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RESEARCH ARTICLE

Expression analysis of MADS-box genes in phenotypically distinct carrot cytoplasmic male sterile lines and their maintainers

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Abstract

The utilization of maternally inherited loss of male fertility has facilitated the development of hybrid seeds for commercial production of carrots. The loss of male sterility is due to alterations in floral organs that render the male reproductive organs unfunctional or may cause the complete elimination of a specific floral organ. Although organ alterations have previously been studied in carrot cytoplasmic male sterile lines to characterize them on phenotypic and molecular basis, the role of MADS-box genes in the development of different floral organs has not been emphasized. In this study, the analogous use of stereo microscopy and qRT-PCR enabled the characterization of the role MADS-box genes play during carrot floral organ development. A total of 25 CMS lines were screened to determine the floral organ alterations. Out of which, four phenotypically distinct floral morphotypes were identified in the CMS germplasm, i.e., sepaloid type, brown anther type, miniature leaf type and lance type. Further, these four phenotypically distinct CMS lines were subjected to quantitative expression analysis of eight floral identity MADS-Box genes. The gene expression results confirmed that variation in the expression of floral identity genes is responsible for organ alteration in male sterile flowers of carrot.

Keywords: qRT-PCR, Cytoplasmic male sterility, Organ alterations, Sepaloid type, Brown anther type.

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Introduction

Carrot (*Daucus carota* L., 2n = 2x = 18) is one of the most exploited root vegetable crops of the *Apiaceae* family whose inflorescence is the distinctive feature, which is a compound umbel made up of several subunits known as umbellets. A standard carrot flower is an epigynous hermaphrodite which has five-lobed calyxes, each flower usually having five stamens, five petals and a two-celled, inferior ovary with a single functional ovule in each locule (Mas et al., 2018). Away from the standard flower types, flowers with altered morphology have also been reported in carrots. These alterations have led to two distinct types of cytoplasmic male sterility systems in cultivated carrots (Banga et al., 1964). The first, comparatively less prominent, is the brown anther type of male sterility, which is characterized by shriveled and brown-to-yellow anthers devoid of functional pollens. This type of male sterility in carrots is not exploited extensively due to its high instability (Luo et al., 2013). The second type of male sterility is characterized by the conversion of stamens into petal-like organs, which is quite stable and has high commercial value in the hybrid seed production of carrots (Nothnagell et al., 2000). Apart from these two cytoplasmic male sterility systems, the presence of a third type of male sterility system has also been documented where stamens have been converted into female reproductive organs and

have been termed as 'carpelloid' type male sterility (Linke et al., 2003). The alterations in the floral morphology of plants are primarily reported to occur due to their inability to physically escape the stress conditions (Esmon et al., 2005).

The genetics of flower development is governed by a complex gene regulatory network (Immink et al., 2010) in which the transcription factors of the MADS domain play an important role (Thangavel and Nayar, 2018). These genes derive their name 'MADS' from the four founding genes which included MCM1 from *Saccharomyces cerevisiae* (Budding Yeast), AGAMOUS from *Arabidopsis thaliana* (Thale Cress), DEFICIENS from *Antirrhinum majus* (Snapdragon) and the SRF gene from *Homo sapiens* (Humans) (Schwarz-Sommer et al., 1990). The involvement of MADS-box genes in floral development is explained through the ABC model of flower development that was given by analyzing 'homeotic' flower mutants of model plant *Arabidopsis thaliana* (Coen and Meyerowitz 1991). The model was later expanded to include class D genes that promote ovule development and class E, or SEPALLATA (SEP) genes that act as cofactors for A, B, C, and D class genes (Theiben and Saedler 2001). Sepals are determined by the class A and E genes in the first (outer) flower whorl. The actions of the class A, B, and E functions define the petals of the second flower whorl. The function of class B, C, and E in the third whorl specifies the stamens (male) organs. The action of class C, class E and class D gene function defines the female organ in the flower's center (Kalia et al., 2023).

In the present study, alterations in flower morphology of CMS lines in carrots will be recorded and the diverse morphotypes of CMS lines and their maintainers will be used for expression analysis at the T3 stage of flower development (Liu et al., 2019) to determine the regulatory network involved in cytoplasmic male sterility and development of flower organs. The study will also analyze the quantitative expression of eight differentially expressed MADS-box genes in carrot CMS lines and their respective maintainers. This study has been undertaken to discover upregulated and downregulated genes associated with the development of diverse morphotypes in the CMS and maintainer germplasm during flower development and will allow us to understand the flower formation process for further research in the development of cytoplasmic male sterility lines in carrot.

Materials and Methods

Experimental plant materials

Experimental material included twenty-five CMS lines along with their maintainers, which were sown in the field for mother root production in the month of September 2020. The recommended package of practices was followed to raise a successful crop (Anonymous 2020). The roots became ready for harvesting in 90 days after sowing. The stecklings were prepared from the harvested mother roots and transplanted under the field in the second fortnight of January 2021 for raising the seed crop. The bolting of the male sterile as well as maintainer lines was observed in 45 to 55 days after planting the stockings in the field. The CMS lines were maintained by crossing with the maintainer plants and the seeds were collected and stored for sowing in the next season.

Phenotypic characterization of CMS lines and their maintainers

About 20 plants were selected at random from each CMS line and its maintainer to ascertain the floral alterations. The cytoplasmic male sterile lines and their maintainers were characterized phenotypically based on the morphological characteristics like florets with single stigma, florets with split stigma, florets with no filament and no anther, florets with filament but no anther, florets with brown anther, florets with white petals, florets with green petals or any other modification.

Stereo microscopy of modified CMS flowers and their maintainers

Flowers from CMS lines that exhibited the most diverse morphotypes during phenotypic characterization along with their maintainers were collected at the mature flowering stage and examined under a stereo microscope. Floral imaging was done using a Leica MZ16 stereo microscope by Leica Microsystems (Switzerland) Ltd, available at Electron Microscopy & Nanoscience Laboratory (EMNL), Punjab Agricultural University, Ludhiana.

Confirmation of male sterility

The flowers were subjected to acetocarmine staining to determine whether or not they were male sterile, given their different morphologies. The presence of viable pollen was checked in both the flowers with morphological variation and their maintainers. The organs were crushed and placed on a microscope glass slide before being stained with a 1% acetocarmine solution (1 g carmine in 100 mL of 45% glacial acetic acid) and viewed under a compound microscope.

RNA isolation and cDNA synthesis

Total RNA was isolated using ReliaPrep™ RNA Tissue Miniprep System (Promega Corporation) by taking 3 biological replicates from young umbels at T3 stage-inflorescence about 3 mm in length (Liu et al., 2019). A Thermo Scientific NanoDrop™ 1000 spectrophotometer was used to measure RNA quantity, and RNA quality was checked using denaturing gel. The first strand of cDNA from the purified RNA was synthesized using GoScript™ Reverse Transcription System (Promega Corporation).

qRT-PCR analysis

The relative gene expression of eight floral identity genes (*DcAG, DcAGL-1, DcAGL-3, DcDEFL-1*, *DcPI, DcAGL9-1, DcSEP1*

and *DcSEP1L*) through, qRT-PCR analyses was performed on four samples with variations in floral morphology from the CMS lines and their respective maintainers. These eight floral identity genes were shortlisted on the basis of their differential expression in CMS and maintainer lines and belonged to three different gene classes (Liu et al., 2019). The gene-specific primers for the aforesaid eight floral identity genes have been given in Table 1. The analysis of relative expression level was done using Applied Biosystems™ StepOne™ Real-Time PCR System. qRT-PCR was performed in triplicates using cDNA as a template. For each sample, three biological replicates and three technical replicates were performed. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to determine the relative expression level of each of the eight selected floral identity genes and the data was considered as the mean ± standard deviation across three biological replicates. The relative expression of the target genes was normalized to the expression of the reference gene and the data was analyzed using ΔCt = Ct mean (target gene) - Ct mean (Actin), ΔΔ Ct =ΔCt (CMS line) - ΔCt (maintainer) and $2^{-\Delta\Delta CT}$ represents fold change in gene expression in flowers with variation in morphology relative to unmodified flower (Relative gene expression). The eight floral identity genes were selected on the basis of differential expression in CMS and maintainer lines and belonged to three different gene classes (Liu et al., 2019). Two genes *DcDEFL-1* and *DcPI* belonged to the B class and were responsible for determining petal and stamen identity, while three genes, each from both C and E classes, were considered for this study. *DcAG, DcAGL-1,* and *DcAGL-3* were associated with the C class, which determines carpel and stamen identity, whereas *DcAGL9-1, DcSEP1* and *DcSEP1L* belonged to E class that acts in combination with all other gene classes.

Table 1: Gene-specific primers used in qRT-PCR analysis

Gene	*Primer*	*Sequence*
DcAG	DcAG-FF	GGAGCTGGGAGGGGTAAGAT
	DcAG-RR	ACGACCACGGCTAGAGAAGA
DcAGL-1	DcAGL-1-FF	CAGGTGCATCTGGGAGTT
	DcAGL-1-RR	GGTCCTGGTGAGACGAGTAA
DcAGL-3	DcAGL-3-FF	GAGGCCACTAGATTACGTCGTG
	DcAGL-3-RR	AGCTCATTCTTCTTGGAGCGG
DcAGL9-1	DcAGL9-1-FF	TCCGTCAAGTAGCTCCACAA
	DcAGL9-1-RR	GTTATTCGCACTCGGACCAG
DcDEFL-1	DcDEFL-1-FF	AGTAACCACTACCAGGCTATGC
	DcDEFL-1-RR	CCATTCAAACTTTCGCCCTTCC
DcPI	DcPI-FF	TCCCTGCATTTACCAGAACTCG
	DcPI-RR	TGCCACTGCCATTCCCATC
DcSEP1	DcSEP1-FF	ATAACCAGCAGCAGCAAC
	DcSEP1-RR	GCCTCCATCCAGGTAGCA
DcSEP1L	DcSEP1L-FF	GCGGTGAAGTAAGTGCCT
	DcSEP1L-RR	GAATTGCAGTTCAGTGGC

Results

In the phenotypic characterization of CMS and maintainer germplasm, four diverse flower morphotypes were identified. CMS line MS 7 X CARROT VAR 5 had four off type plants with 'sepaloid type' flowers (FT 9). Three plants in MS 9 X CARROT VAR 5 were found to bear 'brown anther type' flowers (FT 14). In male sterile plants of CMS line MS 12 X PC 161, four plants were observed bearing 'miniature leaf type' male sterile flowers (FT 19). Lastly, CMS line MS 27 X PC 161 only had a single plant bearing 'lance type' male sterile flowers (FT 25).

Microscopic assessment of floral biology in morphologically distinct CMS lines and their maintainers

To accurately classify the morphological changes and to describe the organ variations, the CMS flowers were examined under a stereo microscope. In the flowers of FT9 (Figure 1(A)), the outermost whorl, which usually consists of calyx-like teeth, was replaced by elongated sepals, which were geometrically spatulate. In contrast, the second whorl sepals, which replaced the petals, were three-lobed and placed in the interstices of the first whorl sepals. The flowers were thus labeled as 'sepaloid type.' The third whorl was completely devoid of any organs. A similar yet distinct 'green petaloidy' has been previously documented (Kitagawa et al., 1994), but elongation of sepals is a novel report. The flowers of FT14 (Figure 1(B)) were identical to those described by Banga et al. (1964) as 'brown anther type' male sterile flowers. The flowers of FT19 (Figure 1(D)) showed no variation for the first whorl organs and bore calyx-like teeth in the outermost whorl. Prominent changes were observed in the third whorl, where the flower appeared to bear a filament-like organ that was thicker at the base and tapering towards the top. Heart-shaped miniature leaf-like organs replaced the anthers, therefore labeled as 'miniature leaf type.' Small spikes were also observed near the base of the filament on the surface facing away from the gynoecium. The flowers of FT25 (Figure 1(E)) resembled those of FT19 with the exception of the replacement of stamens by three-pronged spear-like organs, hence identified as 'lance type' flowers. The formation of three spiked organs instead of functional anthers has been documented in the past (Kalia et al., 2019). The previously reported spikes were palmately lobed and joined at a single point, whereas those documented in the current study arise at different positions along the same axis. All four distinct morphotypes were identical in their fourth whorl organs and had a bicarpellary ovary with two styles. Both the maintainer's CARROT VAR 5 (M9) and PC 161 (M19)

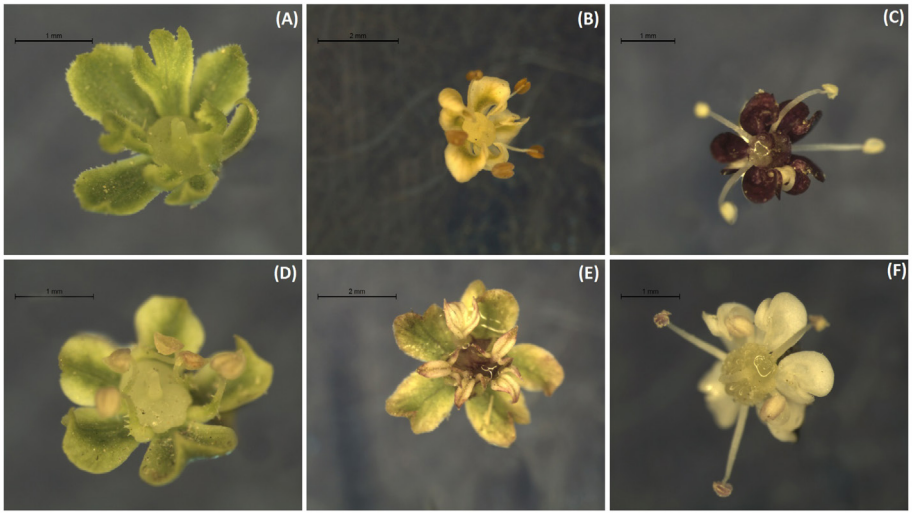

Figure 1: Morphological observation of four phenotypically distinct CMS lines and their maintainers under a stereo microscope. (A) FT9 – 'Sepaloid type', (B) FT14 – 'Brown anther type', (C) M9 – 'Wild type' (Maintainer), (D) FT19 – 'Miniature leaf type', (E) FT25 –'Lance type', (F) M19 – 'Wild type' (Maintainer)

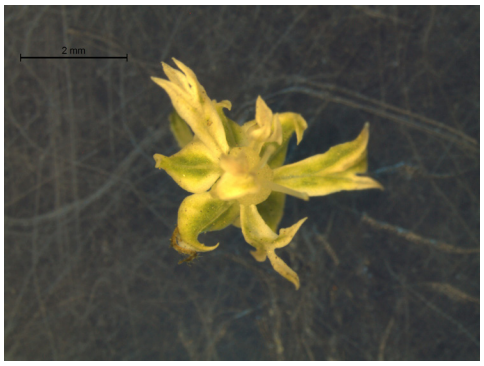

Figure 2: Morphological observation Petaliod Type Male Sterility under a stereo microscope

were found to be identical and were similar to the 'wild type flower' (Figure 1(C) and Figure 1(F)) (Budahn et al., 2014)**.** The flowers were hermaphrodite and epigynous in nature and were composed of four concentric organ whorls. The outermost whorl bore green-colored small calyx teeth, while the second whorl had 5 to 6 petals with reniform geometry. The third whorl had 3 to 5 stamens and the anthers located at the tip of the filaments were producing viable pollen. The gynoecium, having a bicarpellary ovary with two styles was present in the central fourth whorl. Meanwhile, the petaloid type flowers (Figure 2) show a complete transformation of stamens into petal-like structures, leading to dysfunctional male reproductive organs in flowers.

Confirmation of male sterility using 1% acetocarmine staining

Acetocarmine staining was used for confirming flowers with different morphologies for male sterility. The acetocarmine-treated flowers with different morphologies, i.e., FT9, FT14, FT19, and FT25, did not show any staining under the compound microscope, which confirmed that the flowers were male sterile and that the modified organs did not bear any pollen. In contrast, the flowers of maintainer lines (M9 and M19) showed the presence of multiple stained areas, which indicated the presence of viable pollen. These were ovular in shape and the diameter of a single pollen varied between 25 to 35 µm.

Gene expression analysis of eight floral identity genes

The gene expression of eight floral identity MADS-box genes was analyzed in four distinct floral morphotypes, i.e., sepaloid type, brown anther type, miniature leaf type and lance type. The gene expression analysis in the 'sepaloid type' flowers (Figure 3 (A)) was found to be significantly upregulated for six floral identity genes, i.e., *DcAG, DcAGL-1, DcAGL-3, DcPI, DcSEP1* and *DcSEP1L* while for two genes, i.e., *DcAGL9-1* and *DcDEFL-1*, the expression was downregulated. *DcSEP1* had the highest 10-fold upregulated gene expression. There was significant upregulation of every

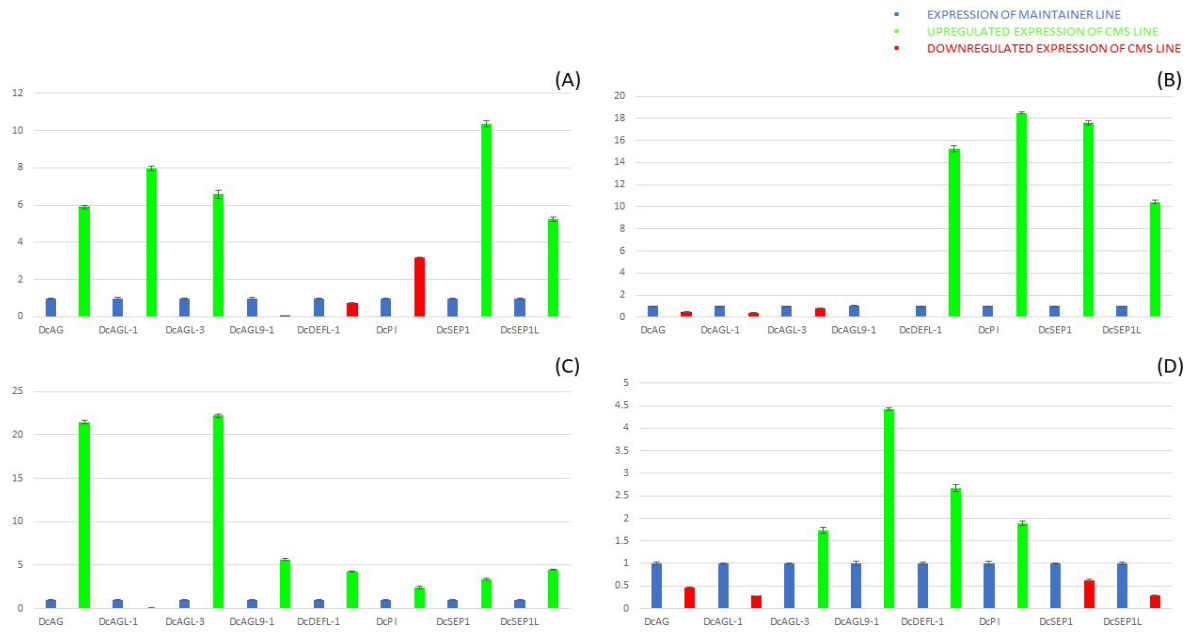

Figure 3: Expression of Eight MADS-Box genes. (A) FT9 – 'Sepaloid type', (B) FT14 – 'Brown anther type', (C) FT19 – 'Miniature leaf type', (D) FT25 –'Lance type'

candidate gene from class C that controls carpel and stamen identity. While *DcAG* and *DcAGL-3* both displayed a 6-fold expression and *DcAGL-1* displayed an 8-fold expression. *DcPI* and *DcDEFL-1*, class B genes responsible for petal and stamen identity, displayed a 3-fold upregulation and a marginal 0.74-fold downregulation, respectively. In the brown anther type male sterile flowers (Figure 3 (B)), class C genes *DcAG, DcAGL-1* and *DcAGL-3* had corresponding 0.5, 0.4, and 0.7-fold expressions and were significantly downregulated. The gene expression in both the class B candidate genes *DcPI* and *DcDEFL-1* was observed to be highly elevated. *DcPI*, with an 18-fold expression, had the highest gene expression, followed by *DcDEFL-1,* with a 15-fold expression. One of the three candidate genes in the class E, i.e., *DcAGL9-1,* had a significantly downregulated fold expression, i.e., 0.01-fold. The expression of the other two genes i.e., *DcSEP1* and *DcSEP1L*, was upregulated by 17 and 10-fold, respectively. In the 'miniature leaf type' male sterile flowers (Figure 3 (C)), only one MADS-box gene i.e., *DcAGL-1* belonging to gene class C was found to be downregulated, which controls the identity of the stamen and carpel, while all other seven floral identity MADS-box genes were significantly upregulated. It was also observed that *DcAGL-3* showed the highest degree of upregulation. The expression of the other two genes in gene class C was upregulated. *DcAG* showed a 21-fold expression, whereas *DcAGL-3* showed a 22-fold expression. 2 and 4 folds, respectively upregulated the expression of the B class genes DcPI and DcDEFL-1. *DcSEP1, DcSEP1L* and *DcAGL9-1*, the three genes from class E showed an expression that was 3, 4, and 5-fold, respectively. Lastly, in the 'lance type' flowers (Figure 3 (D)), *DcAG* and *DcAGL-1*, two of the three class C genes showed downregulated expression of 0.4 and 0.2 folds, respectively, whereas *DcAGL-3* was found to be upregulated by 1.7 folds. The gene expression of the two B class genes, *DcDEFL-1* and *DcPI* was reported to be 2.5 and 2-fold higher than their maintainer. The three genes belonging to class E showed contrasting results. With a 4.5-fold expression, *DcAGL9-1* presented the most upregulated expression among the eight candidate genes. The expression of the other two E-class genes, on the other hand, was found to be downregulated with corresponding fold expression of 0.6 and 0.3. Meanwhile, the gene expression in petaliod type flowers (Figure 2) was found to be upregulated compared to their male counterpart in all MADS-box genes except for DcAP1-1, DcAP1-2 and DcDEFL-3 (Liu et al., 2019).

Discussions

The development of hybrid carrot varieties is greatly aided by the widespread use of cytoplasmic male sterility in carrot breeding. Numerous studies, using different procedures, have attempted to characterize CMS lines on the phenotypic and molecular basis, but only a few studies have emphasized understanding the complete role of MADS-box genes in the development of different floral organs. In the present study, stereo microscopy and qRT-PCR were used in parallel to characterize the role of MADS-box genes during carrot floral organ development. Quantitative RT-PCR was used on account of the high sensitivity of this method to detect transcripts of genes with variable expression.

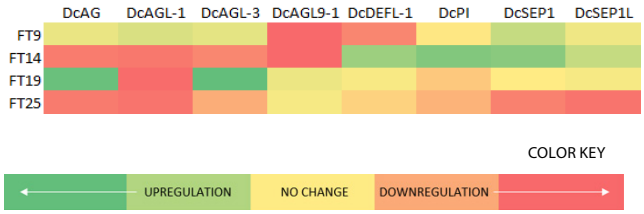

Figure 4: Heat map of eight MADS-Box genes in four different flower types

In the 'sepaloid type' flowers (FT9), characterized by the absence of green-colored calyx teeth, the presence of the elongated sepal-like organs in the outer whorl, the replacement of the petals with three-lobed sepal-like organs and the lack of stamens (Figure 1(A)) two of the eight floral identity genes were found to be down-regulated. One of the two downregulated genes, *DcDEFL-1,* was associated with class B, while the *DcAGL9-1* belonged to class E. As per the ABCDE model, B-class candidate genes are known to influence how petals and stamens are formed (Theissen et al., 2016). The B-class genes are expressed consistently in the third whorl, where stamens usually develop (Whipple et al., 2007). Additionally, studies have suggested that reduced B-class gene expression results in the conversion of petals into sepals (Behrend et al., 2015). nevertheless, sepaloid flowers of *Daucus carota* did not exhibit a downregulated expression of *DcPI*, a B class homologous gene of *CvPI,* that was downregulated in sepaloid flowers of *Calluna vulgaris*. The homeotic mutants of *Arabidopsis* and *Antirrhinum* that showed impaired B-class genes also showed the substitution of petals by sepals. The gene expression results of FT9 were found to coincide with those of Liu et al., (2019) for six candidate genes belonging to different gene classes: *DcAG, DcAGL-1, DcAGL-3, DcPI, DcSEP1* and *DcSEP1L* showed a higher expression compared to its fertile counterpart M9. The findings of Linke et al. (2003) and Liu et al. (2019) suggest that the downregulated transcript levels of MADS-box genes play a key role in the malformation of floral organs of carrot cytoplasmic male sterile lines. Moreover, the eight candidate floral identity genes are known to be upregulated at T3 stage due to the germination of floret primordia and a higher involvement of these genes in the metabolic and cellular processes (Liu et al., 2019). Therefore, the conserved role of *DcDEFL-1* and a highly downregulated expression of *DcAGL9-1* may have affected the organ development in the second and the third whorl, resulting in the formation of sepal-like structures in the second and the absence of male reproductive organs in the third whorl.

In the brown anther-type male sterile flowers (FT14) that are recognized by the presence of yellowish-brown shriveled anthers (Figure 1(B)), four of the eight candidate floral identity genes reported downregulated expression. Three of these four genes, *DcAG, DcAGL-1* and *DcAGL-3*, belonged to class C, whereas the fourth downregulated gene, *DcAGL9-1*, was associated with class B. Therefore, the downregulation of all the C classes and extremely low expression of *DcAGL9-1* were responsible for the development of non-functional, shriveled, brown anthers. The 'miniature leaf' type flowers (FT19) bearing a heart-shaped miniature leaf-like organ in place of the anthers (Figure 1(D)) showed the downregulation of only one MADS-box gene *DcAGL-1* whereas the remaining seven floral identity MADS-box genes were significantly upregulated. Therefore, it could be concluded that the development of miniature leaves to replace the anthers, along with the thickening of the filament in the third whorl, was due to the downregulation of *DcAGL-1*, a C-class gene. Lastly, in the 'lance type' flowers (FT25) having three-pronged spear-like organs replacing the stamens (Figure 1(E)), four of the total eight candidate genes were upregulated, whereas the remaining four candidate genes were notably downregulated. Two of the four downregulated genes, *DcAG* and *DcAGL-1*, were identified as class C genes, while the remaining two downregulated genes, *DcSEP1* and *DcSEP1L,* belonged to gene class E. Since the floral malformation in carrot cytoplasmic male sterile lines is due to reduced expression of MADS-box genes (Linke et al., 2003), it is clear that the formation of lance-shaped organs is mainly due to the downregulation of two floral identity genes belonging to C class accompanying their interaction with two down-regulated E class genes.

The results suggest that flower malformation in carrot cytoplasmic male sterile lines is due to the downregulation of specific floral identity genes. The findings of the study can be supported by the fact that the phenomenon of flower malformation in carrot cytoplasmic male sterile lines is caused by reduced MADS-box gene expression (Linke et al., 2003). The study offers a strong basis to improve the foundation for subsequent investigation into the molecular mechanisms underlying carrot petaloid CMS and flower development. It is concluded that the organ alterations in the cytoplasmic male sterile lines occur due to changes in the magnitude of expression levels in different floral identity genes. The study also illustrates that the development of flowers with organ alteration can be detected before the morphological changes in floral organs occur and immediately after the meristem germination with the aid of gene expression analysis, which the breeders can exploit to screen male sterile lines.

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सारांश

गाजर की व्यावसायिक खेती के लिए हाइब्रिड बीज विकसित करने में मादा पौधे से विरासत में मिले नर बांझपन का उपयोग किया गया है। नर बांझपन फूल के अंगों में बदलाव के कारण होता है, जिससे नर प्रजनन अंग काम नहीं कर पाते हैं या किसी विशिष्ट फूल के अंग का पूर्ण विलोपन हो सकता है। हालांकि, गाजर के साइटोप्लाज्मिक नर बांझ लाइनों में अंग परिवर्तनों का पहले से अध्ययन किया जा चुका है, ताकि उन्हें फेनोटाइपिक और आणविक आधार पर वर्गीकृत किया जा सके, लेकिन विभिन्न फूलों के अंगों के विकास में ड।क्ै.इवग जीन की भूमिका पर जोर नहीं दिया गया है। इस अध्ययन में, स्टीरियो माइक्रोस्कोपी और ड।क्ै.इवग के अनुरूप उपयोग ने गाजर के फूलों के अंगों के विकास के दौरान ड।क्ै.इवग जीन की भूमिका के लक्षण वर्णन को सक्षम बनाया। कुल 25 ब्बै लाइनों की स्क्रीनिंग फूलों के अंगों के परिवर्तनों का निर्धारण करने के लिए की गई। जिनमें से चार फेनोटाइपिक रूप से अलग फूल मोर्फोटाइप ब्बै जर्मप्लाज्म में पहचाने गए थे, अर्थात् सेपलोइड प्रकार, भूरे परागकोष प्रकार, लघु पत्ती प्रकार और भाला प्रकार। इसके अलावा इन चार फेनोटाइपिक रूप से अलग ब्बै लाइनों को आठ फूल पहचान ड।क्ै.ठवग जीन के मात्रात्मक अभिव्यक्ति विश्लेषण के अधीन किया गया। जीन अभिव्यक्ति परिणामों ने पुष्टि की कि फूल पहचान जीनों की अभिव्यक्ति में भिन्नता गाजर के नर बांझ फूलों में अंग परिवर्तन के लिए जिम्मेदार है।"