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GhWDL3 is involved in the formation and development of fiber cell morphology in upland cotton (*Gossypium hirsutum* L.)

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Abstract

Background Cotton fiber is a model tissue for studying microtubule-associated proteins (MAPs). The Xklp2 (TPX2) proteins that belong to the novel MAPs member mainly participate in the formation and development of microtubule (MT). However, there is a lack of studies concerning the systematic characterization of the *TPX2* genes family in cotton. Therefore, the identification and portrayal of *G. hirsutum TPX2* genes can provide key targets for molecular manipulation in the breeding of cotton fiber improvement.

Result In this study, *TPX2* family genes were classified into two distinct subclasses *TPXLs* and MAP genes WAVE DAMPENED2-LIKE (*WDLs*) and quite conservative in quantity. *GhWDL3* was significantly up-regulated in 15 days post anthesis fibers of ZRI-015 (an upland cotton with longer and stronger fiber). *GhWDL3* promotes all stem hairs to become straight when overexpressed in *Arabidopsis*, which may indirectly regulate cotton fiber cell morphology during fiber development. Virus induced gene silencing (VIGS) results showed that *GhWDL3* inhibited fiber cell elongation at fiber development periods through regulating the expression of cell wall related genes.

Conclusion These results reveal that *GhWDL3* regulated cotton fiber cell elongation and provide crucial information for the further investigation in the regulatory mechanisms/networks of cotton fiber length.

Keywords Upland cotton, *GhWDL3*, Fiber length, TPX2, Cytoskeleton, Microtubule-associated proteins (MAPs)

Introduction

Upland cotton (*Gossypium hirsutum*) is the most source of natural fiber for textile industry. However, the advantage of high production is difficult to cover the flaw of relatively poor fiber quality in *G. hirsutum*. Therefore, improving cotton fiber quality is one of the key research directions at present. Fiber length and strength are two key properties in cotton fiber quality, which also have important influences on yarn (Cai et al., 2013). The development of cotton fibers can be delineated into five overlapping stages: the fiber initiation, the primary cell walls (PCW) formation, the PCW synthesis to the secondary cell walls (SCW) synthesis transition, SCW thickening, and dehydration and maturation (Haigler et al., 2012; Huang et al., 2021).

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Cotton fibers are single-celled trichomes, which is a great and important model for plant cell elongation researching and SCW synthesis (Cao et al., 2020). Among them, cellulose accounts for more than 90% in mature fibers of most cotton species, and many cellulose synthases (CESAs) may play a key role in fiber SCW synthesis (Huang et al., 2021). MAP20, a microtubule (MT)-associated protein, was highly expressed in the SCW of hybrid aspen (Rajangam et al., 2008). MTs are involved in the ordered deposition of cellulose microfibrils (Burk et al., 2002). The orientation and dynamic changes in MTs are controlled by the interaction between MTs and microtubule- associated proteins (MAPs) (Lucas et al., 2011; Petrovská et al., 2013; Zhang et al., 2017). MAPs mainly bind to MTs, and they are involved in MTs functions. Tubulin are involved in the regulation of MT assembly and function, and a reduction of α-tubulin can disrupt the MT structure and further cause abnormal cell expansion (Chen et al., 2021a, b). GhMAP20L5 mediated fiber elongation through the interaction with GhTUB13 (Song et al., 2023). MAP65 proteins were active besides anaphase spindle elongation (Mao et al., 2005; Hofmann, 2008). TPX2 proteins that belong to the novel MAPs members mainly participate in the formation and development of MTs functions (Véron et al., 2021). *TPX2* family genes, which consists of two subgroups, *TPXLs* and MT associated protein genes (*WDLs*), have been studied in varies species, including *Arabidopsis* (Smertenko et al., 2021), *Drosophila* (Goshima, 2011), *Eucalyptus grandis* (Du et al., 2016), cotton (Lei et al., 2019), etc. The research found that TPX2 proteins possess an Aurora binding domain, TPX2-signature, and TPX2-C, while TPXL2/3/4/8 proteins own Aurora binding domain and TPX2-signature. Interestingly, TPXL1/5/6/7 and most WDLs only have TPX2-C or TPX2-C^{KLEEK}, and only WDL7/8/9 also have WAND (Smertenko et al., 2021), which demonstrated that the structures of TPX2 proteins already have great differentiation.

TPX2 is as a regulator element in a novel interaction mode to simultaneously bind across longitudinal and lateral tubulin interfaces (Smertenko et al., 2021). TPX2 inhibits the onset of mitosis (Vos et al., 2008; Petry et al., 2013). When AUR1 kinase was co-localized with some TPXLs in MT, it is a hallmark of cell division (Boruc et al., 2019; Tomaštíková et al., 2020). However, the WDLs protein mainly controls MT bundling and the stability of the site of tip-growth in different plants (Du et al., 2016). WVD2 and WDL1 modulate helical organ growth and anisotropic cell expansion in *Arabidopsis* (Yuen et al., 2003). Cotton fibers are countless single cells protruding from the seed epidermis, which belongs to special tip-grow (Champion et al., 2021). *AtWDL4* positively regulates apical hook opening by modulating auxin distribution in *Arabidopsis* (Deng et al., 2021). *AtWVD2* and *AtWDL1*, which regulate the stability or organization of cytoskeleton by binding to bundling MTs, modulated organ growth and anisotropic cell expansion in *Arabidopsis* (Yuen et al., 2003; Perrin et al., 2007). Interestingly, our previous research found that *Gh_D01G220400* (*GhWDL4*) may play a key role in the process of fiber elongation rates (He et al., 2021). Therefore, it is crucial for the functional studies of special *WDLs* genes in cotton fiber development.

Studies have shown that TPX2, TPXLs, as well as other MAPs proteins, play a critical role in the development of cytoskeleton and MT (Petry et al., 2013; Petrovská et al., 2013; Chen et al., 2021a, b), while the function of GhWDLs protein has been poorly studied, especially in cotton fiber and plant cell elongation (Lian et al., 2017; Champion et al., 2021; Deng et al., 2021). Therefore, we firstly analyzed the quantitative evolutionary relationship of the *TPX2* family genes from diploid to tetraploid, wild cotton to cultivated cotton. Then the relative expression levels of *TPX2* family genes was analyzed by utilizing RNA-Seq and quantitative real-time polymerase chain reaction (qRT-PCR) in two upland cotton materials (J02-508 and ZRI015) with large differences in fiber length and strength. The results revealed that some *GhWDLs* showed specific expression levels during fiber development, especially *GhWDL3* at 15 days post anthesis (DPA) in fiber. Furthermore, *GhWDL3* plays a vital role in cell morphology when overexpressed in *Arabidopsis*. Interestingly, fiber cell elongation was promoted in *GhWDL3* silencing cotton plants (CLCrV-*GhWDL3*) by virus induced gene silencing (VIGS) technique, which hints that *GhWDL3* inhibited fiber cell length.

Materials and methods

Database search and sequence retrieval

Plant *TPX2*_importin (PF12214) and *TPX2* (PF06886) gene family members were obtained from pfam database (http://pfam.xfam.org/). *Arabidopsis* TPX2 family protein sequences were downloaded from the *Arabidopsis* Information Resource (TAIR) (https://www.arabidopsis.org/). TPX2 protein sequences of *G. arboreum*, *G. raimondii*, *G. hirsutum*, and *G. barbadense* were applied to acquire from Cotton Functional Genomics Database (CottonFGD) (https://cottonfgd.org/). TPX2 protein sequences of three wild cotton species *G. darwinii*, *G. mustelinum*, and *G. tomentosum* were extracted from National Center of Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Repeated proteins were deleted, and only TPX2 protein sequences with a coverage length of 80 amino acids, identity of 40%, and e-value > 30 were held and checked by NCBI-CDD (NCBI conserved domain database, https://www.ncbi.nlm.nih.

gov/cdd) and SMART (http://smart.embl-heidelberg.de/) for further analysis.

Phylogenetic analysis

Multiple sequence alignments of TPX2 proteins in *Arabidopsis* and the seven species of *Gossypium* (*G. darwinii*, *G. mustelinum*, *G. tomentosum*, *G. raimondii*, *G. arboreum*, *G. barbadense*, and *G. hirsutum*) were done using Alignment Explorer in MEGA-X (Chen et al., 2020). Subsequently, Neighbor-Joining method with bootstrap value of 1 000 was utilized to generate a phylogenetic tree in MEGA-X software.

Analysis of *cis*-elements related to plant hormone in the promoters

Upstream sequences in 2.0 kb of the start codon "ATG" of *TPX2* family genes were obtained from *G. hirsutum* by TBtools (Chen et al., 2020), and the *cis*-elements were determined utilizing the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Transcriptome analysis of two upland cotton genotypes J02-508 and ZRI-015

Two upland cotton genotypes J02-508 (with longer and stronger cotton fiber) and ZRI-015 (with shorter and weaker cotton fiber) were grown in the cotton farm of Institute of Cotton Research, Chinese Academy of Agricultural Sciences (Anyang, Henan province, China). The roots, stems, leaves, and 0, 3 DPA ovules, and 5, 10, 15, 20, 25 DPA fibers of *G. hirsutum* (He et al., 2021) were sent to Biomarker Technologies company for completing transcriptome sequencing. Total RNA of cotton fibers, ovules, and other tissues were isolated by the RNA prep Pure Plant kit (Tiangen, China). The 1 000 ng RNA was reversely synthesized into cDNA by MonScript RTIII Super Mix with dsDNase (Two-Step) (Vazyme, China). Gene expression level was calculated by RSEM software (v.1.3.1), and the transcriptome data of *G. hirsutum* cv. J02-508 and ZRI-015 were obtained. Genes with fragments per kilobase of transcript per million mapped reads (FPKM) > 1 in at least one stage were selected for further analysis. Log_2(FPKM), after normalization, was used to display the gene expression as a heatmap.

Overexpressing *GhWDL2* and *GhWDL3* in *Arabidopsis*

The coding sequence (CDS) of *GhWDL2* (*Gh_D11G347100*) and *GhWDL3* (*Gh_A10G105900*) were cloned and inserted to pBI121 vector using two restriction sites (*Xba*I and *Sma*I), respectively. The primers were designed, and the sequences are presented in Table S1. Subsequently, the GV3101 strain containing constructed *GhWDL2*-pBI121 and *GhWDL3*-pBI121 vector were transformed into *Arabidopsis* plants, respectively, based on the floral dip methods. Three transgenic *Arabidopsis* lines were obtained for PCR confirmation, i.e., the lines OE1, OE2, and OE3, which have high expression levels of *GhWDL2* or *GhWDL3*. The wild-type (WT) *Arabidopsis* plants were used as the controls. The phenotype of stem hair of *Arabidopsis* was observed and analyzed by T4 microscope. The planting conditions of *Arabidopsis* are the same as those described by Chen et al. (2021a, b).

VIGS

To further study whether silence of *GhWDL3* affect the cotton fiber cell elongation, we conducted VIGS experiments. Briefly, a 350–500 bp fragment of the *GhWDL3* were amplified from cDNA using the VIGS primers designed with the CLCrV-VIGS Tool. The PCR products digested with *Spe*I were inserted into *Spe*I-cut CLCrV-VIGS. The CLCrV derivatives fused with the 379 bp target fragments in Supplementary Material-1 were transformed into *Agrobacterium tumefaciens* strain LBA4404. The VIGS approach and planting conditions of ZRI-015 cotton are the same as those described by Gu et al. (2014). Transformed *Agrobacterium* cultures were grown overnight in YEP medium containing rifampicin (100 $mg{\cdot}L^{-1}$) and kanamycin (100 $mg{\cdot}mL^{-1}$) at 28 °C. The *Agrobacterium* cultures were pelleted and resuspended ($OD_{600} = 1$) individually in an infiltration buffer containing 10 $mmol{\cdot}L^{-1}$ $MgCl_2$, 10 $mmol{\cdot}L^{-1}$ 2-(4-Morpholino) ethanesulfonic acid, and 200 $\mu mol{\cdot}L^{-1}$ acetosyringone. The mixed *Agrobacterium* solutions were infiltrated into the abaxial side of cotyledons of 2-week-old cotton seedlings using needleless syringes. To facilitate the infiltration, small wounds were made on the surface of cotyledons or true leaves using small syringe needles (Gu et al., 2014). Two VIGS plants with significantly decreased expression levels were selected to measure the cotton fiber length at maturity.

qRT-PCR

To further verify whether *GhWDL3* regulates fiber elongation in cotton, we analyzed the expression levels of several genes in 15 DPA fiber. qRT-PCR was performed by ABI 7500 real-time PCR system and ChamQ Universal SYBR qPCR Master Mix (Vazyme, China). The cotton ubiquitin gene (*Gh_A10G005800*) were used as the internal reference (Walford et al., 2011). The relative expression levels of genes were calculated by the $2^{-\Delta\Delta C_T}$ methods. The primers used in qRT-PCR analysis were shown in Table S1. All experiments were executed for at least three biological replicates.

Results

Quantitative and phylogenetic analysis of *TPX2* family genes in different cotton species

Studies have shown that TPX2 proteins play a critical role in cytoskeleton and MTs development. To study the quantitative and evolutionary relationships of *TPX2* family members, the protein sequences in *Arabidopsis*, *G. darwinii*, *G. mustelinum*, *G. tomentosum*, *G. raimondii*, *G. arboreum*, *G. barbadense*, and *G. hirsutum* were collected and analyzed. TPX2 family have 301 genes in the eight species, which possess two subgroups TPXLs and WDLs, quantitative analysis showed that the evolutionary relationships of TPX2 family were conservative (Fig. 1A). TPXLs and WDLs, which have 19 genes and 29 genes in *G. hirsutum*, respectively, were evolved into two sub-members TPXL1/5/6 and TPX2/TPXL2/3/4/7/8, which have 8 genes and 11 genes, respectively; and two sub-members WDL1/2/3/4/5/6WVD2 and WDL7/8/9/MAP40, which have 19 genes and 10 genes, respectively (Fig. 1B).

In the process of doubling from diploid cotton to allotetraploid cotton, tetraploid cotton should have twice the number of diploid cotton genes, but the numbers of *TPXL* genes were more one *TPX2* gene (*Gm.A01G020900* or *Gm.A01G021000*, which belong to tandem repeat), one *TPXL3* gene (*Gh_D11G367100*), and one *WDL5* gene (*Gm.A13G2129* or *Gm.A13G2133*, which don't belong to tandem repeat), while it lost two *TPXL5* genes (belong to ortholog genes of *Gt.A05G417700* and *Gt.D12G102900* at Dt04 and At12 sub-genomes, respectively), *WDL5* genes (belong to ortholog genes of *Gb.D07G003280* at At07 sub-genome), and *WDL7* gene (belong to ortholog genes of *Gd.D02G178900* and *Gm.A03G138300* at At03 and Dt02, repectively). Intriguingly, *WDL5* (belong to the ortholog genes of *Ga05G0588*) was all lost on the At05 sub-genome in allotetraploid cotton. Only a few genes were lost or double during the evolution process, which showed that *TPX2* family genes are extremely conservative in quantity and protein sequences.

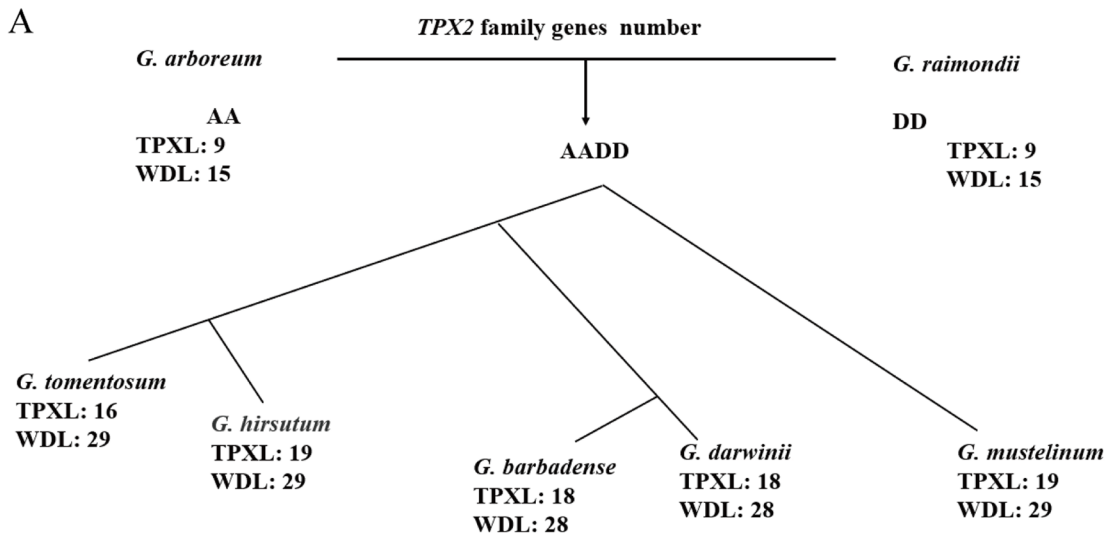
The expression profiles of *TPX2* family genes in *G. hirsutum* cv. J02-508 and ZRI-015

The expression levels of *TPX2* in different tissues were explored using transcriptomic data and qRT-PCR of *G. hirsutum* cv. J02-508 and ZRI-015. The expression levels of all *TPXLs* genes (Fig. 2A) were very low in roots, stems, and leaves, while some *WDLs* genes have high expression levels during the fiber development stages, especially these genes *GhWDL2* (*Gh_A11G342500* and *Gh_D11G347100*, belong to ortholog gene), *GhWDL3* (*Gh_A10G105900* and *Gh_D10G167400*, belong to ortholog gene), *GhWDL4* (*Gh_A01G224600* and *Gh_D01G220400*, belong to ortholog gene). Interestingly, *GhWDL2* and *GhWDL3* are significantly up-regulated at the critical periods from fiber PCW synthesis to SCW synthesis transition (at 15 DPA) in ZRI-015, while they are low expressed in J02-508 (Fig. 2A). Further verifying the expression levels of the four genes by qRT-PCR, *GhWDL3* are significantly up-regulated at 15DPA in ZRI-015, especially extremely significant of *GhWDL3-At10* (*Gh_A10G105900*), which showed that *GhWDL3* may play a role at fiber elongation and cell wall thicken initial stages. *GhWDL2-At11* (*Gh_A11G342500*) are significantly upregulated at 5 DPA and 20 DPA in ZRI-015, while *GhWDL2-Dt11* (*Gh_D11G347100*) has significant higher expression levels at 15–25 DPA in ZRI-015 compared with that of J02-508 (Fig. 2B). Therefore, we further performed functional verification of *GhWDL3-At10*, *GhWDL3-Dt10* and *GhWDL2-Dt11*, which mayregulate the length and strength of cotton fibers.

Up/down-regulation of gene expression is often mediated by the activation of its promoter by upstream transcription factors, so we further focused on its promoter elements. We found that promoters of *TPXL*s are mainly composed of some hormone-related elements, such as abscisic acid responsive element (ABRE), auxin responsive elements (AuxRR-core, TGA-element), methyl jasmonate (MeJA)-responsive elements (CGTCA-motif, TGACG-motif), gibberellin-responsive elements (GARE-motif, P-box, TATC-box), and salicylic acid responsive element (TCA-element). Most promoters of *TPXLs* genes have more than two hormone-acting elements. *GhWDL2-Dt11* have four hormone-acting elements (abscisic acid responsive, auxin responsive, gibberellin-responsive, and salicylic acid responsive), *GhWDL3-At10* have gibberellin-responsive and MeJA-responsive elements, while *GhWDL3-Dt10* only have abscisic acid responsive element (Fig. S1). Combined with the expression levels in cotton fibers, *GhWDL3-At10, GhWDL3-Dt10*, and *GhWDL2-Dt11* may have different regulatory patterns.

Phenotypic analysis of *Arabidopsis* with overexpression of *GhWDL3*

To further confirm the genes function, *GhWDL2* (*Gh_D11G347100*) and *GhWDL3* (*Gh_A10G105900*) was overexpressed in *Arabidopsis*, respectively. Among ten T_3 generation lines, three lines were selected for further analysis. Overexpression of *GhWDL2* has not caused any obvious phenotypes in *Arabidopsis* (Fig. S2), while the overexpression of *GhWDL3* in *Arabidopsis* causes various organs to enlarge in the process of plant growth (Fig. 3).



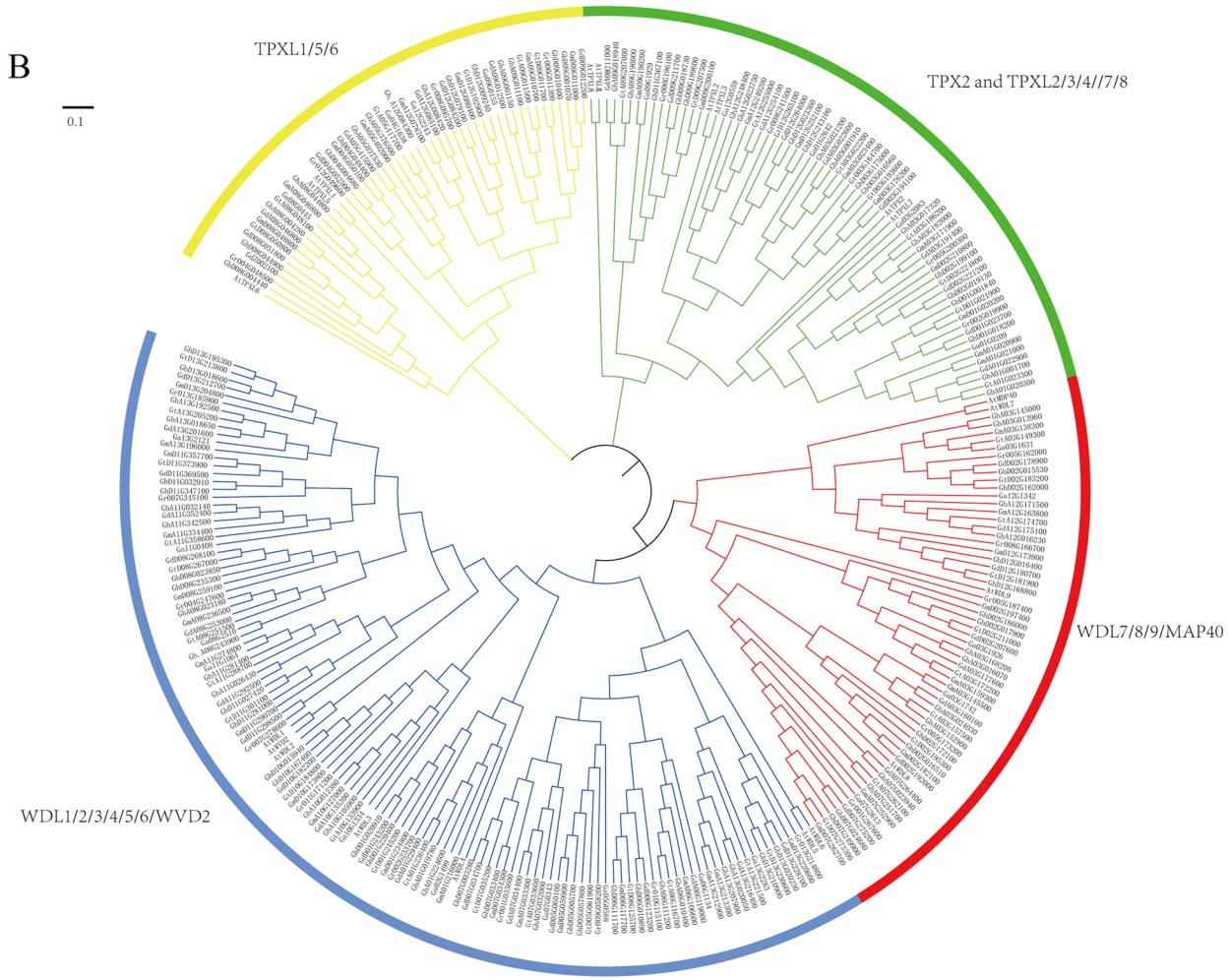


Fig. 1 The quantitative statistics and phylogenetic tree analysis of TPX2 family proteins in cotton. **A** The quantitative statistics of TPXL and WDL subfamily proteins in the evolution from diploid cotton to tetraploid cotton. **B** The phylogenetic tree analysis of TPX2 family proteins from At, *A. thaliana*; Gr, *G. raimondii*; Ga, *G. arboreum*; Gd, *G. darwinii*; Gt, *G. tomentosum*; Gm, *G. mustelinum*; Gb, *G. barbadense*; *Gh, G. hirsutum*

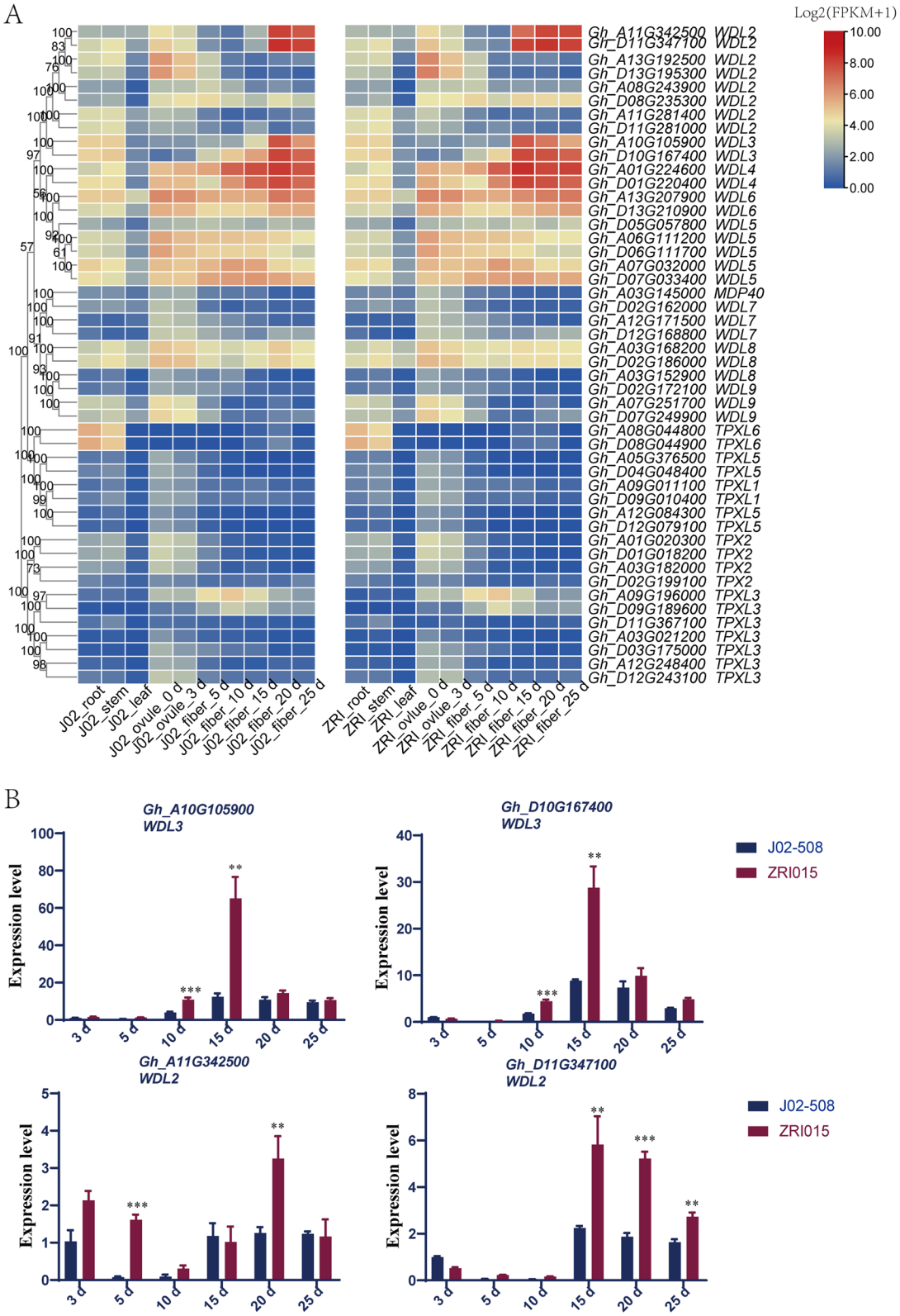


Fig. 2 The expression pattern analysis of *TPX2* family genes in different tissues of upland cotton J02-508 and ZRI015. **A** Transcriptome data of *TPX2* family genes in root, stem, leaf, ovule (0, 3 DPA), and fiber (5, 10, 15, 20, 25 DPA). **B** qRT-PCR analyzed the expression profiling of four candidate genes in ovule (3 DPA) and fiber (5, 10, 15, 20, 25 DPA). Statistical significance was determined using one-way ANOVA combined with Tukey's test. **, $P < 0.01$; ***, $P < 0.001$

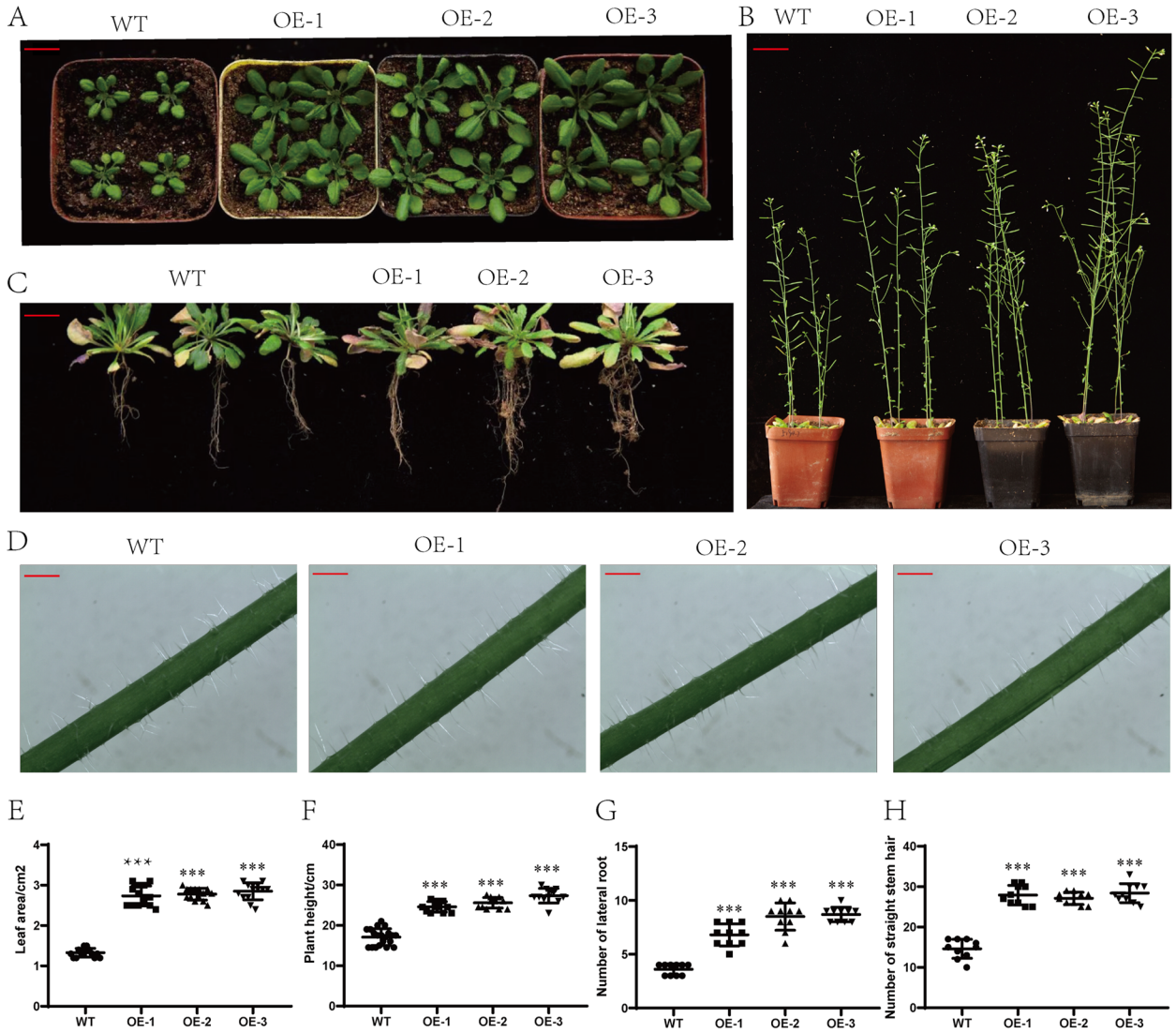


Fig. 3 Phenotypic analysis of *GhWDL3* overexpressing *Arabidopsis*. **A**/**E** Comparison of the leaf area of wild-type and transgenic *Arabidopsis* at the seedling stage (2 weeks). **B**/**F** Comparison of plant height of wild-type and transgenic *Arabidopsis* at the late bolting period stage (6 weeks). **C**/**G** Comparison of lateral roots number in wild-type and transgenic *Arabidopsis* at mature stage (9 weeks). **D**/**H** Comparison of straight stem hairs of wild-type and transgenic *Arabidopsis* at bolting stage (6 weeks). Bars in (**A**) = 1 cm^2. **B**, **C** = 1 cm. Bars in (**D**) = 200 μm. Statistical significance was determined using one-way ANOVA combined with Tukey's test. ***, $P < 0.001$

Therefore, we further explored how *GhWDL3* regulated organ development. *GhWDL3* promoted the development of leaves at the seedling stages of *Arabidopsis*, leading to the enlargement of leaf area (Fig. 3A, E). *GhWDL3* promoted the plant height to become higher at the mature stage of *Arabidopsis* (Fig. 3B/F). At the same time, we found that *GhWDL3* promoted root development, leading to more and longer lateral roots (Fig. 3C/G). Interestingly, WT plants only have some straight hairs in the lower part of the main stem of *Arabidopsis*, while overexpression of *GhWDL3* promotes all stem hairs to become straight (Fig. 3D/H). Sudies have shown that the development of cotton fiber and *Arabidopsis* trichomes have similar regulatory mechanisms (Wang et al. 2021). The above results hinted that *GhWDL3* may regulate cotton fiber cell morphology during fiber development.

GhWDL3 inhibited fiber cell elongation during the critical period of cotton fiber development

To further verify the function of *GhWDL3* in cotton fiber elongation, VIGS experiments were conducted in cotton variety ZRI-015. The expression level of *GhWDL3* was significantly decreased in VIGS plants (Fig. 4A). The fiber

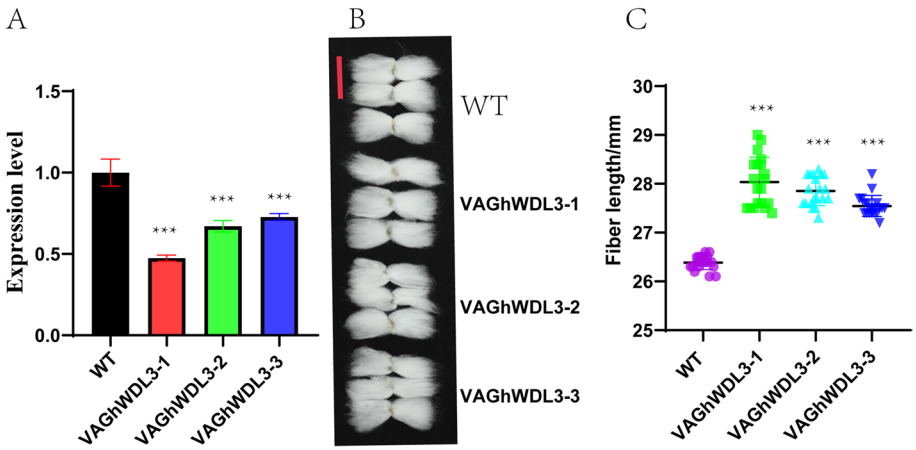


Fig. 4 *GhWDL3* inhibited fiber cell elongation. **A** qRT-PCR analysis of the expression level of *GhWDL3* in 15 DPA fibers of J02-508 (WT) and VIGS-*GhWDL3* lines. *GhUBQ* was used as the internal control. **B** Mature fibers of WT(VA) and VIGS- *GhWDL3* lines. **C** Statistical analysis of mature fiber length of WT and VIGS-*GhWDL3* lines. The error bars in this figure represent the standard deviations (SDs) for at least three independent experiments. Bars in (**B**) = 1 mm. Statistical significance was determined using one-way ANOVA combined with Tukey's test. ***, $P < 0.001$

length of CLCrV-GhWDL3 was significantly increased compared with WT (Fig. 4B/C).

To uncover the mechanisms of *GhWDL3* in regulating fiber elongation, transcriptome analysis was performed of the 15 DPA cotton fiber. There were 7 383 up-regulated and 5 060 down-regulated differentially expressed genes (DEGs) in CLCrV-GhWDL3 lines compared with WT (Fig. 5A). Furthermore, GO enrichment analysis revealed that the down-regulated DEGs in CLCrV-*GhWDL3* lines were mainly enriched in vacuolar part and cell wall (Fig. 5B), the up-regulated DEGs in CLCrV-GhWDL3 lines were mainly enriched in negative regulation of metabolic process (Fig. 5C). So, through analyzing 234 genes in cell wall pathway (Supplementary Material-2), we found 9 crucial candidate genes including 2 CESA8 (*Gh_D10G036700* and *Gh_A10G027600*), 1 EXPB3 (*Gh_D12G156000*), and 6 EXPA4/8/13 (*Gh_A07G108100*, *Gh_A03G056700*, *Gh_A05G266200*, *Gh_D05G280000*, *Gh_D04G012100*, and *Gh_A07G079500*) (Fig. S4A/B). Above results indicated that GhWDL3 may inhibit fiber cell elongation through downregulating the expression of cell wall pathway related genes.

Discussion

The TPX2 proteins, which have mainly two subgroups of *TPXLs* and *WDLs*, that belong to the novel MAPs member, mainly participate in the formation and development of different organs (Smertenko et al., 2021; Goshima, 2011; Du et al., 2016; Lei et al., 2019). In the process of doubling from diploid cotton to allotetraploid cotton, TPX2 family proteins are extremely conservative in quantity and protein sequences, which show that *GhWDL3* may be extremely conservative in functions.

The expression levels of all *TPXLs* genes were very low in roots, stem, and leaves of *G. hirsutum* cv. J02-508 and ZRI-015, only *GhWDL2*, *GhWDL3*, and *GhWDL4* genes have high expression levels in fibers during the fiber cell elongations and cell wall thickening periods. *GhWDL4* may promote fiber elongation rates during fiber development stages (He et al., 2021). *GhWDL2* and *GhWDL3* are significantly up-regulated at the critical periods from fiber PCW synthesis to SCW synthesis transition at 15 DPA in ZRI-015, while their expression levels are relatively low in J02-508, which are consistent with the transcriptome results of previous studies, but contrary to its qRT-PCR results, which may be caused by the unspecificness of the primers in qRT-PCR (Lei et al., 2019). Above results showed that *GhWDL3* may regulate the length and strength of cotton fibers.

The overexpression of *GhWDL3* in *Arabidopsis* caused various organs to enlarge and promoted the increase of plant biomass in the process of plant growth, while the overexpression of *AtWDL3* caused overall shortening of hypocotyl cells and stabilization of cortical MTs in the light (Liu et al., 2013; Lian et al., 2017). The results showed that although *GhWDL3* is orthologous gene of *AtWDL3* (Fig. S3), the structure and function of the genes may be greatly differentiated. Interestingly, *Arabidopsis* only has some straight hairs in the hairy area of the lower part of the main stems, while overexpression of *GhWDL3* in *Arabidopsis* promotes all stem hairs to become straight, which indicated that *GhWDL3* may regulate stems straight hairs in *Arabidopsis*. Studies have shown that the development of cotton fiber and *Arabidopsis* trichomes have similar regulatory mechanisms (Wang et al., 2021). The *wvd2-1* (as the *WDL1* closest

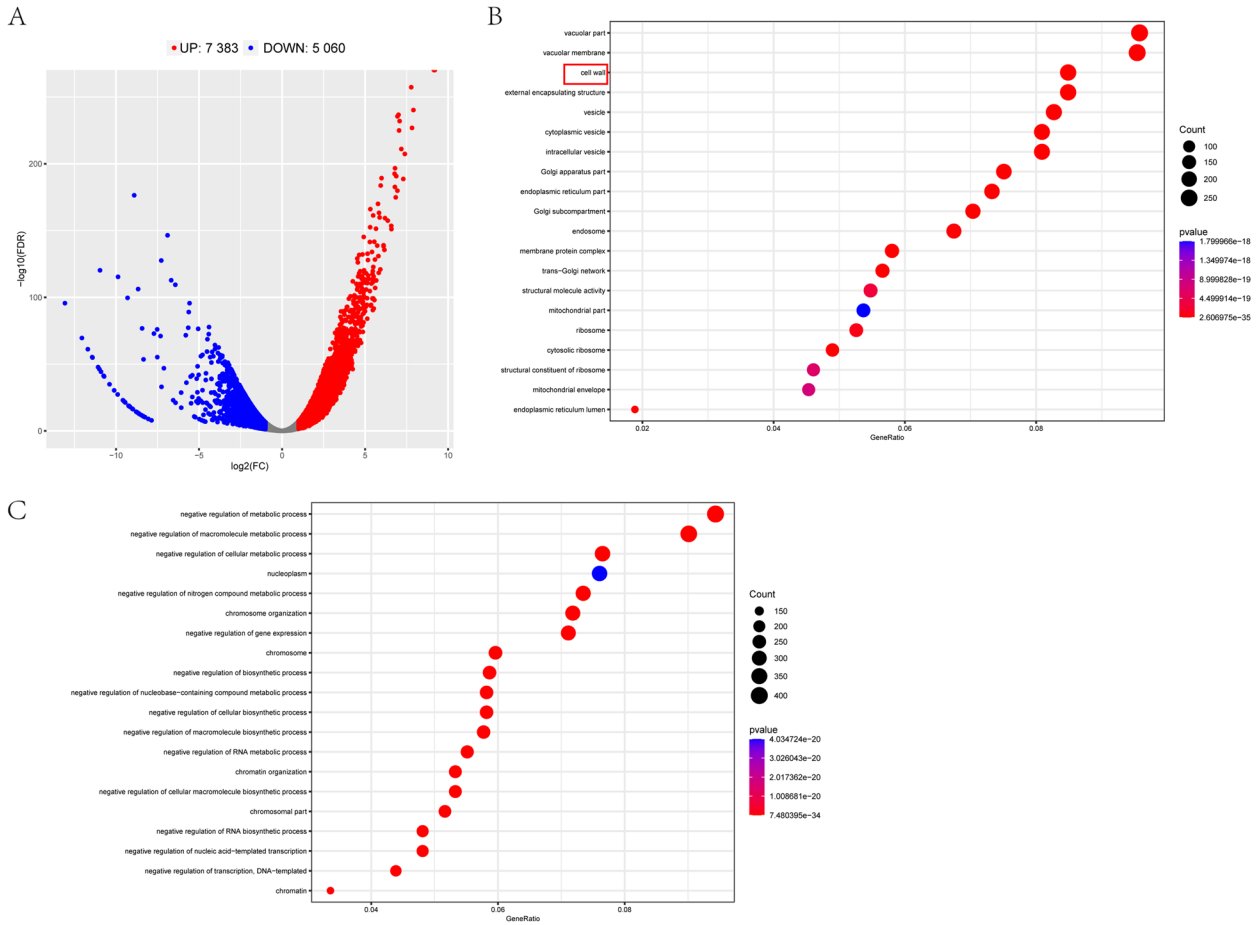


Fig. 5 The transcriptome analysis of the 15 DPA fiber in CLCrV-*GhWDL3* and WT. **A** Volcano plots show the distribution of DEGs of 15 DPA fiber in CLCrV-*GhWDL3* (*GhWDL3* silencing cotton plants) and WT, the red and green dots indicate the up- and down-regulated genes, respectively. **B** GO enrichment analysis of down-regulated DEGs in CLCrV-*GhWDL3* vs. WT, the red box is the cell wall. **C** GO enrichment analysis of up-regulated DEGs in CLCrV-*GhWDL3* vs. WT

paralog) mutant shows aberrantly organized cortical MTs in peripheral root cap cells as well as reduced branching of trichomes, unicellular leaf structures whose development is regulated by MT stability (Perrin et al., 2007). The above results hinted that *GhWDL3* may regulate cotton fiber cell morphology at fiber development periods. Plant hormones play an important role in plant cell elongation and secondary walls formation (Shi et al., 2006; Gou et al., 2007). Ethylene regulates *Arabidopsis* MAP WAVE-DAMPENED2-LIKE5 in etiolated hypocotyl elongation (Sun et al., 2015; Ma et al., 2016). We predicted that *GhWDL3* might affect the cell morphology of stem trichomes in *Arabidopsis*, which showed that WDLs subfamilies proteins may have similar regulatory way.

AtWDL3 was a negative regulator of hypocotyl cell elongation (Liu et al., 2013), while *GhWDL3* promote the enlargement of almost all *Arabidopsis* organs, which indicated that perhaps the functions have differentiated in the process of evolution. Through the analysis of protein sequences, it was found that except for the highly conserved sequence regions, other sequences regions were very different. The fiber length of *GhWDL3* silencing cotton plants was significantly increased compared with control plants. Interestingly, *GhWDL3* also down-regulated the expression levels of cell wall pathway genes *GhCESA8*, *EXPB3*, and *EXPA4/8/13*. The lower expression levels of *GhCESA4/7/8* promoted fiber elongation in anaphase of secondary wall incrassation (Cao et al., 2020; Huang et al., 2021). *GhEXPA4/8* promoted fiber cell elongation at 10 DPA during fiber elongation stages, while *GhEXPA4/8* did not promote fiber cell elongation at 15 DPA of fiber cell wall thickening stages (Zhu et al., 2023). Above results indicated that *GhWDL3* may inhibit fiber elongation during the critical stages of cotton fiber development through regulating the expression of cell wall pathway related genes.

Conclusion

In this study, the evolutionary relationships of *TPX2* family genes were identified in diploid cotton and allotetraploid cotton, which were classified into two distinct subclasses and quite conservative in quantity. *GhWDL3* was significantly up-regulated at 15 DPA fiber in ZRI-015 compared with J02-508. *GhWDL3* promotes stem hairs to become straight when overexpressed in *Arabidopsis*. VIGS analysis showed that *GhWDL3* may inhibit cotton fiber cell elongation through regulating the expression of cell wall related genes. These results revealed that *GhWDL3* inhibited fiber elongation and also provided crucial information for further uncovering the mechanisms of *GhWDL3* in regulating cotton fiber elongation.

Abbreviations

BLASTP	Basic Local Alignment Search Tool for Protein
CESAs	Cellulose synthases
DPA	Day(s) post-anthesis
FPKM	Fragments per kilobase million
MAPs	Microtubule-associated proteins
MTs	Microtubules
qRT-PCR	Quantitative real-time polymerase chain reaction
SCW	Secondary cell walls
VIGS	Virus induced gene silencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42397-024-00167-0.

Additional file 1: Fig. S1. Promoter elements analysis of the *TPX2* family genes in upland cotton.

Additional file 2: Fig. S2. The phenotypes of wild-type and *GhWDL2* transgenic *Arabidopsis* at the initial bolting period stage (4 weeks). Bars = 1 cm.

Additional file 3: Fig. S3. Comparison of protein sequences of *GhWDL3* and *AtWDL3*.

Additional file 4: Fig. S4. Analysis the expression levels of the cell wall pathway related and down-regulated genes in CLCrV-GhWDL3 vs. WT. (A) The transcriptome data of candidate genes *CESA8* and *EXPA3/4/8/13*. (B) qRT-PCR analyzed the expression levels of *CESA8* and *EXPA3/4/8/13*. Statistical significance was determined using one-way ANOVA combined with Tukey's test. **, *P<0.01*; ***, *P<0.001*.

Additional file 5: Table S1. The primers used in this study.

Additional file 6.

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Authors' contributions

Chen BJ, Du XM, and He SP conceived and designed the experiments; Tian ZL, Fu GY, Zhang A, Sun YR, Wang JJ, Pan ZE, Li HG, and Hu DW performed the experiments and collected the data; Du XM and He SP obtained funding; Chen BJ and Xia YY contributed reagents/materials/analysis tools; Chen BJ, Du XM, and He SP revised the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Data will be made available on request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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