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Review

Molecular biology studies on stamen development in ornamental plants

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Abstract: Breeding multi-petal flowers is a significant goal in ornamental plants, with petaloid stamens being a crucial form of multiplicity. Traditional hybrid breeding faces limitations such as long cycles, extensive labour and uncertainty in breeding traits. Utilising molecular biology techniques to investigate the biological functions of key genes involved in stamen development or petalisation in ornamental plants is therefore crucial. This review systematically summarises the genetic regulatory mechanisms of floral organs (including floral bud differentiation and organ development models), MADS-box genes regulating stamen development (such as *AP3* and *PI* genes, *AG* gene and *SEP3* gene), other genes related to stamen development (*TCP* genes, *CYC* genes, *LEAFY* and other genes). The review highlights current limitations in molecular biology research related to stamen development in ornamental plants, analyses potential future research directions, and provides recommendations for future research goals.

Keywords: ornamental plants, floral organ development, stamen petalisation, gene regulation, molecular mechanisms

INTRODUCTION

In floral morphology double-flowered varieties are favoured by consumers in the market due to their numerous petal layers, full flower shape and strong three-dimensional appearance, which align better with modern aesthetic preferences. With the development of the social economy, consumer aesthetic trends and market demands drive the research focus of horticultural scientists.

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Consequently, studies on floral organ development in ornamental plants have gained increasing attention among scholars [1]. There has been substantial research on the molecular mechanisms underlying the formation of double flowers [2, 3]. For ornamental plants, breeding efforts focused on double-flower varieties have always been a key aspect of floral breeding. The dominant traits of double flowers confer significant advantages, making it easier to obtain high-quality double-flower offspring through hybridisation, thereby holding considerable commercial value in seed production [4].

Flowers consist of the pistil, stamens, petals and sepals, with a reduction in stamen number and an increase in petal number as stamen petalisation increases (Figure 1). During plant evolution, homologous genes undergo extensive variation, contributing significantly to the diversity in floral organ morphology and quantity. The formation of floral organs in most plants is influenced by both the genotype and the growth environment [5]. Stamen petalisation is a significant mechanism in the formation of polypetalous flowers, characterised by partial or complete transformation of stamens into petal-like structures, with variations in their petalised forms [6]. During stamen development in plants, the B-class genes *AP3* and *PI*, the C-class gene *AG* and the E-class gene *SEP* collectively determine stamen development and formation [7]. The tetramer protein formed by PI-AP3-SEP3-AG can transform plant leaves into stamen-like organs. Similarly, the PI-AP3-AG protein complex is able to induce *Arabidopsis thaliana* vegetative leaves to develop into stamen-like organs though in a leaf-stamen state [8].

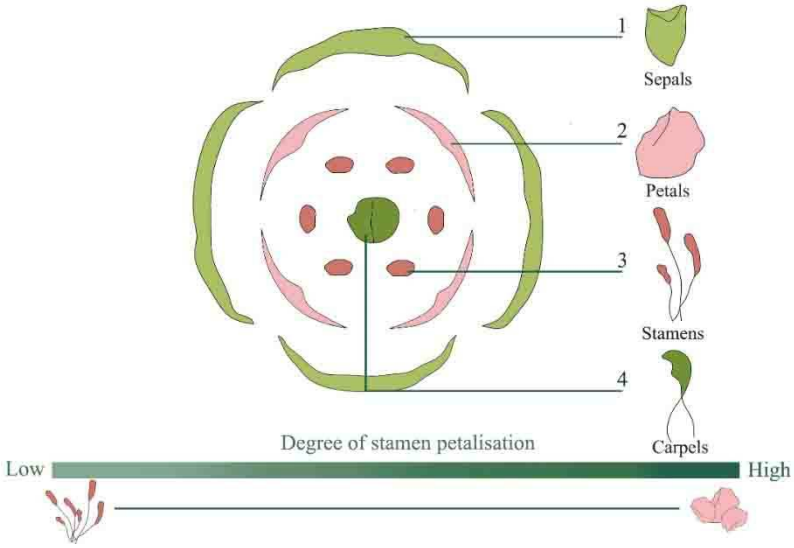

Figure 1. Petaloid transformation of plant stamens

This review systematically summarises the genetic regulatory mechanisms of floral organs, focusing on the regulation of stamen development by MADS-box genes and other genes related to stamen development. A, B, C, D and E class genes involved in plant floral organ development regulate the formation of floral organs in the form of complexes, which can form diverse protein complexes and participate in the development process of floral organs. This review clarifies the functions and relationships of the five classes of genes that regulate stamen development in the floral organ development model. The origin of polypetalous flowers in plants is diverse and there may be evolutionary differences between plants, suggesting possible involvement of other types of genes in the regulation of floral organ development. Further exploration of their expression patterns

and potential functions may be a future research direction of interest. Studying the functions and interactions of these genes helps us better understand the developmental mechanisms of stamens in ornamental plants. This review also identifies some limitations of current molecular biology research on stamen development in ornamental plants, analyses potential future research directions and provides recommendations for future research goals.

GENETIC REGULATION MECHANISMS OF FLORAL ORGANS

Floral Bud Differentiation

The development of plant floral organs follows its own rules, with the establishment of flower morphology beginning at floral bud differentiation [9], which refers to the process whereby a plant transitions from a vegetative to a reproductive growth state. Under favourable conditions, the apical meristem of the bud undergose this transition. Upon receiving the flowering signal, the apical meristem of the bud transforms into an inflorescence meristem, initiating the development of floral organs [10]. During the vegetative growth phase of peony, the apex of the stem (bud) is pointed, but after entering the reproductive growth phase, it becomes relatively flat. Protrusions produced at the edge of the apex are called bract primordia. After the bract primordia are formed, protrusions gradually form on their inner side, which are called sepal primordia. As the floral bud continues to develop, petal primordia gradually appear inside the sepal primordia, and layers of the petal primordia correspond to the layers of petals. The stamen primordia begin to appear after the formation of the petal primordia. The stamen primordia begin to be fully formed after the carpel primordia have developed [1, 6]. Different scholars also noted some differences in the flowering period of different plants, but in terms of their morphological changes, the process of floral bud development is generally similar. That is, floral bud differentiation proceeds from external to internal formation of the bract primordia, sepal primordia, petal primordia, stamen primordia and carpel primordia. Each floral organ primordium undergose continuous growth and development, eventually forming various parts of the flower [6].

Flower Organ Development Model

The development of floral organs and the formation of flower types are regulated by multiple genes and complex pathways. In the model plant *A. thaliana*, the molecular model of floral organ development has evolved from the ABC model to the ABCD model and subsequently to the ABCDE model (Figure 2) as it is continuously supplemented and refined [7]. Prior to the theoretical emergence of the ABCDE model, the well-known model of floral organ development was the ABC model, which marks one of the most significant advancements in the field of plant biology and floral organ research [10]. This model elucidated the roles played by A, B and C class genes in floral organ development as well as their interactions, particularly in the coordinated formation of the four floral organs (sepals, petals, stamens and carpels). For instance, in *A. thaliana*, A class genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*) determine sepal formation. A and B class genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) jointly regulate petal development and formation. B and C class genes, *AGAMOUS* (*AG*), jointly determine stamen identity, while C class genes play a crucial role in the formation of the fourth floral organ, the carpel [11]. Subsequently, the discovery of D class genes *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*), *SHATTERPROOF2* (*SHP2*) and E class genes *SEPALLATA1* (*SEP1*), *SEPALLATA2* (*SEP2*), *SEPALLATA3* (*SEP3*), *SEPALLATA4* (*SEP4*) lead to the advent of the ABCDE model. Among these, D class genes exhibit functional

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similarities with C class genes, while E class genes are a group of functionally redundant MIKC-type MADS-box genes essential for the development and formation of the four floral organs [12].

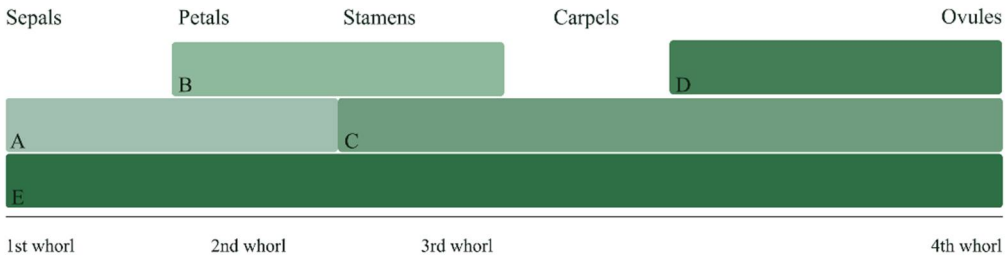

Figure 2. ABCDE model of floral organ development in *A. thaliana*

A, B, C, D and E class genes involved in floral organ development regulate floral morphogenesis through protein complex formation which can assemble into diverse protein complexes participating in the process of floral organ development. In *A. thaliana* the protein complex AP1-AP3-PI-SEP3 regulates leaf development into petals. The protein complex PI-AP3-AP1 or PI-AP3-SEP3 can also transform leaves into petal-like organs. Meanwhile the tetrameric protein complex PI-AP3-SEP3-AG causes the development of leaves into stamen-like organs in plants. The PI-AP3-AG protein complex also results in the transformation of vegetative leaves into stamen-like organs in *A. thaliana*, exhibiting a leaf-stamen state [8, 12]. Based on this, the tetramer model emerges (Figure 3), which provides a clear explanation of the mechanisms underlying the formation of sepals, petals, stamens and carpels in *A. thaliana* [7, 13].

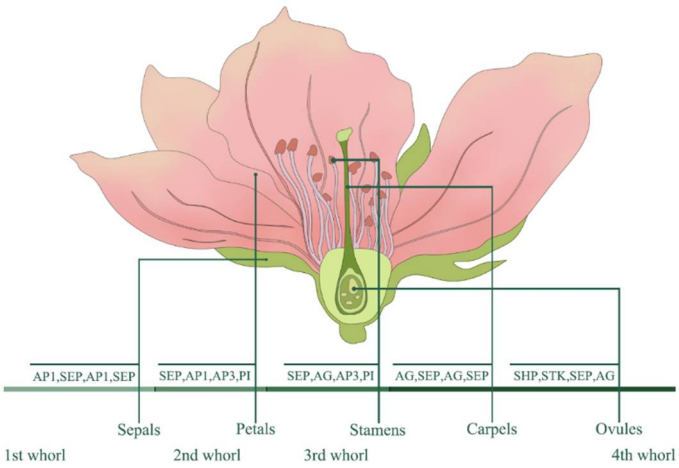

Figure 3. Tetrameric model of *A. thaliana* floral organ development

MADS-BOX GENES REGULATE STAMEN DEVELOPMENT IN ORNAMENTAL PLANTS

AP3 and PI Genes

The *AP3* and *PI* genes, classified as B-class genes in the floral organ development ABCDE model, are MADS-box transcription factors involved in the genetic regulation of flower development. They are essential regulatory genes for the normal development of petals and stamens [8]. Like many MADS-box genes, the proteins encoded by the B-class genes *AP3* and *PI* in *A.*

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thaliana contain a MADS domain, a highly conserved domain consisting of approximately 57 amino acids, which functions in DNA binding and protein dimerisation [14]. During flowering, the expression of *AP3/PI* exhibits temporal and spatial specificity, regulating the expression of different genes and playing distinct roles in various states. Moreover, mutations in *AP3* and *PI* lead to morphological transformations in *A. thaliana* flowers, where petal-like structures convert to sepal-like structures and the third whorl organ stamens develop into carpels. *AP3/PI* genes are expressed in the second and third whorls of flower organs, interacting with numerous genes during flower development and likely primarily regulating the morphogenesis of floral organs [15].

In *Nicotiana tobacum* overexpression of the *A. thaliana AtPI* gene results in abnormal phenotypes in the floral organs. Compared to wild-type tobacco, the transgenic tobacco exhibits primarily reduced corolla size, shortened stamens and malformed fruit, with a longer basal part of the ovary than in the wild type [16]. In *N. tobacum* ectopic expression of the *A. thaliana AtAP3* gene results in a phenotype where stamens replace carpels. However, the AtAP3 protein is predominantly expressed in the second, third and fourth whorls of the floral organs, indicating post-transcriptional regulation of *AP3*. Ectopic expression of *AP3* leads to *PI* functioning in the development of fourth-whorl floral organs, the *AP3* exhibiting positive autoregulation [15]. Overexpression of *AtAP3* in *N. tobacum* results in significantly shorter stamens compared to the wild type, demonstrating specific involvement of the *AP3* gene in stamen development and its crucial role [16].

Numerous scholars have sequentially conducted studies on the regulatory role of the *AP3* gene homologues in floral organ development in various ornamental plants (Table 1). Through a series of experimental validations, these genes show that they primarily function in floral organ development. Additionally, the expression of *AP3* genes exhibits tissue specificity. There are differences in expression levels among different species and different floral organs and the expression levels also vary during different stages of flower development, all contributing to petal and stamen development [17]. Currently *AP3* genes have been isolated and identified in several ornamental plants and these studies are all associated with floral organ development.

Table 1. Overview of functional homologues of *AP3* in different plants

Species	AP3 homologous gene	Main function	Reference
Eriobotrya japonica	*EjAP3*	Petal narrowing; shortening of stamens	[18]
Aquilegia	*AqAP3-1*	Transformation of stamens into carpels; stamen sterilination, delayed development or development into carpels	[19]
Chimonanthus praecox	*AqAP3-2*	Abnormal development of petals and stamens	[17]
Solanum lycopersicum	*CpAP3*	Transformation of petals and stamens into homologous structures	[20]
Lilium longiflorum	*SlAP3*	Shortening of flower petals; shortening of stamen whorls	[21]

The *PI* gene is widely present in plants and its homologues are sequentially cloned in many ornamental plants, with research conducted on its regulation of floral organ development (Table 2). In model plants overexpression of the *PI* gene results in transgenic plants participating in the growth and development of petals or stamens, although their phenotypes vary. Furthermore, there are differences in the expression patterns of genes among different species. Studies indicated that in most species the *PI* gene participates in the growth and development of petals and stamens [1].

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Table 2. Overview of functional homologues of *PI* in different plants

Species	*PI* homologous gene	Main function	Reference
Citrus cavaleriei	*CcMADS20*	Sepal transformation into petal-like structure	[22]
Eriobotrya japonica	*EjPI*	Sepal transformation into petaloid sepal	[23]
Bambusa oldhamii	*BoPI*	Sepal transformation into petal	[24]
Catalpa bungei	*CbPI*	Partial sepal transformation into petal-like structure	[25]

AG Genes

The typical C-class gene *AG* determines the morphogenesis of stamens in the third whorl and carpels in the fourth whorl of floral organs and plays a role in floral meristem formation [26]. During the differentiation of angiosperms and gymnosperms lineages, ancient polyploidisation events are the origin of C-class genes. Simultaneously, they are also the ancestral elements of all stamen and carpel identity genes and D-class genes related to ovule development [27]. In the absence of *AG* gene expression, A-class genes are able to expand and regulate the development of third and fourth whorl floral organs, suggesting a possible mutual inhibition relationship between A and C-class genes [10, 28]. *AG* and B-class genes (*AP3* and *PI*) also exhibit clear antagonistic and independent activities [29]. Research indicates that D-class genes *SHP1* and *SHP2* play a regulatory role in the development of valve and replum in *A. thaliana*. Additionally, they interact with *STK*. In triple mutants of *stk*, *shp1* and *shp2*, abnormal morphogenesis of *A. thaliana* ovules occur, transitioning into leaf-like or replum-like organ structures. In any single or double mutant of the three genes, ovules develop normally, but ectopic expression of any one of them leads to sepals-ovule structures [30]. Due to the functional similarities between C and D-class genes, the latter are also regarded as specialised C-class genes.

The *AG* gene is widely present in plants and several scholars have cloned its homologous genes from various ornamental plants and conducted research on its regulation of floral organ development (Table 3). The ectopic expression of the *AG* gene mostly affected petals, stamens or carpels. Multiple studies indicate a close relationship between *AG* genes and their homologues with stamen development and the formation of multi-petalled flowers. Additionally *AG* gene expression exhibits certain tissue specificity [31]. Furthermore C-class *AG* genes are isolated and identified in many ornamental plants, all of which functionally relate to stamen development.

Table 3. Overview of functional homologues of *AG* in different plants

Species	*AG* homologous gene	Main function	Reference
Lavandula angustifolia	*LaAG*	Early flowering; leaf curling	[32]
Tagetes erecta	*TeAG1*	Overexpression of *TeAG1* and *TeAGL11-1* in *A. thaliana* results in curled leaves and early flowering	[33]
Kerria japonica	*KjAG*	Single-petal flower transforms into multi-petal flower	[34]
Cyclamen persicum	*CpAG1, CpAG2*	*CpAG1* results in stamen transformation into petals, producing flowers with 10 petals; *CpAG2* causes incomplete formation of stamens and carpels.	[35]
Prunus lannesiana	*PrseAG*	Flowers very early, with enlarged stamens and carpels and petals homologously transform into stamens.	[36]
Hosta plantaginea	*HpAG*	Petals are transformed into stamens.	[31]

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SEP3 Gene

The *SEP3* gene belongs to the ABCDE model as an E-class gene and its ectopic expression activates *AP3* and *AG* genes, indicating potential activation effects on B-class and C-class genes. *SEP3* interacts with *AP1* to promote normal flower development [13]. E-class genes act in all floral organ development and cooperate with A-, B-, C- and D-class genes during flower development. The proteins encoded by these genes can form MADS-box protein complexes with those encoded by A-, B-, C- and D-class genes, thereby regulating floral organ development and determining floral organ morphogenesis [12]. *SEP3* regulates auxin response to promote floral organ growth and morphogenesis. Additionally, SEP3 protein is a key component of the transcriptional regulatory network governing floral organ formation. In the quartet model, protein complexes such as AP1-AP3-PI-SEP3, PI-AP3-AP1 and PI-AP3-SEP3 convert sepals into petals or petaloid organs. Moreover, the PI-AP3-SEP3-AG tetramer protein complex transforms plant sepals into stamenoid organs. The specific expression of *SEP3* in floral organs restricts the influence of A, B and C-class genes on floral organ development [8]. It is observed that the protein encoded by the *SEP3* gene acts as a hub for multimeric complexes in floral organ development, playing a crucial role in regulating stamen development.

In *A. thaliana*, the knockout of E-class genes results in significant morphological differences from wild-type plants, where floral organs develop into leaf-like structures or sepals. Besides controlling floral organ identity, *SEP3* also promotes flowering. In *Lavandula angustifolia* and *Ziziphus jujuba*, *SEP3* advances flower opening [32, 37]. Additionally, *SEP3* gene expression exhibits tissue specificity. In *A. thaliana*, *SEP3* is expressed in the second, third and fourth whorls of floral organs while other E-class genes are expressed in the fourth whorl [38].

TRANSCRIPTION FACTORS AND OTHER GENES RELATED TO STAMEN DEVELOPMENT

TCP Gene

TCP genes, as regulators of floral organ size and shape, are related to MADS-box genes [39]. In *A. thaliana TCP* genes are identified as targets of *SEP3* and *AP1* MADS-box proteins [40]. Members of the *TCP* family can be divided into two classes: Class I (also known as *PCF* class) and Class II (also known as *TCP-C* class) [41, 42]. Class II *TCP-C* factors are further subdivided into the *ECE* (*CYC/TB1*) and *CINNATA* (*CIN*) branches [43]. Expression analysis of Class I *TCP* genes (*AtTCP15/14/8/22*) in *A. thaliana* reveals that mutants lacking Class I *TCP* function exhibit poor anther elongation. This indicates that Class I *TCPs* are involved in the anther elongation process. Specifically, *AtTCP15* is found to regulate the expression of the *Small Auxin Up RNA 63* (*SAUR63*) gene family, which is involved in petal and anther development. These findings suggest that the *AtTCP15-SAUR63* module plays a role in regulating anther development [44].

At the cellular level, CYCLOIDEA (CYC2) TCP proteins establish organ cell identity and regulate organ size by modulating cell proliferation or cell expansion. Specifically, *Antirrhinum majus* CYC promotes dorsal petal cell expansion by regulating the expression of the *CYCLIN D3B* gene and inhibits the proliferation of dorsal stamen cells [45]. Conversely, in *Iberis amara*, *TCP1* restricts dorsal petal growth and expansion by negatively regulating cell proliferation [46]. When these transcription factors are expressed in *A. thaliana*, *Antirrhinum majus* CYC has a positive effect on petal growth while *I. amara TCP1* has a negative effect. This discrepancy is consistent across different species. Although the molecular characteristics among different genes are not yet clear, it is evident that different regulatory pathways are involved in the development of floral organs, likely involving protein-protein and protein-DNA interactions [47].

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CYC Gene

CYC-like genes regulate petal morphology and stamen development in most angiosperms, as well as control stem and leaf growth, flower differentiation, and branching. In the Cycadaceae family, CYC-like genes primarily govern morphological characteristics during petal and stamen development [48]. *CYC* genes are expressed in the dorsal region of the symmetric flower meristem, where they modulate stamen degeneration and petal growth through regulation of cell division rates [49]. Phylogenetic analysis indicates that *CYL* genes in Papaveraceae form two paralogous lineages, *PapaCYL1* and *PapaCYL2*. These genes exhibit highly diversified expression patterns and functions across poppy family plants including *Eschscholzia californica* and *Cysticapnos vesicaria*. Silencing of *EscaCYL1* enhances the control of bud branching while *PapaCYL* genes promote stamen initiation and growth. Additionally, *CyveCYL* genes determine sepal and petal characteristics by regulating B-class floral organ identity genes and participate in flower symmetry and perianth development in *Cysticapnos* [50].

Studies demonstrate that the spatial expression patterns of the six members of the *CYC2-like* family in Asteraceae are conserved across the family and collectively regulate capitulum development [51]. *CYC2c* and *CYC2g* play crucial roles in the formation of ray florets in chrysanthemums while CYC2d inhibits dorsal corolla lobe and stamen development in these florets. Further research reveals an interaction between A-class MADS-box genes and *CYC2-like* genes, which may coordinate reproductive organs and ray floret corolla formation, particularly in chrysanthemums capitulum [52]. The B-class MADS-box gene *CDM19* positively regulates the expression of CYC2-like genes *CmCYC2c* and *CmCYC2d*. Through this regulatory mechanism, *CDM19* influences flower shape in chrysanthemums [53].

LEAFY and Other Genes

LFAFY (*LEY*) plays a crucial role in the development of floral organs and stamen differentiation. LFY activates the transcription of *APETALA1* (*AP1*) [54]. The transcription factor *LFY* is a pioneer transcription factor that ensures flower formation by upregulating the floral identity factor *AP1* [55]. *LFY* and *AP1* have a synergistic interaction, enhancing each other's effects and promoting the formation of floral organs [56]. LFY activates the *AP1* locus by binding to specific sites and the structural characterisation of these binding interactions advances our understanding of floral development [57]. *LFY* and *AP1* co-activate several genes including B-class genes (such as *AP3* and *PI*), C-class genes (such as *AG*) and E-class genes (such as *SEP3*). These activated genes determine the specialisation and development of stamens and carpels, ultimately regulating the morphogenesis of all floral organs [58].

In addition to genes determining floral organ identity, other genes such as those involved in floral organ fusion, boundary genes and genes affecting the number and arrangement of floral organs also influence the composition of floral organs. Among these, the *NAC* gene family transcription factor *CUP-SHAPED COTYLEDON* (*CUC*) is typical of floral organ boundary-determining genes [59]. In *A. thaliana* three *CUC* paralogs are expressed at floral organ primordia boundaries. Their function is to inhibit cell division at these boundaries, thereby establishing distinct organ domains. Mutations in these genes cause partial fusion of adjacent sepals or stamens [60]. Variations in floral organ number also affect floral morphology. The molecular mechanisms influencing floral organ number vary, with the *CLAVATA3* (*CLV3*) and *WUSCHEL* (*WUS*) feedback loop regulating stem cell activity to control the total floral organ count. In *A. thaliana* mutations in

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the *WUS* gene lead to a reduction in floral organ count [61] while mutations in the *CLV3* gene result in an increase [62]. At the same time, the *AG-WUS* feedback loop regulates the timely termination of stem cell activity. Mutations in *AG* lead to the persistence of stem cells in *A. thaliana* flowers, resulting in a significant increase in floral organ number [63]. Additionally, the *SUPERMAN* (*SUP*) gene in *A. thaliana* specifically regulates the boundaries between stamens and carpels. Mutations in this gene cause carpels to convert into stamens, leading to the production of extra stamens [64]. Mutations in the *RABBIT EARS* (*RBE*) gene in *A. thaliana* and the *MicroRNA169* gene in *Antirrhinum* result in the loss of petal structure [65]. In *Antirrhinum* simultaneous mutations in the *CYC* and *DICH* genes cause the number of sepals, petals and stamens to increase from five to six [66].

DISCUSSION AND OUTLOOK

The ABCDE model and the tetramer model provide detailed descriptions of the formation of floral organ structures, but these models mainly describe its static aspects. There is still insufficient understanding of the dynamic changes during floral organ development, such as the spatial and temporal regulation of gene expression and the role of non-coding RNAs in this process. Although the ABCDE model highlights the crucial roles of A, B, C, D and E class genes in floral organ development, the complex interaction networks and regulatory mechanisms between these genes have not been fully elucidated. In particular, how various genes coordinate and regulate the formation of floral organs during the formation of multi-gene interaction complexes remains an area requiring further in-depth research and explanation.

Although interactions between the MADS-box genes *AP3*, *PI*, *AG* and *SEP3* in stamen development are known, their complex regulatory networks and molecular mechanisms still require further exploration. In particular, how these genes cooperate at different developmental stages and under varying environmental conditions, as well as their interactions with other gene families, are key areas for future research. This includes studying how to precisely regulate the expression of these genes to achieve specific floral organ development. Future research can utilise chromatin immunoprecipitation-RNA sequencing techniques to analyse the binding of transcription factors such as *PlPI*, *PlAP3*, *PlAG* and *PlSEP3* to the promoter regions of their target genes, identifying the genes they regulate. Electrophoretic Mobility Shift Assay can be used to validate the binding of these transcription factors to the promoter regions of their target genes, further confirming their role in gene regulation. CRISPR-Cas9 can be employed to validate the functions of target genes, with the aim of improving flower morphology and advancing breeding techniques for ornamental plants.

Other genes involved in stamen development in ornamental plants represent a group of genes that define the boundaries of specific organs during plant development, playing a crucial role in the development of floral organs, particularly in the establishment and maintenance of boundaries. The *FFO* gene and its counterparts show functional similarities but also exhibit unique characteristics in their impact on floral organ boundaries. Although the functions and mechanisms of these genes have not been fully explored, they represent a new regulatory pathway, providing new clues for in-depth research into the molecular mechanisms of floral organ development.

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