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Research Paper

The effect of tetraploidy on the expression of genes involved in alkaloid biosynthesis in *Papaver fugax* Poir.

Giti Sotoudeh Ardabili[1], Rasool Asghari Zakaria[1*], Nasser Zare[1], and Leila Ghaffarzadeh Namazi[2]

[1]Department of Agronomy and Plant Breeding, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran.
[2]Meshginshahr Faculty of Agriculture, University of Mohaghegh Ardabili, Ardabil, Iran.
*Corresponding author; Email: rrasghari@yahoo.com

Abstract
Polyploidy, a common phenomenon in the evolution of plant species, plays an imperative role in plant diversification. Bride rose poppy (*Papaver fugax* Poir.) is a valuable medicinal plant due to its various alkaloids, such as fugapavin, mecambrin, romerine, salutaridine, protopine, and thebaine. In this study, we used quantitative polymerase chain reaction (qRT-PCR) to study the differential expression of four key genes, including *CYPB80B1* ((S)-N-methylcoclaurine-3'-hydroxylase), *MLP* (major latex protein), *6OMT* ((S)-norcoclaurine-6-O-methyltransferase), and *salAT* (7(S)-salutaridinol-7-O-acetyltransferase), involved in the biosynthesis of poppy alkaloids. The assessment of extracted RNA samples on 1% agarose gel electrophoresis and NanoDrop showed the proper quality and quantity of isolated RNA samples for cDNA synthesis. Gene expression analysis through qRT-PCR revealed increased expression of *CYP80B1*, *MLP*, and *SalAT* genes and decreased expression of *6OMT* in tetraploid plants compared with diploids. Our results indicated that autotetraploidy induction could be applied, as a useful method, to increase morphine production in *P. fugax*.

Keywords: alkaloids, gene expression, *Papaver fugax*, tetraploidy

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Introduction

The Papaveraceae plants with high production of alkaloids, such as morphine, codeine, thebaine, papaverine, and narcotine (Sariyar 2002; Bayazeid and Yalçın 2021), are well known for their broad medicinal uses (Yu *et al.* 2014). This family consists of approximately 50 genera and 830 species and is mainly distributed in temperate regions of the Northern Hemisphere, Southern America, and

South Africa (Singh 2004). The latest taxonomic revision recognized 11 sections in the *Papaver* genus (Kadereit 1988), the six sections (Carinata, Oxytona, Argemonidium, Rhoeadium, Papaver, and Meconidium) were reported from Iran (Cullen 1965). The Meconidium section is divided into 14 species and 10 varieties, including *Papaver fugax* Poir. (Kadereit 1988). Bride rose poppy (*P. fugax*) is an herbaceous diploid ($2n=2x=14$) plant

(Lavania and Srivastava 1999), with 20-60 cm of height, growing on steep slopes in steppe lands. Its stem is particularly fragile, especially in the lower parts. There are tiny trichomes on its leaves and buds. The fruit is a capsule with a length of 14-16 mm and a diameter of 4-8 mm. Fugapavin, mecambrin, romerine, salutaridine, protopine, and thebaine alkaloids are found in *P. fugax* (Shafiee *et al.* 1997). Codeine and morphine are produced from thebaine (Fairbairn and Hakim 1973; Fakhari *et al.* 2010). It was reported that the amount of codeine in the stem of *P. fugax* is significantly higher than in *P. orientale* and *P. bracteatum* (Fairbairn and Hakim 1973).

Genome doubling is a known phenomenon in the evolutionary history of all extant plants (Yang *et al.* 2011). It has been used in various aspects of genomics research, including evolutionary, functional, and comparative genomics (Chen and Ni 2006). Polyploidy induction is an effective breeding method with a short duration and easy operation (Thao *et al.* 2003; Madon *et al.* 2005). Colchicine as a mitotic inhibitor (Leung *et al.* 2015) has been used for artificial polyploidization in many plants (Dhooghe *et al.* 2011; Tamayo Ordóñez *et al.* 2016) to improve the physiological and morphological characteristics of the plants (Sattler *et al.* 2016). The mechanisms of the production of different phenotypes in polyploid plants are poorly understood. Still, it seems that it is associated with changes in gene expression through increased variability in dosage-regulated gene expression, altered regulatory processes, and rapid genetic and epigenetic changes (Osborn *et al.* 2003). Polyploidy, via increasing the biomass and content of effective ingredients, is usually more valuable in medicinal plants (Gao *et al.* 1996; Miri 2020). Polyploids, particularly allopolyploids, can be caused by chromosomal rearrangements and gene loss (Levy and Feldman 2004; Pires *et al.* 2004; Pontes *et al.* 2004), changes in DNA methylation status (Salmon *et al.* 2005), unequal rates of sequential evolution of duplicated genes (Small *et al.* 1999), and inter-locus concerted evolution of ribosomal DNA repeats (Wendel *et al.* 1995).

One of the most important natural compounds extracted from plants is morphine and codeine, which are used as narcotic analgesic (Heinrich *et al.* 2004). These morphinans are benzylisoquinoline alkaloids identified in the Papaveraceae family (Facchini 2001). Several necessary enzymes catalyze the morphinans biosynthesis in poppy plants. This pathway starts with the condensation of L-tyrosine, which is converted to (S)-norcoclaurine by the enzyme tyrosine/dopa decarboxylase (TYDC) (Lee and Facchini 2011). While 6-O-methyltransferase (6OMT) catalyzes the methylation reaction of (S)-norcoclaurine to (S)-coclaurine, (S)-coclaurine N-

methyltransferase (CNMT), and (S)-N-methylcoclaurine-3'-hydroxylase (CYP80B1) enzymes lead to the production of (S)-N methylococlaurine and (S)-3- hydroxy N-methylcoclauine, respectively. In the next step, (S)-3'-hydroxy-N-methylcoclaurine-4'-O-methyltransferase (4'OMT) catalyzes the conversion of (S)-3- hydroxy N-methylcoclauine to reticuline. The morphine biosynthesis requires the epimerization of (S)-reticuline to (R)-reticuline, performed by enzyme 1,2-dehydroreticuline synthase (DRS) and 1,2-dehydroreticuline reductase (DDR). After, a series of reactions are catalyzed by several enzymes such as salutaridinol synthase (STS), salutaridine 7-oxidoreductase (SOR), and salutaridinol-7-O-acetyltransferase (SAT). Then, the conversion of salutaridinol to thebaine is regulated by enzyme 7(S)-salutaridinol 7-O-acetyltransferase (SalAT). An uncharacterized enzyme demethylates thebaine to yield neopinione and codeinone. The NADPH-dependent codeinone reductase (COR) catalyzes the reduction of codeinone to codeine. Finally, codeine is demethylated by an undefined enzyme into morphine (Mishra *et al.* 2010, O'Connor 2010; Beaudoin and Facchini 2014).

There is little information about the chromosome doubling effect on gene expression modification, particularly in *P. fugax*. So, the present study aimed to investigate the impact of polyploidy induction on the expression of genes involved in morphinans alkaloid synthesis using quantitative real-time PCR (qRT-PCR).

Materials and Methods
Plant materials
The experimental materials consisted of diploid and tetraploid plants of *P. fugax* induced by colchicine treatment of the terminal buds described previously in Sotoudeh Ardabili *et al.* (2021). Briefly, sterilized seeds were aseptically blotted on a hormone-free MS medium and incubated at 25 °C with a 16-h photoperiod of cool white fluorescent light (4000–5000 lux). Terminal buds of 5-day plantlets were treated with a fresh aqueous solution of 0.2% colchicine for 12 h by applying colchicine soaked on terminal buds using the cotton swab method (Dar *et al.* 2017). After washing with distilled water, the plantlets were potted and kept in a growth chamber with 20-25 °C and 16/8 h photoperiod and 75% humidity for one month to adapt, then transferred to the greenhouse under 20-25 °C and 16/8 h of light and darkness conditions. The induced tetraploidy was confirmed through chromosome observations and flow cytometric analysis (Sotoudeh Ardabili *et al.* 2021). The extraction of alkaloids was performed according to Facchini (1995).

Gene expression analysis with RT-PCR
RNA extraction and cDNA synthesis:

Total RNA was extracted from young leaves of diploid and tetraploid plants using the RNeasy Plant Mini Kit (Qiagen, Iran). The concentration of RNA was checked by NanoDrop spectrophotometer and its quality was evaluated by 1% agarose gel electrophoresis. First-strand cDNA was synthesized using the Reverse Transcription Kit (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit) according to the manufacturer protocol. The synthesized cDNAs to be used in real-time PCR reaction were stored at -20 °C.

Primer design and examination of primer specificity and quality: The primers for internal control and examined genes were designed by the Primer3 (http://bioinfo.ut.ee/primer3) software, and then synthesized by Cinaclon Company (Karaj, Iran). *PsACT1* gene, which usually has a relatively constant expression level under control and treatment conditions, was used as an internal control for data normalization in the PCR reaction. The list of oligonucleotide primers is given in Table 1. The specificity of primers was examined and confirmed by the Primer-BLAST software of the NCBI database (http://www.ncbi.nlm.nih.gov/). Also, their quality (lengths, GC percentage, annealing temperature, and complementation to each other) was calculated by Oligo analyzer software.

Table 1. List of oligonucleotide primers used for real-time PCR reactions and the resulting product size.

No.	Genes	Sequences of primers		Amplicon length (bp)
		Forward primer 5'→3'	Reverse primer 5'→3'	
1	*PsACT1*	GCTTGACACTTCCAAAGGCA	CTGGGCACCTGAATCTCTCA	98
2	*CYP80B1*	AGCAGCATAACAACAGCAAGA	GCTCAGACAACGCCCATTCTA	150
3	*MLP*	GCTAATGGGCAAGGAAGCAT	CAGAAGCACAGAGGTGAGAGT	136
4	*U6OMT*	GAAGCATTACCAAAAGGTGGCA	TAGCAAAACCAGCGGCATCA	178
5	*SalAT*	GTCCGTGATGAAATCCACTCCA	GTTGCTGGTAAGAACGCCGA	141

Real-time conditions: We carried out qRT-PCR to investigate the differential expression of four key genes (*CYPB80B1*: (S)-N-methylcoclaurine-3'-hydroxylase, *MLP*: major latex protein, *6OMT*: (S)-norcoclaurine-6-O-methyltransferase, and *salAT*: 7(S)-salutaridinol-7-O-acetyltransferase) involved in the biosynthesis of poppy alkaloids. The expression profile of genes was determined by an RT-PCR detection system (Corbett Rotor-Gene 6000) and SYBR Green I technology. Each reaction was performed in a 10 μL mix,

containing: 0.5 μL of each primer (forward and reverse), 1 μL cDNA, 5 μL Master Mix (Takara kit), and 3 μL nuclease-free water. The PCR program consisted of an initial denaturing at 95 °C for 2 min, followed by 40 cycles with three stages: denaturation at 95 °C for 5 sec, annealing step of each primer pair at 55 °C for 30 sec, and finally, the extension step at 72 °C for 30 sec. For quantitative RT-PCR data analysis, LinReg and REST software (Pfaffl *et al.* 2002) were used: LinReg to determine Ct and efficiency of the reaction for each sample and REST to study significant differences in gene expression in diploid and tetraploid plants. In this study, the data were obtained from three biological and three technical replicates.

Results and Discussion

The assessment of extracted RNA samples on the 1% agarose gel electrophoresis and NanoDrop showed that the quality and quantity of isolated RNA samples were suitable for cDNA synthesis (Figure 1). The real-time PCR product multiplication diagrams showed that the product multiplication was done in linear, exponential, and stationary phases (Figure 2). Also, analysis of the melting curve of the PCR products showed that there is only one peak which indicates specific amplification of the target genes without the incidence of the primer dimer (Figure 3). Also, the specified amplification of products was affirmed by electrophoresis of products on the 2.5% agarose gel (Figure 4). Quantitative expression patterns of studied genes in tetraploid plants are shown in Figure 5. There were significant changes in the expression of all genes involved in the alkaloids biosynthetic pathway. So, *CYP80B1*, *MLP,* and *salAT* were up-regulated in the tetraploid group in comparison to diploid plants, by a fold change of 1.53, 2.87, and 3.305, respectively. By contrast, *6OMT* was downregulated by a fold change of 0.160 (Figure 5).
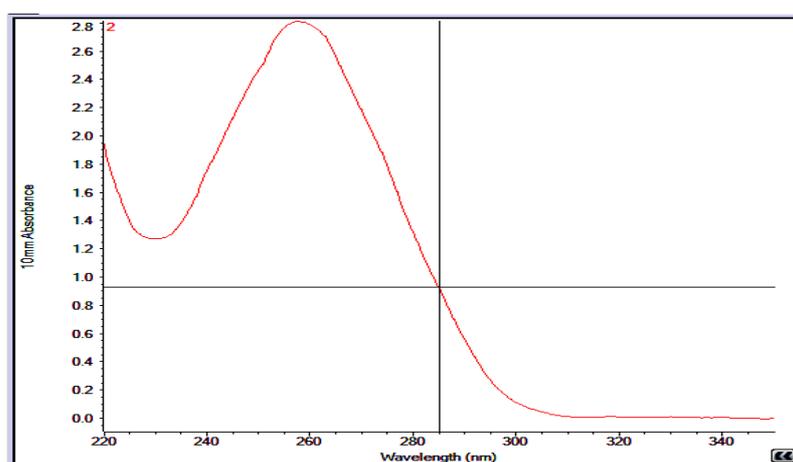

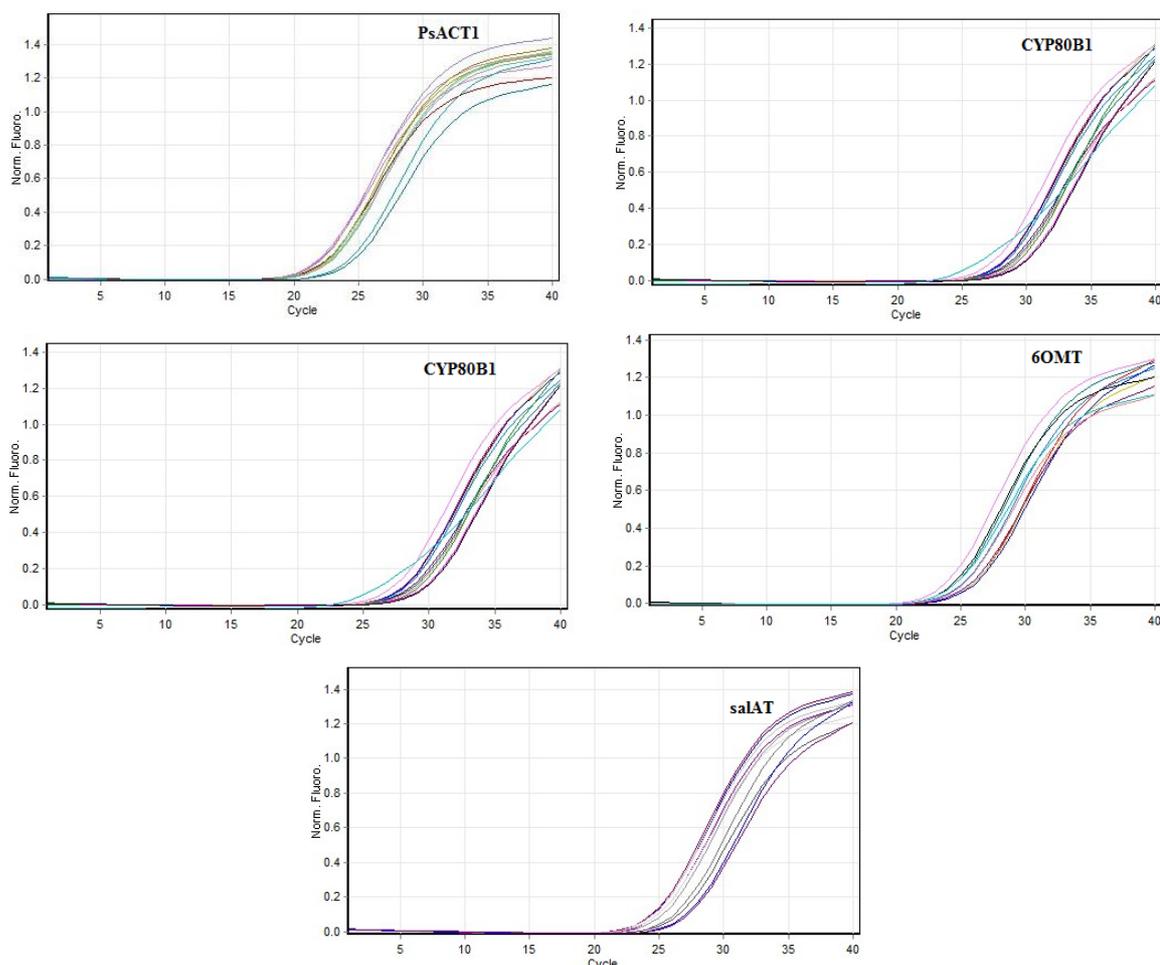Figure 1. Evaluation of the concentration and purity of the extracted RNA.

Figure 2. Real-time PCR product amplification diagram per cycle.

Our data suggested that the expression level for the three studied genes increased after the tetraploidy induction. However, a decreased expression level for one gene was also observed. Therefore, polyploidy induction enhanced the expression of some genes and down-regulated others. Polyploids have larger C-values than their diploid counterparts and are expected to show increased gene expression (Leitch and Bennett 2004; Albuzio *et al.* 2006). Allen *et al.* (2008) reported that overexpression of some genes involved in morphine synthesis (e.g. *SalAT*) increases the production of these alkaloids. Mishra *et al.* (2010) by investigating the gene expression changes of induced autotetraploid poppy (*P. somniferum*) plants through the semi-quantitative PCR method, reported that the high expression levels of genes encoding morphine alkaloids in tetraploid plants lead to the intense morphine production. They demonstrated that with the increase in ploidy level, the expression levels of all studied genes (*MLP*, *SalAT*, *CNMT*, *7OMT*, *4OMT*, 6OMT, *COR*, *CYPB80B1*, *TYDC*, and *SAT*) increased (Mishra *et al.* 2010). Genome expansion and
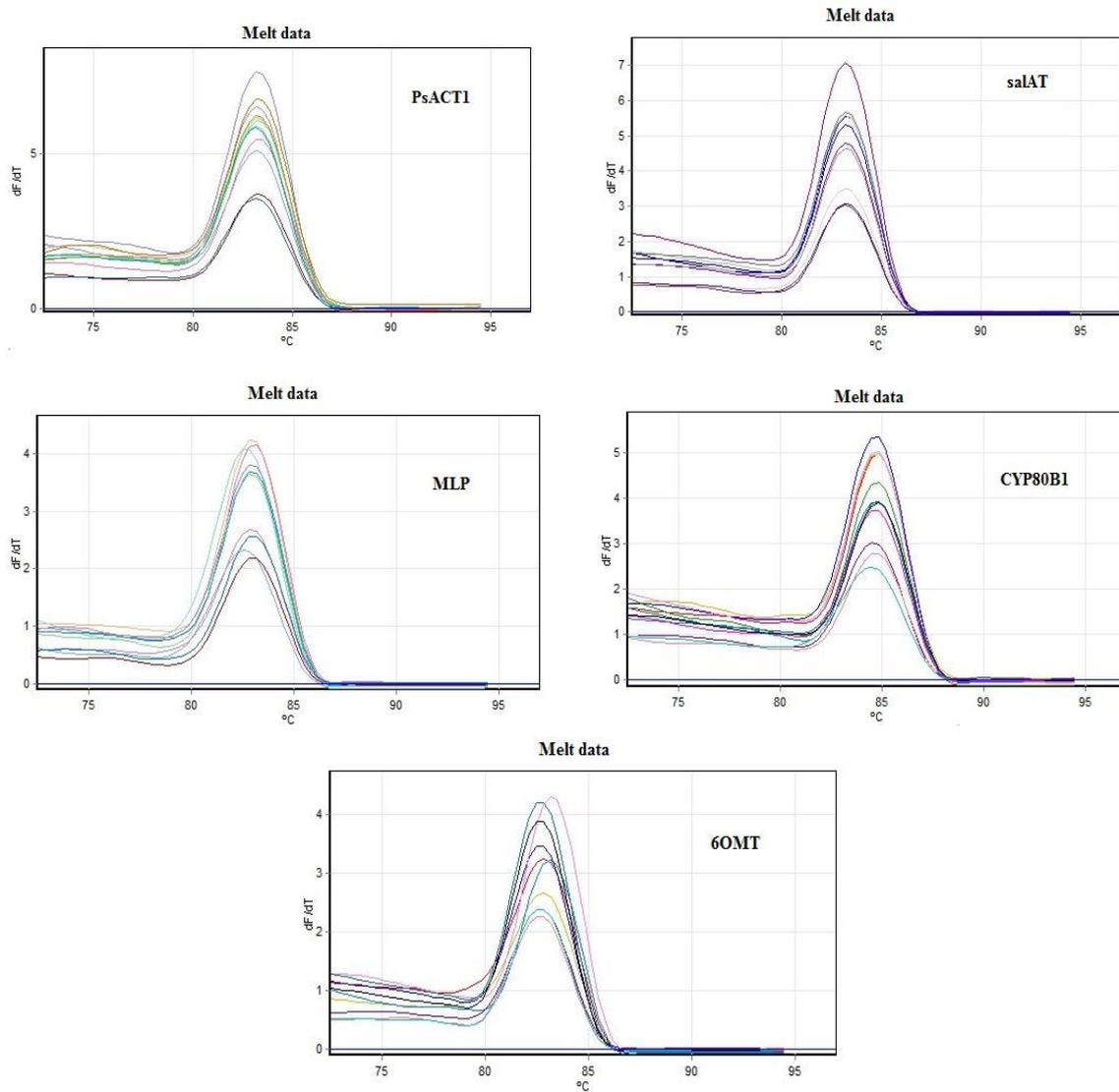
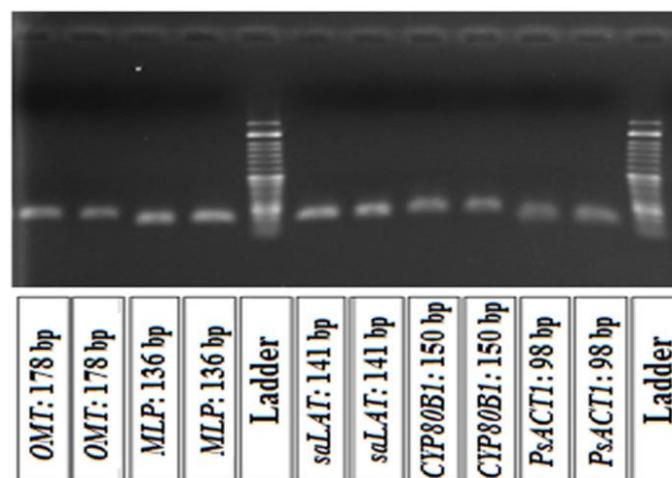Figure 3. Melting curve of PCR products of *PsACT1*, *CYP80B1, MLP*, 6*OMT*, and *salAT* genes.

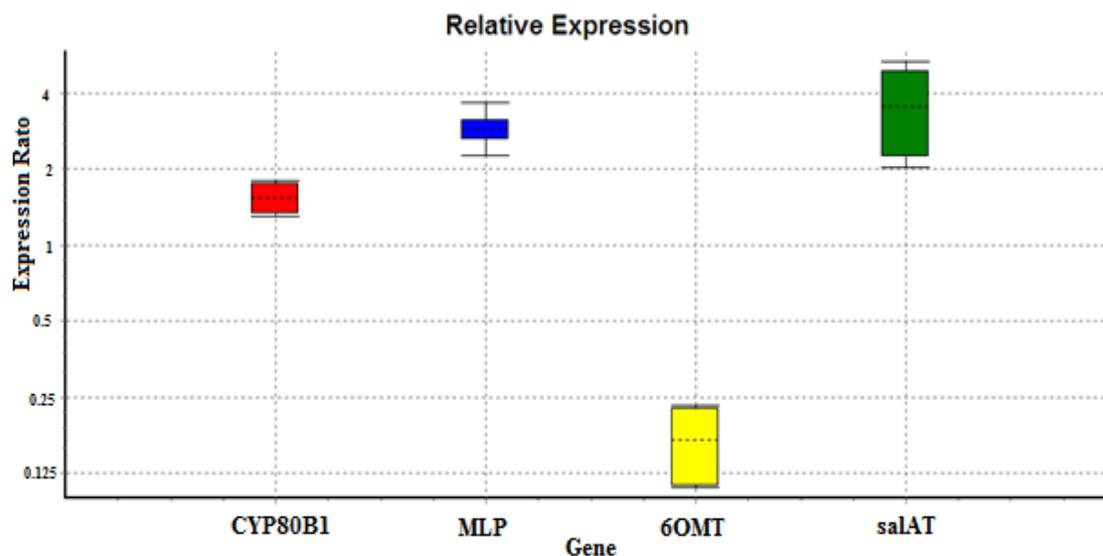Figure 4. Electrophoresis of PCR products on 2.5% agarose gel for each gene with the expected size.

Figure 5. Comparing of expression of *CYP80B1, MLP*, 6*OMT*, and *salAT* genes in diploid and tetraploid plants of *P. fugax*. Bars represent means ± SE (n = 3).

contraction due to polyploid induction have been reported in other polyploid research (Adams and Wendel 2005; Yang *et al.* 2011). Adams and Wendel (2005) also indicated that polyploidy affects gene expression considerably, including silencing and up- or down-regulation of genes. In an analysis of gene expression in colchicine-treated sorghum plants, Murali *et al.* (2013) reported up-regulation in the expression of most of the genes encoding sucrose synthases, and only one gene showed a down-regulated expression. Yang *et al.* (2011) indicated that polyploids show gene-expression bias at the genome level. This happens when genes show a differential expression between ancestral homeologous genomes. Hovav *et al.* (2008) demonstrated that 30% of the gene expression was biased between the cotton A and D

homoeologous genomes. It was also reported that the differential gene expression between the control and colchicine-treated plants may be partially due to the alterations in gene dosage (Murali *et al.* 2013) and the high copy number of genes (Sattler *et al.* 2016). Variations in gene expression caused by changing ploidy levels can be launched via epigenetic changes, subfunctionalization, pseudogenization, and neofunctionalization, which affect gene expression (Salmon and Ainouche 2010).

As reported in the previous study (Sotoudeh Ardabili *et al.* 2021), a significant enhancement in morphine content was observed in tetraploid *P. fugax*. The increase of secondary metabolites production in polyploid plants is a consequence of the overexpression of genes, associated with increasing nuclear

content (Manzoor *et al.* 2019) and due to the intensification of metabolic activity following chromosome doubling (Yun-Soo *et al.* 2004). Enhancement in secondary metabolites through colchicine treatment in tetraploids has been reported in *Tanacetum parthenium* (Majdi *et al.* 2010), *Solanum commersonii* (Caruso *et al.* 2011), *Echinacea purpurea* (Abdoli *et al.* 2013), *Thymus persicus* (Tavan *et al.* 2015), *Datura stramonium* (Belabbassi *et al.* 2016), *Salvia officinalis* (Hassanzadeh *et al.* 2020), and *Galega officinalis* (Khezri *et al.* 2022). Also, according to Sotoudeh Ardabili *et al.* (2021), tetraploid plants of *P. fugax* showed a lower codeine content as compared with diploids. It could be due to the conversion of codeine to morphine (Hagel and Facchini 2010). De Jesus-Gonzalez and Weathers (2003) reported that secondary metabolite accumulation in induced tetraploids of *Artemisia* increased compared with their diploid counterpart. Madani *et al.* (2015) also showed an increment in scopolamine content and a reduction in hyoscyamine content due to ploidy level manipulation in the *Hyoscyamus reticulatus*.

It seems that overexpression of the *SalAT* gene affects the synthesis of thebaine in the biosynthetic pathway of alkaloids in *P. fugax*. In addition, since thebaine is converted to codeinone and oripavine via two different pathways (Hagel and Facchini 2010), it can be concluded that in the induced tetraploid plants, the oripavine synthesis pathway, which eventually leads to morphinone production, is more common than the codeinone synthesis pathway, which yields codeine, and this may be the reason for enhanced morphine and declined codeine content in the induced tetraploid plants. So, it is supposed that the alteration of plant alkaloids is achieved from changes in the expression of genes involved in the biosynthesis of poppy morphinans as a consequence of tetraploidy induction. Therefore, higher production of alkaloids from poppy plants can be achieved through the induction of polyploid plants using antimitotic chemicals.

Conclusion

Given the alkaloid profile, significantly greater morphine content was observed in tetraploid *P. fugax* due to the overexpression of *CYP80B1*, *MLP*, and *SalAT* genes in tetraploids as compared to diploids.

Conflict of interest

There is no potential conflict of interest by the authors.

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تأثیر تتراپلوئیدی بر بیان ژن‌های دخیل در بیوسنتز آلکالوئید در .Papaver fugax Poir

گیتی ستوده اردبیلی[1]، **رسول اصغری ذکریا**[1]*، **ناصر زارع**[1] **و لیلا غفارزاده نمازی**[2]

۱- گروه زراعت و اصلاح نباتات، دانشکده کشاورزی و منابع طبیعی، دانشگاه محقق اردبیلی، اردبیل

۲- دانشکده کشاورزی مشگین شهر، دانشگاه محقق اردبیلی، اردبیل

*مسئول مکاتبه؛ Email: rrasghari@yahoo.com

چکیده

پلی‌پلوئیدی، پدیده‌ای رایج در تکامل گونه‌های گیاهی، نقش مهمی در تنوع گیاهی ایفا می‌کند. خشخاش برگ ریزان (.Papaver fugax Poir) به دلیل داشتن آلکالوئیدهای مختلف مانند فوگاپاوین، مکامبرین، رومرین، سالوتاریدین، پروتوپین و تبائین، گیاه دارویی ارزشمندی است. در این مطالعه از واکنش زنجیره‌ای پلیمراز کمی (qRT-PCR) برای بررسی بیان افتراقی چهار ژن کلیدی در بیوسنتز آلکالوئیدهای خشخاش {((S)-N-methylcoclaurine-3'-hydroxylase) CYPB80B1، MLP (Major latex protein)، (S)- OMT6 norcoclaurine-6-O-methyltransferase) و salAT (7(S)-salutaridinol-7-O-acetyltransferase)} استفاده شد. ارزیابی نمونه‌های RNA استخراج‌شده روی الکتروفورز ژل آگارز ۱ درصد و نانودراپ، کیفیت و کمیت مناسب نمونه‌های RNA جدا شده برای سنتز cDNA را نشان داد. تجزیه و تحلیل بیان ژن از طریق qRT-PCR بیانگر افزایش بیان ژن‌های CYP80B1، MLP و SalAT و کاهش بیان ژن OMT 6 در گیاهان تتراپلوئید در مقایسه با دیپلوئیدها بود. این نتایج نشان داد که القای اتوتتراپلوئیدی می‌تواند به عنوان یک روش مفید برای افزایش تولید مورفین در P. fugax استفاده شود.

واژه‌های کلیدی: آلکالوئیدها، بیان ژن، تتراپلوئیدی، Papaver fugax