

Transcriptome Analysis Identified Dichogamy Regulation Networks in *Alpinia mutica*

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Abstract

In the sexual reproduction of flowering plants, outcrossing has natural advantages in heredity, and is the reproduction method used by most flowering plants. Dichogamy, accompanied by flower sex transition or differential maturation, promotes outcrossing and reduces selfing and interference with male and female functions, impacting the diversity and evolution of flowering plants significantly. However, due to a chronic lack of available research materials, the regulatory mechanism of dichogamy, an important reproductive trait that has been of great interest since Darwin's time, remains unknown. Fortunately, *Aplinia mutica*, a species of wild ginger that has two sexes within the same population and achieve dichogamy through sex transition, provides superb research material. In this study, differential expression gene analysis and group screening were conducted to identify the underlying causes and potential regulatory networks that may be involved in the flower sex transition and the maturity of two sex organs, anther, and stigma. Our findings revealed that genes such as *MYB-TFs*, *NAC-TFs*, *FLA11*, *KSC6*, *NADPH*, *PP1CG2*, and *VTC2*; plant hormones such as jasmonic acid (JA), auxin, and brassinosteroids (BR); environmental factors such as light and temperature; and many other factors, such as phosphorylation and other biological processes, may all affect the sex transition process of *A. mutica*. the regulation of sex transition and dichogamy is a complex process. Since the regulatory mechanism of dichogamy has been an important open question for a long time, our study has made a novel contribution by identifying and examining its transcriptional regulation in plants at the molecular level. This finding holds immense importance for further investigations in this area.

Introduction

Sexual reproduction has been the driving force behind the evolution of angiosperms. Sex expression and conversion determine the mating mode of sexual reproduction of flowering plants (angiosperms), determine the inheritance of plant genetic material, play a crucial role in the evolution of angiosperms, and then influence the evolution and diversity pattern of the entire biological realm (Barrett, 2003; Campbell & Kessler, 2013). The diversity of the mating system is one of the characteristics of flowering plants (Barrett, 2002), most angiosperms are bisexual flowers, which express both male and female, allowing them to engage in both selfing and outcrossing. Outcrossing has become the preferred mode of reproduction for most angiosperms due to its advantages in genetic diversity and progeny fitness (Goodwillie et al., 2005), while selfing is mostly considered to play a role in ensuring reproduction under conditions of limited pollination (Zhou et al., 2012). Therefore, how to promote outcrossing is one of the core issues in the evolution of flowering plant reproductive system (Lloyd & Webb, 1986; Sargent & Otto, 2004). A direct method is to turn bisexual into unisexual (dioecy) (Renner & Ricklefs, 1995). In addition, self-incompatibility (SI) can be used to prevent the generation of selfing (Wright et al., 2013), or the functions of male and female organs (stigma and anther) can be separated in space (heterogamy) and time (dichogamy) to reduce the probability of self-pollination (Bertin & Newman, 1993; Glover & Barrett, 1986). In hermaphrodite flower plants, the sex transition of flowers is one of the key processes to achieve dichogamy and this study focuses on this process.

Surprisingly, there is a group of plants that perfectly combines dichogamy, herkogamy, and dioecy through sex transition. Flexistylis - reported in *Alpinia kwangsiensis* of Zingiberaceae in 2001 (Li, 2002; Li et al., (Li, 2002; Li et al., 2001). Dichogamy represents the temporal separation of male and female functions in plant individuals or populations, unlike most dichogamy species, which only have one type: protandry (PA, male first) or protogyny (PG, female first) (Lloyd & Webb, 1986), *A. Kwangsiensis* has both PA and PG phenotypes and is classified as heterodichogamy (Gleeson, 1982; Li et al., 2001), In the early flowering stage, PG stigma first matures to receive pollen and exercise female function, while PA anther first matures to release pollen and exercise male function. After half the flowering period, the PG anther matures to release pollen, transitioning from female to male, while the PA stigma matures to receive pollen, transitioning from male to female. This is the dichogamy characteristic caused by the difference in maturity time between males and females and the sex transition. At the same time, when the flower is in the female stage, the stigma will bend downward and move towards the pollinating insect's visiting channel, while in the male stage, the stigma will bend upward and move away from the pollinating insect's visiting channel. This is the herkogamy feature brought about by the sigma bending motion. In addition, the proportion of the two phenotypes in the population is 1:1, which conforms to Gregor Mendel's law. Due to the separation of the male and female stages of the two phenotypes, the population shows the characteristics of dioecy (Li, 2002; Li et al., 2001). The combination of these reproductive characteristics greatly promotes outcrossing, minimizing interference between selfing and male and female function.

In addition to its reproductive significance, the greatest value of this unique reproductive strategy lies in providing good materials for the study of rapid sex transition, sex expression control, floral organ movement, and the regulatory mechanisms of dichogamy in plants. Dichogamy, as a reproductive strategy widely existing in flowering plants (Bertin, 1993), is an indispensable part of studying the diversity of flowering plant reproductive systems. Since the Darwin's time(Darwin, 1877), researchers have conducted many explorations into its adaptive significance in ecology and evolution (Bertin & Newman, 1993; Lloyd & Webb, 1986). However, due to the fact that most dichogamy species only have one phenotype, it is difficult to conduct a detailed comparative analysis, and some species with two-sex phenotypes are both rare and mostly tall trees, making it difficult to compare and analyze. Therefore, unlike the reproductive strategies of SI, dioecy, and herkogamy, which have already been analyzed, the regulatory mechanism of dichogamy remains a mystery more than 100 years later. The sex transition during flowering, as one of the key processes of dichogamy, also lacks relevant research on its regulatory mechanism. In addition, many scholars believe that the heterodichogamy with PA and PG phenotypes may be controlled by a pair of alleles. The PA type is expressed in individuals who are homozygous recessive (gg), while the PG type is exhibited in individuals who are either heterozygous dominant (Gg) or homozygous dominant (GG), It may be one of the pathways for the transformation of monoecy into dioecy(Pannell & Verdu, 2006; Pendleton et al., 2000; Renner, 2001). In summary, *A Kwangsiensis* and its relatives, as perennial herbs with flexistylis, provide an excellent opportunity to expand our understanding of the diversity of sexual reproduction system of flowering plants, including dichogamy, herkogamy, and dioecy, especially dichogamy and its core regulation of sex transition in the process.

In this study, due to the difficulty in collecting wild populations of *A. kwangsiensis*, we selected a closely related species, *A. mutica*, which exhibits a similar gender transition pattern (flexistylly) as the research material. We present 46 transcriptome datasets pertaining to *A. mutica*, these datasets encompass a range of timings, phenotypes, and tissues. Subsequently, comparative analyses and differential gene screening were performed to identify potential target genes associated with sex transition and maturation of male and female organ differences during dichogamy. These findings provide important targets and basic data for future studies of dichogamy and deepen our understanding of the diversity of angiosperms' mating systems.

Materials and Methods

Plant Materials

The *A. mutica* used in this study was planted by ourselves in the nursery of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (101°25'E, 21°41'N), and was observed and sampled from July to August 2020. The flexistylly of *A. mutica* involves the dichogamy of both male and female sex organs. Therefore, based on the flowering dynamics of *A. mutica*, we collected anthers and stigmas of PA type and PG type at 4 time points: 6:00, 9:00, 12:00, and 15:00, respectively, each group of samples contained 3 biological replicates. All samples were collected and frozen in liquid nitrogen and stored in a – 80 °C ultra-low temperature refrigerator. While collecting molecular samples, we observed and recorded the dichogamy of *A. mutica* in detail.

RNA-Seq

Subsequently, we extracted and performed RNA sequencing

3µg of total RNA per sample was used as input material for the RNA sample preparation. Beads with oligo (dT) were used to isolate poly(A) mRNA from total RNA. RNA sequencing libraries were constructed from these mRNA using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, USA). Briefly, the Elution 2-Frag-Prime (94°C for 8 minutes, 4°C hold) was used to elute, fragment, and prime the mRNA with Elute, Prime, Fragment Mix (Illumina). First-strand cDNA synthesis was performed with First Strand Master Mix and SuperScript II mix (ratio: 1ul SuperScript II/7ul First Strand Master Mix) (Invitrogen). The second strand was synthesized with Second Strand Master Mix (Illumina) and Ampure XP beads (Illumina) were used to separate the double-stranded (ds) cDNA from the 2nd strand reaction mix. After end repair and the addition of a 3'-dA overhang, the cDNA was ligated to Illumina PE adapter oligo mix (Illumina) and size-selected for 350 ± 20 bp fragments by gel purification. After 15 cycles of PCR amplification, the 350 bp paired-end libraries were sequenced using the paired-end sequencing module (150 bp at each end) of the Illumina HiSeq 4000 platform.

These raw reads were stored in fastq format, and processed through Trimmomatic (Version 0.32)(Bolger et al., 2014). This step removed reads containing adapter, reads containing poly-N and low-quality reads from the raw data and yielded clean data for downstream analyses.

DEGs Identification in Different Types of Anther and Stigma of *A. mutica*

To investigate the transcriptional network's impact on dichogamy during sex transition, we utilized transcriptome data from *A. mutica* across four observation periods (6:00, 9:00, 12:00, and 15:00). Through quantification of gene expression and analysis of differential expression in various groups, we identified differentially expressed genes (DEGs). These DEGs included genes with differing phenotypes at the same time and in the same tissue, genes with the same phenotype at the same time but in different tissues, and genes with the same phenotype but at different times in the same tissues, the grouping is shown in Fig. 1c, d, and Table. S3.

The corresponding trimmed clean reads were aligned to the related reference genome employing TopHat2(Kim et al., 2013) software with default settings. Calculations of gene expression levels were conducted using Cufflinks v2.2.1(Trapnell et al., 2014). Fragments per kilobase of exon per million fragments mapped (FPKM) were used to normalize RNA-seq fragment counts and estimate the relative abundance of each gene. The DEGs were decided based on a P-value < 0.05 and at least a 2-fold change between the two FPKMs (adj. P-value $\leq$ 0.05, $|\log_2FC| \geq 1$).

Screening of dichogamy regulation target gene sets- DEGs Analysis

Next, because dichogamy of *A. mutica* involves both male and female functional maturation and transition, we screened DEGs in the anther (male organ) and stigma (female organ) to identify the target gene set associated with dichogamy in controlling *A. mutica*, which may result in differences between PA and PG types.

In anthers, we set 9 sets of screening conditions based on anther maturity and intersected the DEGs of each group (Fig. 2a). Furthermore, in stigmas, we acquired the DEGs of the two phenotypic stigmas at each time, and then took the intersection of the DEGs of the two phenotypes in different time (Fig. 2b). The genes identified in anther and stigma will be used as possible dichogamy-related target gene sets for further analysis

Screening of the dichogamy regulation target network-WGCNA Analysis

After screening the target gene sets, we intend to determine which DEGs, among many, are most important in maintaining dichogamy, we conducted WGCNA analysis of the target gene sets of anther and stigma.

Two co-expression networks are created using the WGCNA package (version 1.72-1), which is based on the R (version 4.2.3) environment. Genes with a maximum FPKM expression lower than 1 were filtered based on the FPKM expression matrix for all genes, and the FPKM was then converted by $\log_2(\text{FPKM} + 1)$ for the best soft threshold screening (a.k.a Power value). The clustering modules are separated into clusters following the computation of hierarchical clustering between genes to choose the most important modules for various traits (refers to the four types of An, St, PG, and PA). The co-expression network (Weight $\geq$ 0.30) of all modules was exported, and the DEGs of the two groups of An and St were

intersected with the total WGCNA expression network, and the final expression network was obtained after screening according to the weight threshold (0.42). Two network maps were created using the Cytoscape software (version 3.9.1), and then a regulatory network with DEGs as the core was obtained. After the final expression network is generated, we extend a node along the core node gene network for subsequent analysis.

Functional analysis of genes in the core genes network

Following the above work, the genes in the anther and stigma networks were subjected to GO enrichment analysis on tbTools (version 1.120)(Chen et al., 2020), and the corresponding protein sequences were compared with the protein (Araport11) obtained in the TAIR database (<https://www.arabidopsis.org>) using Diamond (version 2.0.14) (<http://github.com/bbuchfink/diamond>) software, and the possible functions of the genes in the core network were determined. The regulatory mechanism of dichogamy and flexistylis of *A. mutica* was inferred using the results of GO enrichment and the function of core differential genes, which also includes similar studies that have been reported today.

Result

Dichogamy in *A. mutica*

Dichogamy feature in *A. mutica*, as it is in its sister species *A. kwangsiensis*, which was the first to report this unique sex transition (Li et al., 2001). According to earlier studies, we observed the state of stigma(St) and anther(An) in flowers at 6:00, 9:00, 12:00, and 15:00 based on their blossoming and sex change processes. (Fig. 1a) Both the PA and PG varieties of *A. mutica* had flowered by 6:00. The PA stigma has already curved upward, away from the pollinator's flower-visiting channel, and its anther has fractured and begun dispersing pollen. The PA is currently in the male stage. The PG stigma, on the other hand, is located in the floral visiting channel and is bent downward, its anther securely closed and not dispersing pollen. The PG is currently in the female stage. At 9:00, the sex of the two types remained constant, with the PA type stigma at its lowest and the PA type pollen disperse at its highest. The PA stigma began to descend and enter the pollination channel at 12:00. Pollinators had taken a significant amount of pollen from the type. The PG stigma starts moving upward in the pollination channels, and the PG anther dehiscence starts dispersing pollen. Individuals of both types are currently undergoing sex transition. At 15:00, the PA type stigma had reached its lowest point, and pollinators had removed its pollen. The PG stigma reached its peak, and the PG anther pollen disperse reached its peak. At this point, both types have completed sex conversion, with PA becoming female and PG becoming male.

DEGs in different types of anther and stigma of *A. mutica*

A large number of DEGs were discovered in comparison groups of different phenotypes, at the same time and in the same tissue, and the number of DEGs peaked at 9:00 a. m. and then gradually decreased (Fig. 1c). The number of DEGs was highest in the comparison group of the same phenotype, same time, and different tissues, but only the number of DEGs in the PG type decreased gradually over time (Fig. 1c).

The situation of DEGs was more unique in the comparison groups of the same phenotype, different time, and the same tissue; the number of DEGs gradually increased over time, but the number of DEGs in the PA type anther with the earliest pollen maturation was greater than that in the PG type (Fig. 1d). The most obvious difference was that only PG type had the most DEGs (a small number of up-regulated genes and a large number of down-regulated genes) during 6:00–9:00, which was significantly higher than the number of DEGs in other groups (Fig. 1d). As a result, we hypothesized that the mechanism of dimorphism and style bending was caused by the close correlation of these DEGs between PA and PG types, particularly between 6:00 and 9:00.

Screening of dichogamy regulation target gene sets

By grouping and screening, the dichogamy target gene sets in anther, and stigma was screened out. In anther, the intersection of 9 groups of screening conditions obtained 67 DEGs as target gene sets (Fig. 2a), and in stigma, the intersection of two phenotype stigma DEGs at different times obtained 52 DEGs as target gene sets (Fig. 2b) (For more information, check Table.S4).

With the help of protein sequence alignment tools Diamond v2.0.14

(<http://github.com/bbuchfink/diamond>), we used *Arabidopsis thaliana* genome as a reference for the sequence alignment (default parameters), and annotated DEGs with *A. thaliana* genes description to confirm the potential functions of the corresponding DEGs in *A. mutica*. Many genes related to pollen maturation, pollen adhesion, anther maturation, cell elongation, photomorphogenesis, auxin metabolism, BR response, and JA response were found in 67 target DEGs of anther and 52 target DEGs of stigma. (Table.1). Among them, the number of DEGs related to light signaling/response was the largest. For example, 5 light morphogenetic genes *PSEUDO-RESPONSE REGULATOR5*(*PRR5*, 2 high light stress response genes *Am15.371* (*HEAT SHOCK PROTEIN17.4*, *HSP17.4*), *Am19.246*(*HSP17.4*) and *Am193.158*(*NADH dehydrogenase subunit 5-NADPH*) were identified (Table. 1). In addition to the genes in the light signaling pathway mentioned above, most of the other genes are involved in BR pathway, auxin pathway, and JA pathway. For example, *Am5.758* (*MYB DOMAIN PROTEIN*, *MYB108*, *MYB78*, *MYB112*), *Am14.1921* (*ABC transporter G family member 6*, *ABCG6*), *Am21.1780* (*PHYTOCHELATIN SYNTHASES 1*, *PCS1*) involved in pollen development, *Am24.2135* (*NAM*, *ATAF1,2*, and *CUC2 046*, *NAC046*) involved in the regulation of flower senescence. *Am22.1248* (*BRASSINOSTEROID INSENSITIVE 1*, *BRI1*) and *Am2.30* (*BRI1 SUPPRESSOR 1*, *BRS1*) involved in the BR pathway, and *Am15.1678* (*FASCICLIN-LIKE ARABINOGLACTAN-PROTEIN 1*, *FLA11*) involved in regulating pollen cell adhesion. Among these genes, there are also a few genes involved in cell wall material synthesis and degradation, which may play a role downstream of the genes related to cell elongation and stigma movement, such as *Am6.468*(*NAC075*), *Am14.1914*(*PROLINE-RICH EXTENSIN-LIKE RECEPTOR KINASE 13*, *PERK13*) (Table. 1).

Screening of the dichogamy regulation target network

Through WGCNA analysis, we further identified the most important target genes in dichogamy.

Finally, we were able to isolate 15 anther core DEGs (Fig. 3c) and 5 stigma core DEGs (Fig. 3d), the anther core DEGs are *Am15.1678* (*FLA11*), *Am193.158* (*NADPH*), *Am2.30* (*BRS1*), *Am3.157* (*CYTOCHROME P450 FAMILY 710 SUBFAMILY A POLYPEPTIDE 1, CYP710A1*), and *Am5.758* (*MYB108*). The core DEGs for stigma are *Am12.2672* (*VITAMIN C DEFECTIVE 2, VTC2*), *Am17.274* (*PROTEIN PHOSPHATASE 2C G GROUP 1, PP2CG1*), and *Am17.286* (*3-KETOACYL-COA SYNTHASE 6, KCS6*), respectively. In addition, 10 and 2 uncharacterized genes, found in the stigma and anther, respectively, may be crucial for maintaining dichogamy and flexistyly. (For more information, check Table. 1).

In terms of expression patterns, 15 core genes in the anther showed opposite expression patterns in the PA and PG types (Fig. 3a, b). The expression was high in the PG type at 6:00 and 9:00 and decreased at 12:00 and 15:00, while it was low in the PA type at 6:00 and 9:00 and slightly increased at 12:00 and 15:00. Other genes in the co-expression network have expression patterns similar to core genes, but there are some differences. The majority of PG genes were highly expressed at 6:00, 9:00, and 12:00, but decreased at 15:00. The PA type expression level peaked at 6:00, then began to fall, reaching its lowest point at 12:00 and remaining there until 15:00.

The expression pattern of core genes in stigma is slightly complex; three genes displayed different expression trends in PA and PG types, while the other two target genes displayed the same pattern (Fig. 3b). The expression of gene: *Am17.286* in both types was highest at 6:00 and lowest at 15:00, but the maximum expression in PA type was lower than that in PG type, and it was down-regulated at 9:00, earlier than 12:00 in PG type. The expression of gene: *Am17.274* in both types was highest at 9:00 and lowest at 15:00, but the maximum expression in the PA type was lower than that in the PG type. The expression of gene: *Am7.1051* in both types was opposite, with consistently low expression in the PA type and a brief increase in the PG type between 9:00 and 12:00 (the high expression in a sample at 15:00 was thought to be an accidental individual difference). The expression of the remaining 2 genes in both types was the same, with high expression at 9:00, and then decreased. The expression patterns of other genes in the co-expression network are different. Most PG type genes are low expressed at 6:00 and high expressed at 9:00, 12:00, and 15:00, whereas the majority of PA type genes are consistently low expressed. The differences in the core genes we screened in stigma are most pronounced between 9:00 and 12:00, which corresponds to the critical stage of *A. mutica*'s sextransition.

The expression patterns of other genes in the WCGNA network are similar (Fig.S2). In anther, genes other than the core genes are mostly highly expressed in PG from 6:00 to 12:00, while they remain consistently low expressed in PA. This trend is similar to core genes, but the downregulation of expression lags behind. In stigma, genes other than the core genes are mostly highly expressed in PG from 9:00 to 12:00, while in PA, they remain consistently low in expression. The period during which the movement direction of stigma is reversed is the most significant difference between the two types.

Functional analysis of genes in the core genes network

The results of GO enrichment analysis showed that nearly half of the genes in the pollen pocket's core network are enriched in terms linked to phosphorylation and cell wall formation (Biological process), and

the majority of them have phosphorylase, hydrolase, and transferase activities (Molecular function). It is worth mentioning that the majority of these genes are engaged in cell wall formation and breakdown. The result of enrichment in the core network of stigma is similar to that of anthers, but what distinguishes it is that the genes of the core network of stigma, whether BP, MF, or CC, are almost exclusively associated with terms related to plant response to environmental stimuli, such as phosphorylation and kinase. Based on the enrichment results, we hypothesize that phosphorylation-related genes may play a significant role in responding to environmental stimuli (such as illumination, mechanical touch, temperature, and so on) and regulating the synthesis and degradation of downstream cell wall substances. The genes (including 10 anther and 2 stigma uncharacterized genes) on the other core nodes were not significantly enriched under the exact GO term, except the *Am2.30 (BRS1)* gene, which was enriched under the catalytic activity (GO: 0003824) and transfer activity (GO: 0016740) terms (Fig.S4, Table. S5). This could be attributed to the fact that most of these genes have unknown functions and have not been thoroughly studied. However, as previously indicated, other node genes radiated by core node genes are enriched for phosphorylation and cell wall material related phrases.

Discussion

Our study gathered and analyzed 12 anther and 10 stigma transcripts of *A. mutica* in different phenotypes and at different times, which is the first investigation of the gene expression pattern of dichogamy, an essential plant reproductive feature, to our knowledge. These findings will aid in identifying and interpreting core genes involved in pollen and anther maturation, pollen release, stigma maturation, stigma movement, and dichogamy.

The maturity and release of plant anther and pollen are essential for male function completion. It is also a complex process that is influenced by a variety of elements, including external (temperature, humidity, light, etc.) and internal (gene expression, plant hormones, etc.). Our research begins with gene expression in these processes. We identified many of the genes (including *MYBs*, *ARFs*, *NACs*, *CYP710A1*, *BRS1*, *NADPH*, *FLA11*, *KCS6*, and others) and their corresponding proteins play significant roles in anther and pollen development, maturity, cracking, and pollen release.

Am5.758 (MYB108) is a basic network node in our WGCNA network. *MYB* transcription factors play a crucial role in the development of plants and pollen. In *Arabidopsis thaliana*, the *MYB108* mutant's stamen growth, pollen fertility, and anther rupture were severely damaged. *MYB108* not only directly controls anther cracking through *AUXIN RESPONSE FACTOR 17 (ARF17)*, but it is also controlled by *MYB21*, which increases anther and pollen maturation. (Mandaokar & Browse, 2009; Xu et al., 2019). Another core gene of interest is *Am15.1678 (FLA11)*. *FLAs* is a glycoprotein with complex multi-domain structure, which is involved in calcium complexation and signal transduction, affecting plant growth and development; *FLA11* is involved in the development of Secondary cell walls (SCWs) and the response to mechanical stress; *FLA3* directly regulates pollen development (Lamport & Varnai, 2013; Lopez-Hernandez et al., 2020; Ma et al., 2022; Ma et al., 2023). The regulatory pathway that controls the initiation and assembly of SCW in plants involves a transcription factor (TF) cascade, with the first

master TFs being *NAC* transcription factors, and the *MYB* TFs being the second master TFs, which directly regulate SCW synthesis genes or regulate downstream *MYBs* to regulate the development of SCW (McCarthy et al., 2009; Nakano et al., 2015). In addition, the expression of these three genes is regulated by both jasmonic acid (JA) and Auxin, and there is negative regulation between the two pathways (Cecchetti et al., 2007). In *Arabidopsis thaliana*, JA directly controls anther cracking through *MYB21/24*, Auxin regulates pollen release by regulating *MYB108* through *ARF17*, excessive *ARF* can inhibit anther cracking (Song et al., 2018), and the expression of *OPR3*, an enzyme in the JA pathway, is significantly upregulated in *ARF* mutant strains, Auxin negatively regulates JA synthesis in plants.. (Cecchetti et al., 2007).

From the expression level perspective, in *A. mutica*, *Am5.758* (*MYB108*) and *Am15.1678* (*FLA11*), 2 core genes, persistently low expressed in the PA type, initially high expressed in the PG type, and decreased in expression from 12:00 to 15:00. This is the stage when PG type anther matures and releases pollen. The expression trend of *Am13.332* (*ARF6*) is consistent with core genes. These 3 genes are all in the Auxin pathway. The expression trends of the 3 genes of the JA pathway, *Am24.2135* (*NAC046*), *Am12.2797* (*12-OXOPHYTODIENOATE REDUCTASE 1, OPR1*), and *Am14.1921* (*JAT3*), are opposite to those of the Auxin pathway. They are initially low expressed in the PG type, upregulated after 12:00, and persistently high expressed in the PA type (Fig.S1). This expression trend demonstrates the negative regulatory relationship between the Auxin pathway and the JA pathway, consistent with previous studies (Cecchetti et al., 2007). At the same time, it also indicates that the accumulation of gene expressions such as *MYBs*, *FLA11*, and *ARFs* is a necessary condition for stamen maturation. In addition, Auxin has been shown to affect the sigma movement of *A. mutica* (Luo et al., 2012).

In addition to the Auxin and JA pathways mentioned above, sterol metabolism may also play an important role in anther maturation. Brassinosteroids (BR) is an important sterol plant hormones, which play an important role in the development and growth of plants, such as seed germination, rhizome elongation and growth, photomorphogenesis, vascular bundle differentiation, tropism and reproductive development (Mandava, 1988). *Am2.30* (*BRS1*) is an important gene in the BR signal transduction pathway, and its phosphorylation activation energy activates the synthesis and signal transduction of BR (Yang et al., 2011). *Am3.157* (*CYP710A1*) is an important gene in sterol metabolism and may also be involved in BR and synthesis regulation. In *A. thaliana*, *CYP710A1* encodes C-22 desaturase and can synthesize stigmasterol using β -sitosterol as its precursor. *CYP710A2* can act on 24-epi-campesterol and β -sitosterol, generating BR and stigmasterol, respectively (Morikawa et al., 2006). An appropriate ratio of β -sitosterol to campesterol may be necessary for normal plant development and sexual reproduction (Benveniste, 2004; Schaller, 2003). In addition, light and JA may also be involved in the regulation of the BR pathway (Martinez et al., 2018; Ren et al., 2009). The expression trend of the above two genes and other genes (*Am22.271* (*THESEUS1, THE1*), *Am20.433* (*THE1*), *Am12.837* (*THE1*), *Am12.3425* (*THE1*), *Am18.1541* (*DON-GLUCOSYLTRANSFERASE 1, DOGT1*), *Am1.367* (*ROTUNDIFOLIA 3, ROT3*), *Am12.454* (*CDG1-like 1, CDL1*), *Am22.2344* (*CDL1*), *Am24.1436* (*CDL1*), *Am13.93* (*PHYB ACTIVATION TAGGED SUPPRESSOR 1, BAS1*), *Am15.2024* (*BRZ-INSENSITIVE-LONG HYPOCOTYLS 4, BIL4*), *Am22.1464* (*BIL4*), *Am17.583* (*RAPID ALKALINIZATION FACTOR 23, RALF23*), *Am6.379* (*SHAGGY-*

LIKE PROTEIN KINASE 32, SK32) of the BR pathway is similar to that of the Auxin pathway genes, and contrary to the JA pathway genes, the BR pathway and JA pathway show antagonistic effect(Fig.S5), which is consistent with previous studies(Ren et al., 2009)

And stigma, we can't find a strong connection between the core genes identified, but there are still some findings worth discussing. *Am17.286(KCS6)*, a core gene identified in stigmas, encodes a key protein of plant synchral long chain fatty acids and is an important part of a pollen coat, which is essential for stigmas to recognize pollen (Fiebig et al., 2000; Mayfield & Preuss, 2000). This gene, which should be highly expressed in pollen, is highly expressed in the PG stigma, 6:00–12: 00, the reason may be that the first time when we collected, the PG stigma has matured and received a large amount of pollen from the PA anther, thus showing the difference between the two phenotypic stigmas. However, the expression trend of this gene in anther is consistent with that of several core genes in anther mentioned above - it is highly expressed when anther is immature and cracking, and its expression is decreased after maturity. It has also been shown that *KCS6* can promote the elongation of cotton fiber cells(Qin et al., 2007), There may be a link between this function and stigma's movement, but it is not clear now. We have also found many genes involved in the JA pathway, Auxin pathway, and BR pathway in the WCGNA network of stigma, indicating that these pathways also play a role in the regulation of stigma movement and maturation. However, we have not found evidence to link these roles.

The core genes we identified also reflect the role of environmental conditions in dichogamy regulation. *Am193.158(NADPH)* is a gene closely associated with the production of reactive oxygen species (ROS), and its light response has been reported in several studies (Martin et al., 2004; Quan et al., 2022). ROS plays an important role in the degradation of tapetum and apoptosis of cell walls during anther maturation, and its high expression in PG anther in the morning is consistent with previous studies. In addition to the late cracking of PG anther, the whole anther and pollen development of PG anther may lag behind that of PA type *Am12.2672(VTC2)* identified in stigma is a core gene in plant ascorbic acid(VC) synthesis(Anisimova et al., 2021), L-ascorbate is associated with responses to growth (Pignocchi & Foyer, 2003), hormone response, flowering, and senescence(Barth et al., 2006), and various environmental stresses, as well as ozone, strong ultraviolet radiation, high temperature, and high light intensity(Linster & Clarke, 2008). The expression of *VCT2* is proved to be light-regulated and controlled by circadian rhythm (Aarabi & Fernie, 2023). *Am17.274 (PP2CG1)* encodes a putative protein phosphate 2C, whose phosphorylation can amplify plant temperature sensing signals and enhance plant temperature response (Lv et al., 2021). Previous studies have shown that light treatment can alter the phenotype of dichogamy(Bi et al., 2012), Our field observations found that the flowering phenology of *Lanxangia tsao-ko* significantly prolongs when experiencing low temperatures, and the maturation of anther and the movement of stigma are also proportionally delayed (unpublished data). At present, we cannot elucidate the specific role of environmental factors in the regulation of dichogamy, but our results also indicate that environmental factors such as light and temperature may be very important

Phosphorylation, as an important mode of post-translational covalent modification, is widely involved in plant growth and development, stress response, and signal transduction processes, and plays an

accurate role in regulating circadian rhythm(Sancar et al., 2009). Our GO enrichment analysis of all genes in the WCGNA network showed that both in anther and stigma, a lot of GO terms (GO:0015914, GO:0015748, GO:0006796, GO:0006793, GO:0016310) with high q-value are related to phosphorylation, indicating that phosphorylation may play an important role in dichogamy regulation (Fig.S4). In addition, calcium ions play an important role in the bending of plant organs, and previous studies have shown that calcium ions can influence stigma movement in *A. mutica*(Luo et al., 2011). our network also contains many genes related to calcium ion pathways, (*Am10.991*(*CALCIUM DEPENDENT PROTEIN KINASE 3*, *CPK3*), *Am15.1822*(*CPK3*), *Am13.1768*(*CPK3*), *Am14.190*(*CPK3*), *Am3.1138*(*CPK3*), *Am6.1925*(*CPK3*), *Am6.727*(*CPK6*), *Am7.1668*(*CPK8*), *Am8.1973*(*CPK8*)), which are briefly highly expressed in PG and 9:00–12:00, while underexpressed in other samples which are briefly overexpressed in PG from 9:00 to 12:00, while underexpressed in other samples (Fig.S6). This trend of expression shows a difference between calcium ions prior to the two phenotypes and is likely related to stigma maturation and downward movement.

In summary, most of the target genes we identified have been reported to play an important role in plant sexual maturation, especially male functional maturation, indicating that our identification is reliable. Based on the above results and existing research, we summarized the regulatory network of factors that may participate in dichogamy regulation (Fig. 4). Dichogamy is a complex character, and its functional realization includes a series of steps such as the development, display, and maturation of male and female sexual organs, among which the regulation of each process may affect the regulation of male and female heterogamy, so it should have a complex regulatory mechanism. Unfortunately, our study failed to identify a relatively fine regulatory network. Many of the genes identified in our study have been shown to play an important role in the maturation and motility of plant sexual functions, but we believe that they may be downstream regulated genes in dichogamy regulation, and the upstream of which still needs to be explored (Fig.S3). Among the core genes identified by us, there are still many genes whose functions are unknown, and their roles in dichogamy regulation are worth further exploration. At the same time, transcriptome analysis of one species cannot directly reflect the sequence differences of PA and PG phenotypes and the diversity of their mechanisms, thus further multi-omics analysis including comparative genomes and validation analysis of expanded populations of more species are needed. Nevertheless, for the first time, our work has conducted targeted research on the molecular regulatory mechanism of dichogamy and its rapid sex transition, laying an important foundation and providing an entry point for future related researches.

Declarations

Data Availability

All raw data of transcriptomes were deposited in National Center for Biotechnology Information (NCBI) under accession number PRJNA1010482.

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Contributions

The research was designed, conceived and supervised by W.W.B, P.X.C and H.A.D. W.W.B, H.A.D, P.X.C, L.P.W, J.L.J and D.S.C collected samples, RNA isolation and DEGs identification. W.W.B, P.X.C, D.S.C, and X.G.C analyzed subsequent data. The manuscript was drafted by H.A.D and P.X.C. All authors contributed to the review of the manuscript before submission for publication and approved the final version.

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Ethics declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Table 1

Table. 1.Alignment of the screened target genes with the Arabidopsis database. An = anther, St = stigma, Am Symbol = Gene numbers for *A. mutica* annotation, Subject ID = Gene ID of the Arabidopsis database.

Group	Am Symbol	Subject ID	Gene Symbol	Function Description
An	Am18.1514	AT1G04250.1	<i>IAA17, AXR3</i>	auxin signaling, senescence
An	Am17.2675	AT5G25890.1	<i>IAA28, IAR2</i>	auxin signaling, elongation
An	Am8.2205	AT3G08940.2	<i>LHCB4.2</i>	light signaling
An	Am10.2654	AT1G22770.1	<i>FB, GI</i>	light signaling
An	Am13.332	AT1G30330.1	<i>ARF6</i>	auxin signaling, auxin response, elongation
An	Am8.2249	AT1G64760.1	<i>ZET</i>	morphology twist
An	Am14.1914	AT1G70460.1	<i>PERK13, RHS10</i>	elongation
An	Am12.2797	AT1G76680.1	<i>OPR1, ATOPR1</i>	senescence, JA signaling
An	Am4.468	AT2G30575.1	<i>GAUT5, LGT5</i>	Pollen tube development
An	Am3.157	AT2G34500.1	<i>CYP710A1</i>	
An	Am13.162	AT3G48520.1	<i>CYP94B3</i>	JA signaling
An	Am9.112	AT2G39660.1	<i>BIK1</i>	auxin signaling
An	Am242.186	AT2G45050.1	<i>GATA2</i>	photomorphogenesis
An	Am24.2135	AT3G04060.1	<i>NAC046</i>	senescence
An	Am5.758	AT3G06490.1; AT5G49620.1; AT1G48000.1	<i>MYB108, BOS1;</i> <i>MYB78; MYB112</i>	pollen development, senescence
An	Am22.1248	AT3G13380.1; AT4G39400.1; AT3G28450.1	<i>BRL3; DWF2, BRI1,</i> <i>BIN1, CBB2; BIR2</i>	BR signaling
An	Am10.730	AT3G54110.1	<i>UCP1, PUMP1, UCP</i>	photomorphogenesis, light signaling
An	Am12.1139	AT3G55250.1	<i>PSA3, PDE329</i>	light signaling
An	Am12.1516	AT4G12800.1	<i>PSAL</i>	light signaling
An	Am2.30	AT4G30610.1	<i>BRS1, SCPL24</i>	BR signaling
An	Am17.431	AT4G33950.2	<i>SNRK2.6, SRK2E,</i> <i>P44, OST1</i>	ABA signaling

An	Am14.386	AT4G34280.1	<i>DHU1</i>	light response
An	Am21.1780	AT5G02190.1	<i>PCS1, EMB24, ASP38</i>	fertility
An	Am5.1356	AT5G02750.1	<i>SGR9</i>	gravity sensing
An	Am15.1678	AT5G03170.1	<i>FLA11</i>	Cell wall, adhesion
An	Am20.1659	AT5G04310.3	<i>PLL12</i>	Cell wall
An	Am12.570	AT5G50800.1	<i>RPG2, SWEET13</i>	pollen development
An	Am193.158	ATCG01010.1	<i>NDHF, NADPH</i>	light signaling
St	Am16.1283	AT1G06040.1	<i>BBX24, STO</i>	light signaling
St	Am15.140	AT1G15820.1	<i>LHCB6, CP24</i>	light signaling
St	Am18.1550	AT1G29930.1	<i>LHCB1.3, AB140, CAB140, CAB1</i>	
St	Am20.2069	AT1G22770.1	<i>FB, GI</i>	light signaling
St	Am6.1047	AT1G50250.1	<i>FTSH1</i>	light signaling
St	Am17.286	AT1G68530.1	<i>KCS6, G2, CER6, POP1, CUT1</i>	pollen development, elongation
St	Am17.274	AT2G33700.1	<i>PP2CG1</i>	salt tolerance
St	Am6.468	AT4G29230.3	<i>NAC075, SND4</i>	Cell wall
St	Am14.31	AT3G22830.1	<i>HSFA6B</i>	Heat Stress response
St	Am15.371	AT3G46230.1	<i>HSP17.4</i>	heat, cold, salt, drought and light stress response
St	Am19.246	AT3G46230.1	<i>HSP17.4</i>	heat, cold, salt, drought and light stress response
St	Am13.207	AT5G52640.1	<i>HSP81.1, HSP83, HSP90.1</i>	disease resistance
St	Am18.637	AT3G50310.1	<i>AIK1, MAPKKK20, MKKK20</i>	osmotic stress response, elongation
St	Am10.154	AT4G14690.1	<i>ELIP2</i>	light signaling
St	Am12.2672	AT4G26850.1	<i>GGP, VTC2</i>	light response

St	Am20.1942	AT4G30270.1	<i>MERI5B, XTH24,</i> <i>MERI-5, SEN4</i>	BR signaling
St	Am6.2398	AT5G11260.1	<i>HY5, TED5</i>	ABA signaling, light response
St	Am14.1921	AT5G13580.1	<i>JAT3, ABCG6</i>	JA signaling, pollen development
St	Am144.28	AT5G24470.1	<i>PRR5</i>	photomorphogenesis, light signaling
St	Am17.1520	AT5G24470.1	<i>PRR5</i>	photomorphogenesis, light signaling
St	Am6.1038	AT5G24470.1	<i>PRR5</i>	photomorphogenesis, light signaling
St	Am24.2245	AT5G24470.1	<i>PRR5</i>	photomorphogenesis, light signaling
St	Am11.1564	AT5G24850.1	<i>CRY3</i>	light response
St	Am12.561	AT5G50260.1	<i>CEP1</i>	pollen development

Figures

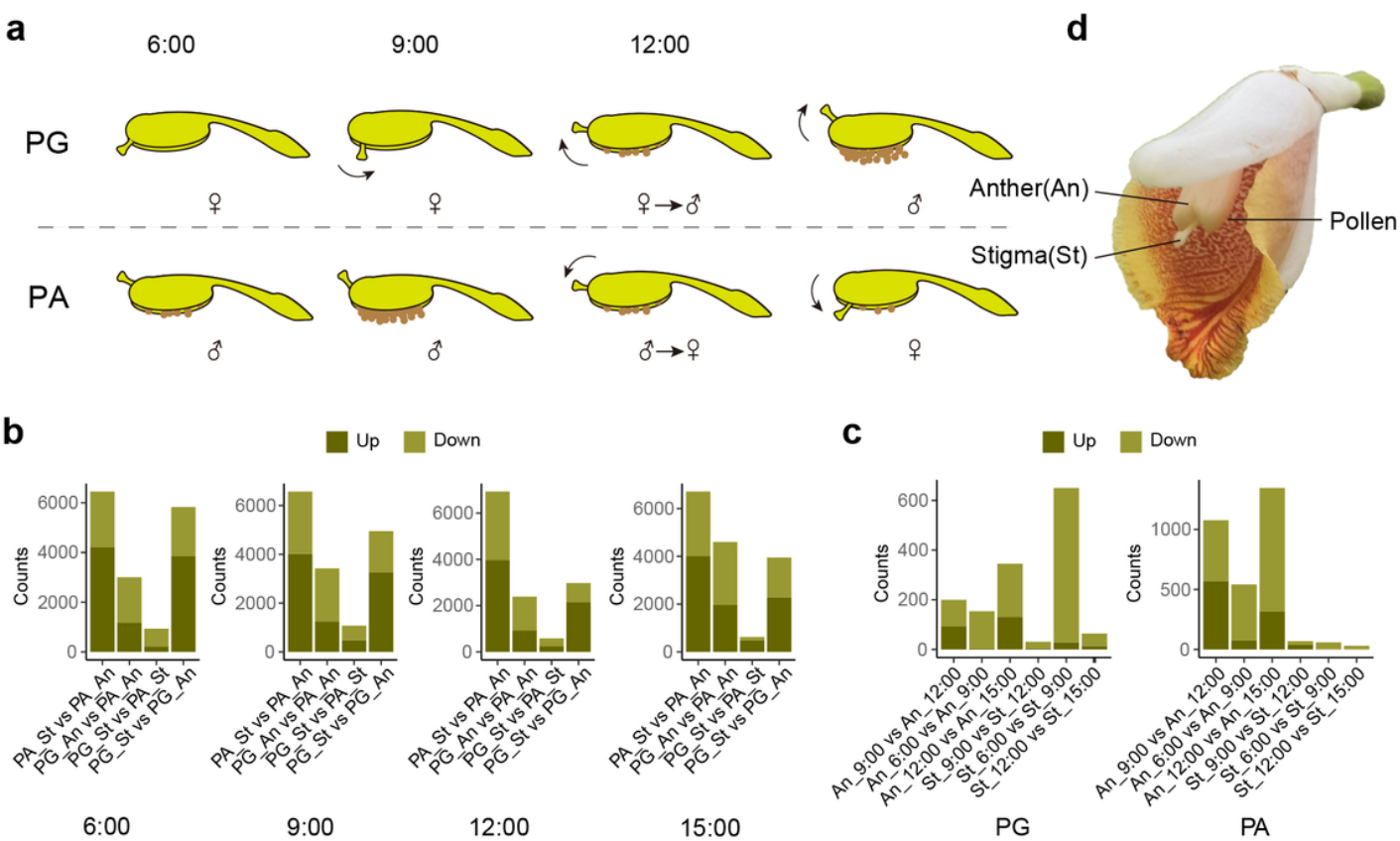


Figure 1

Flexistyly mode, plant photos, and overview of DGEs in *A. mutica*. (a) Flexistyly mode: shown the maturation of anther(An) and the location of sigma(St) at different times in different phenotypes. (b)

Photo of *A. mutica*. (c) DGEs of different phenotypes or tissues at the same time. (d) DGEs of the same phenotypes, same tissues at different time.



Figure 2

Screening of core gene set in anther and stigma. (a) Rose plot of screening conditions in anther. The underlined green words represent the sets as non-differentially expressed genes, the black words represent the sets as differentially expressed genes (b) Venn plot of screening conditions in stigma.

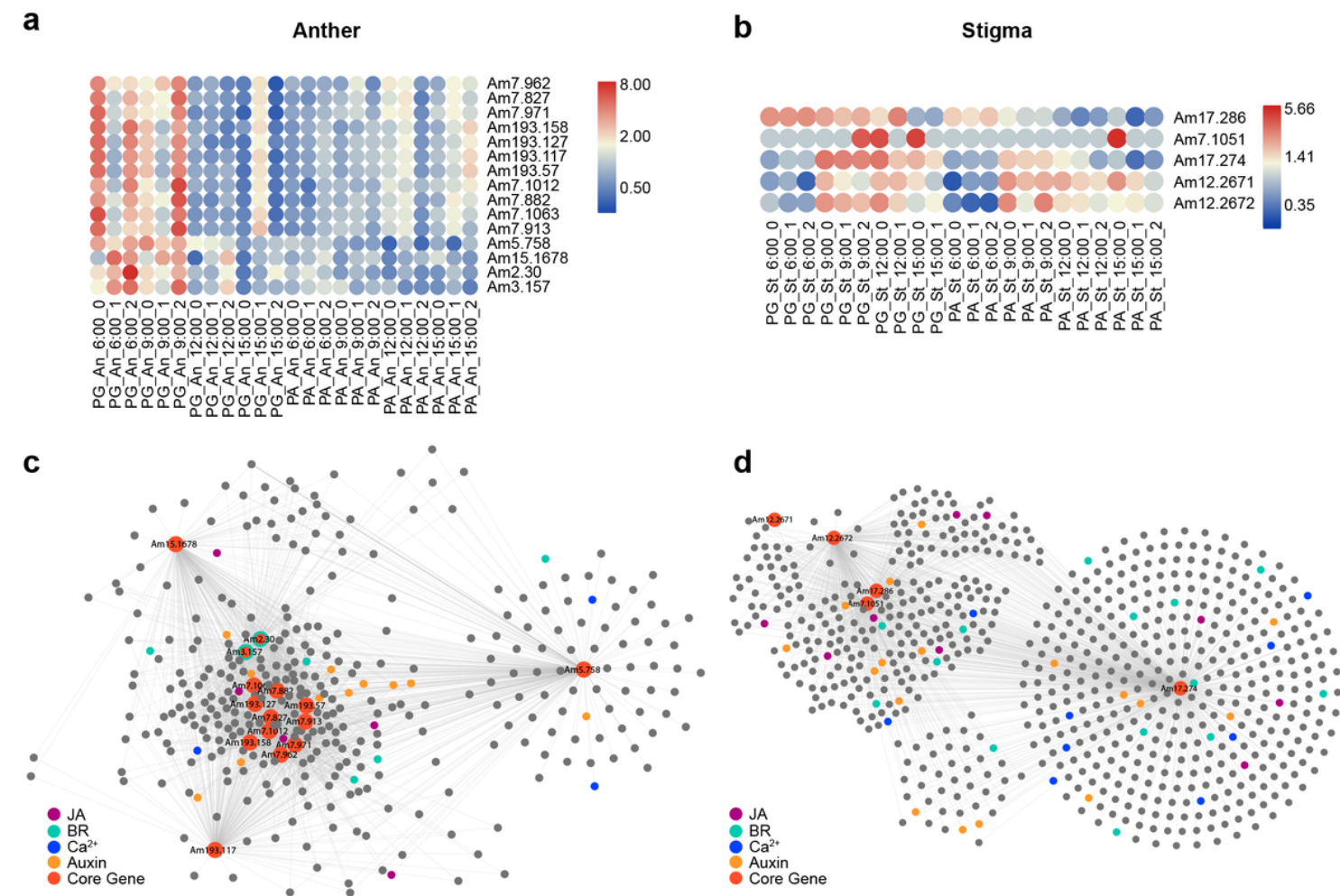


Figure 3

The target network and the expression trend of core genes in the network. (a) Heat map of core gene expression trends in anther. (b) Heat map of core gene expression trends in stigma. (c) The WGCNA co-expression network of core genes in anther. (d) The WGCNA co-expression network of core genes in stigma.

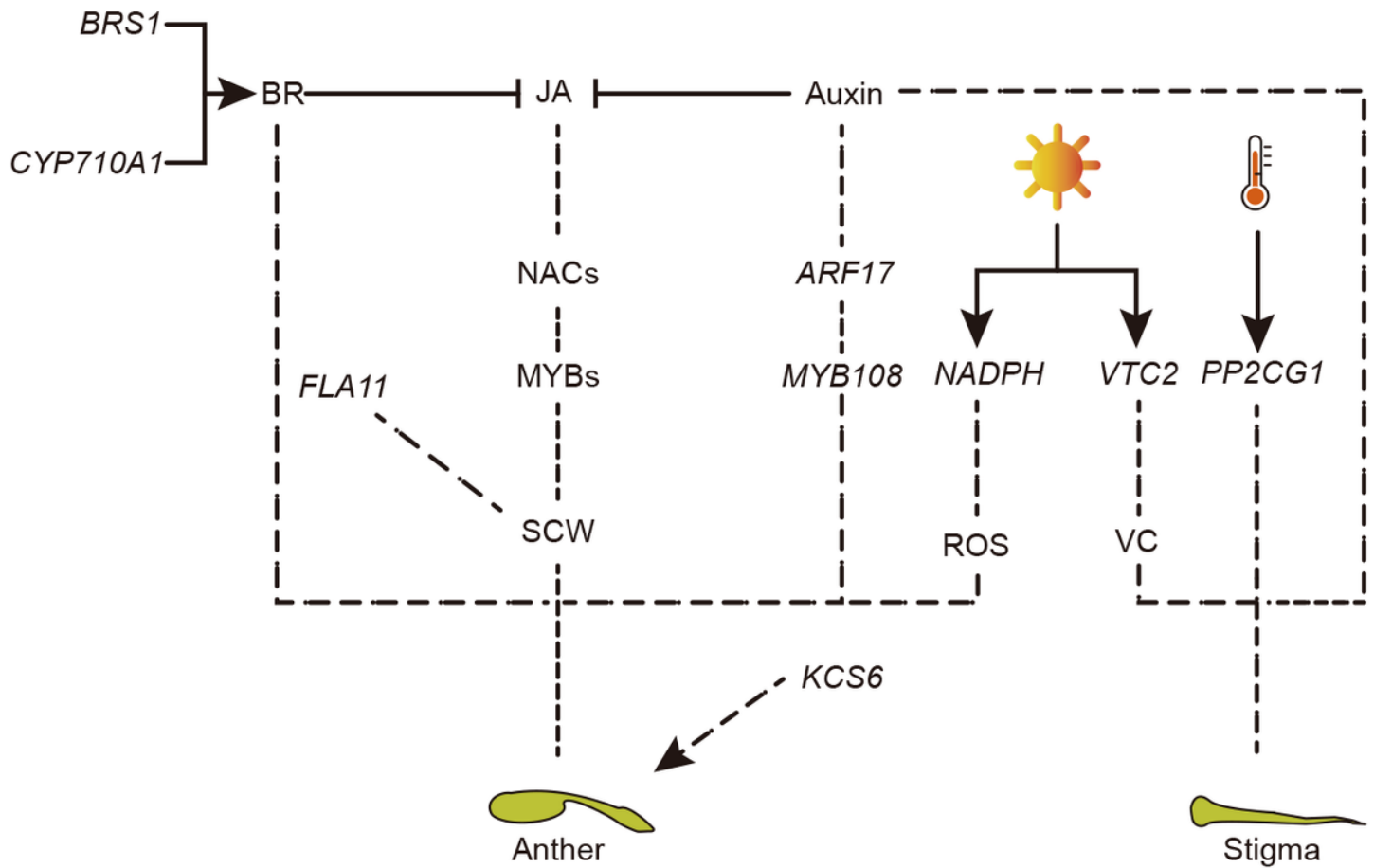


Figure 4

Possible dichogamy regulatory pathways summarized through discussion The solid line with an arrow represents a promoting relationship, the solid line with a vertical bar represents an antagonistic relationship, the dotted line represents a potential but unknown regulatory relationship, the sun pattern represents light, and the thermometer pattern represents temperature.