-PCR System (Invitrogen) employing the manufacturer's protocol. Four-fold diluted cDNA (~50 ng/μl) was used to set up the real-time PCR using the manufacturer's (Applied Biosystems) protocol. Each reaction was set in 10 μl and comprised of 5 μl of TaqMan Universal PCR Master Mix (2X), 0.5 μl Assay-by-Design (20X), and 50-ng cDNA. The 18S rRNA gene was used as the endogenous control. The reactions were run on an Applied Biosystems 7900HT fast real-time PCR system using the following thermal cycling parameters: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The results were analyzed using the SDS version 2.2.1 software. The $\Delta\Delta C_T$ method was used for relative quantification, whereby $2^{-\Delta\Delta C_T}$ yielded the relative quantity (RQ) value that is the amount of a target normalized to the endogenous control gene and relative to the calibrator (*Sampada*).

### Alkaloid analysis by HPLC

A validated method (Singh et al. 2000) was used for alkaloid analysis with some modifications. The HPLC analysis was carried out on Waters equipment: pump 600E; auto-injector-717plus; column oven; detector-2996 PDA. Data acquisition and computation were carried out with Waters Empower software. All the standards and extracts were filtered through a 0.45 μm syringe membrane filter (Millipore) and analyzed by HPLC. Analysis was carried out at 25 °C on a Waters Symmetry C18 (250×4.6 mm, 5 μm) column. Prior to use, solvents were filtered through 0.45 μm, 50 mm diameter membrane filter (Millipore) and sonicated for 15 min in a Microclean-109 bath (Oscar, India). Chromatography was carried out using mobile phase of acetonitrile/0.1 M sodium dihydrogen phosphate/acetic acid buffer (20:80:0.4, *v*/*v*/*v*, pH 3.8). The flow rate during the analysis was 1.0 ml/min. Chromatographic data acquisition was carried out in the range 200–400 nm to ensure specificity of targeted alkaloids and quantitation was performed at 240 nm (Supplementary Fig. 1).

## Results

### Alkaloid profiles of *Sujata* and *Sampada* capsule walls

Chemo-profiling of the capsule wall tissue for five major benzylisoquinoline alkaloids (BIAs) (morphine, codeine, thebaine, narcotine, and papaverine) indicated that the two poppy genotypes, *Sampada* and *Sujata*, were quite contrasting in their alkaloid profiles (Table 1). In general, it was found that except for thebaine, the *Sujata* genotype had a depressed alkaloid phenotype as compared to *Sampada*. In fact, the content of thebaine was found to be significantly higher in the leaf and straw of *Sujata* as compared to *Sampada* (data not shown), making it a suitable starting material for developing thebaine-rich genotypes. In *Sujata* genotype itself, compared to capsule and leaf, the straw was a better source of thebaine with maximal thebaine content found in the early stage straw (data not shown). Except for morphine, the content of other alkaloids was found to decrease with capsule maturity. Although morphine could not be detected in the *Sujata* capsule and straw, it was found to be present in its leaves (data not shown), thereby indicating that it could not be considered to be truly non-narcotic.

### Subtractive hybridization-derived ESTs from *Sujata* and *Sampada* capsule walls

Forward as well as reverse suppression subtractive hybridization was carried out using *Sampada* and *Sujata* capsule tissues. The subtracted cDNAs were cloned and sequenced to generate 20 *Sampada*-abundant (PsSamTSujD) and 11 *Sujata*-abundant (PsSujTSamD) ESTs, which were analyzed through BLAST for homology-based putative functional identification (Supplementary Table 3). Most of these subtracted ESTs showed homology to hypothetical proteins or were novel (showing no significant similarity to any database candidate). However, two significant results were obtained for ESTs, PsSujTSamD233 (Accession number GT571597) and PsSujTSamD9 (Accession number GT571588), which showed similarity to a non-specific lipid transfer protein and hypothetical/cell wall-associated hydrolase, respectively. The differentially expressed genes may account for the variance in the alkaloid and/or latex profiles of the capsule wall of the two genotypes. Interestingly, eight of the *Sampada*-abundant and only one of the *Sujata*-abundant ESTs were found to be potentially new genes having no similarity in the public databases (Supplementary Table 3).

### Comparative gene expression analysis in *Sampada* and *Sujata* genotypes

The primary interest of the study was to identify the genes (from among the selected 750 annotated targets on the array) that are differentially expressed in *Sampada* and *Sujata*

**Table 1** Stage-specific profiles (% dry weight basis) of key benzoisoquinoline alkaloids in capsule walls of contrasting genotypes of *Papaver somniferum*

The data presented is the mean of three replicates. Standard deviation was between 5 and 19 % of the mean values

*ND* not detected

| | Morphine (%) | Codeine (%) | Thebaine (%) | Papaverine (%) | Narcotine (%) |
|---|---|---|---|---|---|
| Early stage capsule | | | | | |
| Sampada | ND | 0.0267 | 0.0442 | 0.0799 | 0.0683 |
| Sujata | ND | 0.0086 | 0.0465 | 0.0375 | 0.0083 |
| Late stage capsule | | | | | |
| Sampada | 0.0026 | 0.0107 | 0.0022 | 0.0313 | 0.0598 |
| Sujata | ND | 0.0025 | 0.0018 | 0.0049 | 0.0005 |

genotypes of *P. somniferum*. The hybridization experiment was performed using a dual channel procedure with dye-swap arrangement. For the comparative analysis, genes with a log fold change value of ≥0.5849 (*FC*≥1.5) were declared as up-regulated while genes with log fold change ≤−0.5849 (FC≤ 0.66) were declared as down-regulated, whereby *Sampada* sample was taken as the control. Based on this criterion, out of the 750 target genes on the chip, 52 genes were found to be down-regulated in *Sujata* (Supplementary Table 4), whereas 46 genes were up-regulated in *Sujata* (Supplementary Table 5) as compared to *Sampada*. The heat map for the differentially expressing genes is provided in Supplementary Fig. 2. However, while selecting the candidates for down-stream analysis and usage, a more stringent threshold was used, whereby genes with a log fold change value of ≥1 (FC≥2) were considered to be up-regulated and those with a log fold change value of ≤−1 (FC≤0.50) were considered down-regulated (with a few exceptions like the known genes/ESTs of *P. somniferum*). On the basis of this more stringent criterion, 21 genes qualified for the "down-regulated in *Sujata*" category and 20 for the "up-regulated in *Sujata*" category (Table 2). The results obtained through the microarray experiment were verified by semi-quantitative RT-PCR analysis of a representative set of genes (Fig. 1). For some of the known genes of *P. somniferum* (Supplementary Table 2, Supplementary Fig. 3) the microarray data was validated through TaqMan chemistry-based real-time PCR and found to have a similar trend. For most of these known genes, although the fold change difference was not significant in the microarray data, real-time PCR indicated significant down-regulation in *Sujata*, which may be due to the higher sensitivity of real-time PCR.

## Discussion

### *Sujata* as a better source of thebaine and a model for latex-related studies

Based on the chemo-profiling data of the rich *P. somniferum* germplasm available in the National Gene Bank for Medicinal and Aromatic Plants at CSIR-CIMAP, Lucknow, India, it has been shown that the released varieties *Sujata* and *Sampada* are the most contrasting and consistent genotypes in terms of latex and alkaloid profiles (Chaturvedi 2012). It is evident from the present study that the thebaine-rich latex-less *Sujata* background could be exploited and improved further if a morphological/phenotypic marker (not requiring lancing) could be added to it to make it distinguishable from the morphine-rich plants in the field. It seems that since both, gamma rays and EMS, have been used to generate *Sujata* from *Sampada*, the mapping of the mutations caused may be very tedious. Besides, it may also have resulted in pleiotropic effects. This is precisely the reason for the various differences (like latex, oil/lipid, alkaloid content, etc.) in the two genotypes.

Latex is the milky cytoplasm of the specialized cells known as laticifers that is often found to contain complex secondary metabolites (Post et al. 2012). Apart from *Sujata's* utility towards curtailing narcotic abuse of opium poppy, such a genotype has a wider application in studying the phenomenon of latex formation, sequestration, and storage in species that produce economically useful latex (like *Hevea brasiliensis*). The comparative analysis of contrasting (for latex) genotypes, *Sujata* and *Sampada*, has provided many clues, which may be applicable for strengthening latex formation in other species also where the latex commands a premium market price.

### Differential gene expression—*Sujata vs Sampada*

Like some earlier studies on *P. somniferum* (Millgate et al. 2004; Zulak et al. 2007), microarray-based analysis was employed in the present study. Interrogation of the custom *P. somniferum* array with mRNA populations derived from the early stage capsules of two contrasting *P. somniferum* genotypes, yielded many differentially expressing transcripts. Homology-based annotation of the differentially expressed genes broadly classified them into major categories like—(1) latex related, (2) oil and lipid related, (3) alkaloid related (Fig. 2), (4) cell wall related, and (5) others. The leads from this study may be further exploited to characterize the *Sujata* phenotype in *P. somniferum*, which has been highly sought after in recent times.

Unlike the *top1* mutant that produces pigmented latex (Millgate et al. 2004), *Sujata* does not exude latex at all upon

**Table 2** Genes in early stage capsule found to be differentially expressed in *Sujata* as compared to *Sampada*

| S. No. | Gene/probe ID | Gene/probe homology-based annotation/description | Log fold change[a] | Fold change[a] |
|---|---|---|---|---|
| Down-regulated in *Sujata* | | | | |
| 1 | Psom1_46 | *P. somniferum* salutaridine synthase mRNA, complete cds (EF451150) | −0.636140 | 0.643432 |
| 2 | Psom10 | *A. thaliana* polyubiquitin (ubq10) (CAB81074) | −1.143303 | 0.452722 |
| 3 | Psom17_46 | *P. somniferum* phospholipase D2 (PLD2) mRNA, complete cds (AF451980) | −0.805242 | 0.572266 |
| 4 | Psom18_46 | *P. somniferum* phospholipase D1 (PLD1) mRNA, complete cds (AF451979) | −0.721258 | 0.606568 |
| 5 | Psom39_46 | Opium poppy mlp mRNA for a major latex protein (X54305) | −2.645241 | 0.159846 |
| 6 | Psom42_46 | *P. somniferum* major latex protein mRNA, 3′ end (M37493) | −1.047264 | 0.483885 |
| 7 | Psom62 | *A. thaliana* invertase/pectin methylesterase inhibitor family protein (NP_201041) | −1.917005 | 0.264804 |
| 8 | Psom91 | *A. thaliana* anthranilate N-hydroxycinnamoyl/benzoyltransferase-like protein (AAK96747) | −1.376992 | 0.385021 |
| 9 | Psom225 | *A. thaliana* laccase 7 (NP_187533) | −2.165886 | 0.222845 |
| 10 | Psom287 | *A. thaliana* putative tyramine N-feruloyltransferase 4/11 (NP_181435) | −1.136828 | 0.454758 |
| 11 | Psom337 | *A. thaliana* putative xyloglucan glycosyltransferase 12 (NP_192536) | −1.085927 | 0.471090 |
| 12 | Psom364 | *A. thaliana* monogalactosyldiacylglycerol synthase 3 (NP_565352) | −2.127673 | 0.228827 |
| 13 | Psom403 | *A. thaliana* EIN3-binding F-box protein 1 (NP_565597) | −0.997913 | 0.500724 |
| 14 | Psom409 | *A. thaliana* putative ethylene receptor (AAC62208) | −1.387772 | 0.382154 |
| 15 | Psom441 | *A. thaliana* oligopeptide transporter (NP_567493) | −1.183977 | 0.440136 |
| 16 | Psom451 | *A. thaliana* gibberellin 2-beta-dioxygenase 2 (NP_174296) | −1.646155 | 0.319490 |
| 17 | Psom548 | *A. thaliana* lysine histidine transporter-like 5 (NP_565019) | −1.897877 | 0.268338 |
| 18 | Psom557 | *A. thaliana* xyloglucan endotransglucosylase/hydrolase protein 4 (NP_178708) | −2.440468 | 0.184224 |
| 19 | Psom561 | *P. somniferum* codeine *O*-demethylase (D4N502) | −1.135481 | 0.455183 |
| 20 | Psom605 | *A. thaliana* sugar transporter like protein (BAE98899) | −1.286526 | 0.409937 |
| 21 | Psom699 | *A. thaliana* glutathione S-transferase-like protein (NP_191835) | −2.133115 | 0.227965 |
| Up-regulated in *Sujata* | | | | |
| 22 | Psom2 | *A. thaliana* seed storage albumin 5 (NP_200285) | 2.930155 | 7.621925 |
| 23 | Psom4 | *A. thaliana* S-adenosylmethionine synthetase (AAA32868) | 1.086164 | 2.123088 |
| 24 | Psom13_46 | *P. somniferum* glutathione S-transferase 3 mRNA, complete cds (AF118926) | 1.788471 | 3.454486 |
| 25 | Psom16_46 | *P. somniferum* catechol *O*-methyltransferase mRNA, complete cds (AY268895) | 0.994108 | 1.991849 |
| 26 | Psom26 | *A. thaliana* seed storage protein CRU4/cruciferin (NP_851127) | 1.838410 | 3.576157 |
| 27 | Psom29_46 | *P. somniferum* putative NADPH-dependent oxidoreductase (cor2) mRNA, cor2-1 allele, complete cds (AF108438) | 1.434421 | 2.702737 |
| 28 | Psom43 | *A. thaliana* germin-like protein subfamily T member 2 (NP_173332) | 2.169185 | 4.497693 |
| 29 | Psom45 | *A. thaliana* unknown protein | 1.920231 | 3.784837 |
| 30 | Psom46 | *A. thaliana* oleosin 2 (NP_198858) | 1.500645 | 2.829692 |
| 31 | Psom116 | *A. thaliana* LTP6 (Lipid transfer protein 6); lipid binding (LTP6) | 2.247507 | 4.748616 |
| 32 | Psom169 | No significant similarity | 1.533245 | 2.894362 |
| 33 | Psom172 | No significant similarity | 1.065553 | 2.092972 |
| 34 | Psom231 | *A. thaliana* class-II DAHP synthetase-like protein (NM_102090) | 1.873061 | 3.663090 |
| 35 | Psom236 | *A. thaliana* malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) (NP_197960) | 1.584639 | 2.999328 |
| 36 | Psom250 | *A. thaliana* arogenate dehydratase 6 (NP_563809) | 1.628673 | 3.092285 |
| 37 | Psom274 | *A. thaliana* stearoyl-acyl-carrier protein desaturase-like protein (NP_175048) | 3.061950 | 8.351007 |
| 38 | Psom437 | *A. thaliana* glucokinase (AAM63104) | 1.033394 | 2.046833 |
| 39 | Psom582 | *A. thaliana* cinnamyl alcohol dehydrogenase 4 (NP_188576) | 2.295911 | 4.910640 |
| 40 | Psom667 | *A. thaliana* homocysteine S-methyltransferase AtHMT-2(AAF23822) | 0.976297 | 1.967410 |
| 41 | Psom709 | *A. thaliana* receptor-like protein kinase (BAF01042) | 1.386778 | 2.614939 |

[a] Criteria for the comparative analysis adopted here was that genes with a log fold change of ≤−1 (FC≤0.50) were considered as down-regulated and those with a log fold change value of ≥1 (FC≥2) were considered as up-regulated in *Sujata*, whereby *Sampada* sample was taken as the control. However, a few exceptions were there based on the perceived importance of the gene function in relation to the differences in the two genotypes

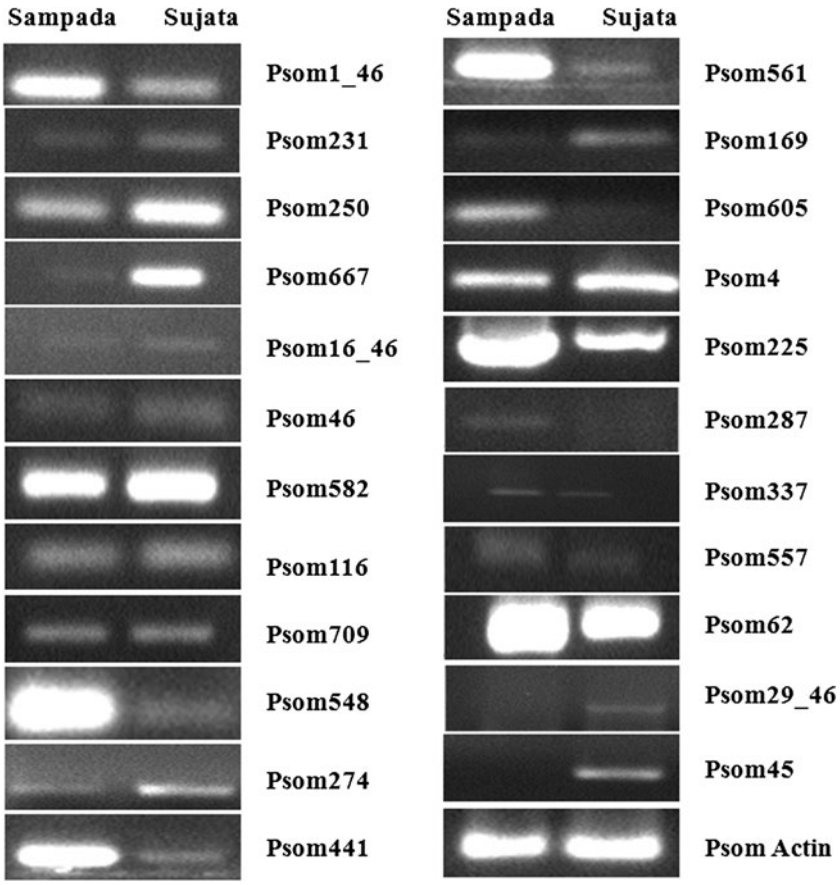


**Fig. 1** Semi-quantitative RT-PCR-based validation of microarray data for differentially expressed genes in contrasting *Papaver somniferum* genotypes, *Sampada* and *Sujata*

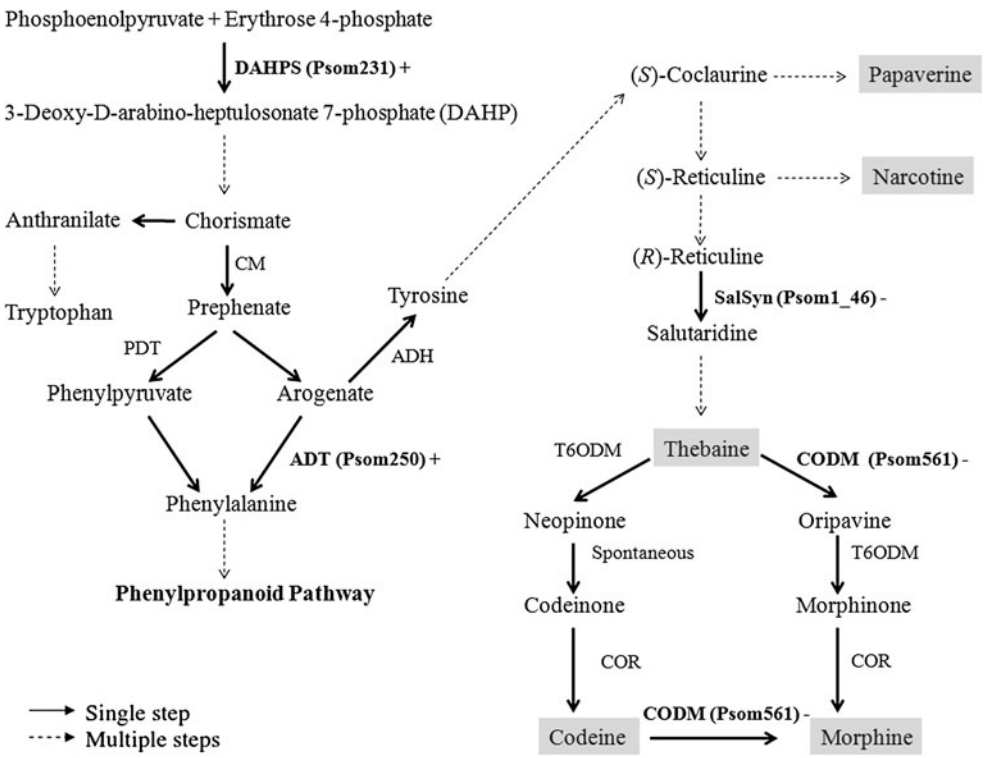


**Fig. 2** The metabolic pathway in *Papaver somniferum* depicting the aromatic amino acid and late morphinan alkaloid biosynthesis. The alkaloids chemo-profiled in the present study have been *shaded* and the genes showing differential expression in *Sujata* and *Sampada* have been marked in *bold*. + indicates up-regulated in *Sujata* and − indicates down-regulated in *Sujata* as compared to *Sampada*. *ADH* arogenate dehydrogenase, *ADT* arogenate dehydratase, *CM* chorismate mutase, *CODM* codeine *O*-demethylase, *COR* codeinone reductase (*cor1* allele), *DAHPS* 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase, *PDT* prephenate dehydratase, *SalSyn* salutaridine synthase (CYP719B1), *T6ODM* thebaine 6-*O*-demethylase

lancing, which is an added advantage. The dominant feature of the opium poppy laticifer is the presence of numerous alkaloid-rich, membrane bound vesicles that occupy most of the cell lumen (Nessler et al. 1985). Major latex proteins (MLPs) are laticifer-specific, low-molecular weight polypeptides and it has been observed that they accumulate early in laticifer development (Nessler and Burnett 1992). Expectedly, MLP (Psom39_46 and Psom 42_46) transcript abundance was significantly lower in *Sujata* as compared to *Sampada*. However, it seems that all MLPs present in *P. somniferum* have still not been completely characterized as evident from the MLP-like protein (Psom126, annotation drawn from *A. thaliana*) that had a different expression pattern and was found to be having higher transcript abundance in *Sujata* as compared to *Sampada* (Supplementary Table 5).

Ethylene has been shown to be an efficient stimulant of latex yield and this stimulation may occur in several ways (Dusotoit-Coucaud et al. 2009). For example, latex biosynthesis requires sucrose as the unique precursor and ethylene stimulation of latex production results in high sugar influx into the latex cells, which implies the role of sucrose transporters (Dusotoit-Coucaud et al. 2010). In view of this observation, the role of sugar transporters like Psom605 (lower transcript abundance in *Sujata* as compared to *Sampada*) assumes significance in opium poppy. On similar lines, other transporters (Psom441 and Psom548) found in the present study to have higher transcript abundance in *Sampada*, as compared to *Sujata*, may also be having significant roles in differentiating the two genotypes. It was interesting to find ethylene response-related genes (Psom403 and Psom409) down-regulated in *Sujata* as compared to *Sampada*. Psom403 has been annotated as an ETHYLENE-INSENSITIVE3 (EIN3) binding F-box protein. It may have a role in latex biogenesis/laticifer formation, which needs to be explored further. This hypothesis is strengthened by the fact that earlier a coronatine insensitive-1 gene predicted to possess F-box and leucine-rich repeat domains has been isolated from *H. brasiliensis* and shown to express in a laticifer-prominent manner and was inducible by jasmonate (Peng et al. 2009). It is also known that the intact coronatine molecule impacts signaling via the jasmonic acid, ethylene, and auxin pathways (Uppalapati et al. 2005) and is also the component of a laticifer formation enhancer (Akiyama 2010). Besides, laticifer differentiation in *H. brasiliensis* has been shown to be induced by exogenous jasmonic and linolenic acids (Hao and Wu 2000). EIN3 is a transcription factor responsible for mediating various ethylene responses and therefore a key positive switch in ethylene perception. The EIN3 binding F-box proteins regulate its turnover through ubiquitination and subsequent proteasomal degradation in absence of ethylene (Binder et al. 2007) but they are themselves also subject to proteasomal degradation (An et al. 2010).

Some oil and lipid-related genes were found to have higher transcript abundance in *Sujata* in comparison to *Sampada*, which is consistent with the higher oil content in the former genotype. Prominent among these were oleosin (Psom46), lipid transfer protein (LTP) (Psom116, PsSujTSamD233—accession number GT571597) and stearoyl-acyl-carrier protein (stearoyl-ACP) desaturase (Psom274). Fatty oils in plants are generally stored in spherical intracellular organelles (oleosomes) that are covered by proteins like oleosin and seeds with higher oil content have more oleosin than those with lower oil content. Although the exact role of oleosin in oil accumulation is yet to be elucidated, it has been recently shown to be a bifunctional enzyme that has both monoacylglycerol acyltransferase and phospholipase activities (Parthibane et al. 2012). LTPs may function as a component of the cuticular lipid export machinery (Debono et al. 2009). The desaturation of stearoyl-ACP is the last step of *de novo* synthesis of fatty acids in plants, but its desaturated product (oleoyl-) is the substrate forming many poly-unsaturated fatty acids, such as linoleic and linolenic acids, because double bonds are further introduced into the monounsaturated acyl-lipids and saturated acyl groups do not generally serve as substrates for desaturation (Luo et al. 2007). Stearoyl-ACP desaturase, which plays a key role in determining the ratio of saturated to unsaturated fatty acids in plants (McKeon and Stumpf 1982) may be indirectly responsible for the higher content of unsaturated linoleic acid in *Sujata* (56 %) as compared to *Sampada* (6 %) (Sharma et al. 2004). In contrast to oleosin, LTP and stearoyl-ACP desaturase, phospholipase D [phospholipase D1 (Psom18_46) and phospholipase D2 (Psom17_46)] that catalyzes the hydrolysis of glycerophospholipids at the terminal phosphodiester bond to form phosphatidic acid and an alcohol originating from the head group (Lerchner et al. 2005) was found to be having lower transcript abundance in *Sujata* as compared to *Sampada*.

It seems that the two genotypes, *Sujata* and *Sampada*, also differ in the composition of their thylakoid membrane lipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol, as MGDG synthase (Psom364), which is the key enzyme for the biosynthesis of both these galactolipids and consequently, biogenesis of chloroplast membranes (Kobayashi et al. 2009), shows significantly lower transcript abundance in *Sujata*.

Surprisingly, the transcript abundance of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase (Psom231), the first enzyme of the shikimate pathway that controls the carbon flux towards the aromatic amino acids (tyrosine, phenylalanine, tryptophan), was found to be significantly higher in *Sujata* as compared to *Sampada* (Fig. 2). It was also found that arogenate dehydratase (Psom250) transcript levels were 3-fold higher in *Sujata* as compared to *Sampada*. Since arogenate dehydratase operates in the predominant route for phenylalanine biosynthesis from

prephenate via arogenate (Maeda et al. 2010), it implies that the major portion of the metabolic flux at the branchpoint arogenate gets diverted towards phenylalanine rather than towards tyrosine in *Sujata*, which may be the major reason for its depressed BIA profile. This hypothesis could be further ascertained through the analysis of expression level of arogenate dehydrogenase that converts arogenate into tyrosine, the entry point for all BIAs.

Interestingly, in the case of codeinone reductase (*cor*), many alleles have been described in literature (Unterlinner et al. 1999). *Cor* alleles *cor1.1-cor1.4* are functional (the two less studied alleles, *cor1.5* and *cor1.6*, were not represented on the array) whereas *cor2* (represented by *cor2.1*), which is 70 % similar to *cor1* alleles, is a non functional allele (silent allele). Since there is a 95–96 % sequence similarity among *cor1.1-cor1.4*, it was difficult to find unique probe regions for each of them. Hence, there might be some degree of cross hybridization among these allelic forms of the gene during expression analysis experiments. However, this was not a problem, as their biochemical analysis revealed no major difference in Km, pH optima, and substrate specificity (Unterlinner et al. 1999). Although *Sujata* early stage capsule accumulated high amount of thebaine, *cor1* transcript abundance in it was similar to *Sampada*. This was unlike the case *P. bracteatum* that also accumulates high amount of thebaine and shows lower *cor1* expression compared to *P. somniferum* (Ziegler et al. 2009). Exceptionally, *cor2.1* (Psom29_46) was found to show significantly higher transcript abundance in the *Sujata* capsule compared to *Sampada*. The exact implication of this phenomenon could not be ascertained immediately. However, it was not totally unexpected as *cor* is an exceptional gene (a rare example of an alkaloid biosynthesis-related gene represented by many allelic forms). Future research must be focused on elucidating the role of silent alleles like *cor2*. It is quite possible that such alleles may show conditional expression (under conditions where the normally active allele does not express optimally).

As expected, the transcript abundance of codeine demethylase (*codm*, Psom561) that catalyzes the conversion of codeine to morphine and thebaine to oripavine was found to be nearly half in *Sujata* as compared to *Sampada*, which may account for the low morphine and high thebaine content in the former genotype. Thus, the *Sujata* genotype could also be used as a starting material for further development of a dream *P. somniferum* variety blocked at codeine *O*-demethylase (CODM) that would allow the direct recovery of codeine from the plant and prevent morphine biosynthesis (Hagel and Facchini 2010). Salutaridine synthase (Psom1_46) was another morphinan alkaloid biosynthetic pathway gene that had significantly higher transcript abundance in *Sampada* as compared to *Sujata*. Anthranilate N-hydroxycinnamoyl/benzoyltransferase (Psom91) transcripts were more abundant in *Sampada* in comparison to *Sujata*. On the contrary, catechol *O*-methyltransferase (Psom16_46) showed higher transcript abundance in *Sujata* as compared to *Sampada*. Catechol is also derived from the shikimate pathway and recently catechol *O*-methyltransferase has been shown to convert it into guaiacol (a small volatile molecule with a smoky aroma contributing to fruit flavor) in the tomato fruit (Mageroy et al. 2012).

Another significant category of genes found to be differentially expressed in *Sujata* and *Sampada* was related to the cell wall with most of them found to be down-regulated in *Sujata*. These were invertase/pectin methyl esterase inhibitor family protein (Psom62), laccase (Psom225, Psom391), tyramine N-feruloyltransferase (Psom287), xyloglucan glycosyltransferase (Psom337), xyloglucan endotransglucosylase/hydrolase (Psom557), pectinesterase (Psom500) and alpha-1,4-galacturonosyltransferase (Psom 628). Xyloglucan endotransglycosylase has been proposed to participate in laticifer cell wall remodeling, although its specific role is yet to be ascertained (Pilatzke-Wunderlich and Nessler 2001). Interestingly, cinnamyl alcohol dehydrogenase (Psom582, Psom 550) was found to be up-regulated in *Sujata* as compared to *Sampada*. These results imply that there are significant differences in the cell walls of the early stage capsules of the two genotypes, which needs to be further explored and the differentially expressing cell wall related genes must be functionally characterized in opium poppy. In our recent RNAi-based study, functional characterization of *P. somniferum* laccase gene explained the weak peduncle character and phenomenon of lodging in *Sujata* (Chaturvedi et al. 2013).

One particular seed storage albumin (Psom2) showed very high transcript abundance in *Sujata* as compared to *Sampada*. This is in line with the result obtained for LTP as plant LTPs share the same structural domain with storage proteins (Henrissat et al. 1988). In the future, some of the novel gene candidates (like Psom169 and Psom172 as well as those obtained from the subtractive hybridization experiment) that were found to be differentially expressed in the two genotypes could provide significant leads for further characterization using rapid forward genetics-based gene function assay tools like virus induced gene silencing that have been standardized for *P. somniferum* (Hileman et al. 2005; Wijekoon and Facchini 2012).

## Conclusion

Currently, *P. somniferum* is one of the model plant species for studying alkaloid biosynthesis in plants (Facchini et al. 2007; Facchini and De Luca 2008). This comparative study between *Sujata* and *Sampada* genotypes has provided several clues in the form of differentially expressing genes for advancing research on *P. somniferum*. A latex-based proteomics approach (2-dimensional map) would enable further elucidation

of the differentially expressing proteins in the two genotypes (Decker et al. 2000). However, there are many other mutants and genotypes of *P. somniferum* available globally (Desgagne-Penix et al. 2009; Singh et al. 2011). It would be really worthwhile to compare them on a common platform to derive maximal information about the opium poppy metabolome towards beneficial exploitation for mankind.

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**Conflicts of interest** The authors declare that they have no conflict of interest.

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