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Modulated gene expression in newly synthesized auto-tetraploid of *Papaver somniferum* L.

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Abstract

Autopolyploidy is advantageous for plant metabolism in terms of elevated rates of synthesis or a higher variability of metabolically relevant compounds. In the present study, successful induction of polyploidy was achieved through applying colchicine soaked cotton on shoot meristem. The ploidy level of the developed tetraploids was confirmed through microscopic observations of stomata and chromosomal studies. Chromosome number in the developed tetraploids were $2x=2n=44$ as compared to the control having $2x=2n=22$. Alkaloid profile of both treated and control plants showed a significant enhancement from 25% to 50% in morphine content. Expression analysis through semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) of various known genes involved in the biosynthesis of morphinanes showed increased expression in tetraploids. Gene expression analysis of different polyploidy series can supplement our understanding related to molecular mechanism involved in increased alkaloid biosynthesis during polyploidization.

Keywords: Alkaloids; Autopolyploidy; Colchicine; Diploid; Opium poppy; Polyploidization

1. Introduction

Polyploidy has been a recurrent process during the evolution of flowering plant that has made a considerable impact on plant species diversity (Wendel and Doyle, 2005). In nature, duplication of genomic content either of the same (autopolyploidy) or divergent (allopolyploidy) genomes are a major force of evolution that affects both genome size and gene copy number (Soltis and Soltis, 2000; Wendel, 2000). In contrast to allopolyploids which are much prevalent in nature, autopolyploids has several potential advantages since the organism can resort to higher number of genes and higher maximum number of allelic variants (Soltis and Soltis, 2000). Plant breeders have taken advantages of autopolyploids for the improvement of agronomic traits of economically important plants (Otto and Whitton, 2000; Bennett, 2004). Studies suggests that novel phenotypes and ecological diversification often emerges with polyploid formation which is not present in their diploid progenitors or exceed the range of contributing species (Otto and Whitton, 2000; Ramsey and Schemske, 2002; Osborn et al., 2003; Soltis et al., 2003). Some of the traits such as pest resistance, increased drought tolerance, apomixis (asexual seed production), flowering time, organ size and biomass, could allow polyploidy to enter new niches or enhance their chances of being selected for agriculture (Osborn et al., 2003). Polyploids are also responsible for enhanced secondary metabolite production (Dhawan and Lavania, 1996).

A large number of aromatic nitrogenous compounds are known to be present in different plant species. Opium poppy (*Papaver somniferum* L.), member of family Papaveracae, contain more than 100 of these compounds. Most of these compounds present in poppy have immense medicinal importance due to the presence of phenanthredene (morphine, codiene and thebaine), benzylisoquinilone (papaverine) and phthalideisoquinilone (narcotine) alkaloids in their latex vessels (Ziegler et al., 2005).

Recently, enhancement in secondary metabolites has been made in *Chamomilla recutita*, Petunia and Salvia through polyploidy (Gao et al., 1996; Griesbach and Kamo, 1998;

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Svehlikova and Repcak, 2000). This has been achieved through colchicine treatment to different plant parts (Saeed et al., 2006). To validate the success in inducing polyploidy, chromosomal count and stomatal morphology has been used for pre-screening of ploidy levels (Masterson, 1994; Saeed et al., 2006; Knight and Beaulieu, 2008). Though polyploidy have been induced in these plants but studies related to consequences of autopolyploidation on gene expression has not been studied.

Keeping in view, the recent significant achievements obtained in various crops through polyploidization, the present study is an effort to induce polyploidy in *Papaver somniferum* L. The development of polyploidy in this study enhanced alkaloid content in the high latex yielding varieties. In addition to this, we have studied modulation of gene expression in autopolyploid system to correlate enhanced alkaloid biosynthesis to gene expression.

2. Materials and methods

2.1. Development of autopolyploid series

In the present experiment, 120 plantlets of opium poppy variety NBRI-5 from a large grown population in the experimental field of National Botanical Research Institute, Lucknow situated at 26.5° N, 80.5° E and 120 m above sea level, were used for polyploidization. The plantlets were treated with freshly prepared aqueous solution of colchicine in two different concentrations (0.25% to one set of 60 plants and 0.40% to another set of 60 plants) in two different modes of treatment comprising 30 plants per mode for both the concentration. First mode of treatment to a set of 30 plants, wet cotton soaked in colchicine solution of concentration 0.25% was kept on plant apical meristem followed by pouring same solution three times a day for two days. Another set of 30 plants were given same concentration of colchicine, but it was through injecting the solution by syringe in the cortical region only once. In third mode of treatment seeds were soaked overnight in the solution of colchicine (0.25 and 0.40%) sown in the field. Recommended cultural practices were followed for raising the crop (Yadav et al., 2006).

2.2. Stomatal and cytological studies

Slides for stomatal studies were prepared from the leaves of control and colchiploid plants. The exact chromosome number of plant material is generally determined by chromosome counting (Dart et al., 2004; Dhooghe et al., 2009). Thus for cytological studies, the young floral buds from control and colchiploid plants were fixed in 1:3 acetic alcohol solution (Carnoy's fixative). The buds were kept for 24 h in fixative followed by storage in 70% alcohol. The anthers of the buds were squashed with acetocarmine (25%) and chromosome numbers were counted from these temporary slides under compound microscope.

2.3. Alkaloids estimation

The opium latex of each control and colchiploid plants were collected. The opium samples were prepared by dissolving 75 mg opium powder in 10 ml DMSO and kept overnight. Estimation of various alkaloids was carried out through HPLC following the standard method (Khanna and Shukla, 1986) using Waters (Milford, USA) High Pressure Liquid Chromatography consisting of M6000 A solvent delivery system, 717 plus auto sampler, μ Bondapak C18 column (3.9 mm × 30 mm), 2487 Dual λ absorbance detector and millennium^{32} software. The mobile phase constituted methanol, glacial acetic acid and Millipore water (40:1:59) per litre to which 1.1 g heptane sulphonic acid was added to get 0.005 molar solution with pH 3.5. Standard curves were made by using standards of different opium alkaloids. Response of both peak height and peak area was used to obtain calibration curve.

2.4. RNA isolation and expression analysis

Leaves from 2nd and 3rd node of the control and colchiploid plants were crushed in liquid N_2 and stored in −70 °C till further use. RNA was isolated using Spectrum total RNA kit (Sigma, USA) followed by treatment with RNAase-free DNase (Fermantas, Life Sciences, Ontario, Canada). Five μg of total RNA was subjected to first strand cDNA synthesis using MMLV-RT Kit (MMLV-RT; MBI Fermentas Inc., Amherst, New York) using oligo(dT) primer. cDNAs were subjected to PCR using primers for tyrosine/dopa decarboxylase (TYDC), (S)-N-methylcoclaurine-3'-hydroxylase (CYPB80B1), codeinine reductase (COR), (S)-norcoclaurine-6-O-methyltransferase (6OMT), (S)-3'-hydroxy-N-methylcoclaurine-4'-O-methyltransferase (4'OMT), reticuline 7-O-methyl transferase (7OMT), (S)-coclaurine N-methyltransferase (CNMT), 7(S)-salutaridinol 7-O-acetyltransferase (SALAT), major latex protein (MLP) and salutaridinol 7-O-acetyltransferase SAT genes. Expression of *PsACT* (actin) was used as an internal control to equalize cDNA quantity in each reaction. The list of oligonucleotide primers used in this study is given in Table 1. The PCR reaction was carried out using the following cycle conditions: an initial denaturation at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final 5-min extension at 72 °C. After PCR, samples were analyzed on 1.2% agarose gel and documented on a gel documentation system.

3. Results

3.1. Stomatal and chromosomal variation

Variation in stomatal size often arises with the formation of polyploids and it is the basic step which indicates polyploidy induction (Silva et al., 2000; Beck et al., 2003; Gu et al., 2005; Stanys et al., 2006; Campos et al., 2009). Successful induction of polyploidy was noticed in our colchiploid plants only in the shoot apex treated plants in which size of stomata were significantly larger (Fig. 1; Table 2). Length of the stomata in

Table 1
List of oligonucleotide primers used for semi-quantitative RTPCR analysis of genes involved in alkaloid biosynthesis.

S.No.	Genes	Forward primer	Reverse primer
1.	Actin	GTATTGTGTTGGCTCTGGTGATGGTGT	GATGGATCCTCACACTGTATCCAGACACTGT
2.	TYDC	GTGGTTCCTAACACATTCGCCATG	TGTCATCCACTGTACCAAGTATG
3.	CNMT	CCGTTGATGCAGAAGATTGGTACAC	GTGAGCATCCATTCTTCACCATTC
4.	SAT	ACCAGTGGTACATCATGCCGTGAAC	CGAACTTAGCCATATCATCCTCAAG
5.	CYP80B1	TCTGCTCTTCCACGTCGAGCAC	TCTTCCATGATCAGTTGGCTAGGATC
6.	4OMT	ATGTGGAAGTATTTAGAAGTTAATC	AGCTTCAATGACAGACTGAATAGC
7.	7OMT	GGCTCATCCACACATACAAGG CATC	GCCGGAGTTCGAATGACATTGTAAC
8.	6OMT	ATCTTCCTCATGTCATAGCTGATTC	TTAATAAGGGTAAGCCTCAATTAC'
9.	MLP	GAACCAGTATCAACCATCATC AATG	GATGTTCATCGTTGCATATGGAC
10.	SALAT	TTCAACTGCCGTTACAG	TCACGGCATGATCCACT
11.	COR	GCTCTGGTGCGCTGATGCTCAC	ACAGCAATCTGGTGAAGCACCTG

colchiploid plants was 57±3.8 μm in comparison to 46± 9.5 μm in control plants. Initially, treated plants showed slow growth in comparison to the control but later on attained similar growth (data not shown). During the growth period, no phenotypic changes were observed except the leaves of the treated plants were slightly thickened. However, no changes in the size and frequency of stomata appeared in syringe treated plants. The syringe treated plants were compressed into compact bunch and the growth was stunted. Seeds which were treated with both the concentrations of colchicine did germinate and also attained a height of few centimeters but died later on. Meiotic studies of pollen mother cells collected from premature floral buds of shoot apex treated as well as control plants confirmed the chromosomal doubling in only three treated plants with each concentrations. Chromosome number in the developed tetraploids were $2x=2n=44$ (Fig. 2) as compared to the control having $2x=2n=22$ (Kapoor, 1995).

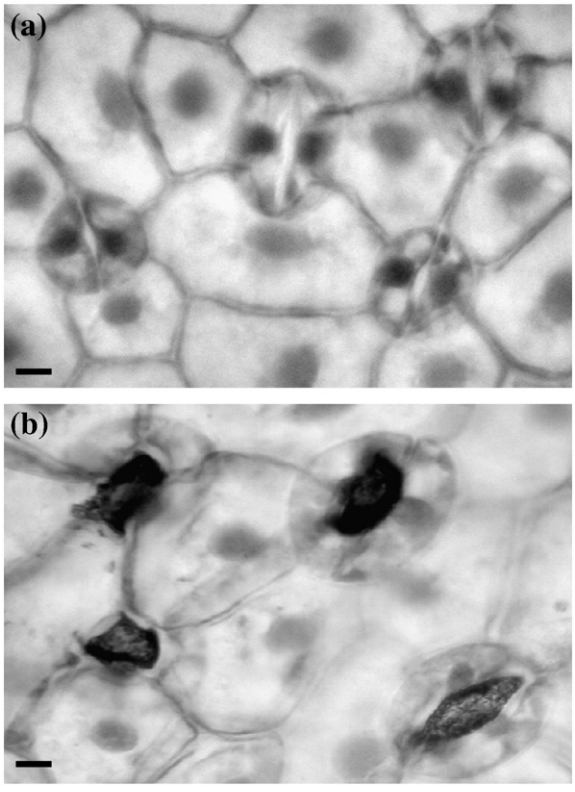

Fig. 1. Comparative size of the stomata (40×) in (a) diploid and (b) induced auto-tetraploid of *P. somniferum* L. Bar in the pictures represent 40 μm size.

3.2. Variation in alkaloid profile

Alkaloid profile of both treated and control plants estimated through High Performance Liquid Chromatography showed a significant enhancement from 25% to 50% in morphine content. At the same time, a reduction in thebaine content in the tetraploid plants with respect to control was observed (Table 3) in plants treated with 0.40% colchicine. Besides enhancement in morphine content in auto-tetraploids, the total alkaloidal content was generally similar.

3.3. Gene expression profile

Expression analysis for various genes involved in biosynthesis of different alkaloids was carried out through semi-quantitative expression analysis in the autotetraploids and the diploid plants. The expression of all the genes (TYDC, CYPB80B1, COR, 6OMT, 4OMT, 7OMT, CNMT, SALAT, MLP and SAT) of the alkaloid biosynthetic pathway increased in tetraploid plants in comparison to diploid (Fig. 3).

4. Discussion

In the present study, successful induction of polyploidy was achieved through applying colchicine soaked cotton on shoot meristem. The other two modes of treatment which were tried were found inefficient in causing polyploidy probably due to fungal infection caused in wounds created by the syringe and lethality in seed treated plants leading to bunchy stunted initial

Table 2
Variation in stomatal size in control and tetraploid plants of *Papaver somniferum L.*

Treatments/Traits		Length (μ)	Width (μ)
Control	Mean+SE	46±9.5	32±3.2
	Range	36–56	28–36
Tetraploid	Mean+SE	57±3.8	37.25±1.7
	Range	52–60	35–39

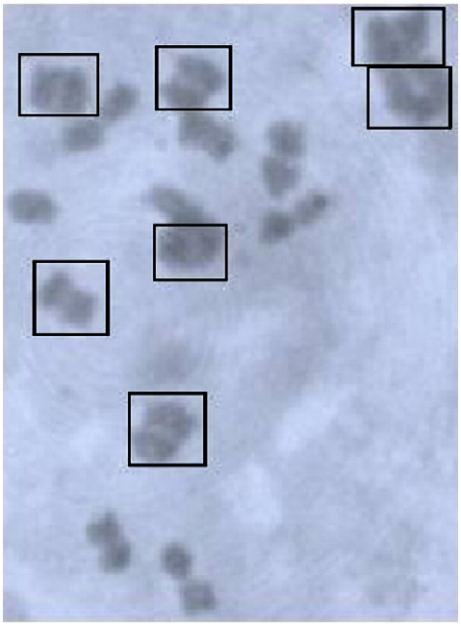

Fig. 2. Chromosome count in auto-tetraploid ($2n=44$) of *P. somniferum* L.

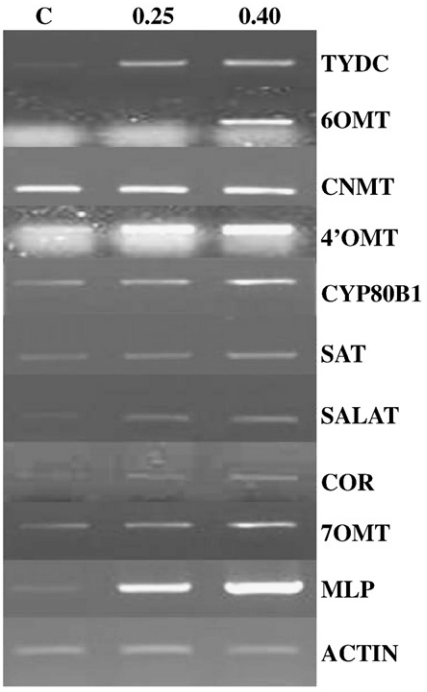

Fig. 3. Differential gene expression in control and colchicine treated plants of *P. somniferum* L. C, 0.25 and 0.40 represents control, 0.25% colchicine and 0.40% colchicine used for treatment respectively.

growth ultimately causing death. Plant growth inhibition and occasional mortality is a known phenomenon in autotetraploids (Saeed et al., 2006). In the present study, slow plant growth was the first indication of successful induction of polyploidy by colchicine treatment confirming the findings of Kerr (2001) and Yan (2001). Size of stomata is treated as an accurate indicator of the polyploidy induction in many plants (Knight and Beaulieu, 2008; Zheng et al., 2009). The microscopic observation showed significant increase in stomatal size in developed tetraploid which is in agreement with previous studies (Masterson, 1994; Stupar et al., 2007; Anssour et al., 2009).

The alkaloids profile showed significant increase in the morphine content in the developed tetraploids over diploids which might be due to enhancement in the metabolic activity following doubling of chromosome number (Strahil et al., 2003; Yun-Soo et al., 2004). Quantitative differences in secondary metabolites following polyploidy induction have been reported earlier in the developed tetraploids of several plants like Petunia, Salvia, Artemisia etc. in which secondary metabolite accumulation was enhanced in comparison to their diploid counterpart (Griesbach and Kamo, 1998; Svehlikova and Repcak, 2000; De Jesus-Gonzalez and Weathers, 2003). It is interesting to note that though thebaine and codeine content of the developed tetraploids in plants treated with 0.25% colchicine remained more of less same as compared to control, it decreased in plants treated with 0.40% colchicines. It might be due to use of these compounds in synthesizing higher amount of final product i.e. morphine of the pathway. Norcotine which is not produced by thebaine and codeine increased even plants treated with 0.40% colchicines. Developed tetraploids showed increased accumulation of morphine, however, the content of the total morphinane alkaloids were similar in both the tetraploids and diploids (Griesbach and Kamo, 1998). These changes in the metabolite profile of the developed autotetraploids might be due to mere multiplication of the basic genome, which could be due to disturbance in metabolic mechanism that regulates the biosynthesis of individual compound (Albuzio et al., 2006). Differential gene expression data obtained through semi quantitative expression analysis of various known genes involved in the biosynthesis of morphinanes showed increased expression. Over expression of these genes may be playing important role in increasing morphine content which has been observed in autotetraploids developed in this study (Fig. 4). Decreased amount of thebaine and codeine content in 0.40% colchicine treated plants may be increased expression of COR leading to increased synthesis of morphine. Higher ploidy

Table 3
Alkaloid profile of control and treated plants of *Papaver somniferum* L.

Treatments/Traits		Opium yield(mg)	Morphine%	Codiene%	Thebaine%	Narcotine%	Papaverine%
Control	Mean+SE	113.2±17.89	12.70±0.47	3.80±0.27	4.97±0.59	7.72±0.55	Nil
	Range	55–160	11.27–14.01	3.24–4.77	3.33–6.45	6.18–9.26	Nil
0.25%	Mean+SE	289.66±1.76	15.17±0.29	4.24±0.25	4.38±0.64	10.30±0.32	Nil
	Range	287–293	14.75–15.75	3.85–4.72	3.15–5.33	9.86–10.92	Nil
0.40%	Mean+SE	159.66±1.20	18.76±0.30	1.92±0.17	0.98±0.08	7.71±0.53	Nil
	Range	158–162	18.20–19.25	1.66–2.25	0.86–1.15	6.93–8.72	Nil

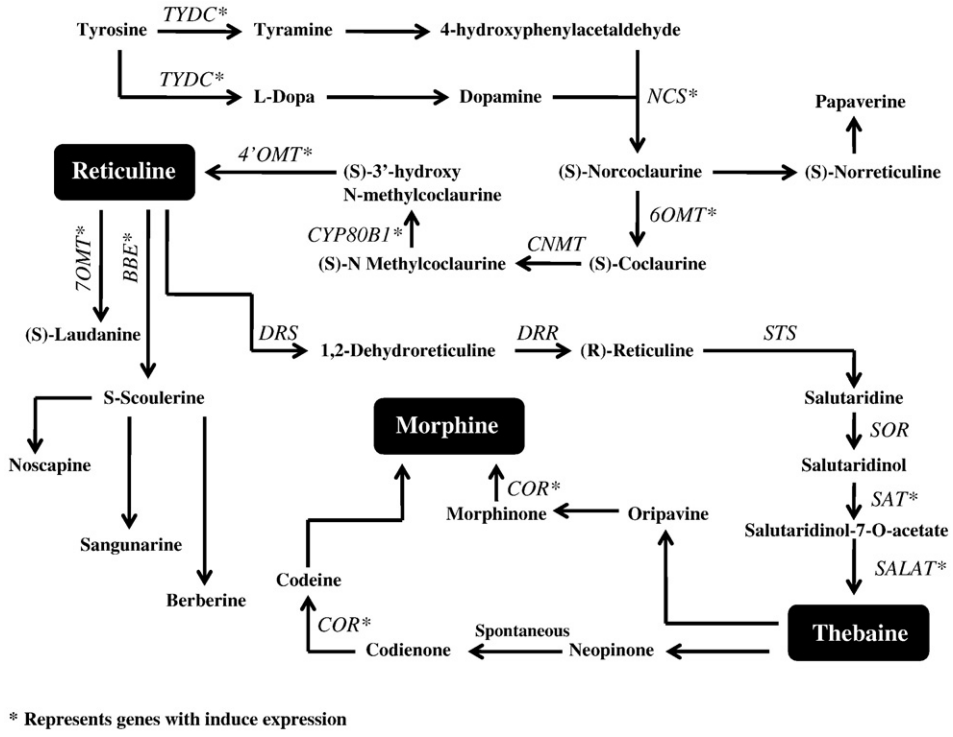

Fig. 4. Alkaloid biosynthetic pathway in *P. somniferum* L. Different genes for which expression was induced in auto-tetraploid are marked with asterisk mark.

cells are larger and would be expected to have a higher level of transcription than lower ploidy cells. Therefore, gross expression of most of the gene would be expected to increase linearly with ploidy on per cell (Albuzio et al., 2006). Similar findings have observed in *Atropa belladonna* in which all of the hyoscyamine converted into scopolamine due to increased expression of gene encoding hyoscyamine-6β-hydroxylase (Yun, 1992).

In conclusion, we have successfully induced tetraploidy in opium poppy plants by colchicine treatment of shoot apical meristem which caused enhancement in opium yield as well as of morphine. The significant enhancement in morphine content was related with increased expression of genes related to biosynthesis pathway. Further analysis of auto-tetraploids progenies through gene expression and metabolom analysis will provide information regarding metabolite flux diversions leading to higher accumulation of alkaloids. Studies of progenies developed in this study with respect to gene expression and metabolite content are in progress which may open up avenues towards the development of hexaploids and amphidiploids for multifold increase in specific alkaloids.

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