MECHANISMS OF DIFFERENT LEVELS OF OPIUM COMPOSITION IN SELECTED LINEAGES OF OPIUM POPPY

Jiri Cerven[1], Aneta Matznerova[1], Kristyna Kundratova[1], Martin Bartas[1], Petr Pecinka[1], Vladislav Curn[2]

[1]University of Ostrava, Faculty of Science, Department of Biology and Ecology, Ostrava, 70301, Czech Republic

[2]University of South Bohemia, Faculty of Agriculture, Center of Biotechnology, Ceske Budejovice, 37005, Czech Republic

Abstract

Opium poppy is cultivated for both alkaloids, that can be used in pharmacology as well as for seed, that is valuable in food production. In both cases, levels of alkaloid production are important, even though desired levels are antagonistic. Using bioinformatical approaches we have found full or partial duplications of genes responsible for different steps in morphine production pathways. We hypothesize, that gene duplications or deletions are responsible for different alkaloid content in different poppy varieties. We have tested this hypothesis by qPCR detection of gene duplications in two contrasting (Buddha – high morphine and Redy – low morphine) varieties of poppy and found partial duplications of SalS, SalR, SalAT, NCS2, CODM genes, and whole 4OMT gene duplication in the Buddha variety. In the future work, we will test this hypothesis on more varieties and confirm our findings by FISH and sequencing.

Keywords: *Opium poppy, Morphine, Gene duplication, Breeding*

1. INTRODUCTION

Opium poppy (*Papaver somniferum*) is a one-year plant, that has been cultivated since ancient times. It has been produced mainly as medicinal plant in the eastern – mainly Asian – countries. Current varieties most likely come from original Asian plant *Papaver somniferum subsp. Setigerum* (Labanca et. al, 2018). Opium poppy has two main production goals, that come from two different products – seeds that are used in gastronomy, and alkaloids that are used in pharmaceutical production. Both goals come with different and often contradicting requirements, which leads to intensive breeding with desired outcomes and also different breeding strategies (Vašák, 2010).

First goal is breeding towards food production, where high number of seed and production of seeds per cultivation area is main parameter. In this goal production of alkaloids is desired to be as low as possible, due to possible illegal production of drugs. Because of this possibility growing of poppy plants is forbidden in many European countries. In breeding towards alkaloid production increase in production of specific alkaloids or general increase in alkaloid production is favourable while seeds are desired to grow in lower numbers (Vašák, 2010).

Biosynthesis pathways for alkaloids in opium poppy are complex, and give rise to huge variety of compounds, from which only some are stable and only some find larger pharmacological use. Namely morphine, codeine, papaverine, and noscapine and some semisynthetic compounds as oxycodone, hydrocodone, buprenorphine and naltrexone. Synthesis of these compounds is driven by large number of enzymes, as can be seen on figure one from wonderful review from Braudoin and Facchini, 2014.

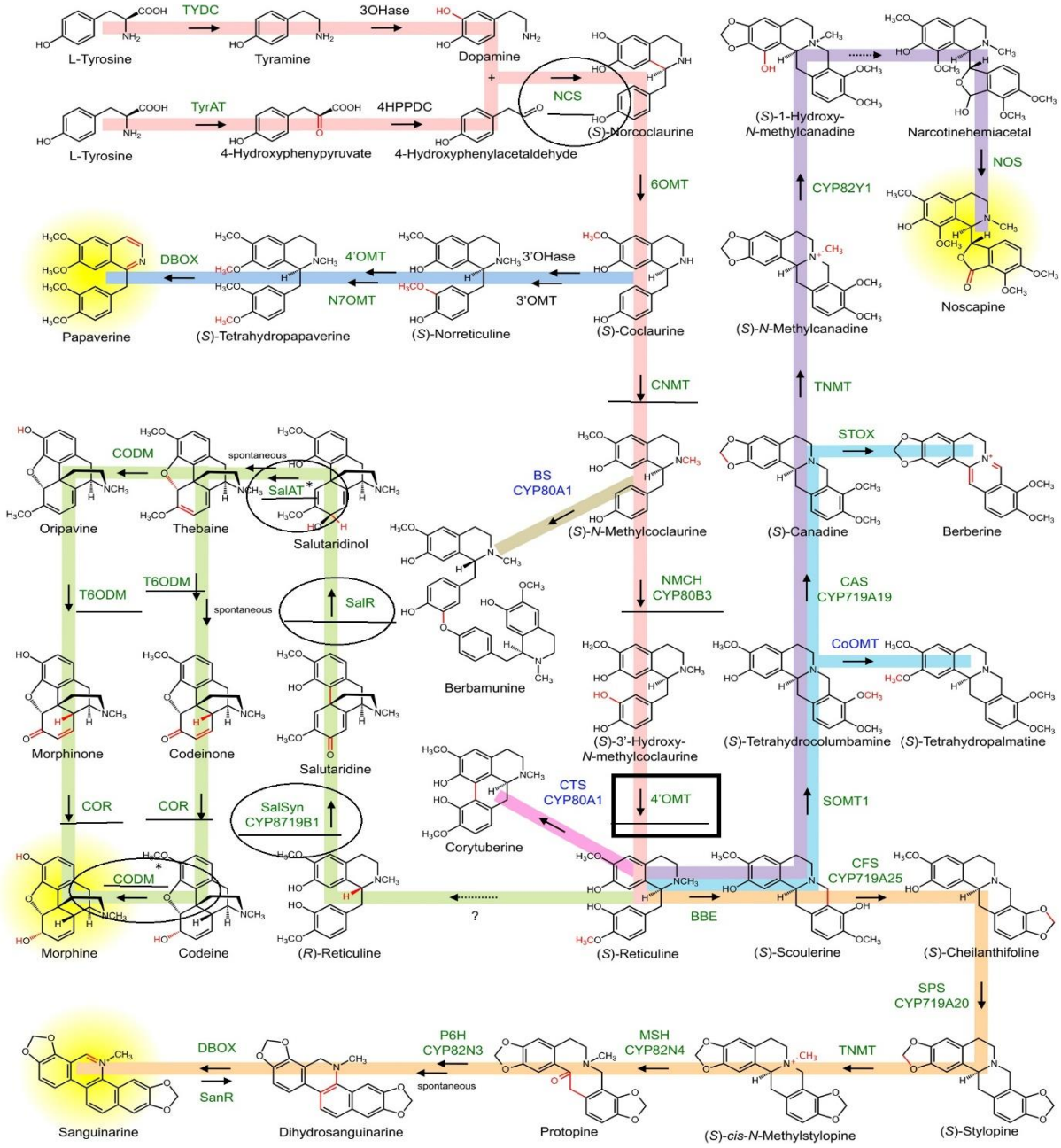


Fig. 1. Biosynthesis pathways for different alkaloids, edited from Beaudoin and Facchini, 2014. Genes, that we have tested for duplication are underlined, those which were found to be partially duplicated are encircled, genes that were found to be fully duplicated are marked in rectangles, genes with not fully statistically significant results (see table 3) are marked by asterisks. Main pharmacologically important products are highlighted in yellow.

Opium poppy is usually diploid (2N) plant, but some lineages have been detected to be tetra (4N) and also octa (8N) ploid. Also, in some lineages partial duplication of chromosomes and genes has been detected (Kurian and Sankar, 2007; Shaghani et al., 2019; Vašek et. al., 2020). In the year 2018 complete genome of Opium poppy has been published in Science by Guo et. al. and updated in the context of benzylisoquinoline alkaloid biosynthesis pathways in 2019 by Singh et. al. In both papers authors highlight, that genomic rearrangements have been crucial in evolution of different lineages of poppy also in context of alkaloid production.

This has led us to hypothesis, that differences in morphine content in many lineages can be caused by the whole or partial duplication (or deletion) of specific genes. In this work we have decided to test this hypothesis on two contrasting lineages of poppy: Buddha high morphine variety (2,18% content in the poppy straw) and Redy low morphine variety (0,16% content in the poppy straw). We have decided to use qPCR as simple and affordable method for detection of gene duplication.

2. MATERIALS AND METHODS

2.1. Materials

Oppium poppy seeds from two varieties morphine rich Buddha (https://grinczech.vurv.cz/gringlobal/taxonomydetail.aspx?id=406377) and morphine low Redy (https://grinczech.vurv.cz/gringlobal/accessiondetail.aspx?id=61609) were provided by Oseva pro. (Opava, Czech republic) seed-gene bank and were cultivated in basic agricultural substrate.

qPCR reactions were performed using EliZyme Green MIX AddRox (Elisabeth pharmacon, Czech republic) mastermix using Roche LightCycler II 480 (Roche, Switzerland).

Primers (Table 1) were synthetized by Elisabeth Pharmacon (Czech republic) in lyophilised state, dissolved in ultrapure water to concentration of 100 µM, dilluted to 10 µM stock concentration and used in 0.5 µM working concentration. Two primers were designed for each gene. One primer spanned central region of the gene and second in 3´end to detect whole or patial gene duplication.

All bioinformatic analyses were performed on NCBI accesion NC_03968.1

Table 1. Sequences of primers for different genes and their regions

Gene center	Sequence	Gene 3´end	Sequence
SALS-F	ACGGAACACCTGATTGGGAC	SALS-F	CTCCGGTGAGACAACAGTCC
SALS-R	CAACGCGCACATGAATGACA	SALS-R	GCGCTTTTAGTGAGTTGCCC
SALR-F	AACGGAACACCTGATTGGGA	SALR-F	CTGGGGGTGCCAAATACACT
SALR-R	ACGTTGGGTTGAAGATCCCG	SALR-R	GCAGTCTTGTCGTGGTCTCT
SALAT-F	TCGGGATCTTCAACCCAACG	SALAT-F	CTGGGGGTGCCAAATACACT
SALAT-R	AGAGCTTCTTCGGTGTGACC	SALAT-R	GCAGTCTTGTCGTGGTCTCT
T6ODM-F	GCCAAACCATCTAGGGAAATGC	T6ODM-F	CTCGAAGAGAATGCCAAACCA
T6ODM-R	GGTCTATGAAAGGGAGCGGG	T6ODM-R	GAAAGGGAGCGGGAAGTGAT
CODM-F	GGTTCAGGTATTAGAGTCTGGAA	CODM-F	CTAAGATCCGGTTCAGGTATTAGA
CODM-R	TCCTCTGTTTGATGTGGTTAACTT	CODM-R	CATATGATTTTCAGATGTGTCGGA
COR-F	GGTAGCGGTCCACGCA	COR-F	GCGGTCCACGCATTGT
COR-R	ACCTACGCCTTTTCAGACCT	COR-R	CTACCTACGCCTTTTCAGACCT
NCS2-F	TTGGGCATCCTTTTCCGCTT	NCS2-F	GCAAGAAACAACACCAGGGC
NCS2-R	ATTTGATCCTGCAGGGTTGTG	NCS2-R	TTTGATCCTGCAGGGTTGTG
CNMT-F	AAGGAGGGGTAACCTCGTGT	CNMT-F	CCCAGAGTCTACCCACCAGT
CNMT-R	TCTGACCGTGGTGGTGAGTA	CNMT-R	AACCTCCAGGCAAGAAAGGT
NMCH-F	ATCCGCTTGAGGTGGTCTTC	NMCH-F	AGTAATTGGGCTCACCAGGG
NMCH-R	ACCCTGGTGAGCCCAATTAC	NMCH-R	GGTCGAGTCAGCCTTGTTTG
4OMT-F	ACTCTGCATTTCCTTGCCTT	4OMT-F	CTCTGCATTTCCTTGCCTTCA
4OMT-R	ACTGGTTTTAGCTTCATGTCGG	4OMT-R	AACCCCAACTGGTTTTAGCTTC

2.2. Methods

Genes of morphine production pathway was searched on the NCBI (Database "Gene") using common gene names obtained from Beaudoin and Facchini, 2014. Queries were restricted to *Papaver somniferum.* All information including gene IDs, gene names, chromosome locations, exon counts, whole gene lengths, and CDS lengths, were collected.

10 seeds of each variety were planted in 5cm diameter flower pots and cultivated in grow chambers (Binder, Germany) in 16/8 light (150 µM fotons/cm^2)/dark period and watered daily by 5ml of tap water. After 21 days of cultivation, total 50 mg of leafs from five plants were pooled for each variety and isolated by EliGene Plant DNA Isolation Kit (Elisabeth pharmacon, Czech republic) by manufacturers instructions and diluted to same concentration of 5 ng/µl (Implen nanophotometer P300, Germany).

qPCR reactions were performed in 40 two-step cycles composed of 15 s 95 °C denaturation and 15 s 60°C annealing/elongation. Global mean was calculated for all Cq values for each variety separatelly and differences from global mean for each measured gene were compared between varieties. Only values, that were greater than 0,5 after normalisation to global mean in two following qPCR measurements were considered significant.

Primer sequences were selected by NCBI Primer-BLAST to span central and 3´end of target gene, optimized to melting temperature 60 ± 1°C, self-complementarity <5, self-3´ complementarity <2, with amplicon sizes 110 ± 20 bp.

3. RESULTS

At first, we have analyzed different opium poppy sequences concerning morphine production pathway in NCBI database. We have found, that some genes are fully or partially duplicated. This can be seen in table 2. Each hit is presented as specific line in the table. Some genes are duplicated on same chromosomes (T6ODM), some are found on different chromosomes (NMCH) and for some exact positions are not yet know (marked as Unplaced). For some genes we can see whole gene duplication (T6ODM, COR) but most of the genes both CDS and whole gene lengths slightly differ. These differences led us for testing of total or partial gene duplication in the qPCR experiments.

Further we have decided to test two opium poppy lineages for gene duplications by qPCR. We have designed primers, that span central and 3´ends of genes so that we can see full or partial gene duplications. Results are summarized in table 3. As we are dealing with possible gene duplications, we didn´t want to normalize our results to common housekeeping reference gene, as we could possibly bring more bias into the results. For this reason, we have chosen normalization to global mean of Cq values (details described in methods section). Generally, in every replication of the experiment, there was always lower global Cq mean in the morphine rich Buddha lineage, suggesting "more DNA" coming from gene duplications is present. Most significant enrichment was found for parts of *SalS*, *SalR*, *SalAT*, *NCS2*, *CODM* genes and whole *4OMT* gene. Interestingly, *SalAT* and *NCS2* were not found to be duplicated in the original annotated genome which is represented by different variety (HN1, Guo L. et. al, 2018) so we can hypothesize, that different genes duplications can be found in different varieties.

Table 2. Genes of morphium production pathway in opium poppy. In the first column, NCBI gene IDs are provided. Sequences found on complementary strand are provided in *italics*. In the second column, gene names are present. Third column contains gene abbreviations. Fourth column contains exon counts of particular genes. In the fifth column, coding sequence (CDS) length in nucleotides is present. Sixth column contains chromosome number and seventh column contains detailed location of the gene. The last column contains the whole gene lengths in nucleotides.

Gene ID	Gene name	Shortcut	CDS length	Chromosome	Location	whole gene length
113321914	salutaridine synthase	SalS	1518	11	NC_039368.1 (128249007..128250966)	1959
113339274	salutaridine synthase-like	SalS	1521	Unplaced	*NW_020631041.1 (8601134..8603100,*	1966
113322262	salutaridine reductase	SalR	939	11	NC_039368.1 (128330274..128331968)	1694
113340174	salutaridine reductase	SalR	942	Unplaced	*NW_020631041.1 (8574428..8576190,*	1762
113321357	salutaridinol 7-O-acetyltransferase	SalAT	1425	11	*NC_039368.1 (128319439..128321744,*	2305
113347789	thebaine 6-O-demethylase	T6ODM	1095	2	NC_039359.1 (59955747..59958211)	2464
113347787	thebaine 6-O-demethylase-like	T6ODM	1095	2	NC_039359.1 (59933446..59935978)	2532
113347785	thebaine 6-O-demethylase-like	T6ODM	1095	2	NC_039359.1 (59911109..59913641)	2532
113311621	codeine O-demethylase	CODM	1083	1	NC_039358.1 (198606455..198608421)	1966
113311630	codeine O-demethylase	CODM	1083	1	NC_039358.1 (198677764..198679676)	1912
113328201	NADPH-dependent codeinone reductase 1-5	COR	966	Unplaced	NW_020619603.1 (7219550..7221550)	2000
113294469	NADPH-dependent codeinone reductase 1-4	COR	966	7	*NC_039364.1 (2467198..2469038)*	1840
113340282	S-norcoclaurine synthase 2	NCS2	2103	Unplaced	*NW_020631041.1 (12957304..12960215)*	2912
113283898	(RS)-norcoclaurine 6-O-methyltransferase	6OMT	1041	5	NC_039362.1 (183999440..184000794)	1355
113294670	(S)-coclaurine N-methyltransferase	CNMT	1056	7	NC_039364.1 (4136348..4138555)	2208
113314340	(S)-N-methylcoclaurine 3′-hydroxylase	NMCH	1464	1	*NC_039358.1 (208684986..208686784,*	1799
113328451	(S)-N-methylcoclaurine 3′-hydroxylase	NMCH	1467	Unplaced	NW_020619603.1 (9494942..9496974)	2033
113283892	(S)-N-methylcoclaurine 3′-hydroxylase	NMCH	1464	5	*NC_039362.1 (183738532..183740333,*	1802
113339850	3'-hydroxy-N-methyl-(S)-coclaurine 4'-O-methyltransferase 2	4OMT	1074	Unplaced	NW_020631041.1 (11170945..11172437)	1493
113327792	3'-hydroxy-N-methyl-(S)-coclaurine 4'-O-methyltransferase 1 [	4OMT	1065	Unplaced	NW_020619603.1 (9452525..9454113)	1589

Table 3. qPCR detection of gene duplication. For each variety mean of all Cqs was calculated, then the Cq value for each gene was subtracted. These differences in Cq values were subtracted between varieties. Values higher then 0.5 (two-fold difference in DNA content is equal to 0,5 Cq difference) were considered significant are marked in red. Values higher than 0.5, where difference is lesser then 0,5 after subtraction of SD are marked by asterisks (*).

Gene name	Mean of differences	SD
SalS mid	0.44	0.38
SalS end	1	0.05
SalR mid	0.59	0.06
SalR end	0.31	0.18
SalAT mid	0.12	0.12
SalAT end	*0.55	0.29
T6ODM mid	0.06	0.01
T6ODM end	0.43	0.24
CODM mid	*0.84	0.80
CODM end	0.20	0.16
COR mid	0.47	0.18
COR end	0.40	0.35
NCS2 mid	0.50	0.15

NCS2 end	2,27	0.68
CNMT mid	0.12	0.07
CNMT end	0.17	0.13
NMCH mid	0.08	0.05
NMCH end	0.10	0.10
4OMT mid	1,37	0.16
4OMT end	0.87	0.06

4. DISCUSSION

As we can see on figure one, genes that were found to be duplicated are found in the morphine branch of the pathways. This confirms our hypothesis, that gene duplications play huge role in different alkaloid content. We can see several of partial duplication and one whole gene duplication in 3'-hydroxy-N-methyl-(S)-coclaurine 4'-O-methyltransferase 2 (4OMT). It is more probable, that duplication of 4OMT is the result for higher morphine production. Duplication of 4´OMT gene also enables faster morphine production, as it is responsible for production of (S)-reticuline, which is important precursor for morphine production. In future work, we will test varieties with high content of other alkaloids, that are important for pharmacological prudction to see, if we can find simillar duplication patterns. We will also confirm our results by other methods, such as FISH and long reads sequencing.

5. CONCLUSIONS

We have confirmed, that gene duplications can be responsible for different alkaloid content in opium poppy. Knowledge of genes, that are duplicated can lead to more efficient breending strategies. In this regard, we have proven qPCR to be simple and reliable method for gene duplication detection.

ACKNOWLEDGMENTS

This research was funded by the Ministry of Agriculture of the Czech Republic, grant number QK1810391, University of South Bohemia GAJU027/2019/Z" and by the University of Ostrava, SGS01/PřF/2020.

We would like to thank to OSEVA pro. for providing seeds for experiments and valuable advices for growing of the plants.

REFERENCES

1. Beaudoin, Guillaume A. W., a Peter J. Facchini. 2014. „Benzylisoquinoline Alkaloid Biosynthesis in Opium Poppy". *Planta* 240 (1): 19–32. https://doi.org/10.1007/s00425-014-2056-8.
2. Guo, Li, Thilo Winzer, Xiaofei Yang, Yi Li, Zemin Ning, Zhesi He, Roxana Teodor, et al. 2018. „The Opium Poppy Genome and Morphinan Production". *Science* 362 (6412): 343–47. https://doi.org/10.1126/science.aat4096.
3. Labanca, Fabiana, Jaroslava Ovesnà, a Luigi Milella. 2018. „Papaver Somniferum L. Taxonomy, Uses and New Insight in Poppy Alkaloid Pathways". *Phytochemistry Reviews* 17 (4): 853–71. https://doi.org/10.1007/s11101-018-9563-3.
4. Kurian, Alice a M. Asha Sankar. Medicinal plants. 2. New Delhi: New India Publishing Agency, 2007. ISBN 81-89422-42-1.

5. Shaghaghi, Arezoo, Abolfazl Alirezalu, Emad Nazarianpour, Ali Sonboli, a Samad Nejad-Ebrahimi. 2019. „Opioid Alkaloids Profiling and Antioxidant Capacity of Papaver Species from Iran". *Industrial Crops and Products* 142 (prosinec): 111870. https://doi.org/10.1016/j.indcrop.2019.111870.
6. Singh, Aparna, Ivette M. Menéndez-Perdomo, a Peter J. Facchini. 2019. „Benzylisoquinoline Alkaloid Biosynthesis in Opium Poppy: An Update". *Phytochemistry Reviews* 18 (6): 1457–82. https://doi.org/10.1007/s11101-019-09644-w.
7. VAŠÁK, Jan. Mák. Praha: Powerprint, 2010. ISBN 978-80-904011-8-1.