

Investigation of genes involved in scent and color production in *Rosa Damascena* Mill

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Abstract

Rosa damascena Mill., commonly known as the King Flower, is a fragrant and important species of the Rosaceae family. It is widely used in the perfumery and pharmaceutical industries. The scent and color of the flowers are significant characteristics of this ornamental plant. This study aimed to investigate the relative expression of *MYB1*, *CCD1*, *FLS*, *PAL*, *CER1*, *GT1*, *ANS* and *PAR* genes under two growth stages (S1 and S2) in two accessions. The *CCD1* gene pathway is highly correlated with the biosynthesis of volatile compounds. The results showed that the overexpression of *MYB1*, one of the important transcription factors in the production of fragrance and color, in the pink accession of sample S2 increased the expression of *PAR*, *PAL*, *FLS*, *RhGT1*, *CCD1*, *ANS*, *CER1*, and *GGPPS*. The methyl jasmonate stimulant had a positive and cumulative effect on gene expression in most genes, such as *FLS* in ACC.26 of the S2 sample, *RhGT1*, *MYB1*, *CCD1*, *PAR*, *ANS*, *CER1*, and *PAL* in ACC.1. To further study, a comprehensive analysis was performed to evaluate the relationship between the principal volatile compounds and colors. Our data suggest that the rose with pink flowers had a higher accumulation content of flavonoids and anthocyanin. To separate essential oil compounds, GC/MS analysis identified 26 compounds in four samples. The highest amount of geraniol, one of the main components of damask rose, was found in the dark-pink flower, 23.54%, under the influence of the methyl jasmonate hormone.

1. Introduction

Rose (*Rosa damascena* Mill.) is a cultivar of rose growing in most parts of Iran and is known for its floral fragrance and excellent color characteristics. It is the most economically valuable rose species in the production of aromatic compounds, which contains high levels of monoterpene alcohols such as geraniol, citronellol, and nerol, as well as phenyl ethyl alcohol [1]. The essential oil of *R. damascena* is a complex mixture of various compounds with a wide range of uses and in the food industry, it prevents food spoilage due to its harmless natural oil and antioxidant, antifungal, and antibacterial properties [2]. In the aromatic materials and perfumery industries, *R. damascena* essential oil is used to create higher-quality perfumes [3, 4]. Additionally, due to its unique properties, *R. damascena* is a suitable choice for studying the different compounds of biosynthesis [5].

R. damascena is divided into two main flowering groups; summer damascene and autumn damascene. The latter has white flowers and less fragrance, while the former has more popularity, more use, and higher economic value [6, 7]. In this study, multiple genotypes of *R. damascena* in Iran are specified, but the exact number is not stated in the given context [3]. Despite limited information on the regulation of floral fragrance biosynthesis, only a few transcription factors, *ODORANT1*, *EOBII*, and *PhMYB* are involved in the regulation of volatile molecule biosynthesis in flowers [8]. The most abundant transcription factors in rose are the *MYB* family [9]. The *MYB1* gene is involved in the biosynthesis of rose fragrance [10], regulation of the relative expression of *PAR*, and the production of the 2-PE component are also involved in the suppression or expression of target genes in color and smell [11]. Certain studies suggested that *MYB* is an important TF that regulates anthocyanin synthesis in

ornamental flowers [12–14]. Sagawa et al. (2016) stated that a transcription factor called *R2R3-MYB* in the *Mimulus lewisii* plant plays an essential role in carotenoid metabolism.

The *GGPPS* gene may be involved in the biosynthesis of volatile monoterpenes in the flowers of *R. damascena*. This gene can be important for adjusting the secondary metabolism of the aromatic components of *R. damascena* [15]. The *CCD1* (carotenoid cleavage dioxygenases) gene pathway is closely related to the biosynthesis of volatile compounds [16]. Increasing the expression of the *CCD1* gene in the petals leads to discoloration and an increase in the amount of ketone compounds in the essential oil [11]. The *CER1* gene is an aldehyde decarboxylase encoder [17], resistant to cold and drought stress, and the up regulation of the expression of this gene corresponds to an increase in the content of alkanes [18]. This gene is involved in the synthesis of the precursor aromatic compounds (VLC fatty acids) in the endoplasmic reticulum (ER) [19].

ANS and *RhGT1* are key genes in anthocyanin biosynthesis [18]. Glycosylation is a prerequisite for anthocyanin biosynthesis, and *RhGT1*, as a new glycosyltransferase, shows a novel pathway for anthocyanin biosynthesis [20]. Anthocyanin and other flavonoid components are produced through the phenylpropanoid pathway. Phenylalanine, the main precursor of flavonoids, is converted into required derivatives by the enzyme Phenylalanine ammonia-lyase (*PAL*). Dihydroflavonols can be transformed into flavonols and anthocyanins. The production of flavonols is catalyzed by the enzyme flavonol synthase (*FLS*), while dihydro flavonol 4-reductase (*DFR*) is the first enzyme that takes part in the step-by-step cascade of anthocyanin synthesis. Stabilization and alteration of anthocyanin molecules affect the final color of the flower [21]. In addition, *ANS* gene is a major gene in anthocyanin biosynthesis, and overexpression of this gene may increase anthocyanin pigments in petals [11]. The main secondary metabolites of *R. damascena* are phenolic acids (gallic, caffeic, chlorogenic, and coumaric) and flavonoids (rutin, quercetin, kaempferol, and apigenin). Kanani *et al.* (2021) reported that the highest content of flavonoids was estimated in the fully open flower stage. In addition, the activity of phenylalanine ammonia-lyase (*PAL*) enzyme showed a positive association with the production of some flavonoids.

Jasmonic acid (JA) and its derivatives are key messenger molecules and play an important role in many biological processes, such as growth inhibition, senescence, and plant defense [22]. Methyl jasmonate, JA derivative, has been used to increase the production of secondary metabolites by inducing defense responses in many species, such as volatile terpenoids in *Amomum villosum* [23], triterpenes in *Euphorbia pekinensis* [24] and Tropane alkaloids in *Hyoscyamus niger* [25].

With these considerations in mind, the current study was conducted to investigate the effectiveness of direction of MVA and MEP pathway genes in the biosynthesis of aromatic compounds and the color of the rose during two important stages of flower development (bud and young flower (fully open flower)) under the influence of two different concentrations of methyl jasmonate. Additionally, relative expressions of *PAL*, *PAR*, *FLS*, *MYB1*, *CER1*, *GT1*, *CCD1*, *ANS*, and *GGPPS* genes were measured in the treated samples. Finally, to gain a deeper understanding of flower growth stages, gas chromatography-

mass (GC-MS) and real-time PCR techniques were employed to figure out phytochemical and molecular changes.

2. Materials and methods

2.1. Plant materials

The petal samples were collected from two accessions of *R. damascena*, in two colors, white and dark-pink, grown at the research station on the Agricultural Campus of the University of Tehran. Flower buds were harvested at two important flower developmental stages (S1 = budding stage, S2 = full opening stage) according to Rasouli et al. (2010, Table 1). Methyl jasmonate treatment was applied at two levels of zero [26] and 300 μ M [27] in an ethanol solution to study their effect on gene expression. The control plants were sprayed with distilled water containing a small amount of ethanol and samples were collected after 48 hours of spraying. The collected petals were immediately placed in paper bags and transported to the laboratory for essential oil extraction and physiological evaluation. For molecular investigations, the petals were frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

2.2. Determination of Total Flavonoid Content (TFC)

Total Flavonoid Content (TFC) was calculated based on the method introduced by Khorasani Esmaeili et al. (2015) with slight modifications. Solutions of 4% NaOH, 5% NaNO₂, and 10% AlCl₃ in distilled water were used to test. AlCl₃ solution was prepared by gradually adding AlCl₃ to distilled water under a the Laminar Hood carefully and steadily. 25 μ L of each sample solution, 100 μ L of distilled water, and 7.5 μ L of 5% NaNO₂ solution (in eight replicates) were added to a 96-well plate. After 6 min, 7.5 μ L of 10% AlCl₃, 100 μ L of 4% NaOH, and 0 μ L of distilled water were added to each well. The plate was covered with aluminum foil, and the absorbance of the solutions was read after 15 min. Absorbance was measured at 510 nm with an ELISA Reader. TFC was expressed as mg of rutin equivalents (CTE) per gram of extract dry matter, using a calibration curve of rutin.

2.3. Determination of Total Anthocyanin Content (TAC)

100 mg of petal tissue was dissolved in 100 cc of acidic methanol (methanol and hydrochloric acid at a volume ratio of 99:1) using a Porcelain Mortar. The desired solution was kept in the dark for 24 h at a temperature of 25°C. Then it was centrifuged for 15 min at 4000 rpm, and the absorbance of the supernatant was read at a wavelength of 530 nm by a spectrophotometer. Using the formula, $A = \epsilon bc$ and considering the extinction coefficient (ϵ) equal to 33000, concentration of the results was presented in μ M per gram of fresh weight. Where A = absorption of the high solution, b = cuvette path length, which is equal to 1, and c = concentration of anthocyanin per mole of fresh weight of the sample [28].

2.4. Essential oil extraction & GC/MS analysis of the EOs compounds

To extract the essential oil by hydro-distillation method, 250 gr of fresh petals were separated using Clevenger Apparatus according to the method recommended by [29]. Then, the volatile oil layer on top of

the aqueous layer was collected in a glass vial. The amount of essential oil was obtained based on the percentage ratio (v/w). The percentage of each component of the essential oil was determined using GC-MS instrument, Agilent 7890A/5975C model, equipped with a mass spectrometer detector and CTC CombiPAL for liquid and headspaces. In the present study, a silica column (Resteck HP-5MS) made of 5% phenyl methyl silox and injector temperature of 325 °C were used with the following features: 60 m length, 320 µm diameter, and 0.25 µm of corn size. The injector ran in the splitless mode with a temperature of 280 °C. The temperature of the oven was set at 50 °C for 2 min, following an increased slope of 3 °C min⁻¹ to 240 °C for 12 min, and then held to 290 °C at a rate of 20 °C min⁻¹. The solvent was delayed for 4 min, for a total run time of 79.833 min. The carrier gas was helium (purity 99.999%) and flowed with a flow rate of 1.0 mL min⁻¹. Peaks were analyzed with Mass lab software at one scan per second speed. Using the calculation of the Quartz coefficient (injection of normal n-alkane hydrocarbons (c11-c28) under the same conditions as the injection of essential oils), identifying essential oil compounds and checking the mass spectrum proposed by the GC-MS libraries, including "NIST05a.L & wiley7n.l", were compared [30-32].

2.5. Extraction of ribonucleic acid (RNA), cDNA synthesis, and design of primers

Extraction of total ribonucleic acid from the tissues of young flower petals and buds was conducted using the manual CTAB method according to the slightly modified instructions [33] and was treated with DNaseI (KiaGen company) to remove genomic contamination. The quantity and quality of RNA were determined by nanodrop and 1% agarose gel electrophoresis, respectively. To synthesize cDNA from RNA, Parstos cDNA synthesis kit was used. Primers were designed for the *Actin* gene (as reference gene: KT965025.1), using Primer3 software. The proposed primers were evaluated using Oligo Analyzer online software in terms of having key features (Self-Dimer, Hetero-Dimer, and Hairpin) and primer binding temperature. The pair of primers for the actin gene as a housekeeping gene and the primer for the studied genes are given in Table 2.

2.6. Real-time PCR analysis

The SYBR® Green Real-Time PCR Master Mix kit of (Parstous company), which has Syber-green, fluorescent dye, was used to perform the qRT-PCR test. The real-time PCR reaction was performed using the Rotor Q-gene device from Qiagen Company, according to the instructions kit. The *Actin* housekeeping gene was considered as internal control and for data normalization. The reaction program included 95°C for 15 minutes and 40 cycles: 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 20 seconds. All reactions were mixed, each with two technical replicates for statistical analysis, and the relative expression of genes was calculated using the formula [34].

2.7. Plotting Heatmap and Dendrogram

In this research, with the aim of investigating the pattern of similarity between physiological parameters and genetic variables, a cluster analysis was conducted. For clustering, the heatmap package in the R software was used, and the distance matrix was calculated using the Ward method [35]. Information

related to genes was placed in columns and samples in rows. Row clustering was represented with a dendrogram, and subsequently, different colors were assigned to genes to display expression values. Ultimately, a heatmap was drawn based on the data matrix and clusters. Colors were adjusted based on data values to make patterns and differences easily observable [36].

3. Result

3.1. Extraction of essential oils and evaluation of volatile compounds (VOC)

The essential oil extracted from 250 grams of fresh *R. damascena* flowers had a delightful smell, which plays a significant role in attracting insects for pollination and is used in the perfumery and cosmetics industries. Separation and identification of essential oil compounds were conducted using GC/MS analysis. In general, 26 compounds were identified in four samples (Table 3). Ten volatile compounds were found in the full opening stage of *R. damascena*, with the principal components being Linalool, Nerol, β -Citronellol, Geraniol, Nonadecene, and Heneicosane, while α -Pinene, Geranyl acetate, Tricosane, and Pentacosane were minor ones. Only a small number of monoterpenes and sesquiterpenes were found, whereas a large number of aliphatic components were found. The effect of methyl jasmonate treatment led to an increase in the volatile compounds such as α -Pinene, Geraniol, β -Citronellol, Nerol, Linalool, β -Myrcene, β -Pinene, Sabinene, and Geranyl acetate in white flowers, and Geraniol and Geranyl acetate in pink flowers. The results showed that the production rate of Monoterpene compounds was 28.32%, Sesquiterpene 2.14%, and aliphatic 48.95% in white petals after 48 hours of the application of the hormone methyl jasmonate. Similarly, the application of this hormone in dark-pink petals led to a decrease of 42.73% in terpenoid compounds and an increase of 40.99% in aliphatic compounds. The highest number of volatile compounds, including Linalool (1.68%), β -Citronellol (7.98%), and Nerol (3.38%), were obtained in pink flowers. Meanwhile, the highest amount of geraniol (23.54%) was obtained from pink flowers under the influence of the methyl jasmonate hormone, which may have potential applications in various industries.

3.2. Total Flavonoid Content (TFC)

The total flavonoid content of the essential oil was determined according to [37] using the aluminum chloride colorimetric method with slight modifications and rutin as the standard. The results were expressed as mg of rutin equivalents per every gram of dry weight of the plant (mg RE/g Dw). According to the results, the highest amount of flavonoid was estimated in the dark-pink petal, so the total flavonoid content (Fig. 3) in the dark-pink and white *R. damascena* petals were 63.6 and 39.8 mg RE/g Dw, respectively.

3.3. Total Anthocyanin Content (TAC)

The results of the total anthocyanin content showed that the amount of TAC in hot-pink petals (37.48 μ mol/g DW) and white petals (2.12 μ mol/g DW) were the same. However, as the number of flavonoids in

pink petals was higher than that of white ones (Fig.1), the amount of anthocyanin in pinks was 18 times higher than that of whites.

3.4. Gene expression analysis

3.4.1. Expression of genes (*MYB1*, *CCD1*, *GGPPs*) involved in fragrance and color in petals in two growth stages

The expression pattern of the *CCD1* gene in two different accessions was found to be almost identical. The expression of this gene in ACC.1 was higher than in ACC.26, and the highest amount of expression was recorded for S1 stage under the influence of methyl jasmonate stimulus (30-fold increase). Data analysis showed a significant interaction effect ($P > 0.01$) between genotype and different growth stages (S1 and S2). These results were in accordance with the biochemical data, as the number of flavonoids and anthocyanins in ACC.1 petal organ was higher compared to ACC.26. Furthermore, the expression of the *CCD1* gene was found to be positively correlated with the number of ketone compounds in the petals. The analysis of *MYB1* gene expression showed a decrease in ACC.26 but an increase in ACC.1 in the S2 compared to the control, suggesting more fragrance and color. The effect of methyl jasmonate was found to be positive and increasing in ACC.1, leading to a 59-fold increase in gene expression in the juvenile stage compared with the control. In contrast, in the bud stage, methyl jasmonate had only a partial effect. In ACC.1, the relative expression of the *GGPPS* gene increased by two times compared to the control in the S2 growth stage, while methyl jasmonate had not significant effect on gene expression. On the other hand, in ACC.26, the relative expression of the *GGPPS* gene only experienced a slight rise in the S2 under the influence of methyl jasmonate, whereas the effect of methyl jasmonate on gene expression was reduced in the first growth stage (Fig. 4).

3.4.2. Expression of *PAL*, *CER1*, and *PAR* genes (involved in aroma) during two flower developmental stages

The results of analyzing the gene expression regarding scent in two control conditions (without methyl jasmonate treatment) and the conditions treated with methyl jasmonate (300 μ M concentration 48 hours before harvest) in *R. damascena* showed that in ACC.26)white flowers(, the highest expression of *PAL* gene was in the bud stage and methyl jasmonate had no significant effect on increasing gene expression. Also, in ACC.1 (bright pink petals), gene expression was higher in the S2 stage, and the effect of methyl jasmonate in both biological stages led to an increase in the expression of the related gene. Sample TS1 had the highest relative expression (2-folds) compared to the control in the *PAR* gene. Methyl jasmonate elicitor was effective in the bud growth stage of ACC.1 while relative expression experienced a decrease compared to the control in other samples. The relative expression level of all *CER1* gene samples decreased in comparison with the control, except for sample TS2, which had an increase in the biological stage of fully open flower in Hot-pink accession under the influence of an abiotic elicitor. The frequency of the *CER1* gene transcript, which is predicted to function in the synthesis of alkanes, was highly induced (20-Folds) by methyl jasmonate treatment (Fig. 5).

3.4.3. Expression of *FLS*, *GT1*, and *ANS* genes (involved in petal color) in two growth stages

The analysis of gene expression connected to scent in *R. damascena* showed that in ACC.26 (white flowers), the highest expression of the *PAL* gene occurred in the bud stage, and methyl jasmonate had no significant effect on gene expression. In contrast, in ACC.1 (bright pink petals), gene expression was higher in the S2 stage, and the effect of methyl jasmonate increased gene expression in both growth stages. The highest relative expression of the *PAR* gene was seen in sample TS1 (a two-fold increase compared to the control). The methyl jasmonate elicitor was effective in the bud growth stage of ACC.1, while the relative expression decreased compared to the control in the other samples. The expression level of the *CER1* gene decreased in all samples except for sample TS2, showing an increase in the fully open flower stage of Hot-pink accession under the influence of an abiotic elicitor. The frequency of the *CER1* gene transcript, which is involved in the synthesis of alkanes, was highly induced (a twenty-fold increase) by methyl jasmonate treatment (Fig. 6).

3.5. Correlation and relationship between Genes and Parameters

3.5.1. Cluster analysis

The heatmap analysis revealed a categorization of the studied genes into two main clusters, prominently visible at the top and bottom. Despite the lack of a clear segregating pattern for these clusters or conditions, the need for enhanced visual analysis was underscored. The first cluster encompassed four genes (*GT1*, *GGPPS*, *MYB1*, and *ANS*), while the second cluster included five genes (*CER1*, *PAL*, *CCD1*, *PAR*, and *FLS*), all grouped based on their high gene expression. The horizontal cluster analysis, comprising five groups, indicated that white (W) and pink (P) petals each corresponded to a distinct cluster, albeit with exceptions. For instance, PS1, representing the bud stage of pink petals, was grouped with white petals, exhibiting low and negligible gene expression, akin to other white petals. An exception was observed in a white petal sample that demonstrated an increase in *FLS* gene expression, potentially attributable to the influence of the methyl jasmonate stimulant. Clustering based on the impact of the methyl jasmonate stimulant revealed that the genes *FLS*, *CER1*, *PAL*, *CCD1*, and *PAR* all fell within a single group, exhibiting the highest gene expression. In conclusion, the PS2 sample emerged as the superior specimen, characterized by a substantial upregulation in the expression of genes *GT1*, *GGPPS*, *MYB1*, and *ANS* (Fig.7).

4. Discussion

The essential oil compounds of *R. damascena* were separated and identified using GC/MS. In total, 26 compounds were found in four samples (Table 3). Ten volatile compounds were found in the headspace of *R. damascena*, which had the highest peaks as shown in Figs. 1 and 2. The main components of the volatile compounds were Linalool, Nerol, β -Citronellol, Geraniol, Nonadecene, and Heneicosane, while α -Pinene, Geranyl acetate, Tricosane, and Pentacosane were spotted as minor components. Only a small number of monoterpenes and sesquiterpenes were found, while numerous aliphatic components were present. Methyl jasmonate treatment led to an increase in the levels of α -Pinene, Geraniol, β -Citronellol,

Nerol, Linalool, β -Myrcene, β -Pinene, Sabinene, and Geranyl acetate in white flowers, as well as Geraniol and Geranyl acetate in pink flowers.

Geraniol and linalool are acyclic monoterpenoids, while nerol is an acyclic monoterpenoid alcohol found in numerous plants' essential oils. These compounds are widely used in the cosmetics and perfume industries due to their desirable scent. Valid medicinal properties, such as tumor cell growth inhibition in geraniol, antifungal effect in nerol, and anticonvulsant, antimicrobial, anti-inflammatory, and neuroprotective properties in linalool, have been reported for these compounds (Liu et al., 2021). Studies have shown that the amount of geraniol varies from 10 to 45 percent, and the climatic conditions, stresses, and growth stages of the plant effectively synthesize this substance in the *R. damascena* petal organ [38]. In the current study, we perceived a 1.5% increase for geraniol under the effect of elicitor compared to the condition without elicitor in the pink flower. Therefore, it is concluded that this abiotic stress had a positive and increasing effect on the amount of geraniol.

Citronellol is a precursor to the aromatic composition of rose oxide and contributes to the sweet smell of essential oil. This compound is more important than geraniol in the perfumery industry [39]. A study on the volatile compounds of roses showed that the principal compounds of these oils are citronellol, geraniol, nerol, nonadecane, heneicosan, and linalool. The study also demonstrated that the growth and location of flowers, i.e., climatic conditions, have a significant impact on the production of essential oils (volatile compounds) [40].

The analysis of *Trachyspermum ammi* L. essential oil compounds showed that the production rate of alpha terpene, the precursor of monoterpenes, increased after 24 hours of applying the methyl jasmonate hormone. The methyl jasmonate elicitor stimulates the biosynthesis pathway of secondary compounds and affects the expression level of the MEP pathway genes one day after the hormone application, which increases the production of monoterpenes [41]. Previous research has shown that citronellol, geraniol, nerol, linalyl acetate, and dihydro citronellol acetate are the main components of rose essential oil, and their amount increases during the flower development process. The quantity of these compounds decreases at the end of flowering during flower aging [15], which is consistent with the studies conducted during this review.

Anthocyanins, which are one of the six broad groups of plant phenols, are the main flower pigments in higher plants [42, 43]. As flavonoids, these water-soluble pigments have a wide range of biological functions and positive effects on human health [44, 45]. In natural conditions, anthocyanins are often found in the form of anthocyanin glycosides [44] and are biosynthesized through the phenylpropanoid pathway, a branch of the shikimate pathway [46]. The color stability of anthocyanins is influenced by various factors, including the structure of the pigment, pH, temperature, oxygen, light, and water activity. The alteration of the anthocyanin structure is pH-dependent [47], and the color change of anthocyanin-containing extracts ranges from purple to red in acidic solutions and from green to yellow in alkaline solutions [48]. In our experiment, the extract from pink petals was bright red (acidic), while the extract from white petals was yellowish, indicating the presence of a significant amount of anthocyanin in the

tested samples. According to a report by Zoi et al. (2008), the production of volatile phenylpropanoid/benzenoid compounds in petunia increased tenfold with anthocyanin pigment. Additionally, the results obtained from Chroho et al. (2022) on improving color retention without affecting the gustatory quality of strawberries show that the addition of polyphenols from *Rosa damascena* Mill. petals leads to an increase in the half-life and a decrease in the thermal degradation of strawberry anthocyanins.

The *Rosa damascena* carotenoid cleavage (di-)-oxygenase 1 (*RdCCD1*) protein contributes to the formation of constituents of the rose aroma [49]. The *CCD1* gene C13 nor isoprenoids such as β -damascenone, β -damaskone, and β -ionone are the key flavor compounds in rose essential oil, which are obtained from carotenoid degradation. Additionally, the *CCD1* gene generates carotenoid – derived volatiles with fruity fragrance, using carotenoids as substrates, through the *CAROTENOID CLEAVAGE DIOXYGENASE* (*CCD*) enzymes [50]. The carotenoid cleavage dioxygenase pathway, which is located downstream of the terpenoid biosynthesis pathway, is closely related to the biosynthesis of volatile compounds [16]. Transcription factors (TFs) act as the main regulators of cellular processes [51] and control gene expression in plants [52]. MYB transcription factors play a crucial role in the positive regulation of many secondary metabolites at the transcriptional level, including flower color formation, flavonoid synthesis [53], and anthocyanin biosynthesis regulation [54]. GGPPS is responsible for the production of geranylgeranyl pyrophosphate (GGPP), which provides a platform for post-translational modification (geranylgeranylation) of proteins [55]. GGPPS is an essential enzyme in the isoprenoid biosynthesis pathway (IBP) and is involved in isoprenoid biosynthesis and metabolism [56]. GGPPS plays a key role in cytoskeleton regulation, signaling pathways, intracellular transport, and protein prenylation [57].

Previous studies have shown that the *MYB* transcription factor, a key regulator, has the highest expression during petunia's release of flower scent compounds in the full flowering stage [58]. Additionally, Shi et al. (2018) found that the *MYB* transcription factor plays an important role in the aroma formation of pear fruit. Several candidate transcription factors, such as *MYB1*, have been linked to metabolite flux in the phenylpropanoid pathway, resulting in the production of flavonols/anthocyanin in monocytogenes, which then produces fragrant white flowers of *Narcissus* and fragrant orange corollas of *Narcissus tazetta* [8]. Increasing the expression of the *CCD1* gene also increases the amount of ketone compounds in the essential oil [59]. Earlier studies have shown that with the increase in the expression of *CCD1* genes, the petals become colorless, which is consistent with our studies. Therefore, in the white accession, the petals become increasingly pale due to the increase in the growth stage. However, the hot pink accession in the S2 stage, under the influence of methyl jasmonate, led to increased gene activity and the relative paleness of the petals. Yu et al. (2022) demonstrated that the increase in geraniol content is directly influenced by the high expression of GGPPS in the petals of 'Old Blush' (OB, with intense aroma). The combination of IPP and DMAPP is the primary source for the synthesis of geranylgeranyl pyrophosphate (*GGPP*), farnesyl diphosphate (*FPP*), and geranyl pyrophosphate (*GPP*), which are from the terpene family [60]. *GGPPS* functions as a protein that plays a

key role in cytoskeleton regulation, signaling pathways, intracellular transport, and protein prenylation [57].

As a key regulator, phenylalanine ammonia-lyase (*PAL*) catalyzes the first step in the biosynthesis of the phenylpropanoid pathway, which is the trans-cinnamic acid production from the deamination of phenylalanine [61, 62]. *PAL* plays an important role in the biosynthesis of flavonoids [63], the synthesis of phenylpropanoid/benzenoid volatiles in flowers [64], and the biosynthesis of secondary metabolic pathways in higher plants [65]. Phenylacetaldehyde reductase (*PAR*) catalyzes the reduction of 2-phenylethylamine to produce 2-phenyl ethanol, a constituent of floral scent in petals [66]. The advantage of *PAR* is the reduction of the required ketone substrates, which is done by the regeneration of NADH and the oxidation of secondary alcohols, such as 2-propanol [67]. *Eceriferum* (*CER1*), the main gene of the alkane biosynthesis pathway, is an alkane-forming enzyme [68, 69] that converts very-long-chain (VLC) aldehydes into alkanes [70] and encodes aldehyde decarboxylase [71], wax biosynthesis [68] and plant flexibility in response to biotic and abiotic stresses [72]. The *CER1* gene is located at the endoplasmic reticulum (ER), the place where the synthesis of fatty acids (precursor of aromatic compounds) and their derivatives [19, 73]. Cuticular wax also consists of VLC aliphatic fatty acid derivatives and aromatic compounds [74]. *CER1*-mediated wax production on the petal organ is necessary for the synthesis of aliphatic components of the pollen wall [69] and the "petal effect" [75]. Previous studies have shown that the *CER1* gene leads to the production of 2-phenyl ethanol, which creates a pleasant scent in the petals [67, 76]. Chan et al. (2011) studies showed that the highest *PAR* transcript peaked in the petal and at the unfurling stage (fully open flower); also, *PAR* is classified as a short-chain dehydrogenase reductase (SDR). Sánchez et al. (2021) revealed that olive (*Olea Europaea*) is a rich source of bioactive polyphenols that can produce hydroxytyrosol, an important precursor of acetone [77]. In this regard, *PAR* is one of the genes related to the synthesis of hydroxytyrosol. The results of the above-mentioned phenethylamine transcript study supply a basis for analyzing the Acteoside synthesis pathway and extracting regulatory genes and key enzymes.

The Anthocyanin synthetase (*ANS*) gene is a key gene responsible for synthesizing the final stage of anthocyanins. The enzyme produced by this gene converts Leucoanthocyanidins into colorless anthocyanidins [78, 79]. Anthocyanin biosynthesis is carried out by a branch of the flavonoid biosynthesis pathway that is also responsible for isoflavonoid and flavonol biosynthesis [80]. Glycosyltransferases (*GT1*) is another key gene involved in anthocyanin biosynthesis [18]. The main important aromatic and color compounds in *R.damascena* are 2-phenyl ethanol alcohol and anthocyanins, which are synthesized by *PAR* and *ANS* genes, respectively [11]. RhGT1 enzyme plays a role in anthocyanin biosynthesis by glycosylating precursors, which stabilizes the critical molecule [20, 81], that has an important function in the glycosylation of secondary metabolites and hormone regulation in plants. Flavonol synthase (FLS) is another key enzyme gene related to flower color [82] and the biosynthesis pathway of flavonoids [16]. It catalyzes the production of flavonol from dihydro flavonol [83] and plays a role in determining the synthesis of flavonol glycosides, which is one of the main branches of the flavonoid pathway [84].

Different studies have been conducted on the expression of the *ANS* gene, which is a key gene in anthocyanin biosynthesis. Zhao et al. (2012) found that increasing *ANS* gene expression in *Paeonia* led to an increase in anthocyanin content in petals [85], which is consistent with the results of the present study in ACC.1. Shimizu et al. (2011) also observed no color change in *Lisianthus* plants due to the absence of the *ANS* gene [86], which is consistent with the results of the present study in ACC.26. Xu et al. (2022) confirmed that the reddening of leaves in red maple is regulated by the GT1 family genes, which affect anthocyanin accumulation [87]. These findings are consistent with previous studies on the high expression of the *FLS* gene in white flowers of tobacco, which leads to the predominant accumulation of flavonol and a lack of anthocyanin [88]. Furthermore, Hu et al. (2023) studied three different colors of *Scutellaria baicalensis* petals (white, pink, and purple) and found that the reduction in anthocyanin biosynthesis in white petals was due to the decreased activity of certain promoter regions of the *ANS* gene. This clarifies the mechanisms behind the color loss phenotype in the white flower of this plant [89]. Rasouli et al. (2020) investigated molecular and phytochemical changes in four landraces of *R. damascena* in three growth stages and found that high expression of the *RhANS* gene, the key gene in anthocyanin biosynthesis, leads to an increase in anthocyanin pigments in petals. The highest amount of anthocyanin was observed in growth (stage b), while an increase in cell acidity in growth (stage c) led to a decrease in anthocyanin content [11].

The clustering of network genes provides valuable insights into gene interactions, practical topics, and potential regulatory networks. Further investigation of these genes and their roles in specific pathways will enhance our understanding of cellular processes. The grouping of *MYB1*, *ANS*, *GT1* and *GGPPS* genes into a single cluster could suggest analogous expression trends or operational correlations. The shared expression patterns could be attributed to similar regulatory mechanisms, overlapping biological pathways, or interactions within cellular processes. Studies have indicated that the interplay between the *MYB1* and *ANS* genes might have a regulatory effect on the biosynthesis of anthocyanins in onions, thereby influencing the coloration of the onion tissue [90]. The *GGPPS* gene holds a crucial role in the carotenoid biosynthesis pathway. This gene instigates the production of ketonic compounds, specifically C13-norisoprenoids, which are a result of the *CCD1* gene's activity. Interestingly, flowering plants determine the color or aroma of their flowers through mutual relationships among their biosynthetic pathways, aligning with the growth of the petals. In this study, a connection is observed between the *CCD1* and *GGPPS* genes, placing them in the same group. The *CCD1* gene takes on the responsibility of altering the petal color and augmenting the quantity of carotenoid components. Conversely, the *GGPPS* gene could potentially be accountable for the generation of volatile components and the development of carotenoid pigments. In a multitude of studies, shared genes that have experienced notable upregulation or downregulation have been employed in the exploration of biological, molecular, and cellular pathways. Broadly speaking, this collection of genes may influence color, aroma, and certain pathways that are instrumental in the operational process of this characteristic. The findings from these studies could enhance our comprehension of genetic control in plants and their reaction to stress.

5. Conclusion

The findings of this research suggest that the *MYB1* and *CCD1* genes play an important role in determining the scent and color of rose flowers. The overexpression of *MYB* transcription factors was found to significantly increase *ANS* gene expression. Additionally, the application of the methyl jasmonate elicitor was found to have a positive effect on the relative expression of most genes, except *GGPPS*. The study also showed that there were 26 different compounds in four samples using GC/MS analysis. The production of monoterpenes, sesquiterpenes, and aliphatic compounds in white flowers was found to increase after 48 hours of methyl jasmonate application. In contrast, the application of methyl jasmonate to pink flowers led to a decrease in terpenoid compounds and an increase in aliphatic compounds. Further research is needed to fully understand the mechanisms behind the association between methyl jasmonate treatment and gene expression.

Declarations

Author contribution statement

Hoda Sadat Kiani - Conducting the entire experiments, data collection, literature search and manuscript writing. Manijeh Sabokdast Noudehi and Majid Shokrpour- Designing a part of the phytochemical experiments. Meisam Zargar & Mohammad Reza Naghavi*- Develop the idea, Project administration, overall supervision of the experiment, and Funding acquisition.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Tables

Tables 1 to 3 are available in the Supplementary Files section

Figures

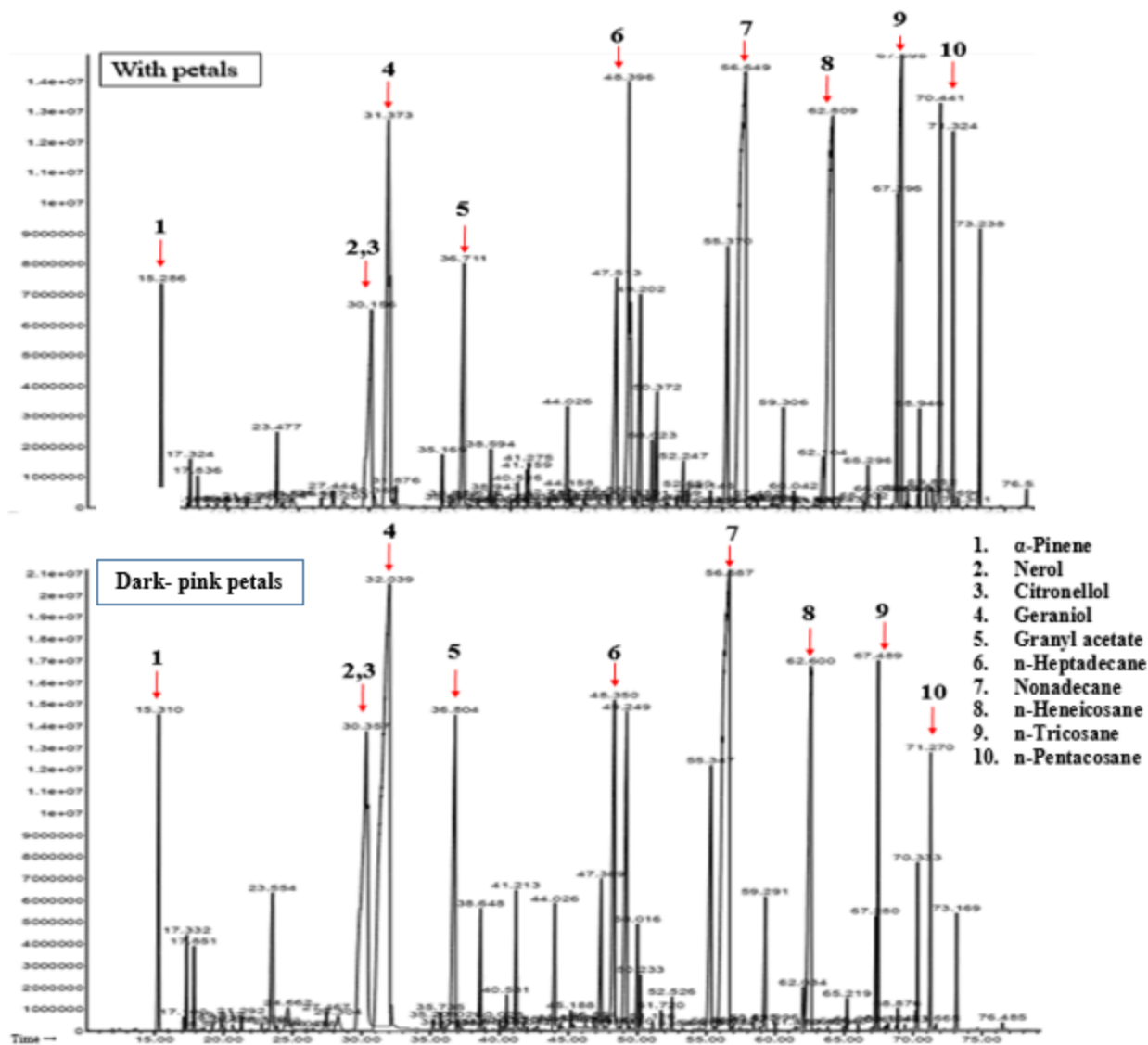


Figure 1

Total Ion Chromatogram (TIC) of white and dark-Pink of *R. damascena* Mill.

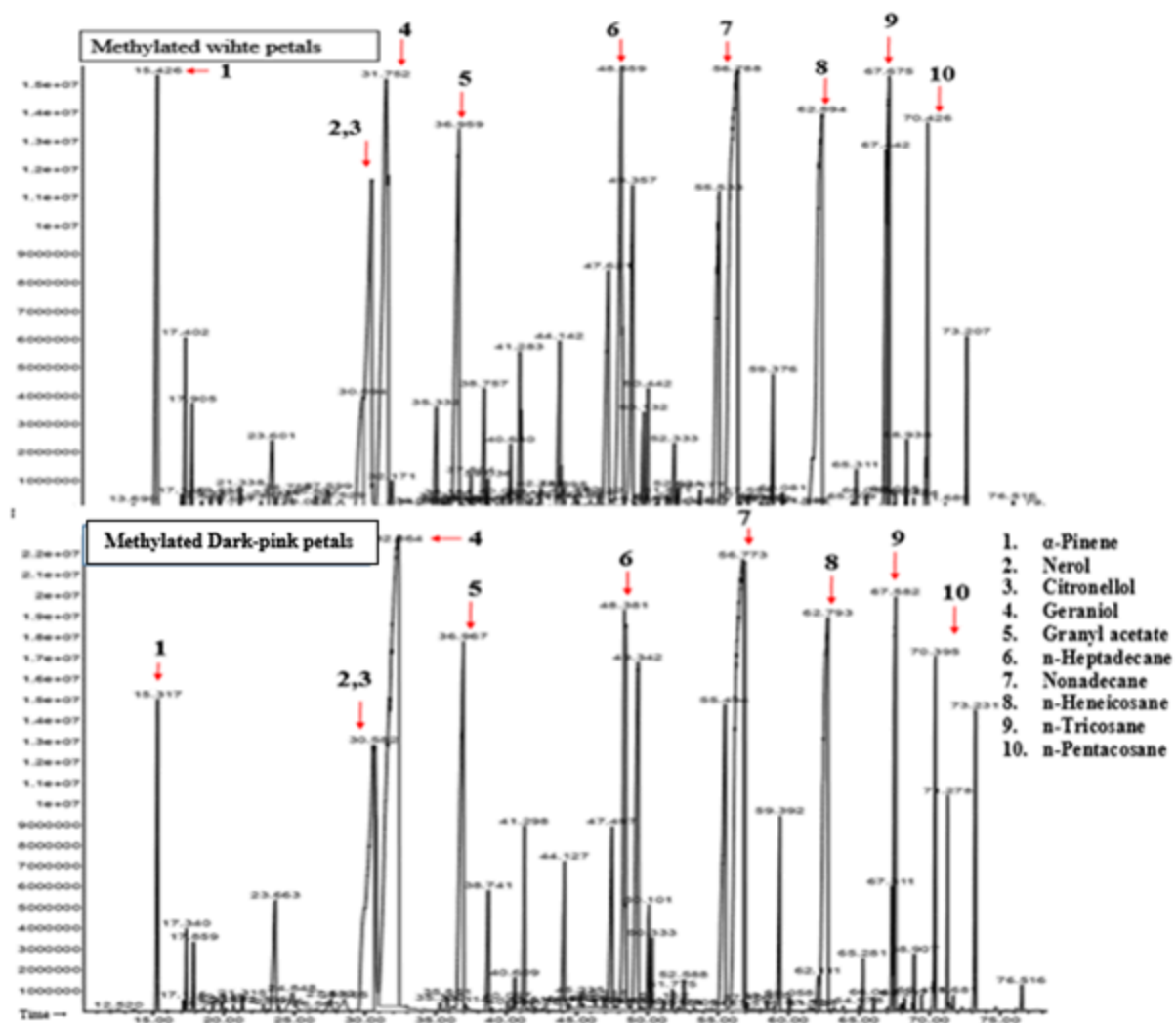


Figure 2

Total Ion Chromatogram (TIC) of white and hot-Pink of *R. damascena* Mill. holding methyl jasmonate elicitor) 300 μ l for 48 h).

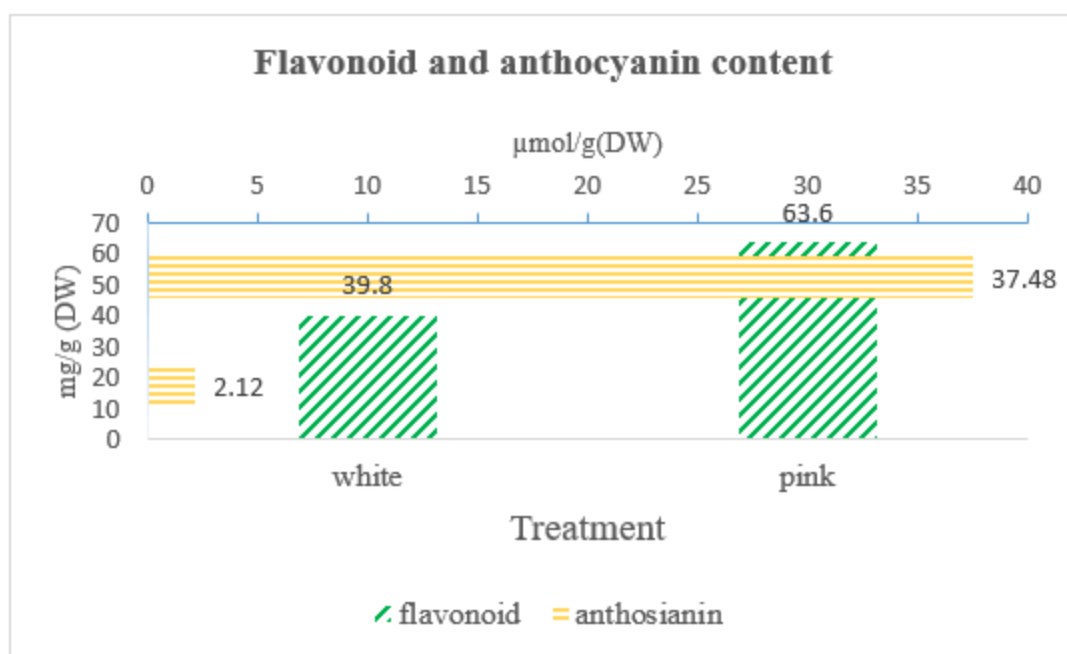


Fig. 3.

Figure 3

Content of total flavonoid and anthocyanin in white and Hot-pink petals of *R. damascena*.

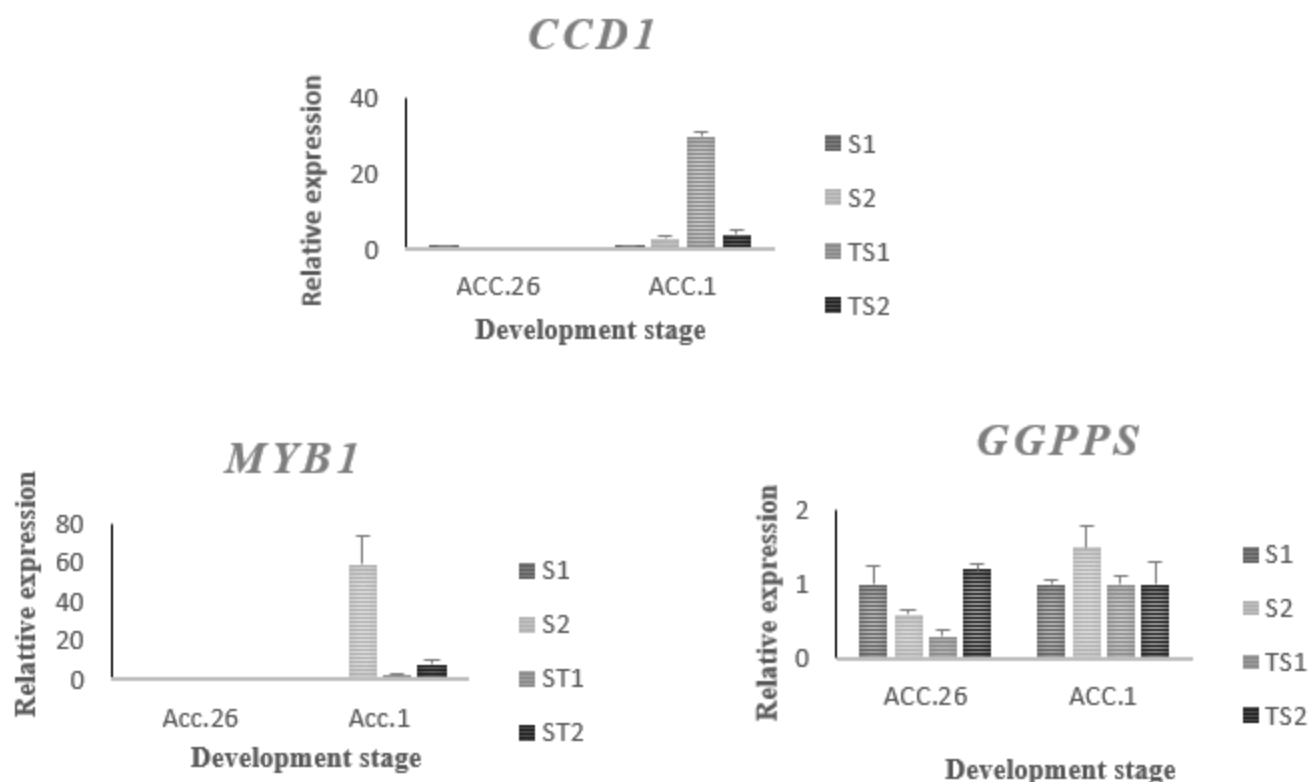


Fig 4.

Figure 4

Quantitative real-time PCR analysis of relative expression level of CCD1, GGPPS, MYB1 (scent and color biosynthetic gene) during flower development (S1 and S2) of two accession numbers (ACC.26 and ACC.1) in *R. damascena*. Relative expression was calculated based on the threshold cycle (CT) method after normalization to the beta-actin gene. Bud stage (S1) was selected as a reference for white (ACC.26) and dark pink (Acc.1) petals. Vertical bars represent standard deviations (n = 3).

[Abbreviations= S1:bud, S2: Full open flower, TS1: methylated bud, TS2: methylated Full open flower]

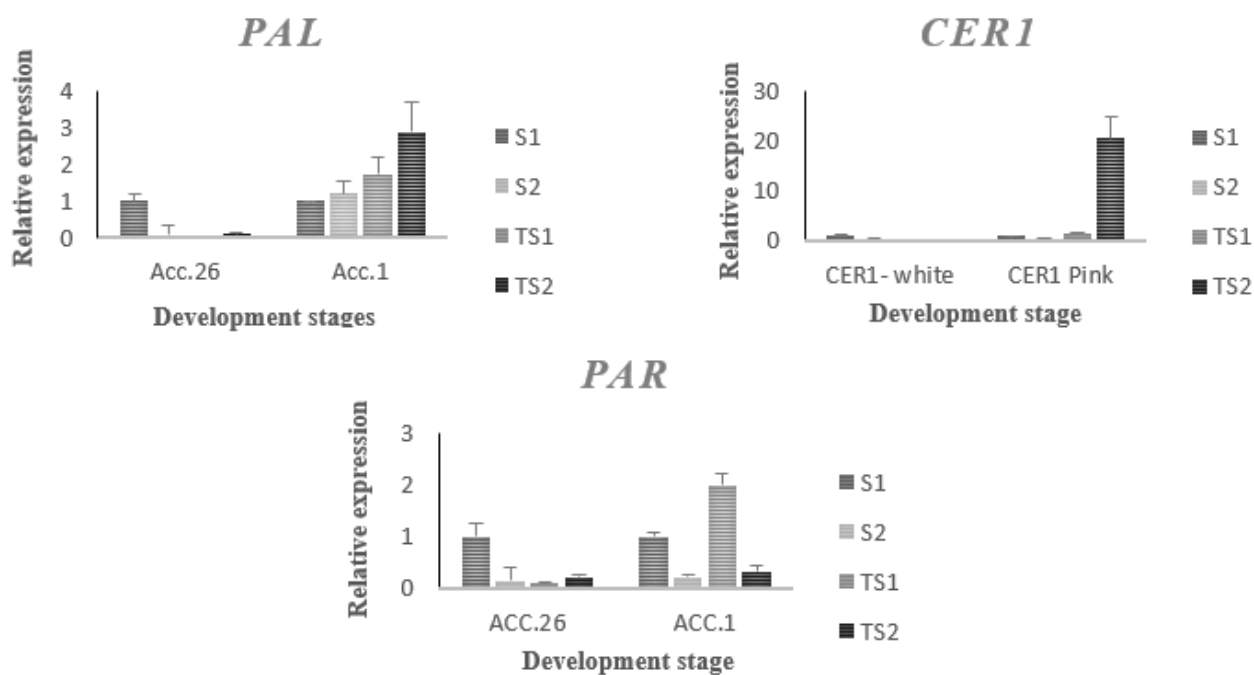


Fig 5.

Figure 5

Quantitative real-time PCR analysis of relative expression level of PAL, CER1, PAR (aroma biosynthetic gene) during flower development (S1 and S2) of two accession numbers (Acc.26 and Acc.1) in R. damascena. Relative expression was calculated based on the threshold cycle (CT) method after normalization to the beta-actin gene. Bud stage (S1) was selected as a reference for white (Acc.26) and dark pink (Acc.1) petals. Vertical bars represent standard deviations (n = 3). [Abbreviations= S1:bud, S2: Full open flower, TS1: methylated bud, TS2: methylated Full open flower]

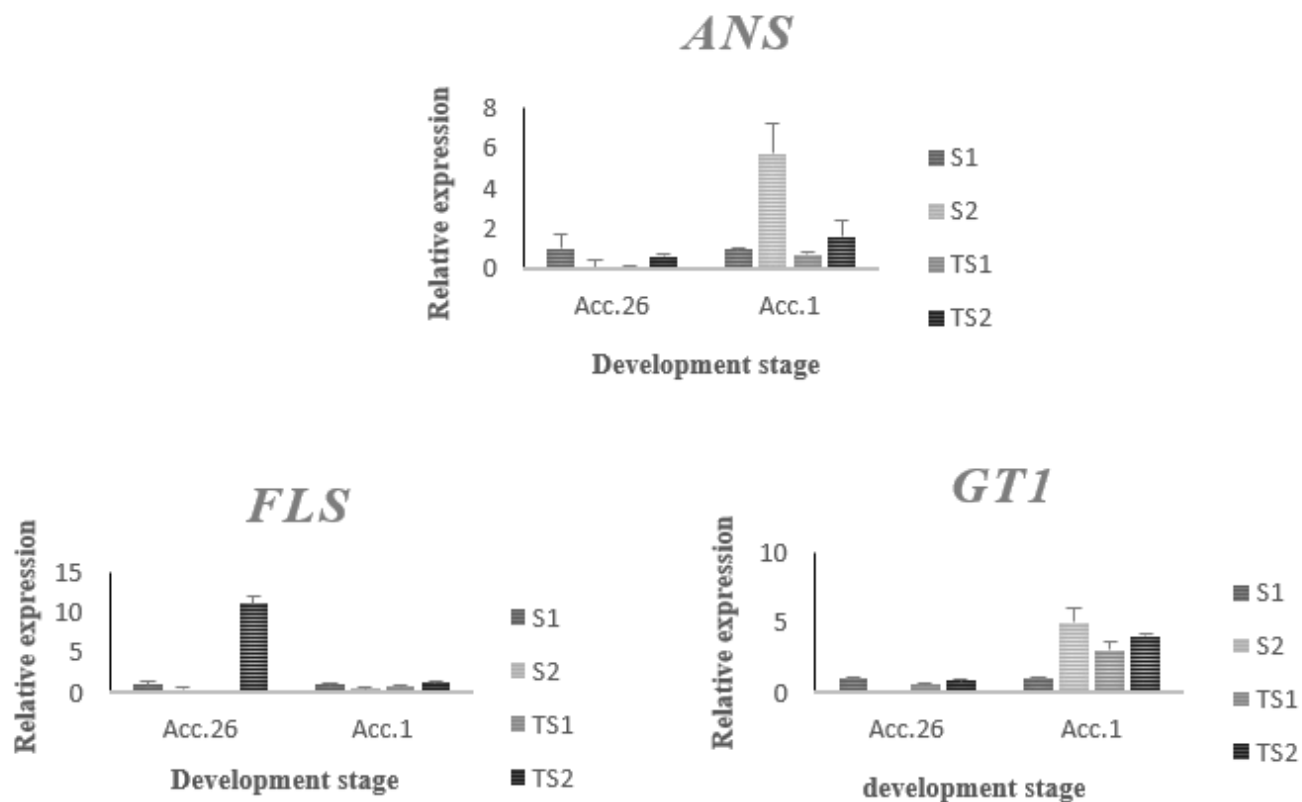


Fig 6.

Figure 6

Quantitative real-time PCR analysis of Relative expression level of FLS, GT1, ANS (color biosynthetic gene) during flower development (S1 and S2) of two Accession numbers (Acc.26 and Acc.1) in *R. damascena*. Relative expression was calculated based on the threshold cycle (CT) method after normalization to the beta-actin gene. Bud stage (S1) was selected as a reference for white (Acc.26) and dark pink (Acc.1) petals. Vertical bars represent standard deviations (n = 3). [Abbreviations= S1:bud, S2: Full open flower, TS1: methylated bud, TS2: methylated Full open flower]

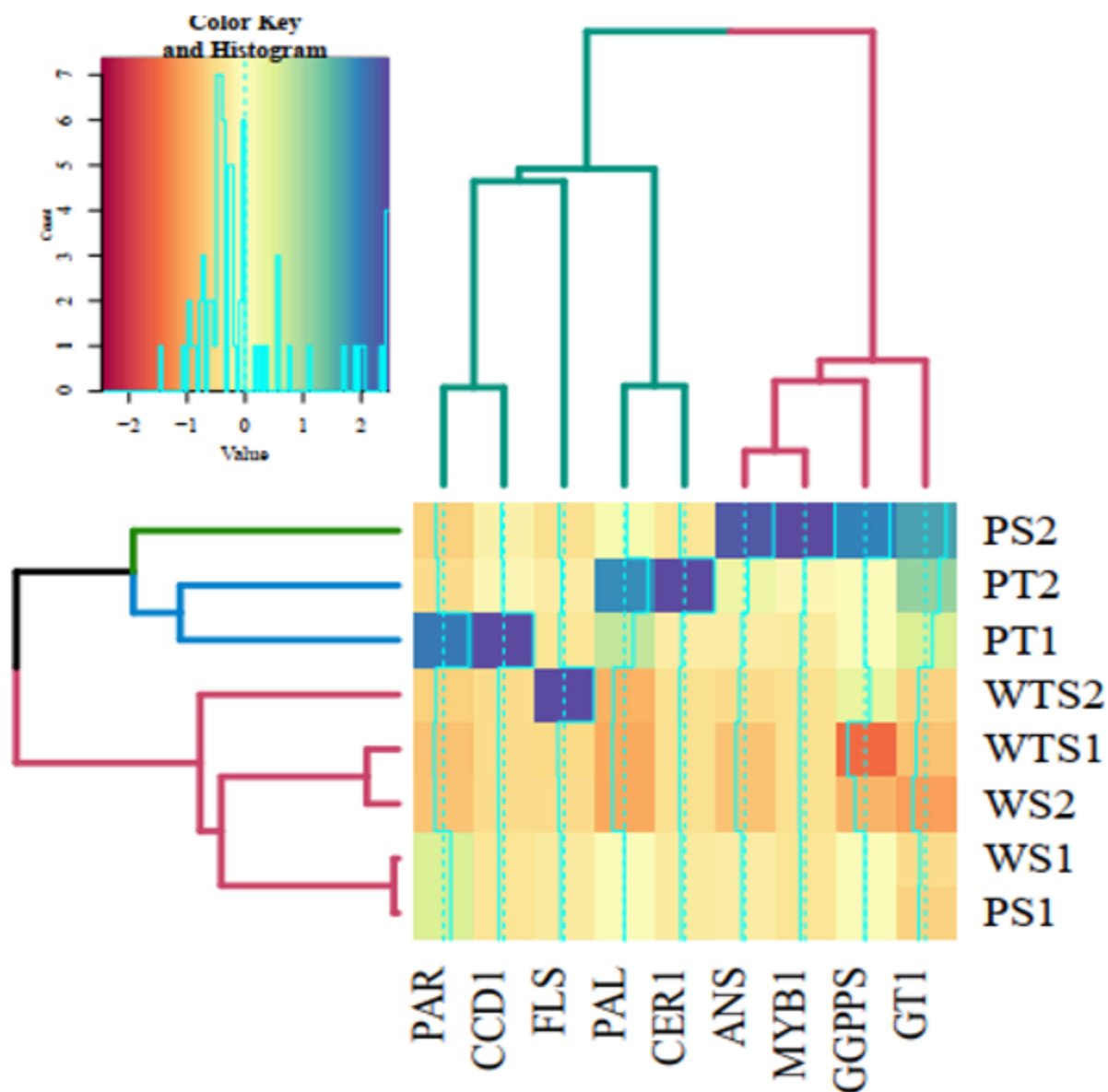


Fig 7.

Figure 7

shows the heatmap and cluster analysis of gene expression in distinct samples of white and pink petals under the influence of applied and unapplied methyl jasmonate elicitor. (PS2: Young stage pink flower; PT2: Methylated young stage pink flower ; PT1: Bud stage pink flower ; WTS2: Methylated young stage white flower; WTS1: Methylated bud stage white flower; WS2: Young stage white flower; WS1: Bud stage white flower; PS1: Bud stage pink flower)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Tables.docx](#)