

Analysis of four orchid protein-protein interactions networks to elucidate the mechanism of floral morphology diversity formation

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Abstract

The diversity of Orchidaceae plants and their complex adaptations to pollinators have made Orchidaceae a hot topic in the study of plant evolution and function. However, our current understanding of the molecular mechanisms behind the diverse floral morphologies of Orchidaceae plants remains limited. To address this gap in knowledge, our study presents an integration of protein network predictions and comparative analysis of four different orchid species (*Apostasia odorata*, *Phalaenopsis equestris*, *Erycina pusilla*, *Dendrobium officinale*) with widely varying floral organ structures. Our research findings suggest that the formation of the orchid labellum is linked to the presence or absence of *AP3-3* and *SEP4* genes, and specific interaction patterns involving these genes in each of the four species may be key to regulating the mechanisms that drive differences in floral morphology. Our analysis indicates that the number and type of interacting proteins of the floral symmetry regulator *DIVARICATA* may play a role in the morphological formation of radial and bilateral symmetry in orchids. Furthermore, both MADS-box and MYB are involved in regulating pollen development. Additionally, we found that the MADS-box, MYB, and GRAS families of genes may regulate flower organ morphology by influencing hormone signaling pathways during flower organ development. To further explore these findings, we utilized the CRISPR/Cas9 technique to knock out the *SYD* gene, and agrobacterium-mediated genetic transformation of *Arabidopsis thaliana* was used to examine the effects. The results showed an increase in petal opening and mating degree, along with changes in the overlapping stamen distribution.

Introduction

The orchid family is one of the largest flowering plant families, with highly specialized floral organ structures compared to other flowering plants, such as specific labellum and gynostegium with fused male and female stamens and petal-like sepals (Zhang et al. 2017). These different floral organ morphologies have high ornamental value and play an important role in insect pollination. The morphological composition of plant floral organs is regulated by MADS-box, MYB, GRAS, TCP, and multiple metabolic pathways by forming complexes at different times (Enrico and Elliot 1991; Immink et al. 2009; Nam et al. 2003; Theissen and Saedler 2001; Kwantes et al. 2012; Geng et al. 2020; Phan et al. 2012; Silverstone et al. 1997; Tyler et al. 2004; Dianella and Michael 2006; Huang and Irish, 2015), leading to floral organ diversity. The mechanism of orchid floral diversity formation has been a puzzle that biologists have explored for long time, however, this has not yet been solved. With the completion of orchid genomic and transcriptomic data, it has become possible to reveal the mechanisms of orchid floral diversity formation through protein interactions.

Proteins interact to achieve specific functions and play key roles in various biological processes such as catalysis, signal transduction, and regulation. Progress has been made in probing protein interactions using Yeast Two-hybrid (Y2H), Bimolecular Fluorescence Complementation (BiFC), and Chromatin Immunoprecipitation (Co-IP) to uncover regulatory patterns between genes and to explain complex biological processes (Fields and Song 1989; Hu and Kerppola 2003; Collas 2010). However, these methods are time-consuming and labor-intensive. For example, the number of interaction pairs detected

at each time point is limited, and large-scale detection at the genomic level is challenging. With the continuous development of molecular biology research, a variety of databases (STRING, GeneMANIA, IMID, etc.) have provided a database for protein interaction prediction (Franceschini et al. 2013; Warde-Farley et al. 2010; Balaji et al. 2012), and reasonable interactions can be predicted for a large number of proteins in a short time by using computational methods such as interolog, gene fusion, and co-expression. STRING database contains predicted and experimentally verified protein-protein interaction (PPI) pairs in various organisms and is a commonly used database (Franceschini et al. 2013). For species included in the database, the interaction pairs of target gene sets can be extracted directly to construct interaction networks; For species not included in the database, BLAST is used to find homologous proteins and construct the PPI network based on the interaction relationship pairs of homologous proteins. The STRING database predicts the interactions of single or multiple proteins in multiple species. For example, an interaction network between miRNA target genes has been constructed in rice to reveal the regulatory mechanism of rice seed vigor (Zhou et al. 2022). PPI networks were constructed to further characterize the dynamic biosynthetic pathways of characteristic transcription factors in rice, and enzymes from the CYP450, TaABA8OH2, PAL and GST families were found to be associated with MYB13-1, MADS-box and NAC transcription factors, which play important roles in water tolerance (Rahimi et al. 2021). Two major classes of ZmARF1-interacting genes were predicted, including C-terminal AUX/IAA proteins and signal transduction proteins, involved in mitogen-activated protein kinase (MAPK) and nuclear factor κ B (NF- κ B) signaling pathways, which play regulatory roles in processes such as stress response and growth (Sun et al. 2020). To investigate the possible mechanism of light signaling in *Arabidopsis thaliana* anther development, the photosystem was screened by interacting with bHLH010, bHLH089, bHLH091, DYT1, and light signal transduction pathway-related proteins through the PPI network to explain the novel roles of light signaling-related genes (Ruan et al. 2019). Studies related to the model of flower development and its specific regulatory networks have become a new research topic. With the mechanisms of flower organ development in the model plants *Arabidopsis thaliana* and *Oryza sativa* becoming more apparent (Ryan et al. 2015; Murai 2013), the regulatory network of flower organ development in orchids also needs further elucidation. In this study, we analyzed the transcriptome data of the flower buds of *Dendrobium officinale*, *Apostasia odorata*, *Erycina pusilla*, and *Phalaenopsis equestris*. We used the STRING database with an interolog to construct the PPI network. Through differential network comparison, we aimed to explore the formation mechanism of floral diversity in these four orchids and provide a reference for the theory of floral organ development in the orchid system.

Materials and Methods

Data acquisition

The raw bud Transcriptome data of *Dendrobium officinale*(srx1021811), *Apostasia odorata* (srx2938655), *Erycina pusilla* (srx2310422), and *Phalaenopsis equestris* (srx1074880) were obtained from the National Center for Biotechnology Information (NCBI) SRA database. The raw data were filtered, and all transcript

sequences were spliced from scratch after removing low-quality read segments using the Trinity software (<https://github.com/trinityrnaseq/trinityrnaseq/wiki>).

Gene structure analysis

The unigene sequence was compared with the NR (<ftp://ftp.ncbi.nih.gov/blast/db/>) and GO (<http://www.geneontology.org/>) databases using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the parameter was an E-value $\leq 1 \text{ e}^{-5}$. KOBAS2.0 (<http://kobas.cbi.pku.edu.cn/help.do>) was used to obtain KEGG orthology results. Amino acid sequence alignment was performed using the Pfam database (<http://pfam.xfam.org/>) and HMMER software (<http://hmmer.org/>) to obtain unigene annotation information; the parameter was E-value $\leq 1 \text{ e}^{-10}$. The genes were retrieved from the NR annotations, and each protein sequence structural domain was viewed from the Pfam annotations.

Gene expression analysis

Gene expression levels were analyzed by clustering the heatmap of genes in R software, and the parameter was taken as $\log_{10}$.

Multiple sequence alignment and phylogenetic tree analysis

Arabidopsis thaliana and rice MADS-box proteins were downloaded from UniPort database. The phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0, where the bootstrap value was set to 500, and the p-distance method was selected for analysis.

Protein interaction network construction

All transcriptome data were imported into the STRING database (<http://string-db.org/>) to predict protein interactions and to construct a PPI network. Visualization was performed using the Cytoscape 3.7.2 software (<http://www.cytoscape.org/>).

Construction of SYD gene knockout vector

Use online website CRISPR-p (<http://cbi.hzau.edu.cn/crispr/>) to knock out targets design, and according to the target design three sgRNA express box primers (Table 1), carrier construction method reference (Zeng et al. 2018). Primer 5 software was used to design two pairs of specific PCR amplification primers Cas9-f/Cas9-r and HYG-f/HYG-r for positive identification.

Table 1
Primer sequence information of sgRNA expression box

Primer name	Primer sequence (5'→3')
gRS1	TTCTCAGCTTGCCCTTGCTTgttttagagctagaaat
OsU6aS1	AAGCAAGGGCAAGCTGAGAACggcagccaagccagca
gRS2	CTGACCTGATCCGGGGTTACgttttagagctagaaat
OsU6bS2	GTAACCCCGGATCAGGTCAGCaacacaagcggcagc
gRS3	TTCAGGCAATCGAGAACTGGgttttagagctagaaat
OsU6cS3	CCAGTTCTCGATTGCCTGAACTgagcctcagcgcag
Cas9-f	CTGACGCTAACCTCGACAAG
Cas9-r	CCGATCTAGTAACATAGATGACACC
HYG-f	ACGCGTCGACATGTCTAAGGGCGAGGAACTC
HYG-r	GGACTAGTTTATTTATAGAGTTCGTCCAT

Genetic transformation of *Arabidopsis thaliana*

The constructed CRISPR/Cas9 expression vector was transferred into *Agrobacterium* EHA105 by heat shock method, and then cultured in LB liquid medium containing kanamycin (50mg/L) (5g/L yeast powder + 10g/L peptone + 10g/L NaCl₂, pH7.0). Incubated overnight in a constant temperature shak at 28°C and 180r/min. When the OD600 value of the bacterial solution was 0.6-1.0 determined by ultraviolet spectrophotometer, centrifuge at 4°C and 4000r/min for 12min. Remove the supernatant and add the same amount of aseptic suspension as the bacterial solution (MS + 10g/L D-glucose + 20g/L sucrose, pH5.0). Mix with vortex oscillator, add 0.04% Silwet-L77 and set aside. The flower buds of newly exposed *Arabidopsis thaliana* were immersed in the above suspended bacteria solution for 2min by flower immersion method, then soaked in a bag for moisturizing, dark for 24h, and then moved to an artificial climate box for normal culture. F1 seeds were collected in hygromycin B resistance screening medium (MS + 0.4mg/L 6-BA + 0.2mg/L NAA + 14g/L sucrose + 10g/L AGAR powder + 80g/L potato + 25g/L banana + 0.5g/L AC + 20 mg/L hygromycin B). pH6.0) for 20 days, and select viable seedlings for transplanting culture.

Identification of positive *Arabidopsis thaliana* plants

Plant Genomic DNA was used to extract *Arabidopsis* leaf DNA after resistance screening. Two pairs of specific primers in the skeleton vector Cas-f/Cas-r and HYG-f/HYG-r were used for PCR amplification. The reaction system (30μL) was as follows: Mix 15μL, DNA template 1μL, upstream and downstream primers 1μL each, ddH₂O 12μL. The specific process is: 95°C, 5min, 95°C, 25s, 57°C, 25s, 72°C, 30s, 72°C, 3min, 4°C, 28 cycles.

Results

Analysis of MADS-box transcription factor protein interactions

MADS-box genes expression and phylogenetic analysis

A total of 83 genes with complete MADS-box structural domains were obtained from the transcriptome data based on NR annotation results. We constructed phylogenetic trees (Fig. 1) of *Arabidopsis thaliana*, *Oryza sativa*, *Dendrobium officinale*, *Apostasia odorata*, *Erycina pusilla*, and *Phalaenopsis equestris*. All MADS-box genes were of the MICK type, including 13 MICK*-type and 70 MICKC-type, mainly containing A-, B-, C/D-, and E- type genes. Combined with the clustering heat map results (Fig. 1), we found that *Apostasia odorata* possessed only two *AP3* genes and three *SEP*-type genes. The gene sequences were further subjected to BLAST comparison, and *AP3-3* and *SEP4* gene expression were missing in *Apostasia odorata*.

Construction and analysis of MADS-box regulatory network in different species

All transcriptome data of *Dendrobium officinale*, *Apostasia odorata*, *Erycina pusilla*, and *Phalaenopsis equestris* were used to construct the PPI network, and the MADS-box PPI subnetworks (Fig. 2) of each of the four species were constructed using Cytoscape software. The results demonstrated that only a few MADS-box genes were involved in the interactions, and the *SEP* class existed in all four species. These *SEP* proteins, which are the network's core nodes, interact with various enzymes, such as histone acetyltransferases (HACs), mitogen-activated protein kinases (MAPKs), the two-component response regulator of cytokinin pathway RR21, and transcription factor E2FB. However, there are differences in the *SEP* protein interactions between different species. *SEP3* interacts with *BRM* and *SYD* proteins in all three species, except *Apostasia odorata*. *SEP1* interacts with *MAPK2*, *ZAT8*, *ARR4*, and *IDD5* in *Apostasia odorata*. *SEP2* and *SEP4* interact with *ARR17* and *MAPK10* in *Dendrobium officinale*. *SEP1* and *SEP2* interacted with *HDA6*, lysine-specific demethylases *REF6*, and *NRPD1* in *Erycina pusilla*. *SEP2* and *SEP4* interact with *C₂H₂*-type zinc finger protein family members *ZAT3*, *ZAT4*, and *ZAT9* in *Phalaenopsis equestris*.

AP3 and *MADS50* only interact with *Dendrobium officinale* and *Apostasia odorata*; however, the proteins interacting with *AP3* are entirely different. Both *AP3-1* and *AP3-2* in *Apostasia odorata* interact with *LFY*. However, *AP3-2* in *Dendrobium officinale* interacts with *NTF3* and the signal factor *MAPK4*. *AP1* exists only in *Dendrobium officinale* and has one interacting protein, *MAPK2*. *AG1* and *AG2* in *Erycina pusilla* and *Phalaenopsis equestris* interacted with *AGL66*, whereas *AG2* in *Apostasia odorata* interacted with *MAPK14* and *HAG1*.

Analysis of pollen development-associated MADS-box protein interactions

To understand the specific regulation of pollen development-related MADS-box genes, the MIKC*-type MADS-box PPI sub-networks (Figs. 3A-B) of different orchids were constructed individually. It was found that there were no protein interactions in *Apostasia odorata* and *Dendrobium officinale*. However, AGL66 in *Erycina pusilla* and *Phalaenopsis equestris* interacted with AG1 and AG2, respectively. AGL104 interacted with MAPKs and FHA2 in *Phalaenopsis equestris* and with the AG class in *Erycina pusilla*. A network with AGL65 as the core was also found in *Erycina pusilla*, which mainly interacted with various enzymes such as MAPKs, HAC1, REF6, HDA6, histone-lysine N-methyltransferase SUVR5, and transcription factors such as E2FB, GATA, and WIP2. This revealed that the PPI network of these proteins in *Erycina pusilla* is more complex.

Analysis of MADS-box gene interactions with plant hormones

PPI subnetwork analysis (Figs. 3C-F) and plant hormone signaling pathway analysis (Fig. 3G) revealed that the SEP class in all four species interacted with the cytokinin pathway-related protein RR21. In *Apostasia odorata*, SEP1 interacts with ARR4, which belongs to the type A ARR gene, but not with type B ARR proteins RR22 and APRR2. Both SEP2 and SEP4 interact with ARR17 in *Dendrobium officinale*. SEP class proteins were also related to the brassinosteroid pathway from the hormone and MADS-box subnetwork, and the brassinosteroid LRR receptor kinase BRI1 was found to interact with SEP proteins via MAPK in all four plants.

Analysis of MYB transcription factor family

Differential analysis of pollen development-related MYB protein interactions

Previous studies, have demonstrated that some MYB members had important regulatory roles in pollen development. A total of 43 MYB genes related to pollen development were screened from the transcriptome data based on Swissprot and NR annotation results to construct PPI subnetworks (Fig. 4). The results illustrated that MYB52, MYB106, CSA, and GAMYB interacted with proteins in all four species. The number of interacting proteins with CSA was low. In *Apostasia odorata*, CSA interacts with elongation factor LOS1 and RNA polymerase NRPB12. CSA interacted with NRPC2 in *Erycina pusilla* and *Phalaenopsis equestris*. The number of GAMYB interacting proteins varied significantly, with only three in *Apostasia odorata*, whereas the other three species exceeded 100. GAMYB mainly interacts with HAC1, GSO1, BRM, F-1, RHs, BRI1, CURL3, and E2FB in *Erycina pusilla*, *Dendrobium officinale*, and *Phalaenopsis equestris*. GAMYB also interacts with HDA6, BAM2, and cell cycle proteins in *Erycina pusilla* and *Phalaenopsis equestris*. In addition, MYB106 interacted with ASHR2, NRPB5A, ACA1, GSO1 and other

enzymes in *Dendrobium officinale* and with XBOS33 and BAM1 in *Apostasia odorata*. MYB52 only interacted with PROA protein in *Apostasia odorata*, and HDA6, MAK3, SYD, BRM, CYCT1-5, and other enzymes were the key differentially interacting proteins in *Phalaenopsis equestris*.

Some MYB proteins do interact with proteins from different species. MYB26 and MYB35 only interact with RNA polymerase and cell cycle proteins in *Phalaenopsis equestris*. MYB15 interact with HDA2, ACA1, ZAT5, GSO1, and MAK3 in *Erycina pusilla*. MYB2, MYB4, and MYB20 had no corresponding interactions in *Phalaenopsis equestris*, *Dendrobium officinale*, and *Apostasia odorata*. MYB80 interacted only with *Phalaenopsis equestris* and *Apostasia odorata*.

Differential analysis of floral symmetry-related MYB protein interactions

According to transcriptome NR annotation and BLAST sequence comparison results, 24 homologous genes of the DIVARICATA (DIV) gene that encode MYB proteins involved in floral symmetry regulation were obtained. Cluster heat map analysis (Fig. 5A) revealed that this gene is expressed in all four species. Further studies on the differences in protein interactions (Figs. 5B-E) among various species depicted that the number of interacting proteins was significantly lower in *Apostasia odorata*, mainly interacting with HS, BIP1, and MED37A. The types of interacting proteins in the other three species were similar, mainly interacting with the zinc finger proteins ZIP3, DNAJ1, DJA6, LOS1, and GRIK1.

Analysis of the regulatory relationship between MYB and plant hormones

Among MYB PPI networks, it was found that BRI1 and its homologous protein CUEL3 directly interacted with MYB. Through the MYB PPI subnetwork and plant hormone signal pathway (Fig. 6), it was found that MYB also interacted indirectly with CYCD3 of the brassinosteroid pathway, ARR of the cytokinin pathway, and PP2C proteins of the abscisic acid pathway in four orchids. The four orchids differed in their MYB species and intermediate interacting protein types. PP2C27 interacts indirectly with MYB via BRI1 or CUEL3, whereas MYB indirectly regulates ARR and CYCD3 through other proteins. CYCD3-1 and CYCD3-2 in *Apostasia odorata* and *Erycina pusilla* indirectly interacted with MYB through transcription factors such as E2FA and E2FB and with ARR via proteins such as SNF12 and CHR12. However, CYCD3-1 in *Dendrobium officinale* and *Phalaenopsis equestris* had RH3B as an intermediate protein, and ARR through intermediate BRM, HAC1, HAC12, and CHR12 proteins interacted with MYB.

GRAS protein interaction network construction and difference analysis

GRAS transcription factors play regulatory roles in signal transduction, male gametogenesis, and flower development. Therefore, the PPI network (Fig. 7) was constructed using all GRAS genes as the core. The results demonstrated that interactions of the RHT1 protein existed in all four species with a high degree of similarity. However, particular interactions were observed between the gibberellin receptor protein GID in *Apostasia odorata* and *Phalaenopsis equestris*. There were interactions between GAI in *Apostasia odorata* and *Dendrobium officinale*. GAI in *Apostasia odorata* interacts with the three hormone signal transduction-related proteins GID1C, GID2, and BZR1. Further analysis revealed that GID1C and GID2 are two signaling factors with opposing roles in the GA signaling pathway, and BZR1 is involved in regulating the brassinosteroid pathway. In addition, in *Dendrobium officinale*, RGL1 interacted with CXE18 and PIF5, whereas RGL1 interacted with GID2 and CXE15 in *Phalaenopsis equestris*.

Construction of DoSYD gene knockout vector

SYD, a key differential interaction gene in the above protein interaction network, was selected as the research object to explore its role in floral diversity and was renamed *DoSYD*. The sgRNA expression box of *DoSYD* gene was constructed by Overlapping PCR method. The PCR result of the round (Fig. 8) showed that the target primer was successfully linked to the 3 promoters and the corresponding sgRNA. Three complete sgRNA expression boxes (Fig. 9) were successfully constructed by using the first round of product mixture as the template of the second round of PCR reaction, and the three sgRNA expression boxes were successively combined into the skeleton vector, and the receptive cells of *Escherichia coli* were successfully introduced. A full single colony was used as the template, and gRS1/U6bS2 or gRS2/U6bS3 were used as primers. Colony PCR was performed, and positive colonies were successfully detected (Fig. 10). Positive colony plasmid was extracted by shaking and sequenced with primers SP-L1 and SP-R on the vector. The results showed that the promoter and sgRNA expression box were successfully connected to the vector, that is, the knockout vector was successfully constructed and could be used for the subsequent genetic transformation experiment.

Positive plants were obtained by genetic transformation

The constructed CRISPR/Cas9-*DoSYD* gene editing vector was activated to transform *Arabidopsis thaliana* flowers by flower dipping method. The F1 generation of *Arabidopsis thaliana* seedlings were transplanted and cultured through the resistance screening medium containing hydromycin B. DNA was extracted from the leaves and PCR amplification was performed by two pairs of specific primers Cas9-f/Cas9-r and HYG-f/HYG-r. Target bands were detected by electrophoresis, and the positive conversion rate was up to 15.5%.

Identification of phenotypic differences of positive *Arabidopsis thaliana* plants

The positive seedling sequencing results indicate the occurrence of an A-C base substitution and two base deletions at the target site. The flower type of positive *Arabidopsis thaliana* plants was changed, and the difference between the positive and wild plant was mainly in petal distribution and stamen division.

The wild-type petals were symmetrically distributed, with angles close to 90 degrees (Fig. 11A), while the mutant petals were asymmetrically divided. It is mainly reflected in that the opening and closing angles of the two petals are greater than 90 degrees (Figs. 11E, G), some even reach 180 degrees (Figs. 11D, F), and different degrees of overlap occurred between petals (Figs. 11H, I, J). The wild type has 6 stamens evenly distributed around the pistil (Fig. 11A), and the mutant 6 stamens were symmetrically distributed on both sides of the pistil (Figs. 11B, C).

Discussion

MADS-box, MYB, and GRAS genes are transcriptional regulators that are important for regulating the morphogenesis of flowering organs during the development of plant flowering organs through PPI or protein binding to target DNA sequences. The differences in the regulation of these transcription factors in different species may be related to differences in the final morphology of flowering organs.

Deletion of AP3-3 genes may affect labellar diversity

AP3 and *PI* genes in the MADS-box family can effectively regulate petal and stamen development (Enrico and Elliot 1991; Liu et al. 2013a), whereas class E genes are significant throughout the four rounds of floral organ formation (Kramer and Hall 2005). Class E genes are required from the initiation of floral meristematic tissue to the designation of floral organs and determination of ovule identity (Smaczniak et al. 2012). The evolutionary tree and heatmap results demonstrated that the expression of the *AP3* and *SEP4* genes was lacking in *Apostasia odorata*, which is consistent with previous findings (Zhang et al. 2017). To identify the specific missing *AP3* genes, all gene sequences were compared using BLAST, and it was found that *Apostasia odorata* lacked the expression of *AP3-3* genes, which might be related to the absence of specialized labellar structures.

Class E genes and their interactions maintain normal floral organ development

The relationships between genes within the MADS-box family have been extensively studied in model plants. Notably, the "ABCDE" model (Enrico and Elliot 1991; Immink et al. 2009; Nam et al. 2003) and the "four-molecule" model (Theissen and Saedler 2001) were applied to most dicotyledons, and the "fading borders" model (Buzgo et al. 2004) can explain some monocotyledons. Nevertheless, the floral structure of orchids is exceptional, and their regulatory mechanisms are different. In *Arabidopsis thaliana*, class E genes can interact with other MADS-box subfamily genes to form multimers that exert regulatory effects. Similarly, in the current study, we found that class E genes were present in the PPI network of MADS-box proteins in *Dendrobium officinale*, *Apostasia odorata*, *Erycina pusilla*, and *Phalaenopsis equestris*, indicating that SEP-like proteins are still at the core of the network in orchids and may regulate multiple developmental stages. However, no relationship was found between SEP and class A, B, C, and D genes,

indicating that the expression pattern of floral organ characteristic genes of the MADS-box gene family varies in different plants. For example, the lack of *SEP2* expression in meristems and the expression of *SEP1* in sepals in *Armeniaca mume* may be due to the different subfunctionalization and neofunctionalization of homologous genes of SEP-like in different species (Zhou et al. 2017).

In addition, SEP-like protein interactions are similar in different orchids, mainly interacting with histone acyltransferases, mitogen-activated protein kinases, two-component response regulators, and other transcription factors. Among these, histone acyltransferases can neutralize the positive charge of lysine in histone tails and drive the initiation of gene transcription by changing the specific conformation of local chromatin (Lee and Workman 2007; Morończyk et al. 2022). Therefore, the interactions between it and SEP-like proteins may alter the transcription initiation time of such transcription factors and affect protein expression, which may indirectly affect the normal development of floral organs and the formation of floral meristematic tissues. Members of the MAPK family are proline-targeted serine/threonine kinases that play an important role in signal transduction by regulating the level of gene transcription in the nucleus in response to changes in the cellular environment (Turjanski et al. 2007). It has been demonstrated that MAPK phosphorylation can regulate transcription factor expression by altering the activity, localization, and stability of transcription factors (Turjanski et al. 2007). E2FB and RR21 proteins are involved in cell division regulation and affect the size and number of floral organs. The expression of these genes and their corresponding reciprocal relationships exist in different species and may be crucial for normal flower organ development. However, additional experiments are required to verify whether or not they play significant roles.

Differences in MADS-box protein interactions may lead to orchid floral phenotypic diversity

The existence of large phenotypic differences between species relies on the differential expression of specific genes and gene interactions. It is evident from the analysis results that specific interactions are present in different species. In combination with the flower phenotypes, it is speculated that the key difference lies in the absence of interactions among *SEP3*, *SYD*, and *BRM* in *Apostasia odorata*. Since the *BRM* and *SYD* belong to SWI2/SNF2 chromatin remodeling ATPases, some experimental studies have found that these two genes are recruited to the regulatory regions of class B and C genes during *Arabidopsis thaliana* flower development and physically interact with the direct transcriptional activators *SEP3* and *LFY* to form multimers, which in turn, regulate the expression of *AP3* and *AG* class genes (Wu et al. 2012). This suggests that *Apostasia odorata* may lack such multimer formation, which affects the normal expression of class B genes and results in the absence of a special labellum.

Histone deacetylases negatively affect gene expression by restoring the positive charge of lysine in histones, which triggers chromatin condensation (Martin et al. 2007). It affects the transcriptional activity of proteins and leads to altered functions by altering local chromatin conformation. It is present only in *Erycina pusilla* and interacts with *SEP1* and *SEP2*, suggesting that this protein may play an important role

in floral organ morphogenesis. *REF6* was the first reported H3K27me3 demethylase in plants that can directly bind and activate the expression of *CUC1*, which is a key gene for regulating cotyledons and other organ boundaries (Lu et al. 2011; Cui et al. 2016). Thus, *REF6* plays an important role in floral organ development and boundary formation. The SEP class genes in *Erycina pusilla* and *Phalaenopsis equestris* interact with *REF6*. It is possible that *REF6* is a downstream target gene of MADS-box proteins and is regulated by their expression. It then achieves flower organ boundary formation through multiple regulations, resulting in more complex petal, sepal, and labellum morphology in *Erycina pusilla* and *Phalaenopsis equestris*.

DIV different interactions may regulate flower symmetry formation in orchids

Regarding floral symmetry, *CYC* and *DICH* genes were first identified in the model plant *Antirrhinum majus* (Luo et al. 1999), and subsequent studies have also identified *DIV* and *RAD* in the MYB family that regulate floral symmetry development (Galego and Almeida 2002; Corley et al. 2005). These four genes synergistically regulate the overall asymmetry or asymmetry of individual petals. Radially symmetric ancestors in different angiosperm lineages have often evolved floral bilateral symmetries (Endress 1999). *Apostasia odorata* lacks a specialized labellum and has sepal-like petals of similar size, typical of radially symmetric flowers. *Dendrobium officinale*, *Erycina pusilla*, and *Phalaenopsis equestris* became bilaterally symmetric because of the production of a specialized labellum, suggesting that *Apostasia odorata* is the ancestor of the evolution of floral symmetry in orchids. In addition to the lowest number of interactions, analysis of the PPI network of *DIV* revealed significant differences in the interaction patterns in *Apostasia odorata*. However, the types of interacting proteins are distinct. Various functional proteins, such as ZIP3, DJA6, LOS1, GRIK1, and the like, have recently been identified in the other three species. The emergence of these identical new interactions may be an important reason for the differences in *DIV* expression, which enables orchids to evolve and maintain bilaterally symmetrical flowers by regulating the expression pattern of *DIV*.

MADS-box and MYB Co-Regulate Pollen Development

The MIKC* genes of the MADS-box family have maintained a relatively conserved role in gametophytes during land plant evolution (Kwantes et al. 2012). Five MIKC* genes (*AGL30*, *AGL65*, *AGL66*, *AGL94*, and *AGL104*) are expressed in pollen in *Arabidopsis thaliana* and regulate pollen development by repressing immature pollen genes and activating mature pollen genes (Verelst et al. 2007a, 2007b). Further studies have depicted that the P- and S-subclasses of MIKC*-type genes are primary regulators of male gametophytic development (Kwantes et al. 2012; Liu et al. 2013b). P-subclass genes were found only in *Apostasia odorata*. Although most orchids have pollen grains, only *Apostasia odorata* has dispersed pollen, similar to that of rice and *Arabidopsis thaliana*. This suggests that the loss of the P-subclass members is related to pollen evolution (Zhang et al. 2017). *Apostasia odorata* has powdered pollen in this study, whereas the remaining three had massive composite pollen. Based on previous studies, further

construction of the interaction network of these key proteins regulating pollen development revealed no interaction between *Apostasia odorata* and *Dendrobium officinale*. These genes may play a relatively singular role in the regulation of pollen development. Therefore, the pollen structure of *Apostasia odorata* is probably at the initial stage of evolution because of the presence of P-subclass genes and the absence of interactions with MIKC*-type proteins. *Erycina pusilla* and *Phalaenopsis equestris* have different degrees of interaction owing to their higher levels of evolution. It is speculated that the evolutionary relationships of pollen are, in that order, *Apostasia odorata*, *Dendrobium officinale*, *Phalaenopsis equestris*, and *Erycina pusilla*.

Multiple MYB members are now found in *Arabidopsis thaliana* and regulate anther development and functional formation (Cheng et al. 2009; Mandaokar and Browse 2009). For example, *GAMYB*, a key activator of the GA signaling pathway, is involved in sporopollenin synthesis by regulating the expression of its downstream genes *CYP703A3* and *KAR* (Kwon et al. 2015). *MYB20* regulates the biosynthesis of phenylalanine and lignin to influence the formation of secondary cell walls (Geng et al. 2020). We did not find interactions of MYB20 or the apparent simplification of the GAMYB interaction network in *Apostasia odorata*. The complex regulatory mechanisms of the remaining three may affect the expression of GAMYB and MYB20 during pollen development, leading to earlier sporopollenin synthesis and secondary thickening of the pollen wall. Sporopollenin starts to attach to the surface of the microspore before the callose wall of the microspore tetrad is entirely disintegrated. Thus, the microspores are not free out and form tetrahymena pollen grains.

Hormone signaling pathways are directly or indirectly involved in the regulation of floral organ development

Studies have revealed that many plant hormone signaling pathways influence flower transformation, organ number, and floral meristem (FM) determination. Several studies have identified various transcription factors whose downstream target genes are related to plant hormone responses (Sessions et al. 1997; Leibfried et al. 2005; Shani et al. 2006). Auxin, cytokinin, and jasmonic acid are potential targets of MADS-box (Kaufmann et al. 2009; Li et al. 2021; Cai et al. 2014). They are directly and indirectly involved in plant growth and development, flower organ morphogenesis, and other processes. Nevertheless, the link between flower organ patterns and hormone signaling is not fully understood. In this study, we found that the SEP class in all four species interacted with B-type ARR proteins in the cytokinin signaling pathway and that some SEP proteins were indirectly regulated by the brassinosteroid pathway-related protein BRI1 via MAPKs. SEP1 was found to interact with ARR4 in *Apostasia odorata*, which is consistent with the finding that *OsMADS1* directly acts on type A response factors during rice flower development (Khanday et al. 2013).

In addition to the MADS-box, responses to different plant hormone signaling factors were found in the MYB and GRAS transcription factors. In *Arabidopsis thaliana*, gibberellin was found to regulate the expression of three MYBs through jasmonic acid, which regulates stamen development (Cheng et al. 2009). *MYB108* and *MYB24* jointly regulate jasmonic acid-mediated stamen maturation (Mandaokar and

Browse 2009). In our study, Multiple MYB members interacted directly with BRI1 and CUEL3, whereas ARR was associated with MYB through linking multiple intermediate proteins. In addition, MYB transcription factors are associated with CYCD3, which regulates flower organ development by influencing cell numbers through cell cycle regulation.

It has been demonstrated that DELLA proteins have an inhibitory effect on signaling processes such as ETH and JA (Lawit et al. 2010; Hou et al. 2010), whereas they facilitate ABA accumulation (Zentella et al. 2007). In this study, these hormone-related proteins were not found to be associated with GRAS members. However, RHT1 and GAI, members of the DELLA subfamily, were found to directly interact with GID1 and GID2 in the GA signaling pathway in *Apostasia odorata*. This finding was consistent with previous studies (Dill et al. 2001; Hirsch and Oldroyd 2009). In *Phalaenopsis equestris*, RHT1 and RGL1 were also found to interact with GID2. This indicates that GAI, RHT1, and RGL1 might be involved in flower formation induction by regulating GA signaling. GAI was also found to be associated with BZR1, a signaling factor of the brassinosteroid pathway that regulates cell elongation and division in *Apostasia odorata*.

DoSYD gene function validation

A mutation in flower phenotype was observed in positive *Arabidopsis* plants, which exhibited increased petal aperture, overlapping petals, and altered stamen distribution, indicating the regulatory function of the gene in the formation of floral diversity. However, it remains uncertain whether the *DoSYD* gene affects the development of the labellum in orchids, and further genetic transformation experiments in orchids are required to observe phenotypic differences. It should be noted that genetic transformation of orchids has difficulty and flowering requires approximately 3–4 years, therefore this experiment is limited to *Arabidopsis* transformation study only.

Declarations

Acknowledgments

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Author Contributions

He Fengmei and Chen Yu conceived and designed the experiments. Chen Yu and Zang Rui discussed the results. Chen Yu, Wang Yiqin, and Sun Bo performed behavioral and molecular experiments. Chen Yu and He Fengmei analyzed the results and wrote the paper. All authors discussed, revised the manuscript, and approved the final version.

Data availability

All data related to this manuscript are available in this paper and its supporting materials.

Conflict of interest

None declared.

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Figures

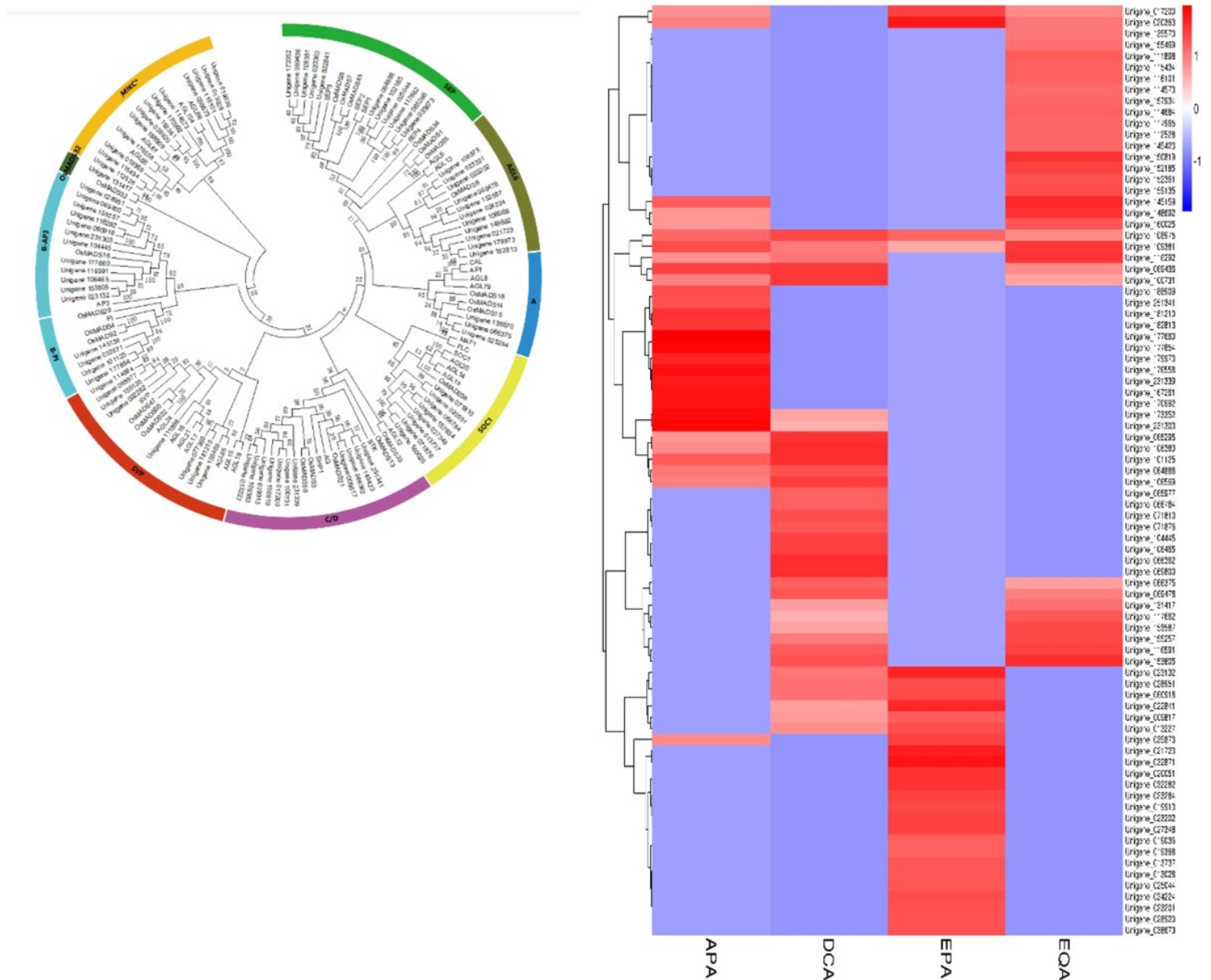


Figure 1

Phylogenetic and clustering analysis of MADS-box genes. **(A)** Phylogenetic tree of MADS-box genes. Unigene + number are MADS-box genes obtained from transcriptome. OsMADS + number are MADS-box genes of *Oryza sativa*. The others are MADS-box genes of *Arabidopsis thaliana*. **(B)** Clustering heat map of MADS-box genes. The different columns represent different samples. APA is *Apostasia odorata*, DCA is *Dendrobium officinale*, EPA is *Erycina pusilla*, and EQA is *Phalaenopsis equestris*. The different rows represent different genes. The colors represent the expression level of genes in the samples with a logarithmic value of 10.

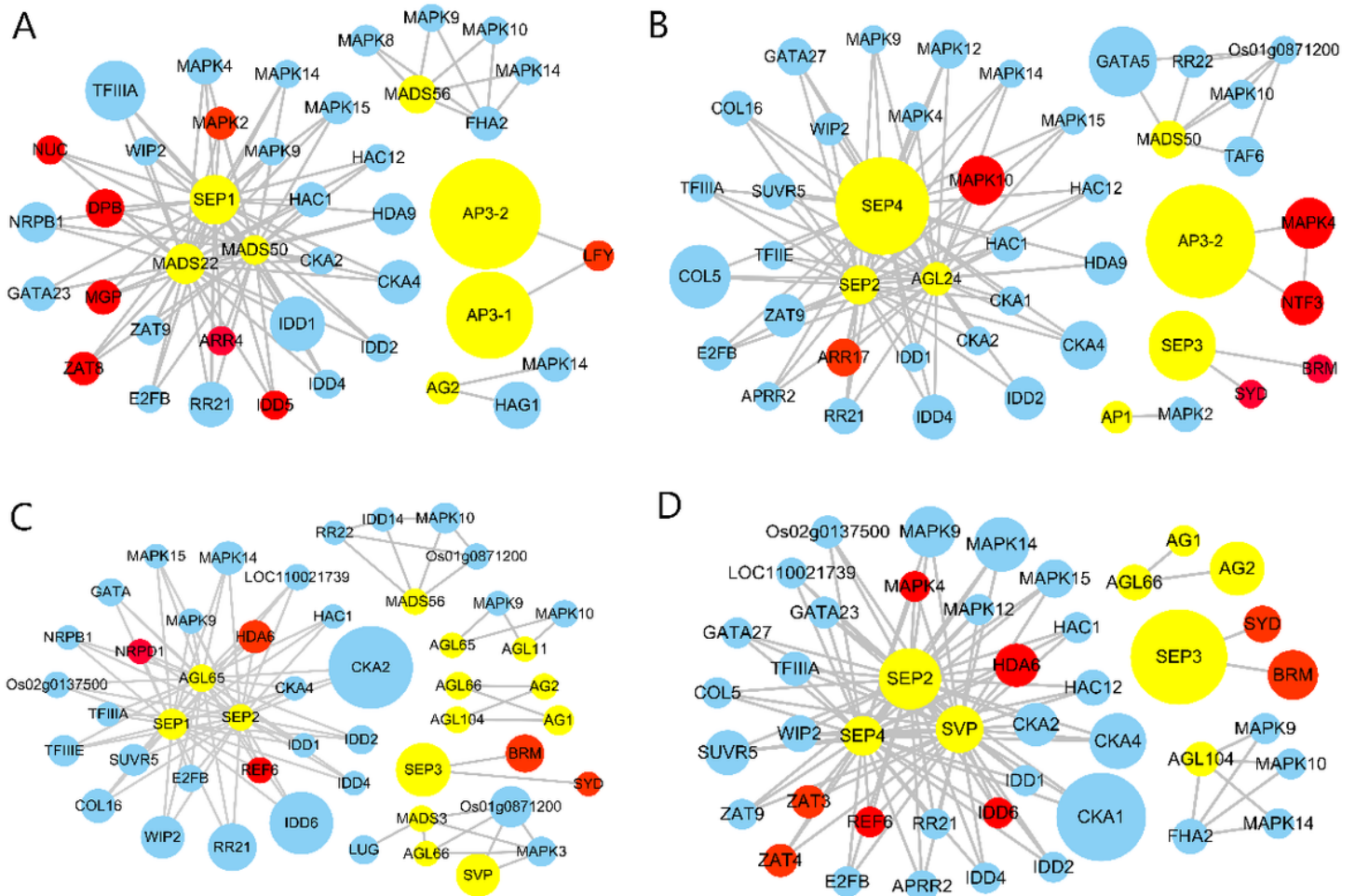


Figure 2

MADS-box PPI networks analysis. **(A-D)** The PPI networks of *Apostasia odorata*, *Dendrobium officinale*, *Erycina pusilla*, and *Phalaenopsis equestris*. The yellow is MADS-box proteins, the blue represents interacting proteins, and the red is the key differential interacting proteins. The size of the circle indicates the FPKM value of the gene, and the larger the FPKM value, the larger the shape.

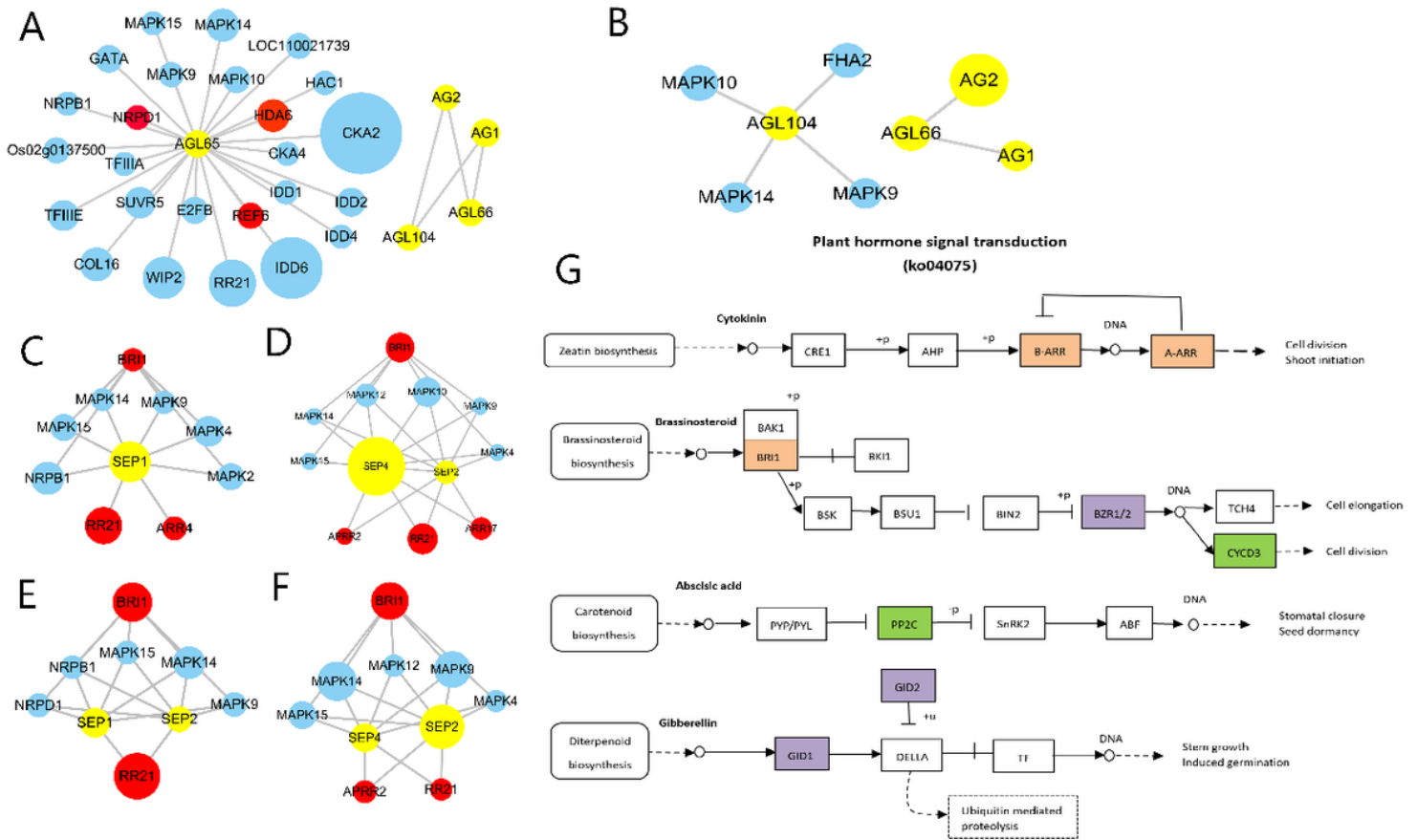


Figure 3

MADS-box PPI subnetworks analysis and KEGG pathway analysis. **(A-B)** The PPI subnetworks of MIKC* type MADS-box proteins in *Erycina pusilla*, and *Phalaenopsis equestris*. The yellow is MIKC* type MADS-box proteins, the blue represents interacting proteins. **(C-F)** The PPI subnetworks of MADS-box and plant hormone in *Apostasia odorata*, *Dendrobium officinale*, *Erycina pusilla*, and *Phalaenopsis equestris*. The yellow is MADS-box proteins, the blue represents interacting proteins, and the red is plant hormone-related proteins. The size of the circle indicates the FPKM value of the gene, and the larger the FPKM value, the larger the shape. **(G)** One KEGG pathway, the plant hormone signal transduction pathway (Ko04075), is involved in the interacting proteins of MADS-box, MYB, and GRAS transcription factors. The orange is the interacting proteins of MADS-box and MYB. The purple is the interacting proteins of GRAS. The green is the interacting proteins of MYB.

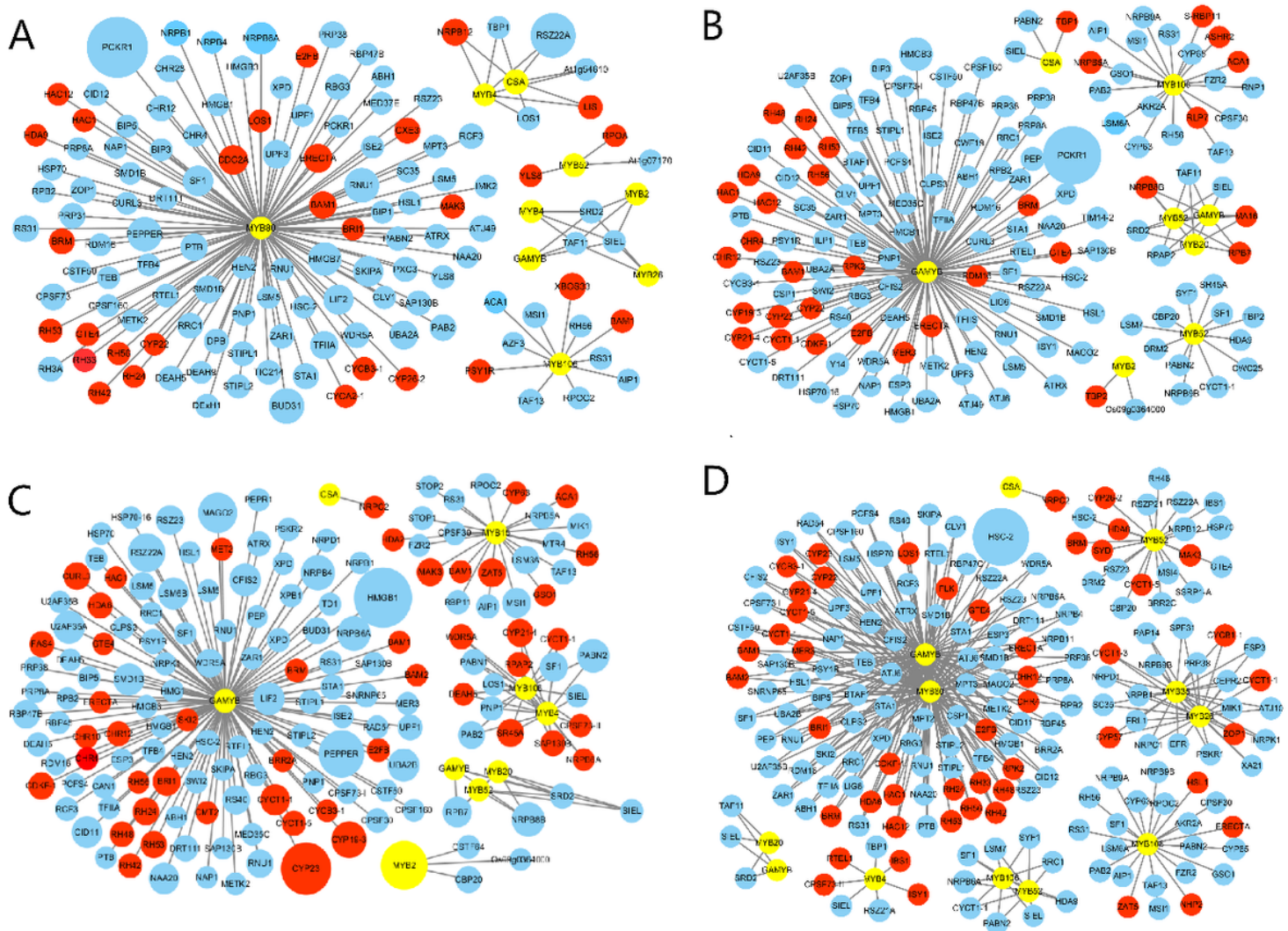


Figure 4

MYB pollen development-related protein PPI networks analysis. **(A-D)** The PPI networks of *Apostasia odorata*, *Dendrobium officinale*, *Erycina pusilla*, and *Phalaenopsis equestris*. The yellow is MYB pollen development-related proteins, the blue represents interacting proteins, and the red is the key differential interacting proteins. The size of the circle indicates the FPKM value of the gene, and the larger the FPKM value, the larger the shape.

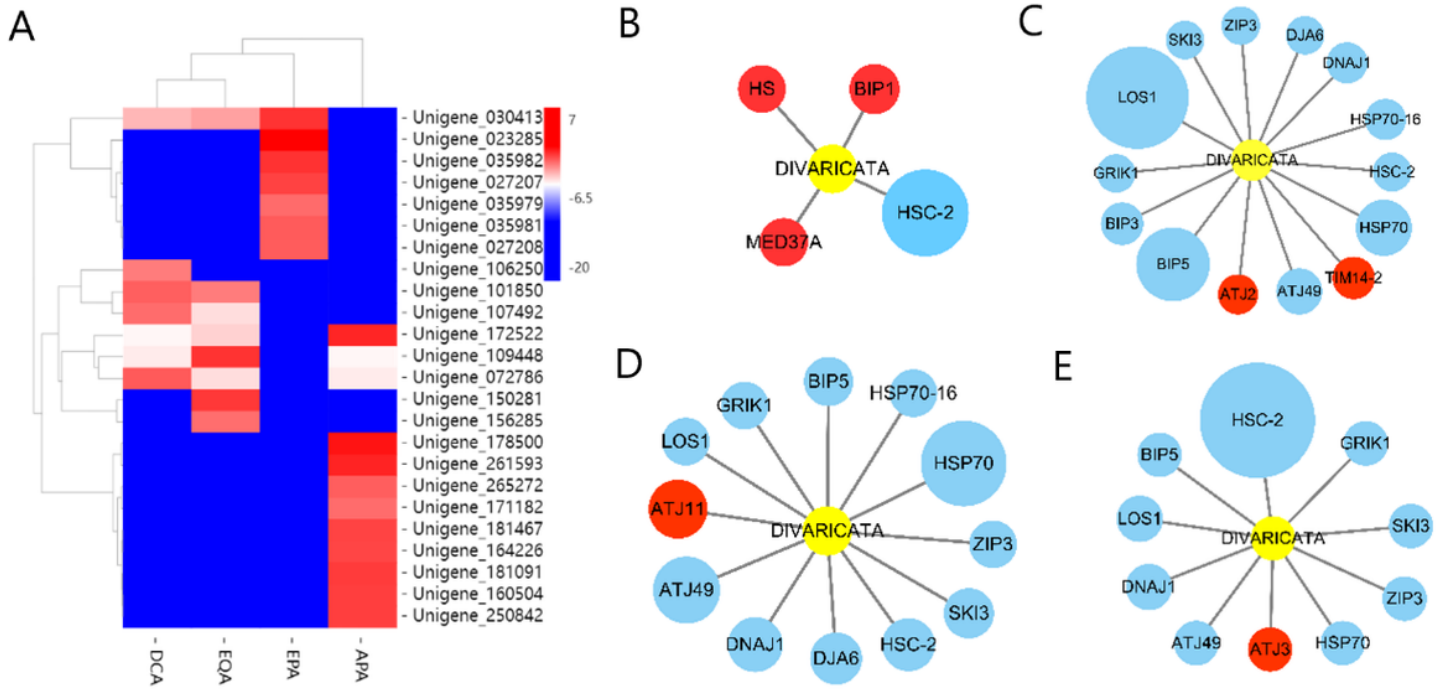


Figure 5

DIVARICATA homologous gene clustering and PPI networks analysis. **(A)** Clustering heat map of DIVARICATA homologous genes. The different columns represent different samples. APA is *Apostasia odorata*, DCA is *Dendrobium officinale*, EPA is *Erycina pusilla*, and EQA is *Phalaenopsis equestris*. The different rows represent different genes. The colors represent the expression level of genes in the samples with a logarithmic value of 10. **(B-E)** The PPI networks of DIVARICATA proteins in *Apostasia odorata*, *Dendrobium officinale*, *Erycina pusilla*, and *Phalaenopsis equestris*. The yellow is DIVARICATA proteins, the blue circles represent interaction proteins, and the red is the key differential interacting proteins. The size of the circle indicates the FPKM value of the gene, and the larger the FPKM value, the larger the shape.

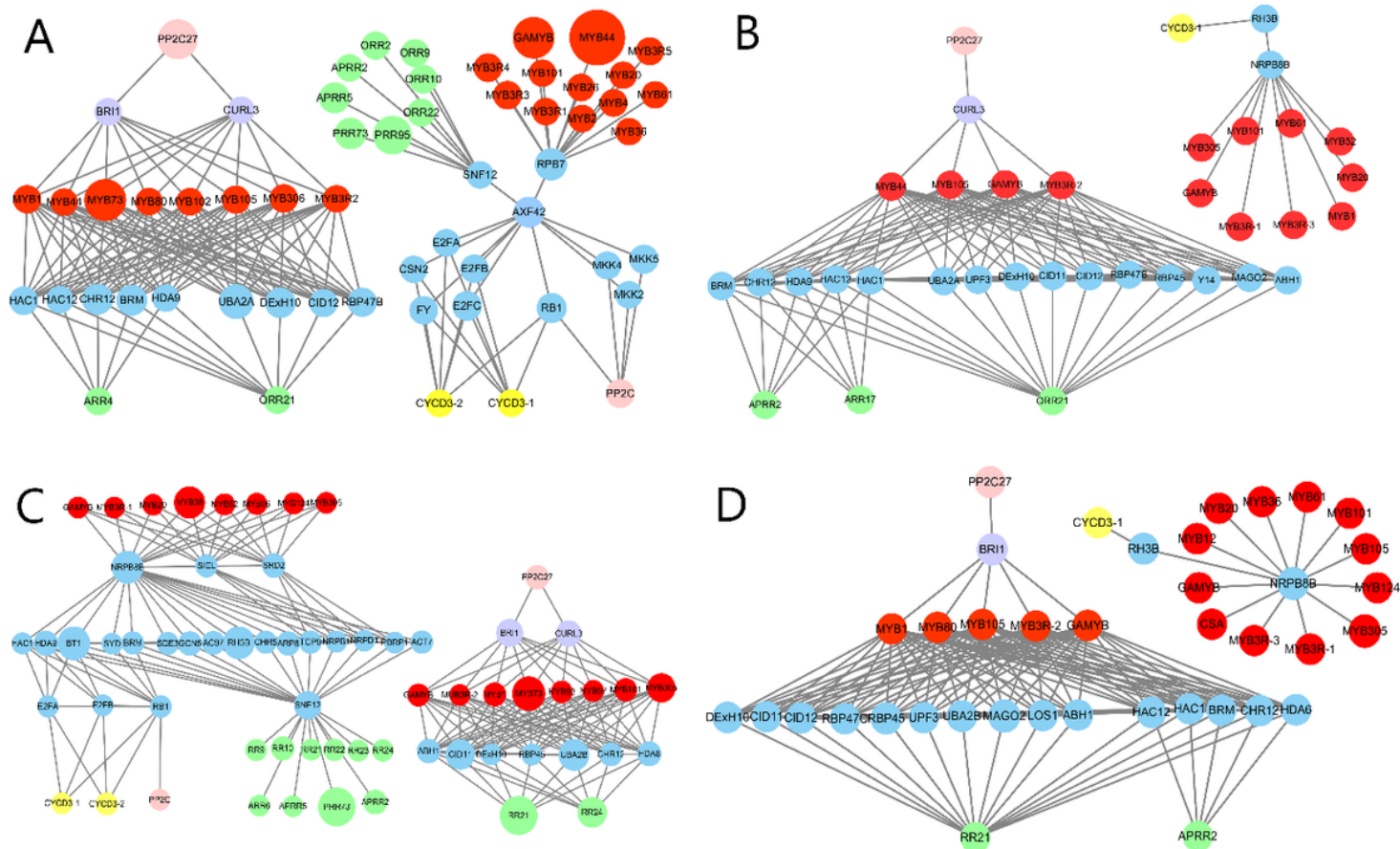


Figure 6

MYB PPI subnetworks analysis. **(A-D)** the PPI networks of MYB and plant hormone in *Apostasia odorata*, *Dendrobium officinale*, *Erycina pusilla*, and *Phalaenopsis equestris*. The red is MYB proteins, the blue represent interacting proteins, and the other colors are plant hormone-related proteins. The size of the circle indicates the FPKM value of the gene, and the larger the FPKM value, the larger the shape.

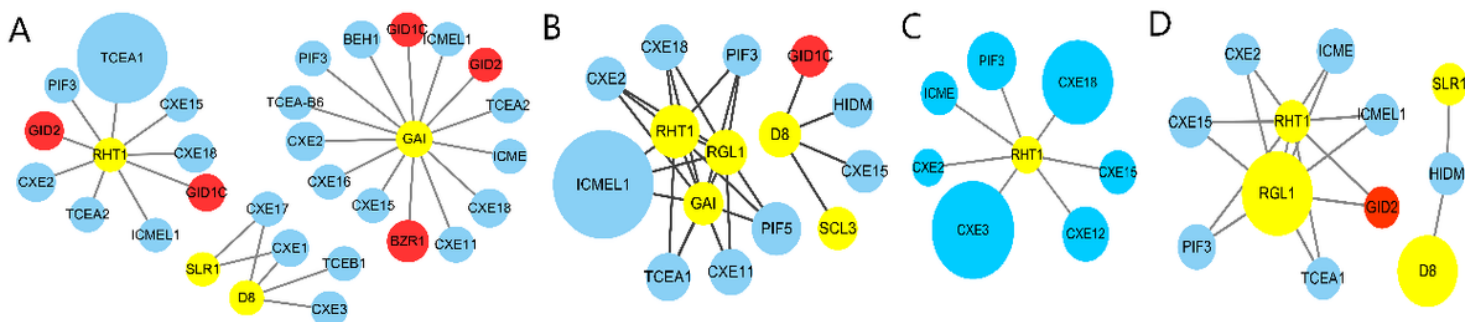


Figure 7

GRAS PPI networks analysis. **(A-D)** The PPI networks of GRAS proteins in *Apostasia odorata*, *Phalaenopsis equestris*, *Dendrobium officinale*, and *Erycina pusilla*. The yellow is GRAS proteins, the blue

represent interacting proteins, and the red is plant hormone-related proteins. The size of the circle indicates the FPKM value of the gene, and the larger the FPKM value, the larger the shape.

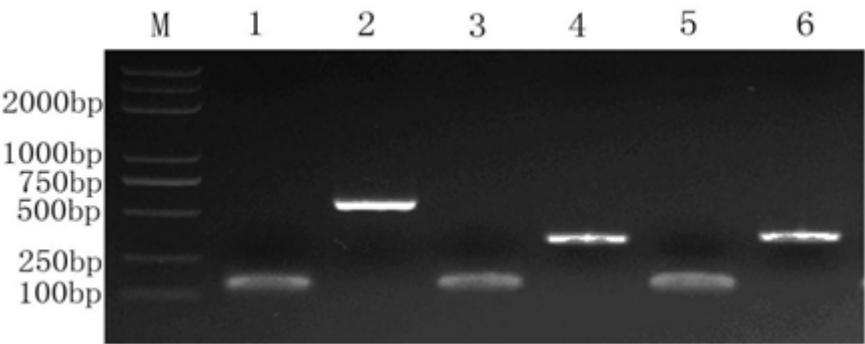


Figure 8

First round PCR agarose gel electrophoresis

Note: M: DNA marker DL 5 000; 1: U6a gRNA; 2: U6a promoter; 3: U6b gRNA; 4: U6b promoter; 5: U6c gRNA; 6: U6c promoter

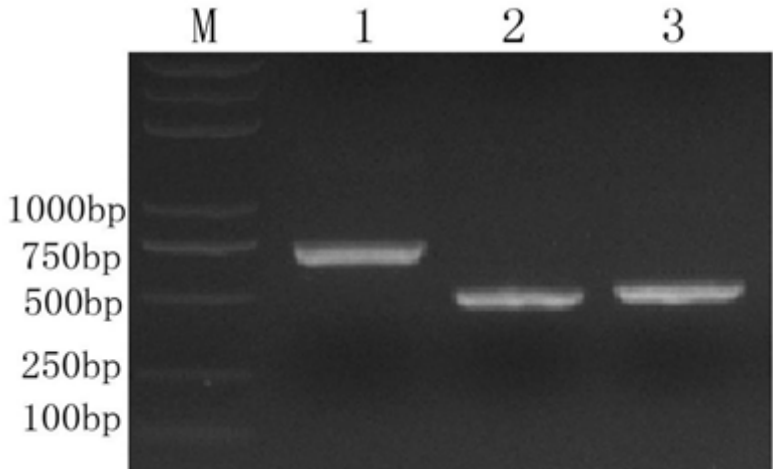


Figure 9

Second round pcr agarose gel electrophoresis

Note: M: DNA marker DL 5 000; 1: Expression cassette 1; 2: Expression cassette 2; 3: Expression cassette 3

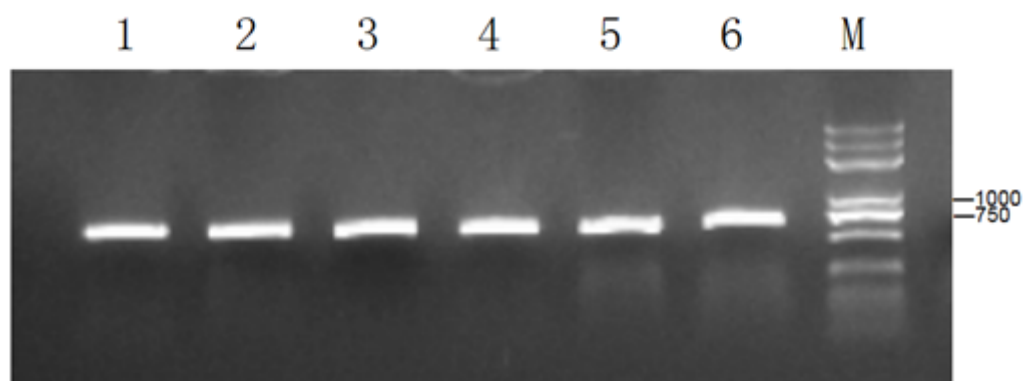


Figure 10

Detection of sgRNA expression box connection by colony PCR

Note: M: DNA marker DL 5 000 1-6 PCR product of e.coli colony

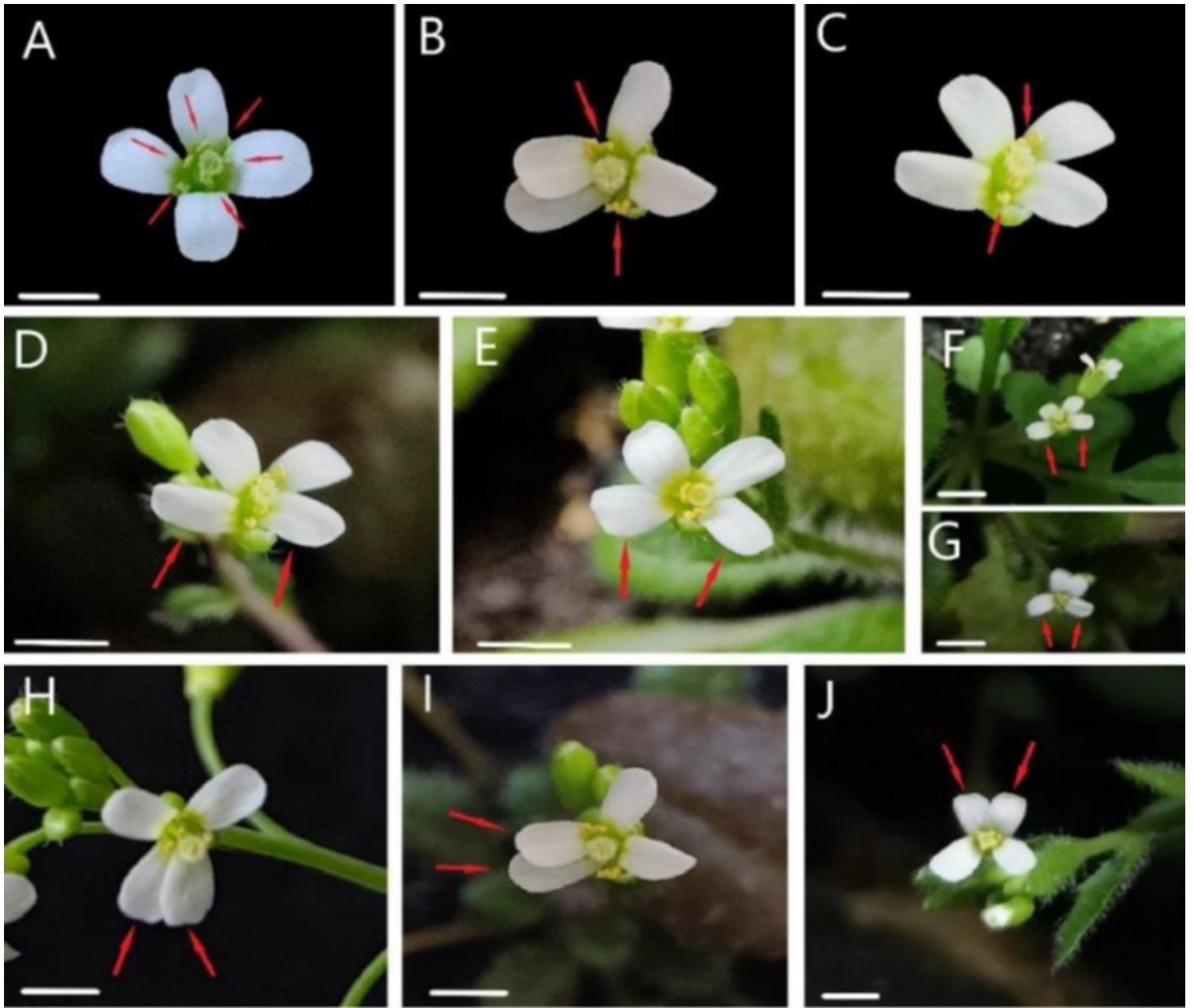


Figure 11

WT and positive Arabidopsis flower phenotype

Note A is the flower of wild type plants, and B-J are the flowers of positive plants. The red arrows point to the specific flower organs that has changed.