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Research article

A cotton (*Gossypium hirsutum*) WRKY transcription factor (GhWRKY22) participates in regulating anther/pollen development

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ABSTRACT

Anther/pollen development is a highly programmed process in flowering plants. However, the molecular mechanism of regulating anther/pollen development is still largely unclear so far. Here, we report a cotton WRKY transcription factor (GhWRKY22) that functions in anther/pollen development. Quantitative RT-PCR and GUS activity analyses revealed that *GhWRKY22* is predominantly expressed in the late developing anther/pollen of cotton. The transgenic Arabidopsis plants expressing *GhWRKY22* displayed the male fertility defect with the fewer viable pollen grains. Expression of the genes involved in jasmonate (JA) biosynthesis was up-regulated, whereas expression of the JA-repressors (*JAZ1* and *JAZ8*) was down-regulated in the transgenic Arabidopsis plants expressing *GhWRKY22*, compared with those in wild type. Yeast one-hybrid and ChIP-qPCR assays demonstrated that GhWRKY22 modulated the expression of *JAZ* genes by directly binding to their promoters for regulating anther/pollen development. Yeast two-hybrid assay indicated that GhMYB24 could interact with GhJAZ8-A and GhJAZ13-A. Furthermore, expression of *AtMYB24*, *AtPAL2* and *AtANS2* was enhanced in the transgenic Arabidopsis plants, owing to *GhWRKY22* overexpression. Taking the data together, our results suggest that GhWRKY22 acts as a transcriptional repressor to regulate anther/pollen development possibly by modulating the expression of the *JAZ* genes.

1. Introduction

In flowering plants, both pistil and stamen play vital roles in plant reproductive inheritance. The pistil (carpels), the female reproductive organ, is composed of a ovary, a style and a stigma where pollen adheres, while the stamen consists of an anther, a space for male gametophyte development, and a filament providing the anther with structural support and nutrients (Li et al., 2013). Within the anther, a highly specialized male gametophyte is composed of two or three cells, of which the larger one is the vegetative cell and the smaller ones are germ cells. Paxson-Sowders et al. (2001) described the main features of male gametophyte development in *Arabidopsis thaliana*. When microspore mother cells complete meiosis, the microspores are free in the form of tetrads in the pollen capsule. Male gametophyte development begins after microspores are released from the tetrads. This development process may be divided into two consecutive phases, developmental phase and functional phase. The developmental phase mainly includes the formation of mature pollen grains (Hafidh et al., 2016). In most plants, mature pollen grains release from anthers when pollen grains possess two or three cells (McCormick, 2004). The functional phase initiates after pollen grains land on the stigma, and includes pollen germination, pollen tube growth, sperm cells transportation to the embryo sac, and finally zygote formation (Hafidh et al., 2016).

Previous studies revealed that some transcription factors are involved in regulating male gametophyte development by forming an extremely complex and highly procedural process, in which the normal expression and the intertwined collaboration of the genes are important for male gametophyte development (Hafidh et al., 2016; Ma and Sundaresan, 2010). As one of the transcription factor superfamilies in plants, WRKY proteins are characterized by the conserved WRKY domain (WRKYGQK) and function by directly binding to a putative cis-element, the W-box (T/CTGACC/T), in the promoters of their target genes (Rushton et al., 2010). In past years, it has been reported that WRKY transcription factors are involved in mediating pollen development. For example, overexpression of AtWRKY27 resulted in plant growth aberration and male fertility defect (Mukhtar et al., 2017). AtWRKY34 is specifically expressed in male gametophyte, and participates in regulating early development of pollen. The *wrky34* mutant exhibits the increased pollen viability, pollen germination and pollen tube growth (Zou et al., 2010). In contrast, overexpression of the

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WRKY2, a WRKY34 close homolog, leads to the decreased pollen viability, pollen germination and pollen tube growth (Guan et al., 2014), suggesting these WRKY proteins may play important roles in pollen development.

Jasmonate (JA, including jasmonic acid and its oxylipin derivatives) is an important plant hormone that acts as the regulatory molecule to influence many aspects of plant growth, development and senescence (Li et al., 2017; Song et al., 2011). Previous studies indicated JA is also involved in mediating pollen development (Jewell and Browse, 2016; Song et al., 2011). In the presence of JA hormone, for example, CORONATINE INSENSITIVE 1 (COI1, a F-box subunit of the Skp-Cullin-F-box ubiquitin ligase complex) binds to jasmonate-ZIM domain proteins (JAZs, the repressors in JA response pathway) to promote the degradation of JAZ proteins. In *coi1* mutants, JAZ proteins are unable to be degraded, resulting in the impaired filament elongation and anther dehiscence (Sheard et al., 2010). PAL (phenylalanine ammonia lyase) and ANS (anthocyanidin synthase) as marker genes are essential for JA-regulated anther/pollen development (Li et al., 2013; Song et al., 2011). ANS catalyzes leucoanthocyanidin into anthocyanidin which acts as a potent antioxidant to reduce intracellular ROS levels in plants (He and Giusti, 2010; Scalbert and Williamson, 2000). Arabidopsis plants with excess anthocyanidin accumulation display the indehiscent anthers during late anther development (Dong et al., 2005). Similarly, *ANS* expression and anthocyanidin content are increased, but ROS activity is decreased in the *GhMYB24* transgenic plants, resulting in male sterility and indehiscent anthers (Li et al., 2013). On the other hand, JAZs may mediate various transcription factors (such as WRKY, MYB and bHLH proteins) to affect the diverse JA responses (Liu et al., 2017; Qi et al., 2015; Song et al., 2011). It was found that overexpression of NtWRKY50 leads to the altered JA content, increases expression of defense-related genes and enhances plant resistance to *Ralstonia solanacearum* (Liu et al., 2017). *BnWRKY33* as a *Sclerotinia sclerotiorum*-responsive gene positively regulates plant resistance to this pathogen by enhancing the expression of the genes involved in camalexin synthesis and the genes regulated by JA (Liu et al., 2018). NtWRKY-R1 mediates JA signaling transduction for regulating the balance of actin polymerization and depolymerization and the expression of the genes related to nicotine synthesis (Jin et al., 2018). Also, the bHLH transcription factors as the targets of JAZs could form a complex with MYB proteins in regulation of stamen development and seed production in Arabidopsis (Qi et al., 2015). In addition, a study revealed that AtMYB21 and AtMYB24 interact with JAZ1, JAZ8 and JAZ11 to regulate plant male fertility (Song et al., 2011). However, little is known in detail of how the WRKY transcription factor regulates expression of JAZs to mediate male fertility so far. In this study, we provide biochemical and genetic evidence to demonstrate that a cotton WRKY transcription factor (GhWRKY22), belonging to pollen-specific group I of the WRKY family, negatively regulates anther/pollen development by inhibiting the expression of the *JAZ* genes.

2. Material and methods

2.1. Plant materials

Seeds of Arabidopsis (Columbia ecotype) germinated on Murashige and Skoog (MS) medium (a commercial product purchased from Duchefa Biochemie Company, the Netherlands, product No. P14894.01) supplemented with 30 g/L sucrose and 8.0 g/L agar powder (PH5.8) under 16 h light/8 h dark photoperiod at 22 °C for 10 d, and then the seedlings were transferred into soil in a growth chamber under same conditions for further growing to maturation. Arabidopsis transformation was performed by the floral dip method. In brief, the target plasmid vector was introduced into *Agrobacterium tumefaciens* (strain GV3101), and a single colony of the transformants was inoculated in liquid Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g NaCl, PH7.0) with 50 mg/L kanamycin for growth until 0.6–0.8 OD value. Then, the cultures were centrifuged and resuspended in a solution containing 0.02–0.03% Silwet L-77 (Yeasen, 41008ES10). The inflorescences of Arabidopsis plants were immersed in the agrobacteria solution for 30 s, and then these plants were cultured in a growth chamber under normal conditions (16 h light/8 h dark, 22 °C) until seed maturation. The transgenic seeds were selected by kanamycin resistance (50 mg/L), and homozygous lines of T3 generations were used for further phenotypic analysis. The primer pairs used in the experiments are listed in Table S1.

Seeds of cotton (*Gossypium hirsutum* cv. Coker312) germinated on one-half MS medium in dark for 5 d and light for 3d at 28 °C. Hypocotyls of the cotton seedlings were cut into approximately 0.5 cm fragments, and transformed by *Agrobacterium*-mediated DNA transfer for gaining the callus cells (Wang and Li, 2009). In brief, the target plasmid vector was introduced into *Agrobacterium tumefaciens* (strain LBA4404), and a single colony of the transformants was inoculated in liquid LB medium with 50 mg/L kanamycin for growth until 1.0 OD value. Then, the cultures were centrifuged and resuspended in a liquid MS medium. The approximate 0.5 cm fragments of cotton hypocotyls were immersed in the agrobacteria solution for 10 min, and then the hypocotyl explants were cultured on a selective medium (MS medium supplemented with 30 g/L glucose, 0.1 mg/L KT, 0.1 mg/L 2,4-D, 2.8 g/L phytagel, pH 6.4) containing 50 mg/L kanamycin and 500 mg/L cephalosporin in a culture room (16 h light/8 h dark) at 28 °C for generating the transgenic callus cells, which were used for further chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis.

2.2. Subcellular localization

The coding sequence of *GhWRKY22* was introduced into the binary vector pBI121-GFP with a *GFP* (green fluorescence protein) gene to generate pBI121-GhWRKY22:GFP vector under control of CaMV 35S promoter. The construct was transferred into *Agrobacterium tumefaciens* (strain GV3101), and a single colony of the transformants was inoculated in liquid LB medium with 50 mg/L kanamycin for growth until 1.0 OD value. Then, the cultures were centrifuged and resuspended in infiltration buffer [10 mM $MgCl_2$, 10 mM MES free acid monohydrate (amresco), 200 μM acetosyringone]. Finally, the agrobacteria suspension was injected into the lower epidermis of tobacco (*Nicotiana benthamiana*) leaves. The tobacco plants with the injected leaves were cultivated for 48–72 h in a growth chamber (16 h light/8 h dark, 22 °C) as described previously (Qin et al., 2017). GFP fluorescence of GhWRKY22:GFP fusion protein was visualized in leaf cells under a confocal laser scanning microscope (SP5, Leica, Germany). Meanwhile, the cells were stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for showing cell nucleus. Leica TCS SP5 software was employed to record and process the digital images taken (Li et al., 2013).

2.3. Transactivation assay

The coding sequence of *GhWRKY22* was amplified by PCR using *Pfu* DNA polymerase and gene-specific primers (GhWRKY22-up and GhWRKY22-dn) (Table S1), and then cloned into pGBKT7 vector. The construct was transferred into yeast stains AH109 and Y187, respectively. AH109 transformants were screened on SD/–Trp/–His medium (SD minimal medium lacking tryptophan and histidine) and SD/–Trp/–Ade medium (SD minimal medium lacking tryptophan and adenine), and Y187 transformants were employed to test the β-galactosidase activity by a flash-freezing filter assay. The yeast cells containing empty pGBKT7 vector were used as the negative control.

2.4. Assay of GUS activity under the control of GhWRKY22 promoter

GhWRKY22 promoter (−1 ~ −1837 from the start code ATG) was cloned into a pBI101 vector to generate the GhWRKY22p:GUS construct for *GhWRKY22* promoter driving *GUS* expression. The construct was

introduced into Arabidopsis by the floral dip method described as above (see Methods 2.1). The transgenic *GhWRKY22p:GUS* plants (T3 generation) were used to assay the GUS activity under the control of *GhWRKY22* promoter. GUS staining was performed by the method described previously (Wang and Li, 2009).

2.5. Seed setting rate test

The seed setting rates of wild type, and *GFP* and *GhWRKY22* overexpression transgenic plants were tested by examining the percentage of each silique with seeds formed in primary inflorescences. At least 150 siliques in 15 independent plants of each line were counted. Excel software was used for statistical analysis of data, and the student *t*-test was performed for statistical inference.

2.6. Pollen viability assay

The viability of pollen grains was assayed by double staining with fluorescein diacetate (FDA) which staining the viable pollen grains and propidium iodid (PI) which staining the non-viable pollen grains as described previously (Mandaokar and Browse, 2009). The ratio of the green fluorescence by FDA and the red color by PI represents the pollen viability. More than 1500 pollen grains from 25 independent flowers of different plants for each transgenic line were randomly selected for measurement of pollen viability. The stained pollen grains were viewed under a Nikon microscope equipped with UV light and a charge-coupled device (CCD) camera (Nikon Digital Sight DS-5Mc). The data were analyzed in Excel software, and the student *t*-test was performed for statistical inference.

2.7. In vitro pollen germination assay

Pollen grains from freshly anther-dehisced flowers of wild type, and *GFP* and *GhWRKY22* overexpression transgenic Arabidopsis plants germinated on basic medium as described previously (Wang et al., 2015). More than 500 pollen grains from 10 independent flowers of different plants for each transgenic line were randomly selected for measurement of pollen germination. The differences in pollen germination between *GhWRKY22* transgenic plants and controls (wild type and *GFP* transgenic plants) were assayed after the pollen grains germinated for 4 h. The pollen grains were viewed under a Nikon microscope with a charge-coupled device (CCD) camera (Nikon Digital Sight DS-5Mc). The germinated pollen grains were calculated in image J software (https://imagej.nih.gov/ij/), and data were analyzed in Excel software. The student *t*-test was performed for statistical inference.

2.8. Quantitative RT-PCR analysis

Total RNA was isolated from flowers of mature Arabidopsis plants using RNAiso Plus Reagent (TAKARA). About 2 μg of RNA was reversely transcribed into cDNAs. The cDNAs were used as the templates in quantitative RT-PCR analysis, using gene-specific primers (Table S1). Real-time PCR analysis was performed by the method as described previously (Li et al., 2017), and the relative values were expressed as the log2 scale of the ratio of the gene expression levels in the transgenic flowers vs. those in control flowers.

2.9. Assay of ChIP-qPCR

Ten-day-old seedlings of *GhWRKY22:GFP* and *GFP* transgenic Arabidopsis and callus cells (see Methods 2.1) of *GhWRKY22:GFP* and *GFP* transgenic cotton were used as materials for ChIP assay as described previously (Chen et al., 2017). Anti-GFP polyclonal antibody (Abcam) and protein A agarose/salmon sperm DNA (Millipore) were used for immunoprecipitation. Then, the precipitated DNA was extracted using equal volume tris-phenol and chloroform. The mixtures were centrifuged (12000 rpm, 5min), and the supernatant was collected into a new tube. The above steps were repeated for three times. Then, DNA in the supernatant was precipitated by adding twice volume of anhydrous ethanol for quantitative PCR (qPCR) analysis. The ChIP experiments were performed three times. Chromatin precipitated without antibody was used as negative control, while the isolated chromatin before precipitation was used as input control. ChIP-qPCR results were presented as a percentage of input DNA and the samples of GFP transgenic plants as the controls. The primers used for ChIP-qPCR amplification are listed in Table S1.

2.10. Yeast one-hybrid assay

Promoter fragments with the W-boxes of *GhJAZs* (*GhJAZ8-A-P1, GhJAZ8-A-P2, GhJAZ8-A-P3, GhJAZ8-A-P4, GhJAZ13-A-P1, GhJAZ13-A-P2*) were cloned into the pBait-AbAi vectors, respectively, and then transferred into the yeast Y1H Gold according to Yeast Protocol Handbook (Clontech) to form the W-box-specific reporter strains used as baits. The transformed yeast cells were selected on dropout medium (SD/-Ura) with 400 ng/ml aureobasidin (AbA) which could completely suppress the basic growth of the transformed yeast cells. The coding sequence of *GhWRKY22* was cloned into pGADT7 vector and then transformed into the bait-specific reporter strain. These yeast cells were plated on SD/-Leu/-Ura medium containing 400 ng/ml AbA for analyzing DNA-protein interaction. The empty pGADT7 was transformed into the bait-specific reporter strain as negative control (Yanai, 2013). The primers used in the experiments are listed in Table S1.

2.11. Yeast two-hybrid assay

The coding sequence of *GhMYB24* was cloned into pGADT7 vector as a prey, and the coding sequences of *GhJAZs* (*GhJAZ4-A*, *GhJAZ7-A*, *GhJAZ8-A* and *GhJAZ13-A*) were cloned into pGBKT7 vector as baits, respectively. Then, the prey and bait were co-transferred into the yeast Y2H Gold (Clontech) by the method described earlier (Chen et al., 2017). In brief, 100 ng target construct plasmid was added in a tube with 50 μL Y2H receptive state yeast cells, and then 500 μL PEG/LiAc solution [50% PEG, 100 mM LiAc, 1 × TE buffer (50 mM Tris-HCl, 1 mM EDTA pH 8.0)] was added in the tube. The mixtures were incubated in a water bath at 30 °C for 30min, heated at 42 °C for 15 min, and then incubated in a shaker (220 rpm, 30 °C) for 90 min. Finally, the transformed yeasts were selected on double dropout medium (DDO medium, SD/–Leu–Trp). Then the single colony of transformants growing on DDO medium were streaked on quadruple dropout medium (QDO medium, SD/–Trp/–Leu/–His/–Ade), using transformants containing empty pGBKT7 and pGADT7-GhMYB24 vectors as negative controls, and transformants containing pGBKT7-53 and pGADT7-RecT vectors as positive controls (Zhang et al., 2010). The primers used in the experiment are listed in Table S1.

2.12. Data analysis

Each experiment was done at least in triplicates, and the data were statistically analyzed by the Student's *t*-test. Mean values and standard deviation (SD) are shown at least from three independent experiments.

3. Results

3.1. GhWRKY22 protein is localized in the cell nucleus and acts as a transcriptional repressor

GhWRKY22 protein (XM_016889204) containing two conserved WRKY domains and one C2H2 type of finger potential zinc ligand belongs to group 1 of WRKY transcription factor family. To assay the subcellular localization of GhWRKY22, we constructed the GhWRKY22:GFP (green fluorescent protein) fusion protein plasmid

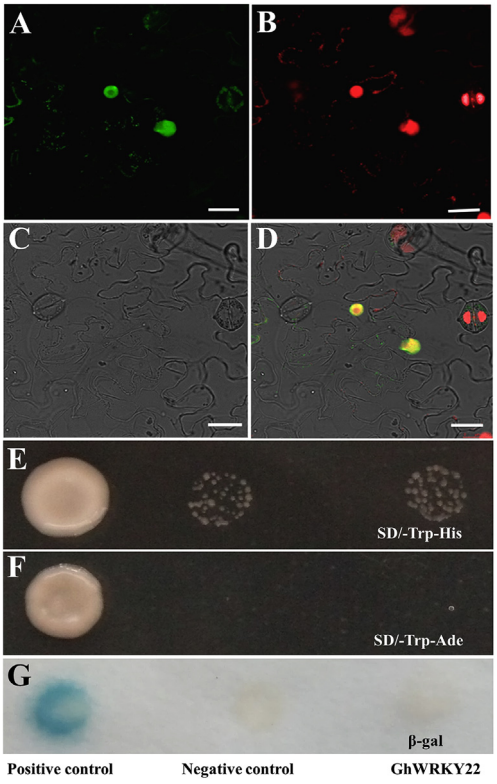

Fig. 1. Subcellular localization and transactivation assay of GhWRKY22. (**A**) GFP signal in the nuclei of tobacco epidermal cells. (**B**) Nuclear staining with 4,6-diamidino-2-phenylindole (DAPI) of the same tobacco epidermal cells in image A. (**C**) Bright field photograph of images A and B. (**D**) Images A and B were merged with its bright-field photograph. (**E**) Yeast transformants were streaked on SD/-Trp-His medium. (**F**) Yeast transformants were streaked on SD/-Trp-Ade medium. The results showed that the Yeast transformants could not grow on the both dropout medium. (**G**) Flash-freezing filter assay of the β-galactosidase activity. The yeast transformants harboring empty pGBKT7 vector were used as negative control. Bar = 25 μm.

vector (see Methods 2.2), and transiently expressed the fusion protein in leaves of tobacco (*N. benthamiana*). The transformed tobacco leaf epidermal cells expressing GhWRKY22:GFP fusion proteins were also stained using 4', 6-diamidino-2-phenylindole (DAPI) that specially reveals the cell nucleus. As shown in Fig. 1A–D, the GFP fluorescence was accumulated mainly in the cell nucleus, and overlapped with DAPI staining, indicating that GhWRKY22 protein is targeted to the cell nucleus.

To determine whether GhWRKY22 has the transcriptional activity, we employed a yeast GAL4-responsive reporter system. The effector construct (pGBKT7-GhWRKY22) was transferred into yeast strains AH109 and Y187, respectively. The transformed AH109 yeast cells were streaked on SD/–Trp/–His and SD/–Trp/–Ade medium, while the transformed Y187 yeast cells were employed to do a flash-freezing filter assay. As shown in Fig. 1E–G, the transformed cells could not grow on the dropout medium and not turned blue in the flash-freezing filter assay, demonstrating GhWRKY22 protein could not activate *LacZ* reporter gene expression in yeasts. The above data indicated that GhWRKY22 does not have the transactivation activity, suggesting it may act as a transcription repressor.

3.2. *GhWRKY22 is dominantly expressed in late developing pollen*

To investigate the expression pattern of *GhWRKY22* gene in cotton, we performed quantitative RT-PCR to analyze gene expression in cotton tissues. As shown in Fig. 2A, *GhWRKY22* is expressed in all tissues examined, and shows its relatively higher expression level in late

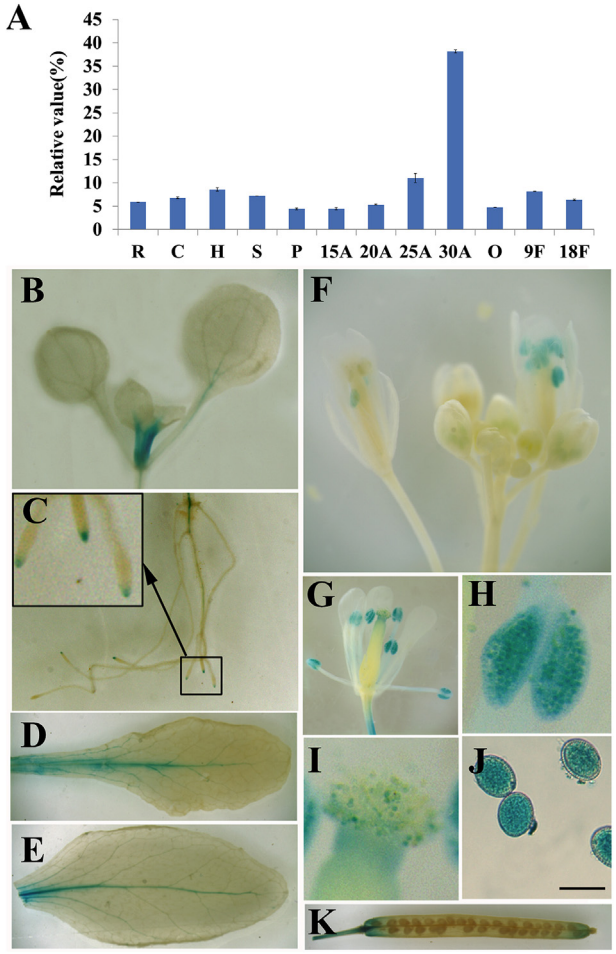

Fig. 2. Analysis of expression pattern of *GhWRKY22* gene. (**A**) Quantitative RT-PCR analysis of *GhWRKY22* expression profiling in cotton tissues (R, roots; C, cotyledons; H, hypocotyls; S, stems; P, petals; 15A–30A, 15- to 30-day-old anthers; O, ovule; 9F and 18F, fibers at 9 and 18 days after anthesis). Relative values of *GhWRKY22* expression are shown as percentage of *GhUBI1* expression activity. (**B–K**) Histochemical assay of GUS activity under the control of *GhWRKY22* promoter in the *GhWRKY22p:GUS* transgenic Arabidopsis. (**B**) A seedling; (**C**) Roots; (**D**) A rosette leaf; (**E**) A cauline leaf; (**F**) Flowers; (**G**) A mature flower; (**H**) Anthers; (**I**) A stigma with germinated pollen grains; (**J**) Pollen grains (**K**) A silique. Each experiment was repeated at least three times, and error bars represent standard deviation (SD). Bar = 200 μm.

developing anthers. During anther development, the expression level of *GhWRKY22* is gradually increased in anthers, and reaches its peak value in 30-day-old anthers. To investigate the *GhWRKY22* expression profiling in more detail, we analyzed expression activity of the β-glucuronidase (*GUS*) reporter gene driven by the *GhWRKY22* promoter in the *GhWRKY22p:GUS* transgenic Arabidopsis plants. As shown in Fig. 2B–E, weak to moderate GUS staining was detected in the vascular system and young tissues of leaves, roots and stems, and moderate to relatively strong GUS activity was also observed in root tips. Especially, very strong GUS signals were mainly detected in mature anthers (Fig. 2G), but no or weak GUS activity was found in young anthers (Fig. 2F). Furthermore, strong GUS signals were mainly concentrated in pollen grains, but no or very weak GUS staining was observed in the stigma (Fig. 2H–J). At later stages of reproductive development, weak GUS activity was also found in siliques (Fig. 2K).

To further determine the expression profiling of *GhWRKY22* in pollen development, we cut the *GhWRKY22p:GUS* transgenic Arabidopsis anthers into cross sections for observing GUS activity in anther tissues. As shown in Fig. S1A, almost no GUS signal was found in

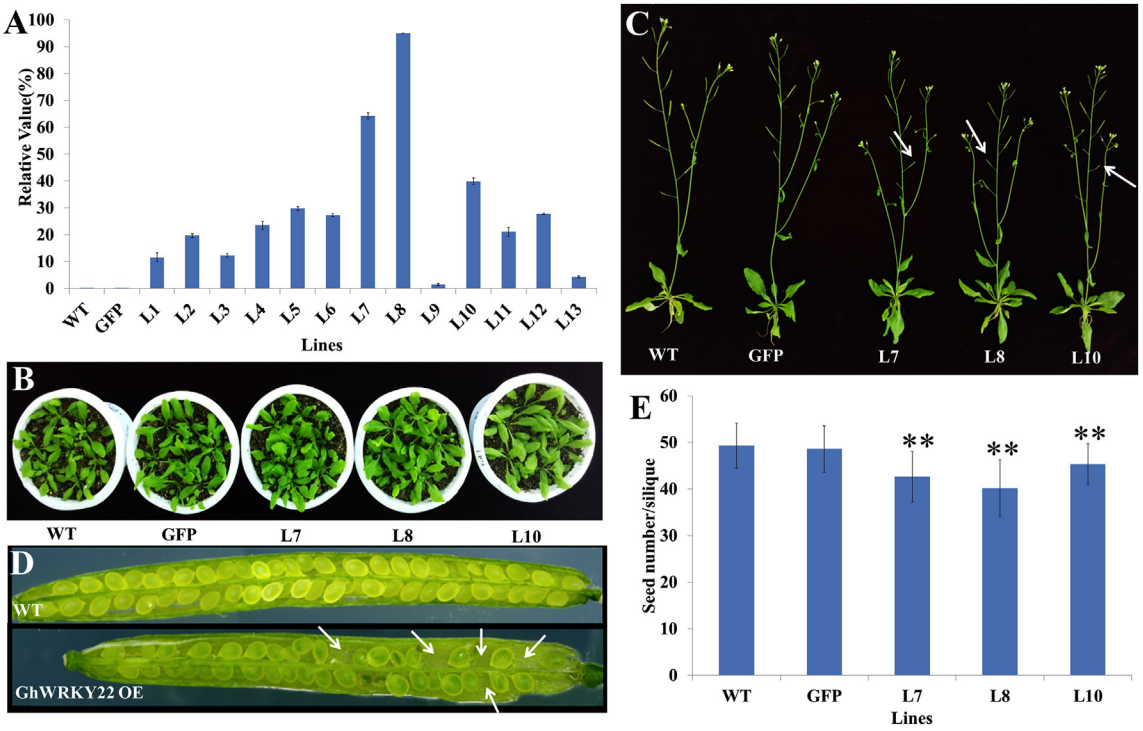

Fig. 3. Phenotypic assay of *GhWRKY22* overexpression transgenic Arabidopsis plants. (**A**) Quantitative RT-PCR analysis of *GhWRKY22* expression in the *GhWRKY22* transgenic lines and controls (wild type and *GFP* transgenic plants). Relative values of *GhWRKY22* expression are shown as percentage of *AtACTIN2* expression activity. (**B**) Seedling phenotype of the *GhWRKY22* transgenic lines and controls at vegetative growth stage. (**C**) Plant phenotype of the *GhWRKY22* transgenic lines and controls at reproductive growth stage. White arrows indicate abnormal siliques in the transgenic plants. (**D**) Phenotype of siliques in the *GhWRKY22* transgenic lines and wild type controls. White arrows indicate unfertilized ovules/undeveloped seeds in the transgenic silique. (**E**) Statistical assay of seed number per sillique in the *GhWRKY22* transgenic lines and controls. WT, wild type; GFP, *GFP* transgenic line; L1–L13, *GhWRKY22* transgenic lines. Each experiment was repeated at least three times, and error bars represent standard deviation (SD) from the mean data of each genotype. Student's *t*-test demonstrates that there were very significant differences (**P < 0.01) between the *GhWRKY22* transgenic lines and controls.

Table 1
Statistical analysis of percentage of seedlings with kanamycin resistance in reciprocal cross between GhWRKY22 overexpression transgenic plants and wild type Arabidopsis.

Pollination ways	Percentage of seedlings resistant to kanamycin			X^2-test
Pollinated with wide type pollen grains	WT(♂) × L7 (♀)	WT(♂) × L8 (♀)	WT(♂) × L10 (♀)	*P*-value
	50.88 ± 3.27	51.27 ± 1.80	50.28 ± 2.67	P > 0.05
Pollinated with transgenic pollen grains	L7(♂) × WT (♀)	L8(♂) × WT(♀)	L10(♂) × WT (♀)	*P*-value
	34.71 ± 0.54	34.07 ± 0.25	35.59 ± 0.17	P < 0.05

Note: Pollen grains in over 20 flowers of each line were collected for cross, and more than 200 hybrid seeds were examined in each line. WT, wild type. L7, L8 and L10, three *GhWRKY22* transgenic lines. Data are mean ± SD. P > 0.05, there is no significant difference from the expected 1:1 ratio for normal Mendelian segregation; P < 0.05, there is significant difference from the expected 1:1 ratio for normal Mendelian segregation.

the early developing anther tissues. As anthers further developed, weak GUS activity was detected in microspores (young pollen) (Figs. S1B–E), and strong GUS staining was found in mature pollen grains (Fig. S1F). The above results suggested that *GhWRKY22* is dominantly expressed in the late developing pollen.

3.3. Overexpression of GhWRKY22 in Arabidopsis affects plant male fertility

To investigate the role of GhWRKY22 in plant reproductive development, we introduced *GhWRKY22* gene into Arabidopsis. Thirteen *GhWRKY22* overexpression transgenic plants were obtained, and three transgenic lines (L7, L8 and L10) with different expression levels of *GhWRKY22* were selected for further analysis (Fig. 3A). At the vegetative growth stage, the transgenic plants displayed no morphological changes, compared with the controls (Fig. 3B). At the reproductive growth stage, however, the transgenic plants produced the stunted siliques, compared with the controls (Fig. 3C). A further observation revealed that a lot of unfertilized ovules/undeveloped seeds were found in siliques of the *GhWRKY22* overexpression transgenic Arabidopsis plants, compared with those in controls (Fig. 3D). Statistical analysis showed that the number of seeds in some siliques and the number of normal siliques in some plants of the transgenic lines were significantly decreased, relative to those in the controls (Fig. 3E, Table S2).

To investigate whether the decreased fertility in the *GhWRKY22* transgenic plants is mainly caused by abnormal development of female gametes or male gametes, we employed reciprocal cross between the *GhWRKY22* transgenic plants and wild type. L7, L8 and L10 lines are all the heterozygotes in presence of the selectable marker kanamycin (data not shown) and included in the experiments to simplify the segregation. More than 20 flowers of each line were selected for cross. The hybrid offspring seeds were cultured on the selective medium with kanamycin. As shown in Table 1, when pollinated with wide type pollen grains, all lines had seedling ratios of kanamycin resistant (kan^r) versus kanamycin sensitive (kan^s) at 1:1, correlating with the expected Mendelian ratios for segregation of heterozygotes. On the contrary, kan^r seedlings

were significantly < 50% in all lines when pollinated with pollen grains of the *GhWRKY22* transgenic lines. Taken together, the above data indicated that the *GhWRKY22* transgenic plants generated more abnormal male gametes, but their female gametes were normal, compared with the controls, demonstrating that the decreased fertility in the *GhWRKY22* overexpression transgenic plants is mainly caused by the partial male sterility.

Additionally, another phenotypic variation of the *GhWRKY22* transgenic Arabidopsis was observed by measuring filament length. Flowers of the *GhWRKY22* transgenic plants contained shorter filaments which could not reach the stigmas, compared with the controls (Figs. S2A–E). Approximately 81.8% and 83.8% of flowers from wild type and *GFP* controls contained four long filaments within a flower. In contrast, the *GhWRKY22* transgenic flowers had much more stamens with the shortened filaments. Only 57.5%, 54.4% and 56.7% of flowers from the *GhWRKY22* transgenic lines 7, 8 and 10 (L7, L8 and L10), respectively, had four long filaments, and the remains showed a portion of short filaments (i.e. each flower has one, two or three normal length filaments) (Fig. S2F). On the other hand, the observation on the dehiscence of anthers showed that the *GhWRKY22* transgenic plants produce normal dehiscent anthers.

3.4. Pollen viability and pollen germination rate are declined in the *GhWRKY22* transgenic plants

To investigate the effect of *GhWRKY22* overexpression on male gametogenesis, we tested pollen viability and pollen germination of the *GhWRKY22* transgenic Arabidopsis plants (L7, L8 and L10), using wild type and *GFP* transgenic plants as controls. Pollen viability of three *GhWRKY22* transgenic lines (L7, L8 and L10) and control plants was examined by double staining with fluorescein diacetate (FDA) and propidium iodide (PI). As shown in Fig. 4A–E, strong green fluorescence was observed in most pollen grains of the controls, whereas red fluorescence was detected in many transgenic pollen grains, indicating that these *GhWRKY22* transgenic pollen grains are not viable. Statistical analysis showed that > 90% of pollen grains were viable in the controls, whereas only 79.71% (L7), 75.15% (L8) and 82.17% (L10) of the transgenic pollen grains were viable (Fig. 4F), indicating that pollen viability was significantly declined in the *GhWRKY22* transgenic plants.

Additionally, we tested pollen germination of both transgenic lines and controls *in vitro* (see Methods 2.7). As shown in Fig. 4G–K, a lot of pollen grains of the *GhWRKY22* transgenic lines did not germinate, and the transgenic pollen tubes grew slowly, compared with those of the controls. Statistical analysis showed that > 66% of the control pollen grains germinated, but only 34.55% (L7), 32.95% (L8), 46.35% (L10) of the transgenic pollen grains germinated *in vitro*, respectively (Fig. 4L), after pollen grains germinated *in vitro* for 4 h. The above results indicated that pollen germination rate is remarkably reduced in the *GhWRKY22* transgenic plants.

3.5. GhWRKY22 is involved in JA signaling pathway by regulating expression of JAZ genes

To investigate whether GhWRKY22 plays a vital role in regulation of male fertility by JA signal pathway, we examined expression of the genes involved in JA-response pathway in the *GhWRKY22* transgenic Arabidopsis plants. As shown in Fig. 5, the genes related to JA biosynthesis (*AtAOS, AtLOX1, AtLOX2, AtAOC1, AtAOC2, AtAOC3, AtAOC4, AtOPR3* and *AtDAD1*) and JA signaling (*AtCOI1*) were up-regulated in flowers of the transgenic plants, but expression levels of *AtJAZ1* and *AtJAZ8* were down-regulated in the *GhWRKY22* transgenic flowers, relative to wild type and *GFP* transgenic plant controls. However, expression of the other *AtJAZs* that are predominantly expressed in flowers was not changed in the *GhWRKY22* transgenic plants (Fig. S3A). Considering the GUS data (Fig. 2), the changes in expression of the genes by quantitative RT-PCR analysis in the flower are likely to reflect only changes in their expression in the anther/pollen. The above results indicated that overexpression of *GhWRKY22* in Arabidopsis affects the expression of the genes involved in JA signal pathway.

Additionally, we employed the ChIP-qPCR system to examine whether *AtJAZ1* and *AtJAZ8* are the direct targets of GhWRKY22 in the *GhWRKY22* transgenic Arabidopsis. The *in vivo* interactions between GhWRKY22 and the W-box elements in *AtJAZ1* and *AtJAZ8* promoters were performed using ChIP-qPCR assay. As shown in Fig. S3B, GhWRKY22 could bind to the P4 fragment of *AtJAZ1* promoter and the P2 fragment of *AtJAZ8* promoter via the W-box sequence, but not to other fragments, suggesting that GhWRKY22 may directly regulate expression of *AtJAZ1* and *AtJAZ8* in the transgenic plants.

As GhJAZ13-A shares high sequence similarity with AtJAZ1, we investigated whether *GhJAZ13-A* is the direct target of GhWRKY22 in cotton. Yeast one-hybrid assay was employed to determine the interaction between GhWRKY22 and *GhJAZ13-A* promoter. As shown in Fig. 5B, GhWRKY22 could bind to P1 and P2 fragments of the *GhJAZ13-A* promoter. Furthermore, we also tested whether GhWRKY22 can bind to promoters of the other anther/pollen-preferential genes, such as *GhJAZ8-A*, *GhJAZ8-D* and *GhJAZ14-D*. As shown in Fig. 5B, *GhJAZ8-A* may be another target of GhWRKY22. To further examine whether *GhJAZ13-A* and *GhJAZ8-A* are the direct targets of GhWRKY22 *in vivo*, we did ChIP-qPCR assay in the *GhWRKY22* transgenic cotton callus cells (see Methods 2.1). As shown in Fig. 5C, GhWRKY22 could directly bind to *GhJAZ13-A* and *GhJAZ8-A* promoters, consistent with the yeast one-hybrid assay. Collectively, the above data suggested that GhWRKY22 may directly regulate the expression of *GhJAZ13-A* and *GhJAZ8-A*.

3.6. GhJAZ proteins interact with GhMYB24

To investigate whether down-regulation of *AtJAZ1* and *AtJAZ8* affects expression of *AtMYB21*, *AtMYB24* and their downstream genes, we examined the expression of *AtMYB21* and *AtMYB24* and their downstream genes in anthers of *GhWRKY22* overexpression transgenic Arabidopsis. As shown in Fig. 6A, expression of *AtMYB24*, *AtPAL2* and *AtANS2* (except *AtMYB21*), which were reported to play critical roles in anther development, was enhanced in anthers of the transgenic plants, possibly owing to the decreased *AtJAZ1* and *AtJAZ8* expression.

To investigate whether GhMYB24 interacts with GhJAZ proteins in cotton, a yeast two-hybrid system was employed to assay the interactions among GhMYB24 and four cotton GhJAZ proteins (GhJAZ4-A, GhJAZ7-A, GhJAZ8-A and GhJAZ13-A), which were reported to be highly expressed in anthers (Li et al., 2017). The prey vector pGADT7-GhMYB24 and bait vectors pGBKT7-GhJAZs were co-transformed into yeast cells for assaying the interactions of GhMYB24 with GhJAZ proteins (see Methods 2.11). As shown in Fig. 6B, the transformants with GhMYB24 and GhJAZ8-A/13-A constructs grew well on QDO nutritional selection medium, but the transformants with GhMYB24 and GhJAZ4-A/7-A constructs did not grow on QDO nutritional selection medium, indicating GhMYB24 could interact with GhJAZ8-A and GhJAZ13-A, but not with GhJAZ4-A and GhJAZ7-A in yeast cells.

4. Discussion

In self-pollinating plants, normal filament elongation and anther dehiscence are important for plant fertility. Our previous study reported that GhMYB24 is involved in regulation of anther/pollen development. GhMYB24 overexpression transgenic plants have large numbers of stamens with short filaments and non-dehiscent anthers (Li et al., 2013). In this study, our data showed *GhWRKY22* is predominantly expressed in late developing anther/pollen. Ectopic expression of *GhWRKY22* in Arabidopsis resulted in the shortened filaments and the reduced male fertility in the transgenic plants. Different from the GhMYB24, however, the transgenic plants expressing *GhWRKY22* displayed normal dehiscent anthers. On the other hand, overexpression of

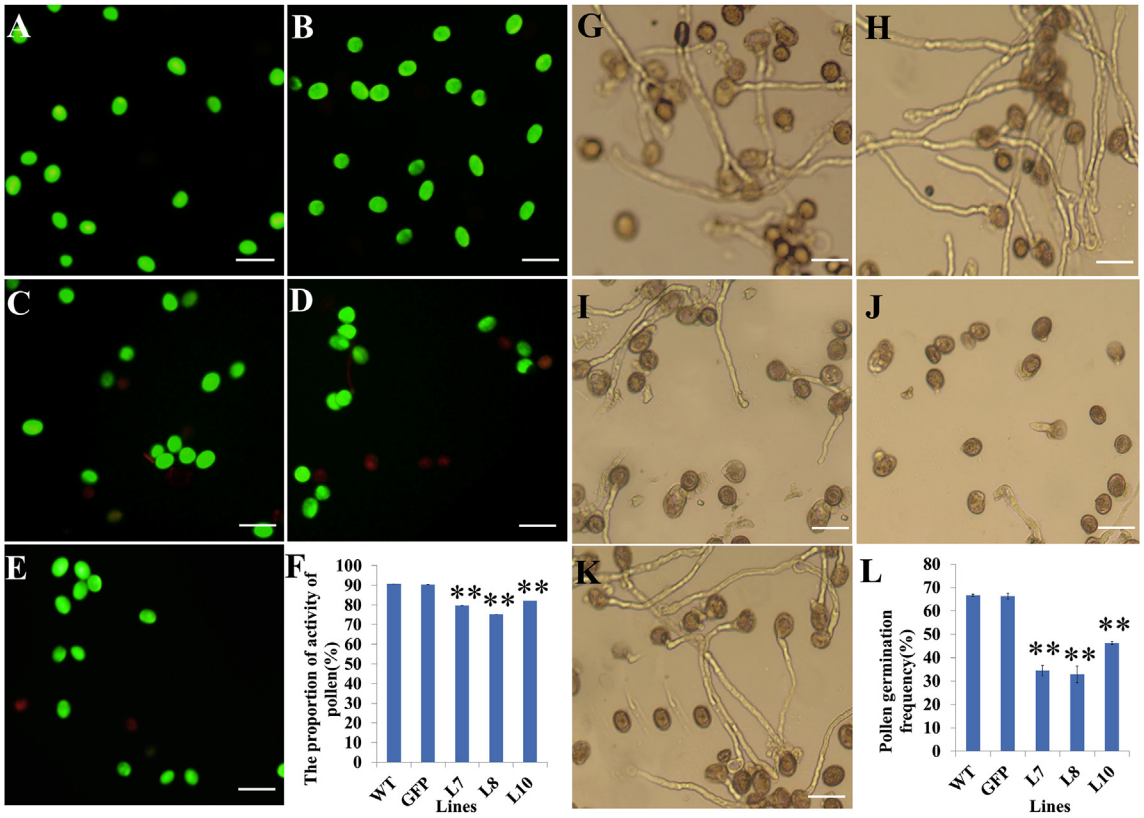

Fig. 4. Assay of pollen viability and pollen germination in *GhWRKY22* overexpression transgenic Arabidopsis. (**A–E**) Assay of pollen viability. (**A**) Wild type; (**B**) *GFP* transgenic line; (**C–E**) *GhWRKY22* overexpression transgenic lines L7, L8 and L10, respectively. Viable pollen displayed green fluorescence and non-viable pollen grains were red. (**F**) Statistical analysis of the percentage of viable pollen in the *GhWRKY22* transgenic lines and controls (wild type and *GFP* transgenic line). (**G–K**) Assay of pollen germination. (**G**) Wild type; (**H**) *GFP* transgenic line. (**I–K**) *GhWRKY22* transgenic lines L7, L8 and L10, respectively. (**L**) Statistical analysis of the percentage of germinated pollen grains in the *GhWRKY22* transgenic lines and controls. WT, wild type; GFP, *GFP* transgenic line; L7, L8 and L10, three *GhWRKY22* transgenic lines. Each experiment was repeated at least three times, and error bars represent standard deviation (SD) from the mean data of each genotype. Student's *t*-test demonstrates that there were very significant differences (**P < 0.01) between the *GhWRKY22* transgenic lines and controls. Bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

GhWRKY22 in Arabidopsis resulted in the reduced pollen viability and germination rate in the transgenic plants (Fig. 4), like those of *GhMYB24* overexpression transgenic plants (Li et al., 2013). These results suggested that the repressed filament elongation, reduced pollen viability and the abnormally pollen germination may jointly lead to the decreased male fertility and the increased unfertilized ovules/undeveloped seeds in the *GhWRKY22* transgenic Arabidopsis.

It has been reported that Arabidopsis WRKY2 and WRKY34 are required for male gametogenesis. WRKY34 is temporally phosphorylated by MPK3 and MPK6 at early stages of pollen development. During pollen maturation, WRKY34 is dephosphorylated and degraded (Guan et al., 2014), suggesting WRKY34 may mainly function in early pollen development. Further study revealed that WRKY2 and WRKY34 interact with VQ motif-containing protein VQ20 to form complexes to co-modulate multiple genes involved in anther/pollen development, pollen germination and pollen tube growth (Lei et al., 2017). Interestingly, MPK3 and MPK6 can not interact with VQ20 in yeast two-hybrid assay, although several VQ proteins are identified as substrates of MPK3 and MPK6 (Pecher et al., 2014). Different from WRKY2 and WRKY34, however, GhWRKY22 may take part in regulating anther/pollen development by inhibiting the expression of JAZs in JA signal pathway. Our results revealed that expression of the genes related to JA biosynthesis and JA signaling (such as *AtCOI1* etc.) was up-regulated, but expression of *AtJAZ1* and *AtJAZ8* was down-regulated in the *GhWRKY22* overexpression transgenic plants, relative to the controls. Considering that seeds were sowed in plates and then vernalization in 4 °C for two days to ensure the consistent germination and to facilitate antibiotics screening of the transgenic seedlings, the growth conditions of the transgenic lines and controls were completely consistent, and expressions of all the genes analyzed in the transgenic plants were compared with the controls. Therefore, we concluded that the differences in expression levels of the genes were caused by *GhWRKY22* overexpression, suggesting that GhWRKY22 takes part in JA signal pathway for regulating anther/pollen development. It was reported that both WRKY transcription factors and JA participate in regulating anther/pollen development. However, the regulation mechanism of WRKY proteins in anther/pollen development via JA signal pathway still remains largely unknown in detail. In this study, yeast one-hybrid and ChIP-qPCR assays demonstrated that GhWRKY22 could directly bind to *GhJAZ* promoters to regulate the expression of *GhJAZs* for affecting anther/pollen development (Fig. 5). Furthermore, the phenotype of *GhWRKY22* transgenic Arabidopsis plants is similar to that of *wrky2wrky34vq20* mutant. That is to say, GhWRKY22 may act as a transcription repressor to regulate anther/pollen development via influencing JA signaling. Similarly, it was revealed that WRKY2 and WRKY34 also act as negative regulators (Lei et al., 2017). However, the WRKY2 and WRKY34 may modulate pollen development by interacting with VQ20 in the different regulatory pathway. Collectively, the data presented in this study showed that GhWRKY22 may play different role from the reported WRKY transcription factors in anther/pollen development.

Besides participating in plant defense system, JA is also involved in plant growth and development, seed germination and pollen fertility (Cheng et al., 2009; Chua et al., 2010; Li et al., 2017; Song et al., 2011).

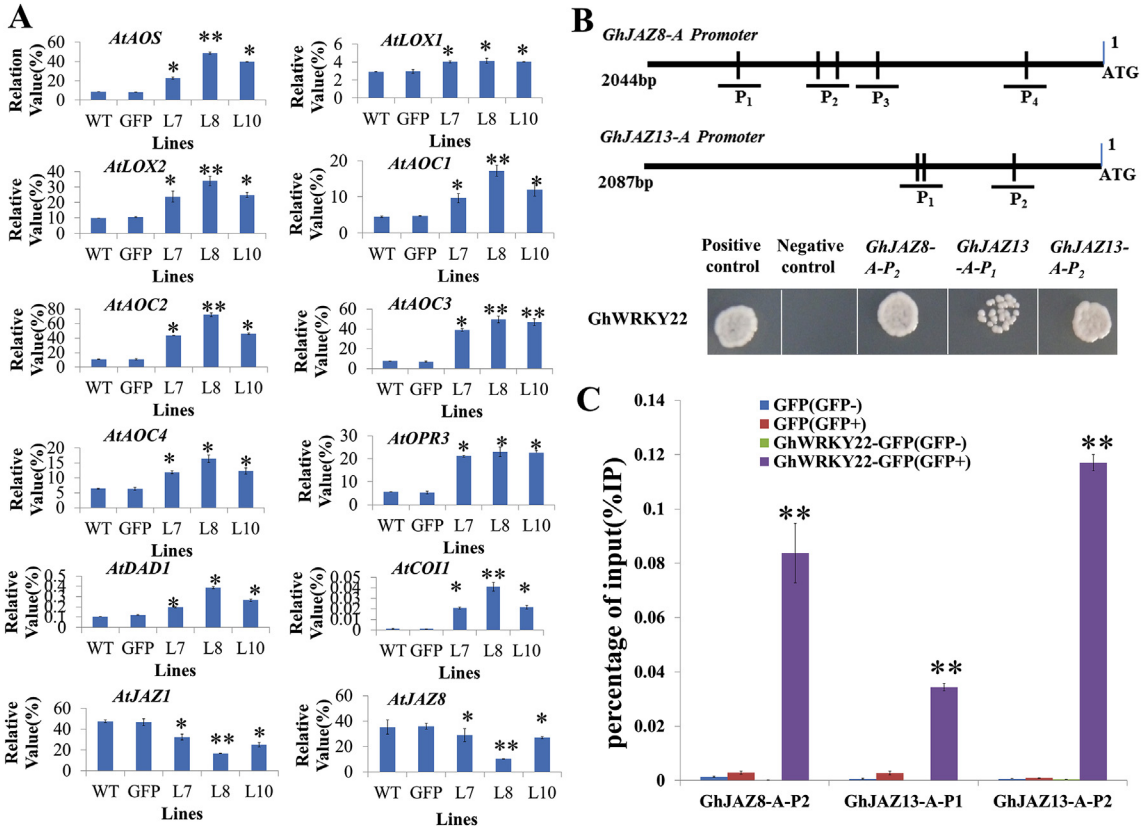

Fig. 5. Assay of GhWRKY22 protein binding to *GhJAZ8-A/13-A* promoters. (**A**) Quantitative RT-PCR analysis of expression of the genes related to JA signal pathway in the *GhWRKY22* transgenic Arabidopsis plants (L7, L8 and L10) and controls (wild type and *GFP* transgenic line). Relative values of expression of the genes are shown as percentage of *AtACTIN2* expression activity. WT, wild type. GFP, *GFP* transgenic line. L7, L8 and L10, three *GhWRKY22* transgenic lines. (**B**) Yeast one-hybrid assay of the interactions among GhWRKY22 and *GhJAZ* promoters (*GhJAZ8-A* and *GhJAZ13-A*). Yeast cells harboring pGADT7-GhWRKY22 and pAbAi-*GhJAZ-Ps* were assayed on SD/-Leu selection medium containing 400 ng/ml AbA (Aureobasidin A), using both pGADT7-p53 and pAbAi-p53 vectors as positive control and both empty pGADT7 and pBait-AbAi vectors as negative control. *GhJAZ8-A* and *GhJAZ13-A* promoter regions contain W-box clusters. The number and relative position of W-boxes in respective promoters relative to start codon (ATG) are indicated by black upright lines, and the fragments detected in yeast one-hybrid and ChIP-qPCR assays are indicated by P1–P4 lines. (**C**) ChIP-qPCR assays of GhWRKY22 binding to *GhJAZ8-A/13-A* promoters in the *GhWRKY22:GFP* transgenic cotton callus cells. The sequences were detected by ChIP-qPCR assays using an anti-GFP antibody. ChIP-qPCR results are presented as a percentage of input DNA. GFP (GFP-), the *GFP* transgenic cotton cells without anti-GFP antibody; GFP (GFP+), the *GFP* transgenic cotton cells with anti-GFP antibody; WRKY22-GFP (GFP-), the *GhWRKY22:GFP* transgenic cotton cells without anti-GFP antibody; WRKY22-GFP (GFP+), the *GhWRKY22:GFP* transgenic cotton cells with anti-GFP antibody. Each experiment was repeated at least three times, and error bars represent standard deviation (SD). Student's *t*-test demonstrates that there were significant (*P < 0.05) or very significant (**P < 0.01) differences between the *GhWRKY22* transgenic lines and controls, or between the anti-GFP antibody samples and without anti-GFP antibody samples.

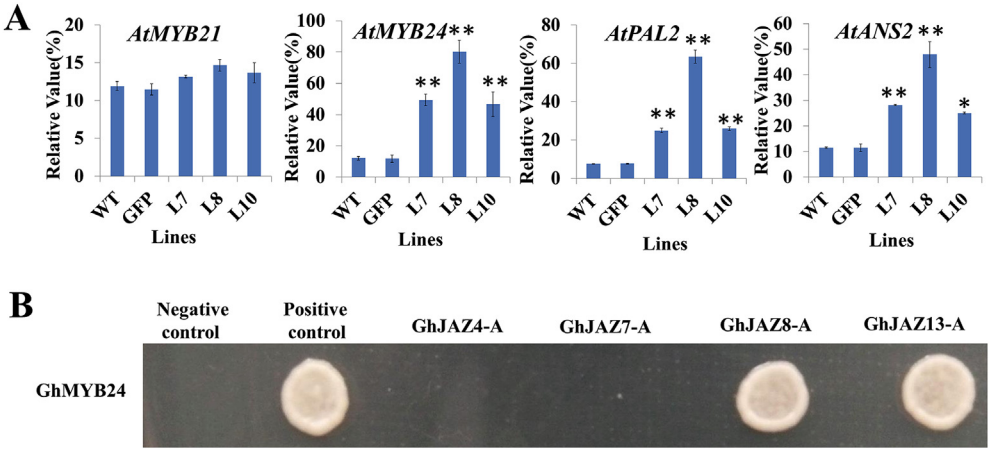

Fig. 6. Yeast two-hybrid assay of interactions among GhMYB24 and GhJAZ proteins. (**A**) Quantitative RT-PCR analysis of expression of the genes related to JA signal pathway in *GhWRKY22* transgenic Arabidopsis plants (L7, L8 and L10) and controls (wild type and *GFP* transgenic line). Relative values of expression of the genes are shown as percentage of *AtACTIN2* expression activity. WT, wild type. GFP, *GFP* transgenic line. L7, L8 and L10, three *GhWRKY22* transgenic lines. (**B**) Yeast two-hybrid assay of protein-protein interaction. Four cotton JAZ proteins (GhJAZ4-A, GhJAZ7-A, GhJAZ8-A and GhJAZ13-A) were individually fused with GAL4 DNA binding domain in pGBK-T7 vector, and GhMYB24 was fused with the activation domain in pGAD-T7 vector for analyzing protein-protein interaction. Yeasts harboring pGADT7-GhMYB24 and pGBKT7-GhJAZs were streaked on QDO nutritional selection medium, using empty pGBKT7 vector and pGADT7-GhMYB24 as negative control, and pGBKT7-Murine p53 and pGADT7-SV40 Large T-antigen as positive control. Each experiment was repeated at least three times, and error bars represent standard deviation (SD). Student's *t*-test demonstrated that there were significant (*P < 0.05) or very significant (**P < 0.01) differences between the *GhWRKY22* transgenic lines and controls.

JAZ proteins are identified as substrates of the SCF^{COI1} complex that repress JA response, and play the crucial roles in JA signal pathway (Chini et al., 2007). Previous study revealed GbWRKY1 in sea island cotton (*Gossypium barbadense*) as a negative regulator takes part in the JA-mediated defense response and plant resistance to the pathogens *Botrytis cinerea* and *Verticillium dahliae* by activating JAZ1 expression (Li et al., 2014). AtWRKY33 is required for resistance toward the necrotrophs *Alternaria brassicicola* and *Botrytis cinerea*. Loss of WRKY33 function results in the down-regulation of JA-associated response (Birkenbihl et al., 2012). However, no evidence revealed that WRKY transcription factors participate in anther/pollen development through JA signaling pathway so far. As GhWRKY22 shares relatively high homology with AtWRKY33, we speculated that GhWRKY22 may regulate anther/pollen development also by JA signaling pathway. Moreover, previous studies revealed that the transcription factors MYB21 and MYB24 are the direct targets of JAZs in Arabidopsis. Excess expression of *MYB21* and *MYB24* results in retarded stamen development of Arabidopsis (Song et al., 2011). Arabidopsis MYB108 acts together with MYB24 to regulate JA-mediated stamen maturation (Mandaokar and Browse, 2009). However, little is known whether these regulatory mechanisms of MYB transcription factors can apply to cotton plant. In this study, our data suggest that GhWRKY22 as a transcription repressor directly binds to the *JAZs* promoters to regulate the expression of these *JAZ* genes. Additionally, GhMYB24 interacts with some GhJAZ proteins possibly to participate in JA signal transduction during cotton anther/pollen development. In summary, the data presented in this study reveal that a cotton WRKY transcription factor, GhWRKY22, acts as a transcriptional repressor to regulate anther/pollen development by suppressing the expression of *GhJAZs* in cotton.

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Author contribution

X.-B.L., Y.W. and Y.L. conceived and designed the research; Y.W., Y.L., S.-P. H., Y.G., N.-N.W. and R.L. performed the experiments; Y.W. and X.-B.L. analyzed data and wrote the paper. All the authors read and approved the manuscript.

Conflicts of interest

The authors declare no any competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.06.005.

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