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In silico COMPARATIVE TRANSCRIPTOME ANALYSIS OF *Papaver somniferum* CULTIVARS

Tuğba Gürkök Tan[1]✉, Mine Türktaş[2], Gülşen Güçlü[3]

[1] Cankiri Karatekin University, Food and Agriculture Vocational School, Department of Field Crops 18100, Cankiri, Turkey
[2] Gazi University, Department of Biology, 06100 Ankara, Turkey
[3] Sivas Cumhuriyet University, Health Services Vocational School, Department of Health Programmes, Sivas, Turkey

ABSTRACT

Papaver somniferum is a medicinal plant of the Papaveraceae family that has traditionally been used for diet or its therapeutic value for thousands of years. Mainly, morphine and noscapine alkaloids exhibit anti-analgesic and anti-cancer effects. However, gene expression patterns and regulatory elements, such as transcription factors between different tissues, still need to be detected. In this study, comparative in silico transcriptome analyses were conducted to examine the tissue-specificity of the benzylisoquinoline alkaloids (BIAs) biosynthetic genes and transcription factors (TFs) between morphine and noscapine cultivars. Analysis showed that BIA biosynthetic genes are expressed in a different pattern between two varieties. Results showed that some members of plant-specific secondary metabolites related to TF families, such as MYB, MADS-box, bHLH, NAC, and WRKY, are differentially expressed between tissues and varieties.

Key words: BIA pathway genes, *Papaver somniferum* L., tissue specificity, transcription factors, transcriptome

INTRODUCTION

Papaver somniferum L. (opium poppy) is known as a source of commercially critical secondary metabolites for the pharmaceutical industry [TMO 2017]. *P. somniferum* synthesizes benzylisoquinoline alkaloids (BIAs) such as codeine, morphine, noscapine, and papaverine [Desgagné-Penix et al. 2010]. The opium poppy metabolomic and transcriptomic analyses showed the genetic and metabolic variances liable for the differential production of specific BIAs; hence, primary alkaloid content varies among different cultivars [Desgagné-Penix et al. 2012].

BIA biosynthesis starts with the conversion of two L-tyrosine molecules to dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) *via* tyrosine/DOPA decarboxylase (TYDC) and tyrosine aminotransferase (TyrAT) [Facchini and De Luca 1994, Facchini et al. 2007]. Following several steps, the last common intermediate (S)-reticuline is synthesized, which is later converted to several other final products such as morphine, noscapine, sanguinarine, or papaverine [Facchini et al. 2004].

Reticuline epimerase (REPI) [Farrow et al. 2015], salutardine synthase (SalSyn), salutardine reductase (SalR), salutardinol 7-O-acetyltransferase (SalAT) and finally codeinone reductase (COR) or codeine-O-demethylase (CODM) function in morphine branch [Grothe et al. 2001, Ziegler et al. 2006, Gesell et al. 2009]. On the other hand, berberine bridge enzyme (BBE), scoulerine 9-O-methyltransferase (SOMT), and noscapine synthase (NCS) catalyze the noscapine branch.

It was reported that the BIAs are synthesized in stem tissue and transported to the capsule, where they

✉ t.gurkok@karatekin.edu.tr

are accumulated [Ziegler et al. 2009]. Several studies have been conducted to indicate which plant tissue BIA biosynthesis transcripts are expressed or which enzyme catalyzes the different BIAs. Desgagné-Penix et al. [2012] performed a transcriptome study from stems of eight different cultivars to find out the diverse genes playing a role in BIA biosynthesis. However, most of these studies were based on the expressed sequence tag (EST) databases, the reads were at low depths, and there was no comparison between stem and capsule tissues.

Transcription factors (TFs) activate or repress the transcription and play significant roles in the regulation of developmental and metabolic processes [Sukumari Nath et al. 2019]. Since TFs have diverse and vital roles in plants, there is an increasing interest in TF studies. Previous studies have revealed that several TFs act as transcriptional regulators on secondary metabolite biosynthesis in plants [Yamada and Sato 2013, Agarwal et al. 2016]. Recently, various TF family genes were identified that are related to BIA biosynthesis [Deng et al. 2018]. To date, in *P. somniferum*, WRKY and MYB TFs were identified using the EST database. Mishra et al. [2013] reported that WRKY TFs play a role in biotic stress. Besides, MYB TFs in opium poppy were isolated by Kakeshpour et al. [2015]. However, no study has been conducted to clarify the tissue-specific expressions of TFs in opium poppy. Here, we performed the *in silico* gene expression analysis of BIA biosynthetic genes and TF genes in two cultivars differing in BIA content using different RNA-seq libraries. The comparison was performed between the stem and capsule tissues. This study aims to observe whether there are differences between the tissues and the cultivars in terms of BIA biosynthesis regulation.

MATERIAL AND METHOD

Data

To investigate the expression levels of the BIA biosynthesis genes and TF genes in stem and capsule tissues, the raw RNA-seq data were retrieved in the NCBI Short Read Achieve (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under accession number SRX3742160 (Stem), SRX3742161 (Capsule) for noscapine rich cultivar (HN1) and ERX651056 (Capsule), ERX651082 (Stem) for morphine rich cultivar Munich.

Data filtering and assembly

The reads with adapter contamination and the reads with uncertain nucleotides that constitute more than 10 percent of reads (N > 10%) were discarded. Low-quality nucleotides (base quality <20) were filtered by the Illumina CASAVA software (version 1.8.2, Illumina, Hayward, CA). High-quality reads were subjected to assembly using Cufflinks software version v2.2.1 [Trapnell et al. 2010] with library-type parameters and mapped to the *P. somniferum* genome using HISAT2 v2.0.5 with default parameters [Kim et al. 2015].

Analysis of differentially expressed genes and functional annotation

Cuffdiff was used to calculate Fragments Per Kilobase of transcript per Million mapped reads (FPKMs) of transcripts in each sample for differential expression analysis. Transcripts with p-value ≤ 0.05 and transcripts with p-value < 0.05 and fold change of ≥ 2 based on log2 expression value were assigned as differentially expressed (DEGs). Annotation of the BIA-related genes and TF genes was performed with ANNOVAR [Wang et al. 2010].

Identification of transcription factors

To identify the *P. somniferum* TFs, the sequences were homology searched against the plant TF database (http://planttfdb.cbi.pku.edu.cn/) [Jin et al. 2016]. The amino acid sequences of the transcripts were used as queries to search against the TFs database employing the BLASTP program (E value of 1×10^{-3}). To confirm the authenticity of TFs, the Hidden Markov Model (HMM) profile for each of the identified TF families was downloaded from the Pfam protein family database (http://pfam.sanger.ac.uk/) and executed to identify the presence of conserved domains in identified putative TF sequences.

RESULTS

Transcriptome sequencing and de novo assembly

The quality scores of Q20 and Q30 (over 90%) percentages indicated the quality of sequencing data. Over 95% of reads from each Munich_Capsule and 76% from Munich-Stem were able to be mapped to the reference genome [Guo et al. 2018].

In Munich, a total of 1327 DEGs were detected between the capsule and stem tissues, while 1827 DEGs were screened out between the stem and capsule in HN1.

Identification of BIA-related genes

The results revealed that there were significant differences between the two cultivars. A set of differentially expressed transcripts encoding genes associated with BIA biosynthesis was identified. The transcriptome library analysis belonging to the morphine-rich variety, Munich, indicated that several genes were expressed differentially. In Munich, 36 genes were found as differentially expressed between capsule and stem.

Twenty-six of them were involved in the BIA biosynthetic pathway. Among these genes, *(RS)-norcoclaurine 6-O-methyltransferase-like, S-norcoclaurine synthase 2-like* were represented by four variants. Seventeen transcripts were found from the beginning of the pathway to the intermediate molecule (S)-reticuline.

On the other hand, ten transcripts for the morphine pathway and seven transcripts for the noscapine pathway were detected. The remaining three transcripts were on the route of laudanine and papaverine synthesis. The log2 fold change ranged from +10 to –6 (Tab. 1). Except for *S-norcoclaurine synthase 2, S-norcoclaurine synthase 2-like* (XLOC_002989 and LOC113355180) and non-functional NADPH-dependent codeinone reductase 2-like (LOC113276337) genes, all the genes revealed up-regulation in capsule vs stem. The *(S)-scoulerine 9-O-methyltransferase-like* (LOC113322507) was detected as the most differentially expressed transcript in a capsule with ten folds, which was found on the noscapine pathway.

Table 1. The most up and down-regulated genes in the Munich cultivar in the BIA pathway

Transcript	Gene ID	log fold	P value	Gene description	Abbreviation of the gene
TCONS_00004760	XLOC_002989	–6	3,00E-04	S-norcoclaurine synthase 2	NCS2
XM_026597971,1	LOC113355180	–3	9,15E-03	S-norcoclaurine synthase 2-like	NCS2
XM_026525926,1	LOC113276337	–3	2,45E-02	non-functional NADPH-dependent codeinone reductase 2-like	COR2
XM_026570943,1	LOC113322791	7	5,00E-05	salutaridinol 7-O-acetyltransferase-like	SALAT
XM_026570190,1	LOC113322152	7	5,00E-05	methyltetrahydroprotoberberine 14-monooxygenase-like	CYP82X2
XM_026570358,1	LOC113322295	8	5,00E-05	methyltetrahydroprotoberberine 14-monooxygenase-like	CYP82X2
XM_026570405,1	LOC113322330	9	5,00E-05	(RS)-norcoclaurine 6-O-methyltransferase-like	6OMT
XM_026570953,1	LOC113322802	9	5,00E-05	(RS)-norcoclaurine 6-O-methyltransferase-like	6OMT
XM_026570116,1	LOC113322097	9	5,00E-05	trifunctional (S)-stylopine synthase/(S)-nandinine synthase/(S)-canadine synthase-like	CYP719A21
XM_026570601,1	LOC113322507	10	5,00E-05	(S)-scoulerine 9-O-methyltransferase-like	SOMT1

Table 2. The most up and down-regulated genes in the HN1 cultivar in the BIA pathway

Transcript	Gene ID	log fold	P value	Gene description	Abbreviation of the gene
XM_026524323,1	LOC113274906	–7	4,53E-02	salutaridine reductase-like	SALR
XM_026571589,1	LOC113323301	–6	1,80E-03	bifunctional protein STORR-like	STORR
XM_026552907,1	LOC113303821	–6	2,00E-03	reticuline oxidase-like	BBE1
XM_026535627,1	LOC113286971	–6	5,00E-05	berberine bridge enzyme-like 8	BBE-like
XM_026533273,1	LOC113283892	–6	5,50E-04	(S)-N-methylcoclaurine 3'-hydroxylase isozyme 1-like	NMCH
XM_026531902,1	LOC113282805	3	2,40E-03	berberine bridge enzyme-like 8	BBE-like
XM_026568781,1	LOC113320885	3	2,02E-02	(S)-tetrahydroprotoberberine N-methyltransferase 1-like isoform X2	TNMT
XM_026569401,1	LOC113321489	3	3,23E-02	S-norcoclaurine synthase 2-like	NCS2
TCONS_00052836	LOC113281310	5	4,77E-02	(+)-neomenthol dehydrogenase-like	SALR
XM_026597809,1	LOC113354490	6	5,00E-05	(S)-coclaurine N-methyltransferase-like	CNMT

In HN1, a total of 36 transcripts were found to be differentially expressed between capsule and stem. Among them, 28 of the transcripts were detected as down-regulated, which indicated that expression levels of most genes were higher in the stem. Eight transcripts showed upregulation in comparison of capsule vs stem. The fold change of the transcripts ranged from –7 to +6 (Tab. 2). The most down-regulated transcript was *salutaridine reductase-like* (LOC113274906), which converts salutaridine to salutaridinol in morphine biosynthesis, while the highest increase was detected in *(S)-coclaurine N-methyltransferase-like* (LOC113354490). These transcripts displayed diverse expression levels. Among 36 transcripts, 20 of them were belonging to the pathway from tyrosine to the (S)-reticuline. Only six transcripts existed in the noscapine pathway.

The cultivars showed different expression profiles. Interestingly, the number of up-regulated BIA biosynthetic genes was higher in the capsule of Munich, whereas the number of up-regulated genes was higher in the stem of HN1 (Fig. 1).

Identification of differentially expressed TF genes

Both in Munich and HN1 cultivars, several TFs were found to be differentially expressed between capsule and stem. In HN1, 179 transcripts, and in Munich, 174 transcripts were identified as differentially expressed TFs. The results revealed that the MYB and bHLH members were the most abundant TF family in both cultivars. In the Munich cultivar, 20.11% of the transcripts belong to the MYB, and 10.34% of the transcripts belong to the MADS-box TF family (Fig. 2a). In HN1 culture, MYB domain-containing transcripts accounted for the most significant proportion (20.67%) of the identified TF families, followed by bHLH-encoding genes (8.93%). (Fig. 2b).

Expression patterns showed that 58.62% of the TFs transcripts were down-regulated in Munich, while 52.51% were down-regulated in HN1. The results indicated that the expression of the genes is higher in the stem than in the capsule for both cultivars. In Munich and HN1 cultivars, the transcripts belonging

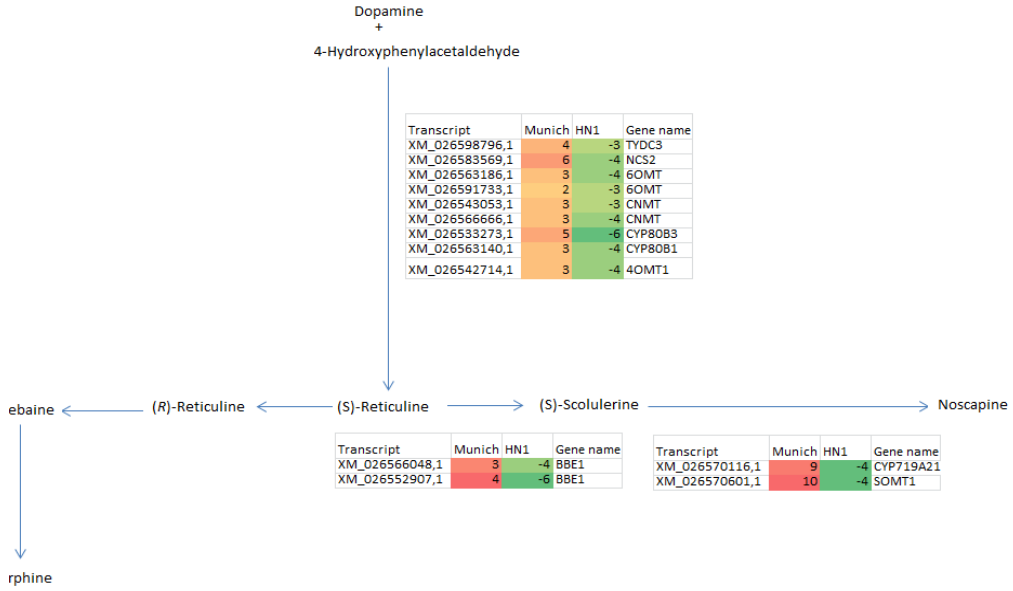

Fig. 1. The significant differentially expressed genes in the BIA pathway

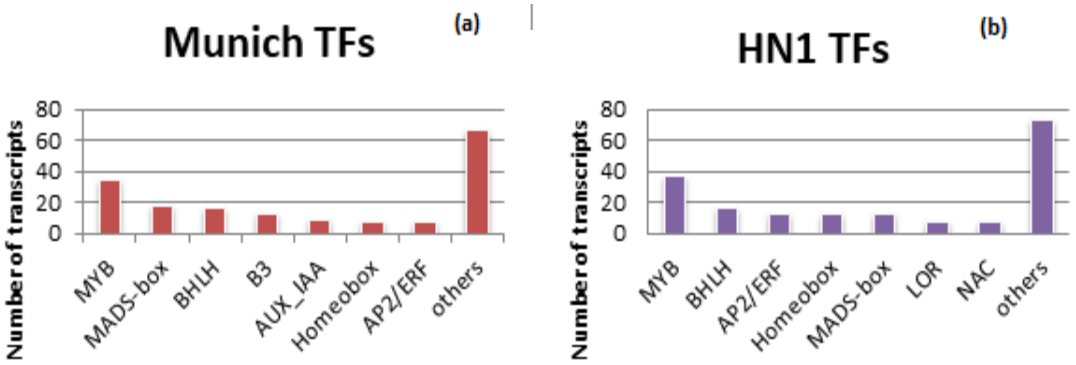

Fig. 2. The number of TF transcripts expressing in Munich and HN1 cultivar

to MADS-box members exhibited the most remarkable changes between the capsule and stem tissues (Tables 3 and 4); opposite MADS-box TFs gene expression patterns were observed between tissues of cultivars.

There were 36 shared TF transcripts between Munich and HN1. Interestingly, all of these transcripts showed opposed expression profiles. Only the *LOB domain-containing protein 21-like* (LOC113280508) gene displayed a correlated expression level. Among 36 shared TF transcripts, the most abundant ones were MYB, bHLH, and MADS-box TF members. Further, considering the expression levels, there were notable differences in MADS-box TF members *floral homeotic protein AGAMOUS-like* (LOC113284331), floral homeotic protein AGAMOUS-like isoform X3 (LOC113308598), and Homeobox TF member *WUSCHEL-related homeobox 3-like* (LOC113324235). These TFs were expressed highly in the stem in the Munich cultivar, whereas in HN1, their expression was higher in the capsule.

DISCUSSION

BIA biosynthesis and its regulation have been studied in both genomic and transcriptomic areas [Boke

Table 3. The highest expression changes for Munich cultivar TFs

Transcript	Gene name	log fold	*p*-value	Gene description
XM_026598368,1	LOC113355500	–10,51	5,00E-05	floral homeotic protein AGAMOUS-like
XM_026557086,1	LOC113308598	–10,35	5,00E-05	floral homeotic protein AGAMOUS-like isoform X3
TCONS_00060641	LOC113287477	–10,34	5,00E-05	floral homeotic protein AGAMOUS-like isoform X1
XM_026533766,1	LOC113284331	–9,72	5,00E-05	floral homeotic protein AGAMOUS-like
XM_026572554,1	LOC113324235	–8,94	5,00E-05	WUSCHEL-related homeobox 3-like
XM_026538707,1	LOC113289445	6,69	3,38E-02	GATA transcription factor 7-like
XM_026532934,1	LOC113283604	7,06	2,14E-02	MADS-box protein JOINTLESS-like
XM_026533584,1	LOC113284171	7,13	3,67E-02	transcription factor HHO2-like
XM_026538717,1	LOC113289465	7,61	5,00E-05	MADS-box protein JOINTLESS-like
XM_026552632,1	LOC113303583	9,04	5,00E-05	homeobox protein knotted-1-like LET6

Table 4. The highest expression changes for HN1 cultivar TFs

Transcript	Gene ID	log fold	*p*-value	Gene description
XM_026602483,1	LOC113358798	-8	3,97E-02	auxin response factor 19-like
XM_026597362,1	LOC113353891	-7	5,00E-05	zinc finger Ran-binding domain-containing protein 2-like
XM_026597209,1	LOC113353685	-7	5,00E-05	zinc finger Ran-binding domain-containing protein 2-like
XM_026525429,1	LOC113275860	-6	5,00E-05	transcription factor PIF7-like
XM_026547532,1	LOC113298719	-6	3,87E-02	protein SHOOT GRAVITROPISM 6-like isoform X2
TCONS_00016750	AG104	7	1,00E-04	Agamous-like MADS-box protein AGL104
XM_026548289,1	LOC113299289	7	1,60E-03	protein PFC0760c-like
TCONS_00125683	LOC113322699	8	5,00E-05	ethylene-responsive transcription factor WRI1-like
XM_026562092,1	LOC113313330	8	4,44E-02	MADS-box transcription factor 23-like isoform X2
XM_026604757,1	LOC113361550	9	5,00E-05	homeobox-leucine zipper protein ATHB-40-like isoform X1
XM_026572554,1	LOC113324235	10	5,00E-05	WUSCHEL-related homeobox 3-like

et al. 2015, Gurkok et al. 2014; Gurkok et al. 2016 Alagoz et al. 2016]. Thanks to plant breeding approaches, it is known that many poppy varieties produce different parent alkaloids. Analyzing cultivars that produce different major alkaloids may be useful for understanding the regulation of the BIA pathway [Facchini et al. 2004]. Unfortunately, knowledge about what regulatory mechanisms are involved in the BIA pathway or the evolutionary process of this pathway is still limited. In all organisms, one of the regulatory mechanisms is TFs, so finding the relations between metabolite and gene expression in different tissues can shed light on BIA biosynthesis and regulation.

BIA related genes

In this study, the capsule and stem tissues of the two cultivars showed different expression patterns. The results showed that 14 of the differentially expressed transcripts (24%) were similar between the two cultivars. The shared transcripts were mostly related to 6OMT, CNMT genes, which are crucial for the biosynthesis of (S)-reticuline, the common precursor to several BIAs. Surprisingly, when the capsule was compared to the stem, not only the common genes but also other BIA-related genes showed adverse expression levels between HN1 and Munich cultivars (Fig. 1). In opium poppy, BIAs such as morphine or noscapine are predominantly produced in the stem and accumulated in the capsule [Ziegler et al. 2009]. However, further studies are needed to clarify whether noscapine biosynthesis occurs in the capsule or the body.

It was reported that the *SalR* gene functions on the route of morphine biosynthesis [Zeigleret al. 2006]. In our study, consistent with this, the *SalR* gene showed higher expression levels in Munich than HN1. In this case, although the dominant alkaloids or pathways are different in diverse poppy cultivars, all the genes are able to work actively, but their expression is changing.

Different transcripts encoding the same gene, such as NCS2 in cultivar Munich (Tab. 1) or BBE-like in cultivar HN1 (Tab. 2), showed different expression patterns. It can be explained by the presence of many transcripts having multiple alleles or duplicates. As mentioned earlier, methyltransferase genes in the BIA pathway have acquired different functions in the evolutionary process through mutations such as duplication, loss, or gain of function [Winzer et al. 2012, Guo et al. 2018].

Transcription factor genes

The synthesis and accumulation of BIAs occur in different types of tissues. Besides, some BIA production and accumulation are tissue-specific. While morphinan alkaloids like morphine and codeine are accumulated in capsules, benzophenanthridine alkaloids like sanguinarine are accumulated in the roots of opium poppy [Hagel and Facchini 2013]. Hence, these processes might be regulated by different regulatory elements, such as noncoding RNAs and TFs. There are significant shortcomings in tissue-specific gene expression analysis of transcription factors of different alkaloid accumulating cultivars in opium poppy.

Here, we analyzed the tissue-specific expression levels of TF transcripts in two cultivars of opium poppy. MYB transcription factors, which are one of the most prominent gene families in plants, are involved in many biological processes, such as growth and development regulation of primary and secondary metabolism [Cao et al. 2020]. In this study, MYB members were the most represented DEGs among detected TF families. According to the results, the *MYB26-like* (LOC113301406) gene showed the highest expression level in both the HN1 capsule and stem. Previous results displayed that the MYB26-like gene has a role in the biosynthesis of phenylpropanoids [Uimari and Strommer 1997]. Genome-wide analysis pointed out that MYB-encoding genes showed duplication events in many plants [Chen et al. 2017]. In our analysis, MYB TF members comprised the most abundant TFs in both cultivars. The data indicated that MYB has a specific role in BIA biosynthesis in opium poppy.

Previous studies on TFs have shown that WRKYs are the most abundant TFs in poppy [Mishra et al. 2013]. However, in our study, the number of MYB TFs was higher than others. It might be because those studies were conducted on pathogen infection. The study indicated that the number of MADS-box TF genes showing significant expression levels is higher in the Munich cultivar than in the HN1 cultivar. To date, no study has been performed to clarify the association of MADS-box TFs with BIA biosynthesis or accumulation in opium poppy. Several studies were carried out about the role of MADS-box TFs in development [Drea et al. 2007]. It was previously reported that MADS-box TFs are involved in secondary metabolism regulation in *Hevea brasiliensis latex* [Li et al.

2016] and in tomatoes [Zhao et al. 2019]. In our study, the significant number of MADS-box TFs and their expression patterns in the Munich cultivar suggest that this TF gene family may also be related to morphine biosynthesis.

In a previous study, Agarwal et al. [2016] analyzed stem transcriptome data of 10 different opium poppy cultivars that accumulate different major alkaloids and indicated that WRKY TFs are the most abundant TFs. Also, they especially indicated the differentially expressed TF transcripts between standard cultivar (BR086) and high papaverine accumulating mutant (pap1). In their study, MYB TF family members also displayed significantly different expression patterns. However, in our study, MYB members were the most abundant among differentially expressed genes in both Munich and HN1 cultivars.

Especially in the Munich cultivar, MADS-box TFs had both high DEG numbers and low expression levels in the capsule (Fig. 2a, Tab. 3). For this reason, it seems that MYB and MADS-box transcription factors have profound importance in the production or accumulation of morphine. While Winzer et al. [2012] reported that the WRKY TF gene family has a role in noscapine biosynthesis, according to our results, MYB, MADS-box, and BHLH TF members might have significant roles in the regulation of noscapine synthesis and its accumulation. It might be due to the tissue-specific expression profiles of TFs in the cultivars.

CONCLUSIONS

In our study, it has been observed that the expression levels of genes encoding transcription factors involved in BIA biosynthesis differ in different tissues or are expressed tissue-specifically. The differentially expressed genes in different organs of different varieties can give clues about their functions. It indicates that transcription factors have essential roles in secondary metabolism. To elucidate the molecular regulatory players involved in the control of BIA biosynthesis and accumulation, most of the TFs need to be identified on a genome-wide scale.

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