Identification and Characterization of the MADS-Box Genes and Their Contribution to Flower Organ in Carnation (Dianthus caryophyllus L.)

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Abstract: Dianthus is a large genus containing many species with high ornamental economic value. Extensive breeding strategies permitted an exploration of an improvement in the quality of cultivated carnation, particularly in flowers. However, little is known on the molecular mechanisms of flower development in carnation. Here, we report the identification and description of MADS-box genes in carnation (DcaMADS) with a focus on those involved in flower development and organ identity determination. In this study, 39 MADS-box genes were identified from the carnation genome and transcriptome by the phylogenetic analysis. These genes were categorized into four subgroups (30 MIKC+, two MIKC*, two Ma, and five My). The MADS-box domain, gene structure, and conserved motif compositions of the carnation MADS genes were analysed. Meanwhile, the expression of DcaMADS genes were significantly different in stems, leaves, and flower buds. Further studies were carried out for exploring the expression of DcaMADS genes in individual flower organs, and some crucial DcaMADS genes correlated with their putative function were validated. Finally, a new expression pattern of DcaMADS genes in flower organs of carnation was provided: sepal (three class E genes and two class A genes), petal (two class B genes, two class E genes, and one SHORT VEGETATIVE PHASE (SVP)), stamen (two class B genes, two class E genes, and two class C), styles (two class E genes and two class C), and ovary (two class E genes, two class C, one AGAMOUS-LIKE 6 (AGL6), one SEEDSTICK (STK), one B sister, one SVP, and one Ma). This result proposes a model in floral organ identity of carnation and it may be helpful to further explore the molecular mechanism of flower organ identity in carnation.

Keywords: floral organs identity; Dianthus caryophyllus L.; MADS-box genes; ABC model

1. Introduction

The MADS-box gene family playing an important role in the regulation of plant growth and development is well known as a key transcription factor (TF). MADS-box genes identified as floral homeotic genes contain a highly-conserved MADS box DNA-binding domain of approximately
58–60 amino-acid sequences in the N-terminal region, which bind to CArG boxes (CC[A/T]GG) [1–4]. MADS-box genes were classified into two major types: type I and type II genes, based on the phylogenetic relation of the conserved MADS box domain [5,6]. Sixty-two type I and 46 type II genes were identified and characterized in Arabidopsis thaliana [7]. Among them, type I genes can be further divided into three subgroups, Ma, Mb, and My, while type II, known as M-type, can be classified into two subgroups, MIKCc and MIKC*, based on their structural characteristics [8,9]. It has been reported that type I MADS-box genes encode SRF-like domain proteins, that type II group genes encode MEF2-like genes of animals and yeast, and that MIKC-type genes are found only in plants [10,11]. The MIKCc proteins contain four common domains, including MADS (M), weakly-conserved intervening (I), conserved keratin (K), and the highly-variable C-terminal (C) domain, which usually contains conserved subfamily-specific sequence motifs [12]. I domain is responsible for DNA binding specificity and dimerization of these proteins. In addition, K domain contributes to mediating dimerization, and C domain functions in transcriptional activation and in the formation of multimeric protein complexes. Compared with type II, type I group genes show a relatively simple gene structure. They are shorter, and usually only have one or two exons without the K domain [13]. With further study of MIKCc type genes, they were subclassified into 12 groups, based on their phylogenetic relationships in A. thaliana. Nevertheless, the knowledge of the function of type I genes remains limited.

In plants, previous reports reveal that the MADS-box family plays a vital role in many developmental processes, such as flower organ identity [14], control of flowering time [15–19], fruit ripening [20], and the development of vegetative organs [21]. Moreover, MIKCc-type MADS-box genes are involved in flowering time control and floral organ identity. The well-known ABC model of flowers, which explain different floral organs identities, are controlled by the combinations of various types of genes [14]. Subsequently, ABC model developed into ABCDE model: sepals (A + E), petals (A + B + E), stamens (B + C + E), carpels (C + E), and ovules (D + E) [22]. In the newly-developed model, class A contains APETALA1 and FRUITFULL (AP1 and FUL); class B contains PISTILLATA and APETALA3 (PI and AP3); class C contains AGAMOUS (AG); class D contains STK; and class E contains SEPALLATA genes 1–4 (SEP1, SEP2, SEP3, and SEP4) [22].

In addition to class ABCDE genes, there are many other MADS genes in regulating flower development in A. thaliana, such as, FLOWERING LOCUS C (FLC), SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1), SVP, AGAMOUS-LIKE 24 (AGL24) [17], MADS AFFECTING FLOWERING (MAF1/FLM) [23] and AGAMOUS-LIKE 15/18 (AGL15/AGL18) [19]. Among them, the FLC gene encoding a specific MADS domain protein has the function of inhibiting flowering [24]. The SOC1 gene can also regulate the flowering time by acting on the vernalization pathway [15]. SVP is considered as an important control factor of flowering time influenced by ambient temperature [16]. Moreover, the AGL16 gene targeted by microRNA 824 (miR824) contributes to the repression of plant flowering time [18]. AGL17 genes show unusually diverse expression patterns with member genes expressed in roots, in pollen [25], and in both [26]. These genes function as either positive (SOC1, AGL24) or negative regulators (FLC, SVP) of flower meristem identity genes together with other subfamilies, such as AGL15, AGL12, and AGL17. By contrast, type I genes were reported to only participate in the development of seed and female gametophyte [27–29].

The carnation is one of the most popular flowers [30]. If more flower shapes can be developed and the flowering time and flower morphology is easier to control, the economic value of carnation will be greatly enhanced. Thus, it is extremely essential to explore how the MADS-box gene family controls floral organ development and regulates flowering time. Recently, more and more MADS-box genes were identified and characterized in various plant species, such as Arabidopsis [7], tomato [31], rice [32], maize [33], cucumber [34], soybean [35], Chinese cabbage [36], sesame [37], and radish [38]. However, few studies of the genome-wide characterization of MADS-box genes in carnation were available. Fortunately, the advent of the carnation genome sequencing makes it possible to analyse MADS-box genes [39]. In this study, MADS-box members from carnation genome were systematically
analysed and their gene structures, conserved motifs, phylogeny, and subcellular localization were presented for the first time. Additionally, preliminary prediction of gene functions were also verified, and the expression of the MADS-box family in carnation were detected with real-time PCR (RT-PCR). These results will offer an insight into the molecular mechanisms underlying flowering and floral organogenesis in carnation through analysis of the expression pattern of MADS-box genes.

2. Material and Methods

2.1. The Identification of MADS-Box Genes in Carnation’s Genome

We download 101 MADS-Box family genes in Arabidopsis from the TAIR website [40] in Table S1 and 71 MADS-Box family genes in the rice genome from the Rice Genome Annotation Project [41], respectively, in Table S2. All the downloaded protein sequences of MADS-Box family genes were used as query sequences by blastp searches (e-value 1e-6) against the carnation’s protein sequences to predict the carnation MADS-Box family genes. In addition, the Hidden Markov Model (HMM) profile for the MADS-box domain (Pfam accession number: PF00319) was also used to search (e-value 1e-7, score 30) against the genome protein sequences by using HMM search tool to ensure the completeness of MADS-box genes as far as possible. Then the genes obtained by the two methods mentioned above were intersected to guarantee the appropriate selection of genes. Each gene predicted was subsequently verified through the National Center for Biotechnology Information (NCBI) [42], SMART [43] and Pfam database [44] to confirm the completeness of the MADS-box domain.

2.2. Conserved Sequence and Structure Model Analysis

To search for the conserved motifs localized within the 39 DcaMADS protein sequences, the multiple expectation for motif elicitation (MEME) tool [45] was used with default parameters. A maximum of 15 motifs were searched. This study took advantage of carnation genome annotation file [46] to extract the carnation MADS-box gene structure information, and then the picture was drawn with the R language.

2.3. Phylogenetic Analysis of MADS-Box Proteins

Multiple sequence alignments were performed between MADS-box protein sequences from carnation, Arabidopsis, and rice by using the ClustalX-2.0 software package with default parameters [47]. A phylogenetic tree was constructed with aligned MADS-box protein sequences with Clustal X by using the neighbour-joining (NJ) method with 1000 iterations for the bootstrap values. The rectangular phylogenetic tree was generated using MEGA6 software package [48].

2.4. RNA Sample Preparation and Quantitative PCR Expression Analysis

Young stems, young leaves, flower buds (diameter 0.5–0.8 cm), and individual flower organs (before blooming) of the carnation cultivar Master were collected for gene expression assays with quantitative real-time RT-PCR (qRT-PCR). The carnation cultivar Master was planted in the experiment yard of Huazhong Agriculture University (Wuhan, China). Total RNA of each sample was extracted using an EASYspin Plant RNA kit reagent (Aidlab Biotechnologies, Beijing, China) according to the manufacturer’s instructions. The PCR amplification was carried out in a 96-well plate with the following cycling parameters: heating for 2 min at 95 °C, 40 cycles of denaturation at 95 °C for 10 s, annealing for 20 s at 60 °C, and extension at 72 °C for 35 s. Three biological replicates were included per sample. The qRT-PCR was conducted using SYBR Primix Ex Taq kit (TaKaRa, Dalian, China) in an Applied Biosystems Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). To confirm results’ reliability, each sample was conducted with three biological and three technical replicates. The housekeeping gene DcaGAPDH (glyceraldehyde-3-phosphate dehydrogenase) was selected as an internal quantitative control (Table S3). The relative expression values were calculated using the comparative CT(2−△△CT) method. The primers used in the analysis are listed in Table S3.
2.5. Subcellular Localization

The full-length candidate complementary DNA (cDNA) sequence was amplified from cDNA of the carnation cultivar Master by PCR. A suitable restriction site sequence was added to the ends of the primers. The primers used in the analysis were listed in Table S4. Products were cloned into vector pGFP 1301 using dual-enzyme digestion. Plasmids were isolated and transformed into Nicotiana benthamiana by injection transformation [49]. The transformed leaves were incubated for three days, then observed and photographed on a microscope (BX61, Olympus, Tokyo, Japan).

3. Results

3.1. The Identification and Annotation Information of Carnation MADS-Box Domain Genes

To define the candidate MADS-box genes, carnation genome and transcriptome protein sequences were searched by using a HMM profile in the Pfam database. A total of 46 putative genes in carnation were identified and all these candidate carnation MADS-box proteins were named DcaMADS1 to DcaMADS46 (Table 1), respectively. To ensure the reliability of these sequences, all the identified sequences were verified through the public databases, including NCBI, Pfam, and SMART, and searching these protein sequences against A. thaliana on the TAIR database by BLASTP. Among them, the complete open reading frames (ORF) sequences of five genes (DcaMADS6, DcaMADS8, DcaMADS18, DcaMADS28, and DcaMADS29) were obtained in the carnation transcriptome (Figure S1). In this study, the DcaMADS8 and DcaMADS31 genes’ names were still used for uniformity, while they were named, respectively, CMB1 and CMB2 [50]. Although DcaMADS36 and DcaMADS39 share the SRF-like domain, their sequence structure does not have a specific motif. Therefore, they are not suitable to be classified into any subgroup of MADS-box gene family, and they were eliminated from further analysis. Interestingly, three candidate genes. DcaMADS3, DcaMADS4, and DcaMADS5, display no difference in their protein sequence; consequently, only DcaMADS3 sequence was selected for further study. A total of 39 sequences were selected in carnation without sequences incompletion. Compared with other species, carnation had a relatively small number of MADS-box gene families; 39 MADS-box genes were categorized into four subgroups (30 MIKC\(^c\), 2 MIKC*, 2 \(\alpha\), 5 \(\gamma\)) and 30 MIKC\(^c\)-type proteins were subdivided into 12 subclasses.

Table 1. The complete list of the carnation MADS-box genes.

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Table 1. Cont.

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3.2. Analysis of the Gene Structure and Conserved Sequence

To better understand the structural diversity and gene evolutionary relationship, the intron–exon pattern of coding sequences of individual MADS-box genes in carnation was analysed (Figure 1). Many previous studies reported that type II genes contained multiple introns, whereas $M\alpha$, $M\beta$, and $M\gamma$ genes usually contained fewer of them [32,51]. This study obtained the similar findings that $M\alpha$ and $M\gamma$ genes contained small numbers of introns in carnation, with the exception of DcaMADS41, containing as many as 11 introns. The structure of type II gene was more complex than that of type I in carnation. The maximum of 13 introns was observed in a single gene in DcaMADS25 (SVP). The structure of typical MIKC$^C$ genes with 1–6 exons and conserved C-terminal motifs in carnation were found, which was similar with previous study [52]. To further understand the intron–exon structure of carnation MIKC$^C$ genes, we analysed the length of MIKC$^C$ genes (Figure 2a). The result indicated that lengths of exon 3, 4, 5, and 8 between genes were consistent and stable, whereas the lengths of exon 1, 2, 6, and 7 were not. The presence of exons 9 or 10, 11, 12, 13, and 14 were peculiar to some specific genes.

All 39 DcaMADS proteins were identify the motifs by the MEME motif search tool [53]. In total, 15 motifs were identified and were named motif 1–15 (Figure 3 and Figure S2). Among these motifs, the conserved motifs 1 and 3, which specify the MADS domain, were observed in most DcaMADS proteins. This motif KR[K/R]X4KK (motif 1 (Figure S2)) at positions 22–30 of the MADS-box domain plays an important role in the translocation of MADS-box proteins into the nucleus [54]. Motif 2 and 5, which specifies the K domain, were found in most MIKC$^C$ group proteins. The sequences of DcaMADS20, DcaMADS21, DcaMADS42, DcaMADS43, and DcaMADS44 and DcaMADS45 proteins were incomplete, with DcaMADS20 and DcaMADS21 lacking K domain and DcaMADS42, 43, 44 and 45 lacking the MADS domain. Interestingly, only DcaMADS42, DcaMADS43, and DcaMADS44 proteins shared the same 4, 6, 8 and 10 motifs (Figure 3).
Figure 1. The exon-intron structure of DcaMADS genes. The lines indicate introns, and the blue boxes indicate exons.

Figure 2. (a) Exon length distribution analysis of the carnation MADS-box genes. Exon length values were extracted from the carnation genome annotation file and then drawn the boxplot with the R language. Each box represents the exon size range in which 50% of the values for particular exon are grouped. The median is shown as a black line. (b) The classification and proportions of DcaMADS genes.
Figure 3. Conserved motifs of 39 carnation MADS-box proteins identified using the multiple expectation for motif elicitation (MEME) program. Motifs 1 to 15 are indicated by different coloured boxes. The names of all members and combined probability values are shown on the left side; motif sizes are shown at the bottom.

3.3. Phylogenetic Analysis of Carnation MADS-Box Domain

To know more about the phylogenetic relationships among carnation MADS-box genes, a phylogenetic tree between 39 carnation MADS-box genes and 101 A. thaliana MADS-box genes was constructed by NJ method (Table S1). It is obvious that DcaMADS genes were divided into four clades with reference to the classification of the A. thaliana, and then they were named subfamilies MIKC\(^\text{C}\), MIKC*, M\(\alpha\), and M\(\gamma\) (Figure 4). Additionally, to further confirm phylogenetic relationships, another phylogenetic tree was constructed by using MADS-box proteins from carnation and rice (Figure S3 and Table S2). The result of two phylogenetic trees were same. Of the 39 DcaMADS proteins, 32 members from DcaMADS1 to DcaMADS38 with high similarity could be unambiguously classified into MIKC type II, whereas the remaining seven members (DcaMADS39-DcaMADS46) were classified into type I according to their relation with AGL proteins [7,32]. In type II, two proteins (DcaMADS37 and DcaMADS38) were subgrouped into MIKC\(^*\), whereas the other 30 members (DcaMADS1-DcaMADS35) were classified into MIKC\(^\text{C}\). These 30 MIKC\(^\text{C}\)-type proteins were subdivided into 12 subclasses with E (SEP) having six members, AGL6 (one member), C/D (AG/STK) (three members), SOC1 (three members), A (AP1/FUL) (two members), AGL12 (one member), AGL17 (one member), AGL15 (two members), SVP (three members), and B (AP3/PI) (five members), B sister (two members) and FLC (one member). Type I proteins were further classified into two subgroups: M\(\alpha\) (two members) and M\(\gamma\) (five members) (Figure 2b). The number of MADS genes from subgroup SEP and AP3/PI in carnation was larger than that in other species, like in tomato [31], in cucumber [34], and in petunia [55] (Table S5).
Figure 4. The phylogenetic tree of the 39 DcaMADS proteins was generated by the neighbour-joining (NJ) algorithm with 1000 iterations for the bootstrap values using Molecular Evolutionary Genetics Analysis (MEGA version 6.0) software [48]. The subgroups are marked in different colours. (a) Phylogenetic tree of Dianthus caryophyllus and Arabidopsis type II proteins. (b) Phylogenetic tree of Arabidopsis and D. caryophyllus type I proteins.

3.4. Expression of DcaMADS Genes in Floral Organs

MADS-box genes participate in various processes of plant growth and development. To know more about their expression patterns, the expression of 35 carnation MADS genes in stems, leaves, and flower buds were examined. No expression of DcaMADS32, DcaMADS42, DcaMADS43, and DcaMADS46 genes in the above-mentioned samples was observed, which may be attributed
to the fact that their expressions were too low to be detected. The expression level of A-, B-, C-, D-, and E class genes were higher in flower buds than that in stems and leaves (Figure 5), especially the genes DcaMADS27, DcaMADS28, DcaMADS12, and DcaMADS1. This was same with DcaMADS9 (AGL6), DcaMADS33 (B sister), and DcaMADS37 (MIKC* groups). While only one gene in the Mα group, namely DcaMADS41, was highly expressed in flower buds. To obtain more knowledge the gene expression in five tissues involved in the development of reproductive organs were examined.

![Expression analysis of 35 DcaMADS genes in different tissues, including stems, leaves, and flower buds. Each bar shows the standard deviation of triplicate assay.](image)

(a) The expression levels of 16 ABCDE DcaMADS genes which mean the class A, class B, class C/D and class E in the well-known ABC model of flowers. (b) The expression levels of genes in AGL6 (1), SOC1 (3), AGL12 (1), AGL17 (1), AGL15 (2), SVP (3), B sister (1), FLC (1), and MIKC* (2) subgroups. (c) The expression levels of four type I DcaMADS genes.

**Figure 5.** Expression analysis of 35 DcaMADS genes in different tissues, including stems, leaves, and flower buds. Each bar shows the standard deviation of triplicate assay.
3.4.1. SEPALLATA

E genes in the ABCE model play a significant role in floral organ development [55–58]. Six SEP (DcaMADS1, DcaMADS2, DcaMADS3, DcaMADS6, DcaMADS7, and DcaMADS8) genes were identified and analysed from carnation indicating that DcaMADS1, DcaMADS2, and DcaMADS3 were highly expressed in ovary tissues. DcaMADS6, DcaMADS7, and DcaMADS8 were expressed mainly in the sepals, and DcaMADS7 and DcaMADS8 were additionally expressed in petals (Figure 6).

3.4.2. AGAMOUS-LIKE 6

There have been reports that AGL6 regulates floral organ identity [59]. We isolated one AGL6 gene (DcaMADS9) from carnation. The DcaMADS9 gene had the highest expression in ovary tissues among five above-mentioned tissues. This finding was consistent to that of experiments with melon [60] (Figure 6).

3.4.3. AGAMOUS/SEEDSTICK

The AG gene mainly functions in specifying stamen and carpel identity and STK in ovaries [61–63]. We identified two AG genes (DcaMADS11 and DcaMADS12) that were expressed exclusively in stamens, styles, and ovaries, and the STK gene (DcaMADS13) exclusively in ovaries (Figure 6). These results are consistent with those found in other species plants, such as Arabidopsis [63], rice [64], tomato [61], etc., indicating that AG subfamily members specified stamen and carpel identity [7,31,32].

3.4.4. SUPPRESSOR OF OVEREXPRESSION OF CO1

SOC1 is an important transcriptional regulation factor controlling flowering time [64,65]. We identified three SOC1 genes (DcaMADS14, DcaMADS15, and DcaMADS16) with various expression patterns in vegetative and reproductive organs of carnation. The DcaMADS14 gene was found to be expressed primarily in sepals. The DcaMADS15 gene was expressed in all tissues, but slightly higher in stamens and petals than in other tissues. Moreover, the DcaMADS16 gene was only markedly detected in stamen tissues (Figure 6).

3.4.5. APETALA 1/FRUITFUL

AP1/FUL genes are typical class A floral organ identity genes, which were expressed in inflorescence [66,67]. In addition, they are involved in specifying sepals and petals [68,69]. Two class A genes were identified (DcaMADS17 and DcaMADS18) to have the same transcript patterns in stems and in leaves. However, they were highly expressed in sepals of carnation (Figure 6). Our results are also in line with the findings of one previous study reporting that the FUL gene was expressed in stems and leaves of Arabidopsis [70].

3.4.6. AGAMOUS-LIKE 12

Only one AGL12 gene (DcaMADS20) was detected in carnation. This gene was expressed in all tested organ tissues (Figures 5 and 6), similar to their Arabidopsis counterpart AGL12 [21]. Moreover, AGL12 was also been detected to be strongly expressed in stems.

3.4.7. AGAMOUS-LIKE17

We identified one AGL17 (DcaMADS21) gene which was reported with strong expression in different tissues, such as in roots [71], in pollen [25,72], or in leaf guard cells [68], and trichomes [5]. However, its expression level in all tissues of carnation merely displayed slight differences with low expression found in style and ovary tissues (Figure 6).
3.4.8. AGAMOUS-LIKE 15

AGL15 can effectively regulate plants senescence in Arabidopsis [19]. In our study, two AGL15 genes (DcaMADS22 and DcaMADS23) were detected and their expressions in different tissues were different. The gene of DcaMADS22 was expressed mainly in ovary tissues, while DcaMADS23 was specifically expressed in stamen tissues (Figure 6).

3.4.9. SHORT VEGETATIVE PHASE/AGAMOUS-LIKE 24

Some genes from the same subfamily have different functions. For example, AGL24 (SVP subfamily) as a flowering promoter in Arabidopsis [16,17], while the SVP [72] gene in barley serves as a floral repressor. Three genes (DcaMADS24, DcaMADS25, and DcaMADS26) in this subfamily were found and their expressions in various organs were analysed (Figure 6). The two genes DcaMADS24 and DcaMADS26 were widely expressed in various tissues. DcaMADS24 had a high expression level in stamens, while DcaMADS26 high expressed in sepals. In addition, DcaMADS25 was expressed in petal and ovary tissues.

3.4.10. APETALA 3/PISTILLATA

B class genes play an important role in controlling petals and stamens during flower development [73,74]. We found two PI genes (DcaMADS27 and DcaMADS28) and two AP3 genes (DcaMADS29 and DcaMADS30) that were expressed exclusively in the flower buds. DcaMADS31 in carnation has a high homology with TM6 in tomato. Transcripts for these genes were abundant in the petals and stamens of carnation. We also found that relatively high expression of DcaMADS27 and DcaMADS28 were widely expressed in various tissues. DcaMADS24 had a high expression level in stamens, while DcaMADS26 high expressed in sepals. In addition, DcaMADS25 was expressed in petal and ovary tissues.

3.4.11. B sister

The DcaMADS33 gene was identified as a sister group of B genes and was, therefore, named B sister (Bs) genes [75,76]. GOA/TT16 is an Arabidopsis B sister gene and was reported to function in the endothelial cells and the seed coat controlling flavonoid biosynthesis [77,78]. DcaMADS33 was found to be expressed strongly in ovary tissues in carnation, similar to the counterpart in Arabidopsis (Figure 6).

3.4.12. FLOWERING LOCUS

The function of FLC gene is to inhibit flowering [79,80]. Five FLC genes were identified in Arabidopsis and in poplar [81], respectively. However, only one FLC gene (DcaMADS34) was detected in carnation, which was expressed in sepal of carnation (Figure 6).

3.4.13. MIKC*

Two MIKC* genes (DcaMADS37 and DcaMADS38) were detected in genome of carnation. Both of them were found to be expressed in stamens, which are consistent with the findings in radish and Arabidopsis [38,82,83]. However, these genes showed differential expression in other tissues. For example, DcaMADS37 was highly expressed in sepal, but DcaMADS38 was not expressed in sepals. The two genes were expressed widely in other floral organ tissues (Figure 6).

3.4.14. Mα

Two Mα genes (DcaMADS40 and DcaMADS41) were detected in all flowers’ organs in this study. However, they exhibited distinct expression patterns. DcaMADS40 had slightly higher expression in sepal than in other tissues, whereas DcaMADS41 was found to have high expression only in ovaries (Figure 6).
Figure 6. Organ specific expression analysis of 35 DcaMADS genes at different flower whorls. Se: sepals, Pe: petals, St: stamens, Sty: styles, Ov: ovaries. Each bar shows the standard deviation of triplicate assay.
3.4.15. Mγ

This study isolated five Mγ genes (DcaMADS42, DcaMADS43, DcaMADS44, DcaMADS45, and DcaMADS46) through phylogenetic tree analysis. The two Mγ genes (DcaMADS44 and DcaMADS45) were expressed in all of the flowers’ organs (Figure 6). Until now, there have been few reports on the function of the Mγ gene in plants.

3.5. Subcellular Localization of DcaMADS

The subcellular localization of DcaMADS proteins was investigated via injection transformation with green fluorescent protein (GFP) fused with 15 DcaMADS proteins in N. benthamiana, belonging to eight subfamilies (A, B, C, E, SOC1, AGL12, AGL15, and SVP subfamilies), which were highly expressed in flower organs. All of the GFP-DcaMADS signals were localized to nuclei (Figure 7). This result is similar to that found in the experiments with other plants, in which several MADS-boxes as transcription factors have been found to be localized to the nucleus [12,27,83,84]. The motif KR[K/R]X4KK (motif 1 (Figure S2)) at positions 22–30 of the MADS-box domain plays an important role in the translocation of MADS-box proteins into the nucleus [54]. This motif of DcaMADS proteins in carnation is highly conservative.

![Figure 7. Subcellular localization of DcaMADS fused with green fluorescent protein. Plasmids containing fusions of GFP and DcaMADS driven by the CaMV35S promoter were transiently expressed.](image)

4. Discussion

In recent years, more and more studies of the MADS-box family in various species, such as in Arabidopsis [7], poplar [81], rice [32], grape [85], cucumber [34], soybean [35], Prunus mume [86], apple [51], Erycina pusilla [87], Brassica rapa [36], and radish [38], have been reported. MADS-box genes in various species showed great difference. However, the MADS-box gene family in Caryophyllaceae has not been reported. In our study, seven Type I MADS-box genes (2 Mα, 5 Mγ) and 32 type II MADS-box genes (2 MIKC* and 30 MIKCc) were identified in carnation. The phylogenetic relationships and expression patterns of the two type genes varied greatly. This study will be hopeful to understanding DcaMADS genes’ contributions to organ development in carnation.

4.1. AP3/PI and SEP Subfamily with Duplication Influences Evolution and Divergence

AP3/PI subfamily: B function gene is one of the most frequently studied MADS-box gene controlling floral organ. The evolution of this subfamily gene involves a large number of gene duplication events at different taxonomic levels [88,89]. One large-scale gene duplication event occurs before the formation of angiosperms, resulting in two evolution lines of paleoAP3 and PI. The other
gene duplication event occurs before the formation of core eudicot plants, giving rise to the birth of two evolution lines of *euAP3* and TM6 deriving from the *paleoAP3* gene through replication [90,91]. These different evolution lines can be distinguished by their C-terminal domain motifs. The C-terminal domain of TM6 gene is found to have PI-derived motif and paleoAP3 motif (which specifically belong to *paleoAP3* gene). Both the PI-derived motif and euAP3 motif are found in the C-terminal domain of the *euAP3* gene, while the PI-derived motif is only found in the C-terminal domain of the PI gene. In carnation, *DcaMADS27* and *DcaMADS28* are members of the PI evolution line; *DcaMADS29* and *DcaMADS30* belongs to the *euAP3* evolution line and *DcaMADS31* belongs to TM6 (Table S6 and Figure S4). The class B subfamily genes in carnation were involved in two duplication events that contributed to the three evolution lines (PI evolution line, euAP3 evolution line, and TM6), which may cause these genes to function differently. Previous study showed that the class B subfamily genes were conserved in determining petal and stamen characteristics, but different evolutionary lines had dynamic changes in time and space [92]. There are three evolution lines in carnation: the PI evolution line and *euAP3* evolution line have two genes, which have different expression, and these genes may play different roles in stamen development in carnation. Our results provide the basis for studying the functional differentiation of carnation class B genes.

**SEP** subfamily: Phylogenetic evolution analysis showed that **SEP** subfamily genes experienced multiple gene duplication events during their evolution. The first duplication event generated **SEP3** (*SEP3* was previously named AGL9, and **SEP1**/**SEP2**/**SEP4** were formerly named AGL2/AGL4/AGL3). The evolution lines of **SEP1**/**SEP2**/**SEP4** underwent two gene duplication events, producing **SEP1**/**SEP2**, **FBP9**/**FBP23**, **SEP4** evolution. This study revealed that *DcaMADS1* belongs to **SEP1**; *DcaMADS2* and *DcaMADS3* are members of the **SEP3** evolution line; *DcaMADS6*, *DcaMADS7*, and *DcaMADS8* belongs to **SEP4** (Table S6 and Figure S5). There are some reports about E-functional genes expressed in different tissues of various species such as *Arabidopsis* [93], the petunia **FBP2** gene [55,94], the **TM5** gene [95], the **LeMADSRIN** gene [96], and the *Gerbera hybrida* **GRCA1/2** gene. The number of **SEP** subfamily genes, their expression pattern and functions vary with the species and so these genes may also have different functions in carnation. What the functions of these genes of **SEP** subfamily in carnation are requires further study.

### 4.2. A Model in Flower Organ Identity of Carnation

Transcription factors of MADS-box genes play specific roles in flower organ development, especially in specifying floral organ identity, which have been revealed in the model eudicots *Arabidopsis* [6,97] and *Antirrhinum* [25]. Different subfamilies have different expression patterns. In this study, we investigated MADS-box in carnation, which can regulate flower development and different organ formation. Some genes in every subfamily have extremely low, or no, transcript abundances in flower organ tissues, while other subfamily genes display diverse expression patterns representing the distinct roles of the different groups. Gene functions of A-, B-, C-, D-, and E classes in carnation showed similarities and differences to that in *Arabidopsis* and other species. In order to further clarify the function of these genes, we conducted an in-depth study of the expression of these genes in different flower organs of carnation. All genes were recalculated for expression with the same gene of lower expression as a reference (Figure S6) and these genes with relative expressions more than 30 were selected. A model of gene expression patterns in carnation (Figure 8) is proposed based on the well-known ABC model of *Arabidopsis*.

In the sepal of carnation, five genes (three class E genes (*DcaMADS1, DcaMADS2*, and *DcaMADS7*), two class A genes (*DcaMADS17* and *DcaMADS18*)) were strongly expressed, suggesting that these genes might function together to control the sepal in carnation flowers (Figure 8), in accordance with the **API/FUL** gene and **SEP** gene together involved in regulating the development of sepals and petals of *Arabidopsis* [70]. Based on these results, we proposed several questions which need a further study: Why were three E genes in sepals detected? Is the complex regulating sepal development the combination of four proteins together?
In petals of carnation, five genes (two class B genes (DcaMADS27 and DcaMADS28), two class E genes (DcaMADS1 and DcaMADS2), and one SVP gene (DcaMADS25)), strongly expressed, play an important role in carnation (Figure 8). A complex of one AP1, one SEP protein, and two class B proteins (AP3 and PI) determines petal identity in the floral quartet model of Arabidopsis [98]. Compared with Arabidopsis, why are class A genes not strongly detected in the petals of carnation? This phenomenon may be due to the special structure of carnation or there may be other genes undetected that are functioning in the petal.

Figure 8. The high expression pattern of the DcaMADSs gene in carnation. The genes in rectangular boxes point to their expression being strongly detected, and the amount of expression decreased in turn.

In the stamen of carnation, two B class (DcaMADS27 and DcaMADS28), two C class (DcaMADS11 and DcaMADS12), and two E class (DcaMADS1 and DcaMADS2) genes were strongly expressed (Figure 8), while previous studies show that a complex of one SEP, one AP3, one PI protein, and one AG protein determines stamen identity in Arabidopsis [14]. Two genes have been added in carnation compared to the classic quarter model and what is the function of these two extra genes? We speculated that two B class or two C class genes create a functional redundancy or these six genes can form new complexes involved in the regulation of the stamen in carnation. We also found DcaMADS1 (TM6) of class B genes in carnation were expressed in sepal, petals, and stamens, and previous studies found that TM6 plays a vital role in regulating stamen identity [99], DcaMADS1 did not present obviously in this trend, which may be due to it not playing a role in stamen development. These hypotheses require more experiments to be performed.

In the carpel of carnation, four genes (two class C genes (DcaMADS11 and DcaMADS12), two class E genes (DcaMADS1 and DcaMADS2)), strongly expressed, play an important role in styles (Figure 8), and two class E genes, two class C genes, one AGL6, one STK, one B sister, one SVP, and one Mα were detected in ovaries suggesting their involvement in floral carpel development. Previous studies found that the combination of SEP genes with C-class and E-class genes regulated the development of the carpel [99]. This study showed that not only DcaMADS13 (STK), but also DcaMADS9 (AGL6), DcaMADS33 (B sister), DcaMADS41 (Mα), and DcaMADS25 (SVP), were detected strongly in ovaries (Figure 8). These genes may be play an important role in floral organ identity.

Carnation is more diverse and complex than the simple Arabidopsis flower. Many of DcaMADS genes in different tissues exhibit expression patterns similar to those in Arabidopsis and other plant species, whereas other genes have their unique expression profiles. This difference may be related to difference species or the genetic evolution over a long history. The real reason requires us to make a deep exploration.
**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4425/9/4/193/s1, Table S1: *Arabidopsis* MADS sequences for the phylogenetic tree, Table S2: Rice MADS sequences for the phylogenetic tree, Table S3: Primers for quantitative PCR of DcaMADSs, Table S4: Primers for subcellular localization of DcaMADSs, Table S5: The number of B and E class genes in different species, Table S6: Sequences of B and E class genes for the phylogenetic tree, Figure S1: The ORF of DcaMADS6, DcaMADS8, DcaMADS18, DcaMADS28, and DcaMADS29, Figure S2: Conserved motifs of 39 carnation MADS-box proteins identified using the MEME program. The information of Motifs 1 to 15, Figure S3: The phylogenetic tree of the 39 DcaMADS genes was generated by the neighbour-joining (NJ) algorithm using Molecular Evolutionary Genetics Analysis (MEGA version 6.0) software. The subgroups are marked in different colors. (a) Phylogenetic tree of D. *caryophyllus* and Rice type II proteins. (b) Phylogenetic tree of Rice and D. *caryophyllus* type I proteins, Figure S4: The phylogenetic tree of the class B genes in carnation was generated by the neighbour-joining (NJ) algorithm using MEGA (version 6.0) software, Figure S5: The phylogenetic tree of the class E genes in carnation was generated by the neighbour-joining (NJ) algorithm using MEGA (version 6.0) software, Figure S6: Organ specific expression of 35 DcaMADS genes at different flower whorls.

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