Recessive Loci Pps-1 and OM Differentially Regulate PISTILLATA-1 and APETALA3-1 Expression for Sepal and Petal Development in *Papaver somniferum*

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Abstract
The involvement of PISTILLATA (PI) and APETALA (AP) transcription factors in the development of floral organs has previously been elucidated but little is known about their upstream regulation. In this investigation, two novel mutations generated in *Papaver somniferum* were analyzed - one with partially petaloid sepal and another having sepaloid petals. Progeny from reciprocal crosses of respective mutant parent genotypes showed a good fit to the monogenic Mendelian inheritance model, indicating that the mutant traits are likely controlled by the single, recessive nuclear genes named “Pps-1” and “OM” in the partially petaloid sepal and sepaloid petal phenotypes, respectively. Both paralogs of PISTILLATA (PapsPI-1 and PapsPI-3) were obtained from the sepal and petals of *P. somniferum*. Ectopic expression of PapsPI-1 in tobacco resulted in a partially petaloid sepal phenotype at a low frequency. Upregulation of PapsPI-1 and PapsAP3-1 in the petal and the petal part of partially petaloid sepal mutant and down-regulation of the same in sepaloid petal mutant indicates a differential pattern of regulation for flowering-related genes in various whorls. Similarly, it was found that the recessive mutation OM in sepaloid petal mutant downregulates *PapsPI-1* and *PapsAP3-1* transcripts. The recessive nature of the mutations was confirmed by the segregation ratios obtained in this analysis.

Introduction
The MADS-box gene family encodes a series of transcription factors involved in controlling vegetative development in plants, flowering time and the formation of flowers [1,2,3]. Floral organ identity genes were first described in the model angiosperms *Antirrhinum majus* and *Arabidopsis thaliana*, leading to the proposal of the ABC model of flower development [4]. Most of the genes corresponding to these functions, with the exception of *APETALA2*, are members of the MADS-box family of transcription factors [5]. PISTILLATA (*PI*) and its homologs are classified as B-class genes of the MADS-box family and function together with another B-class gene, APETALA3 (*AP3*), by forming heterodimers for regulating petal and stamen development in eudicots [6,7,8,9,10]. The functions of these genes appear to be conserved across the orthologs analyzed among the core eudicots [8,11,12] and monocots [13,14,15]. A considerable amount of knowledge is available about the molecular mechanisms specifying petal identity in *Arabidopsis* and other core eudicot species however there is little functional evidence regarding homologs with similar roles in petal-identity specification outside of the core eudicots [16] leading to a significant knowledge gap concerning plant organ differentiation, growth and development outside of the most well-studied model systems.

Opium poppy (*Papaver somniferum*) has a long history of practical, medicinal use spanning thousands of years and it continues to be one of the world’s most important medicinal plants due to its unique ability to synthesize the drugs morphine, codeine and thebaine and a variety of other biologically active cyclopeptanophenantherene and benzylisoquinoline alkaloids in its seed pods. Drea et al. [16] described the roles of several MADS-box genes involved in petal specification by demonstrating the duplication and sub-functionalization of *AP3* lineage in *P. somniferum*. In poppy, one gene copy influences petal development while the other is responsible for stamen development, contrasting the described role of *AP3* in *Arabidopsis* where *AP3* influences both petal and stamen development. Drea et al. [16] also investigated two paralogs of PISTILLATA (*PapsPI-1* and *PapsPI-2*) and showed that the *PapsPI-1* gene encodes a product containing the PI-motif as well as a sequence extension at the C-terminus whereas the predicted product of *PapsPI-2* lacks the consensus PI-motif [17] at the C-terminus. This truncation is due to a single nucleotide insertion in the 3’ coding region followed by a 2-nucleotide deletion 22 bp downstream that generates a stop codon. Although this domain has been shown to be essential for protein function in *Arabidopsis* PI [18], the *Pisum sativum* PI gene also lacks this conserved domain but has been shown to be capable of rescuing the *Arabidopsis* *pi*-mutant phenotype [19]. In the present investigation we analyzed different genes involved in flower development by utilizing partially petaloid sepal (*Pps-1*) and sepaloid petal (*OM*) mutants that were obtained from the normal sepal and petal phenotypes of I-14 and I-268, respectively.
respectively. The development of Pps-1 has been described earlier [20,21]. In the Pps-1 mutant, a part of the sepal is converted into petal rather than forming a complete sepal (Figure 1) whereas in OM the whole petal is converted into a sepal (Figure 2). These analyses indicate the involvement of different recessive mutations for erroneous interconversion of sepals and petals.

Materials and Methods

Plant Material

Plant material consisted of the Pps-1 genotype of P. somniferum with partially petaloid sepals, which spontaneously originated from the downy mildew (DM)-resistant genotype I-14. The parent genotype I-14 is characterized by narrow leaves with very deep leaf incisions and white flower petals [20]. In Pps-1, the margins of the sepals are modified into petal-like characters (Figure 1). Apart from this, true breeding genotypes I-268 and OM (having mutation ‘OM’) were selected to test the hypothesis that specific genes are involved in organ conversion. OM was detected in the open pollinated population of the genotype I-268 of opium poppy in which the petals are converted into sepal-like organs (Figure 2).

All inbred lines (at least 6 selfing cycles) of mutants and their parents were grown and maintained in the research farm of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India since 2007 and the true-breeding characters were maintained.

Breeding

Previously, the segregation of Pps-1 mutation at a ratio of 3:1 has been shown [20], confirming the involvement of single recessive gene in regulating the partially petaloid character. For segregation analysis of the second mutant, the parent genotype I-268 having normal white petals was crossed with OM mutant having sepaloid petals. The crossing was carried out normally and reciprocally taking both as male or female parents. The F1 and F2 generation plants were scored for sepaloid petal character. The collected seeds were sown in the field in randomized block design with 3 replications and observations were collected on a single plant basis. Chi-square analysis was applied to test the goodness-of-fit for frequency distributions in the F2 generations (Table 1). A 3:1 segregation of the OM character indicated the involvement of nuclear recessive mutation.

RNA isolation for cDNA preparation and cloning of PapsPI gene

Total RNA was isolated from 100 mg of ground tissue samples (from fully developed buds) before anthesis using Trizol reagent (Invitrogen, Cleveland, OH, USA). RNA was converted into cDNA using the ThermoScript RT-PCR System (Invitrogen, USA) and gene-specific primers [16] were used to amplify the PapsPI gene. Amplicons were cloned in pGEM-T easy vector system (Promega) and a total of 20 clones were sequenced for each type of tissue sample (normal sepals of I-14, partially petaloid sepals of Pps-1 and normal petals of both genotypes).

Phylogenetic analysis

Amino acid sequences were aligned (and phylogeny was reconstructed using Bootstrap maximum likelihood method MEGAS [22]) with MUSCLE multiple sequence alignment [23,24].
Table 1. Segregation pattern of the sepaloid petal (OM) mutant in different generations of the reciprocal crosses involving parent genotype I-268 having wild type phenotype.

| Generation | Segregation ratio | Number of observed plants | Mutant type | Chi-Square | P |
|------------|------------------|---------------------------|-------------|------------|---|
| Wild type  | Mutant type OM   |                           |             |            |   |
| P          | 1                | 0                         | 133         | 0.53       | 0.50–0.30 |
| P2         | 106              | 0                         | 110         | 0.53       | 0.50–0.30 |
| 6 I-268    | All plants exhibit white petals with light pink margin | F1 (P1 P2) | 82         | 1.18       | 0.30–0.20 |
| 6 I-268    | All plants exhibit white petals with light pink margin | F2 (P1 P1) | 56         | 0.56       | 0.30–0.20 |
| 6 I-268    | All plants exhibit white petals with light pink margin | F2 (P2 P2) | 14         | 3.1        | 0.10 |

Expressions analysis using quantitative and semi-quantitative RT-PCR

Quantitative RT-PCR was carried out using SYBR Green chemistry (Applied Biosystems, USA) as described earlier [25]. Gene-specific primers were designed with Primer Express software (v2.0; Applied Biosystems, USA) and custom-synthesized from Sigma Aldrich, India. The reactions were carried out in 5 biological replicates on the 7900HT Fast Real Time PCR System (Applied Biosystems, USA) and the specificity of the reactions was verified by melting curve analysis with the thermal cycling parameters: initial hold (50°C for 2 min); initial denaturation (95°C for 10 min); and 40 amplification cycles (95°C for 15 s; and 60°C for 1 min) followed by additional steps (60°C for 15 s, 95°C for 15 s and 37°C for 2 min). Relative mRNA levels were quantified with respect to endogeneous control genes (actin [EB740770] in case of P. somniferum or ubiquitin [U66264.1] in case of Nicotiana tabacum) [26,27]. Sequence Detection System (S.D.S.) software version 2.2.1 was used for relative quantification of gene transcript using the ΔΔ Ct method. Threshold cycle (Ct) values obtained after real time PCR were used for calculation of ΔΔCt value (target-endogeneous control). The quantification was carried out by calculating ΔΔCt to determine the fold difference in gene expression [Δ Ct target - Δ Ct calibrator]. Relative quantity (RQ) was determined by 2 −ΔΔCt. Semi-quantitative RT-PCR was performed by following the protocol of Misra et al [26]. Primers were designed on the basis of P. somniferum (for PapsPI-1, PapsPI-2, PopsAP3-1 and PopsAP3-2) gene sequences. Details of the primers used in the semi-quantitative RT-PCR have been provided in Table S1.

Tobacco transformation

Specific primers were designed to prepare the overexpression construct for the PopsPI-1 gene. XbaI (forward primer) and BamHI (reverse primer) restriction sites were introduced at either sides of the coding sequence. The amplified PCR-product was cloned in pGEM-T Easy vector and the sequence was confirmed. The plasmid containing the coding region was digested with XbaI and BamHI and cloned into pBl121 under the control of the CaMV 35S promoter to yield the final construct 35S::PapsPI-1. Binary vectors with and without the transgene were separately trans-formed into GV3101 strain of Agrobacterium and used to generate transgenic tobacco plants as described [28,29]. Transformants were observed after 3-4 weeks of selection on kanamycin (200 μg ml⁻¹). Regenerated shoots were excised and rooted. Plantlets with well established root system were hardened for 2 weeks, subsequently transferred to soilrite mix (Keltech Energies Limited, India) and irrigated with diluted MS media. Fully acclimatised plantlets were grown in the greenhouse and genomic DNA samples of transgenic tobacco lines were screened by PCR using NPTII and PopsPI-1 specific primers to verify the transfer of transgene cassettes into the transgenic lines. The non-transformed plants and empty vector-transformed plants did not show any amplification (Figure S1).

Results

Expression patterns for genes involved in flowering

Expression level was determined for the four genes of the ABC model in sepal and petals of both the Pps-1 mutant and the wild-type (I-14) through semi-quantitative RT-PCR. Among the genes analyzed, the most significant difference was observed for PopsPI-1 whose expression was significantly higher in the partially petaloid sepal relative to normal sepal of I-14 (Figure 3). Differential expression was not detected in the petals of flowers produced by
The present study also generated another copy of the PapsPI homologous genes in phylogenetic analysis the PapsPI-1 of which, nine were before the stop codon in PapsPI-3. Mutations were also detected in tissues of both I-14 and Pps-1. Additionally, seventeen point single base deletion (adenine). This copy was obtained from 1 sepals and petals of both genotypes) were identical, and the AB13927) [16]. All sequenced amplicons (20 clones each from petals of both genotypes. PCR was carried out using gene-specific normal sepals of I-14, partially petaloid sepals of Pps-1 and normal petals of both genotypes. Expression was higher than PapsPI-1 in the normal sepal phenotype demonstrating the recessive nature of the typical mutant character (OM). This also indicated the absence of cytoplasmic control of the mutant trait (sepaloid petal). The segregation pattern of the F2 populations of both reciprocal crosses also provided a good fit of the monogenic Mendelian ratio (P = 0.80-0.70) for the normal wild type (I-268) and the mutant (OM) characters indicating that the mutant trait is controlled by a single recessive nuclear gene “OM” [Table 1]. Interestingly, PapsPI-3 expression was higher than PapsPI-1 expression in the normal sepals of I-268 and the sepaloid petal of OM, whereas in the normal petal of (I-268) PapsPI-1 expression was higher than PapsPI-3 expression. PapsPI-1 expression in the normal petal was higher than that in the sepals [Figure 8]. Relative expression of PapsPI-1 in the sepal and sepaloid petal (I-268 and OM, respectively) was comparable to the PapsPI-1 expression in the sepals of I-14 and sepal or the petaloid-devoid sepal part of the partially petaloid sepal of Pps-1.

Cloning of PapsPI gene copies/paralogs and phylogenetic analysis

PapsPI-1 was cloned from cDNA transcribed from the RNA of normal sepals of I-14, partially petaloid sepals of Pps-1 and normal petals of both genotypes. PCR was carried out using gene-specific primers derived from GenBank sequence EF071994 (amino acid ABO13929) [16]. All sequenced amplicons (20 clones each from sepals and petals of both genotypes) were identical, and the PapsPI-1 sequence (KF550916) from this investigation was 99% similar to the earlier reported PapsPI (EF071994) [16]. Interestingly, the present study also generated another copy of the PapsPI gene (PapsPI-3, deposited under Accession No. F550917) that had a stop codon introduced at the 151 amino acid position due to a single base deletion (adenine). This copy was obtained from partially petaloid sepals (of Pps-1), normal sepals (of I-14) and petal tissues of both I-14 and Pps-1. Additionally, seventeen point mutations were also detected in PapsPI-3 as compared to PapsPI-1, of which, nine were before the stop codon in PapsPI-3. In phylogenetic analysis the PapsPI-1 sequence of this investigation (KF550916) and the one reported earlier (AB013929) [16] clustered together but the sequence of PapsPI-2 reported earlier (nucleotide EF071995, amino acid AB013928) [16] was different from that of PapsPI-3 reported in this investigation (KF550917; Figure 4).
PapsAP3-1 was able to heterodimerize with PapsPI-1 like in Arabidopsis and Antirrhinum [7,16,33]. Although PapsPI-1 is required to specify petal as well as stamen identity, PapsAP3-1 functions primarily in the specification of petals and PapsAP3-2 functions primarily in the specification of stamens [16]. Accordingly, we could observe the differential expression of PapsAP3-1 in the

Figure 4. Unrooted maximum likelihood tree comparing the amino acid sequences of P. somniferum PI-1(KF550916), with PI-1 (PISTILLATA) reported from other species. The symbols for the plants are provided along with the GenBank accession numbers in brackets. Agapanthus praecox Ap (BAC66962); A. praecox, Ap (AAU15475); Akebia trifoliata At (AAT46101); Ananas comosus Ac (ADY39748); Aconitochilus formosanus Af (ACD85087); A. formosanus Af (ACD85088); Aquilegia vulgaris Av (ABP01801); Brassavola nodosa Bn (ACD85092); Cricus sativus Cs (ABB22779); C. sativus Cs (ABB22780); Cymbidium faberi Cf (ADW76860); Dendrobium hybrid cultivar Dhc (ACD85096); Dendrobium moniliforme Dm (ABW96391); Galeola falconeri Gf (ACD85100); Habenaria petelotii Hp (ACD85102); H. petelotii Hp (ACD85103); Liparis distans Ld (ACD85106); Ludisia discolor Ld (ACD85107); L. discolor Ld (ACD85108); Michelia alba Ma (AFN68719); Magnolia amoena Ma (AFN68770); M. championii Mc (AFN68738); M. championii Mc (FN68738); M. coca Mc (AFN68755); M. conferta var. chinigii Mc (AFN68728); M. crassipes Mc (AFN68722); M. crassipes Mc (AFN68723); M. cylindrica Mc (AFN68762); M. cylindrica Mc (AFN68763); M. dandyi Md (AFN68727); M. delavayi Md (AFN68745); M. ducloxi Md (AFN68720); M. ducloxi Md (AFN68721); M. figo Mf (AFN68733); M. fordiana Mf (AFN68737); M. grandiflora Mg (AFN68766); M. grandiflora Mg (AFN68767); M. hookeri Mh (AFN68758); M. hookeri Mh (AFN68759); M. insignis Mi (AFN68730); M. insignis Mi (AFN68731); M. insignis Mi (AFN68732); M. liiflora Mi (AFN68769); M. officinalis subsp. biloba Mosb (AFN68745); M. officinalis subsp. biloba Mosb (AFN68746); M. paenetalauma Mp (AFN68724); M. paenetalauma Mp (AFN68726); M. salicifolia Ms (AFN68734); M. salicifolia Ms (AFN68735); M. sprengeri Ms (AFN68743); M. sprengeri Ms (AFN68744); M. stellata Ms (AFN68750); M. wufengensis Mw (AFN75882); M. wufengensis Mw (AFN75883); M. yunnanensis My (AFN68740); M. zeii Mz (AFN68760); Oncidium hybrid cultivar Ohc (ACD85113); Papaver somniferum Ps (KF550916); P. somniferum Ps (KF550917); P. somniferum Ps (ABO13927); P. somniferum Ps (ABO13928); Papiopeidium hybrid cultivar Phc (ACD85117); Passiflora edulis Pe (AER30449); Persoia americana Pa (ABO62863); Phaius tancarvilleae Pt (ACD85121); Phalaeonopsis hybrid cultivar Phc (AAV28175); P. hybrid cultivar Phc (AAV28490); P. hybrid cultivar Phc (AAV28491); Populus tomentosa Pt (AGL09298); Thalictrum dioicum Td (AAW78031); T. dioicum Td (AAW78032); Trochodendron aralioides Ta (ABQ59546). doi:10.1371/journal.pone.0101272.g004

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partially petaloid sepal of Pps-1 genotype compared to the normal sepal of I-14. This confirms the involvement of PapsPI-1 and PapsAP3-1 in petal development in *P. somniferum*.

Further, to find out the role of *PISTILLATA* in the petaloid and normal (petaloid-devoid) part of the partially petaloid sepal, the expression of *PapsPI-1* and *PapsPI-3* was compared in petals, sepal, the petaloid portion of the partially petaloid sepal and normal (petaloid-devoid) part of the partially petaloid sepal. Two *PI* gene paralogues (*PapsPI-1* and *PapsPI-3*) were detected in this investigation instead of *PapsPI-1* and *PapsPI-2* as reported earlier [16]. The nucleotide sequence of *PapsPI-3* is different from both *PapsPI-1* and *PapsPI-2*. The expression of *PapsPI-3* was also observed to be always higher when compared to *PapsPI-1* in the normal sepal of I-14 and the normal (petaloid-devoid) part of the partially petaloid sepal of Pps-1, whereas a significantly higher expression of *PapsPI-1* was observed compared to *PapsPI-3* in the petals of both the genotypes and in the petaloid part of the partially petaloid sepal of Pps-1. As described earlier, protein produced by the gene *PapsPI-2* does not dimerize with *PapsAP3-1*, *PapsAP3-2*, *PapsPI-1* or *PapsPI-2*. However, this does not obviate the possibility of interaction with other MADS-box gene products that affect its function [16]. Hence the role of *PapsPI-3* cannot be ruled out in partially petaloid sepal character of the Pps-1 flowers.

Ectopic expression of *Antirrhinum* Glo (*GLOBOSA*) in tobacco leads to petaloid sepals, and ectopic expression of both Def (DEFICIENS) and Glo leads to the almost complete conversion of sepals to petals [34]. Glo and Def are *PISTILLATA* and *APETALA3* orthologs from *Anterhimum majus*. Ectopic expression of a single homeotic gene, the *Petunia* gene GREEN PETAL, has also been described as sufficient to convert sepals to petaloid organs [35]. In this investigation, *PapsPI-1* was expressed ectopically in tobacco under the CaMV 35S promoter and of the twenty transgenic plants (that flowered) screened, only one was observed to be producing flowers with partially petaloid sepal character (*Figure 7*). When some of the transgenic plants were analyzed, all showed *PapsPI-1* gene integration as well as expression in sepals (*Figure S2*). But, the expression of *PapsPI-1* in the sepals of transgenic plant producing flowers with the partially petaloid sepal was highest. Hence, in the case of the mutant Pps-1 of *P. somniferum* the overexpression of *PapsPI-1* in the sepal leads to their conversion to a petal-like phenotype, as corroborated in part by ectopic expression of *PapsPI-1* in tobacco. However, considering the small difference in the relative quantity (RQ) values between the partially petaloid sepal phenotype (II: 5.712) and the transgenic plant having normal flowers (Ta: 5.378), other reasons responsible for the low frequency of *PapsPI-1* transgene phenotype in tobacco cannot be ruled out. It is possible that the very small difference in expression (about 6%) might be the tipping point for initiating a developmental switch. But without other lines showing this phenotype, this is only a speculation and it is also possible that the random insertion of the construct in “Ta” might have caused a gene disruption resulting in the phenotype unrelated to the expression of *PapsPI-1*. The analysis of the transgenic lines Ta, Tb, and Tc, not showing the desired phenotype, preclude the possibility of any undesirable effect of the CaMV 35S promoter, which has been described in the past for not yielding desired...

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**Figure 5.** Comparison of quantitative expression levels of *PapsPI-1* and *PapsPI-3* in the petals and sepal of the genotypes Pps-1 and I-14. The Y-axis represents relative quantities equilibrating the expression of *PapsPI-1* in I-14 sepal as 1RQ value. Data represent mean ± standard error of 3-5 biological replicates. In X-axis, the names of the genotypes (Pps-1 and I-14) are followed by the organs (S: sepal; P: Petal; Se: Sepaloid part of the sepal and Pe: Petaloid part of the sepal) and *PapsPI* gene expression (1: *PapsPI-1* and 3: *PapsPI-3*). Shaded bar represents Pps-1 genotype. doi:10.1371/journal.pone.0101272.g005

**Figure 6.** Comparison of quantitative expression levels of *PapsAP3-1* in the sepal of genotypes Pps-1 and I-14. The Y-axis represents relative quantities equilibrating the expression of *PapsAP3-1* in I-14 sepal as 1RQ value. Data represent mean ± standard error of 3-5 biological replicates. On X-axis, S: sepal; Se: Petaloid-devoid part of the partially petaloid sepal. doi:10.1371/journal.pone.0101272.g006
phenotypes, especially for transcription factors expressed under its control [36]. One specific example of misexpression of a component of the flowering regulatory network is ectopic overexpression of the *LFY* gene from *Arabidopsis* [37]. Heterologous expression of transcription factors can also be negatively influenced by the species chosen for overexpression [38]. There may be several reasons for our observing very low frequency of abnormal phenotypes, but the occurrence of a petaloid sepal phenotype while overexpressing the *PapsPI-1* gene cannot be ruled out. The *Pps-1* recessive mutation described earlier [20], was confirmed in this investigation to be controlling the expression of *PapsPI-1* and *PapsAP3-1*, which is higher in petals of both the plants as well as in the petaloid part of Pps-1 sepal compared to sepal of I-14 and normal (petaloid-devoid) part of the mutant Pps-1 sepals. The functional significance of the heterodimer formed by PapsAP3-1 and PapsPI-1 in determining the petal structure [16] cannot be ignored and in this analysis we observed overexpression of *PapsAP3-1* and *PapsPI-1* in the petaloid part as compared to the normal (petaloid-devoid) part of the sepal in Pps-1. Genes encoding products that function as key regulatory components,
such as transcription factors, as well as those participating in large multi-protein complexes (e.g. MADS-domain proteins) [29], appear to be preferentially maintained owing to the requirement for a stoichiometric balance with other components in the pathway [30,40]. Hence, it seems that the recessive PpS-1 locus might be influencing the expression of PapsAP3-1 and PapsPI-1 in sepals during development (Figures 5, 6). As the proteins encoded by PapsAP3-1 and PapsAP3-2 can heterodimerize with PapsPI-1, but PapsAP3-2 can also homodimerize [16], the role of PapsPI-3 cannot be ruled out for petaloid conversion of sepals although this type of gene (PapsPI-3) has been described to be having limited role in petal morphology as compared to PapsPI-1.

Hose in Hose mutants of primrose and cowslip have been found to show dominant homeotic conversion of sepals to petals [41]. The demonstration that in some cases up-regulation of a single B-function MADS box gene can lead to the development of petaloid sepals is consistent with the inheritance of the Hose in Hose as a single dominant locus [41]. In contrast, the CHORIPELATA and DESPENTEDADO mutants of Antirrhinum are inherited as recessive mutations, which also result in the conversion of sepals to petals [42]. In the present study, the mutations controlling up-regulation of PapsAP3-1 and PapsPI-1 in conversion of sepals to petal (Pps-1) and down regulation of PapsAP3-1 and PapsPI-1 in the conversion of petal to sepal (OM) were confirmed to be recessive in nature. In conclusion, this study indicates a differential pattern of regulation for flowering-related genes in various whorls.

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