Brief Communication

GhPRE1A promotes cotton fibre elongation by activating the DNA-binding bHLH factor *GhPAS1*

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Cotton is one of the most important natural fibre resources in the textile industry that has been cultivated to produce long and spinnable fibres. These cotton fibres are elongated single cells that are derived from the outer epidermis cells of ovules, which are an ideal model to study cell elongation in the plant kingdom (Lee *et al.*, 2007). Phytohormones, such as brassinosteroid (BR), have also been shown to promote fibre elongation. Additionally, many genes that regulate fibre elongation have been functionally identified, especially transcription factors. Still, our understanding of fibre elongation remains limited (Huang *et al.*, 2021; Yang *et al.*, 2020). Previously, *PACLOBUTRAZOL RESISTANCE 1* (*GhPRE1*) was shown to be a target gene for fibre evolution, since it encodes an atypical Helix–Loop–Helix (HLH) protein. In allotetraploid cotton, *GhPRE1D* is silenced due to a TATA-box deletion from its promoter, and the retained *GhPRE1A* is specifically expressed in fibre cells, which plays an important role in fibre elongation (Zhao *et al.*, 2018). However, the regulatory mechanism of *GhPRE1A* on fibre elongation still remains elusive.

To dissect the molecular mechanism of fibre development, we used *GhPRE1A* as bait to screen its interacting proteins in a cotton fibre Y2H library. Y2H, BiFC and Co-IP experiments verified that GhPRE1A interacted with ATBS1 INTERACTING FACTOR 3 (GhAIF3) (Figure 1a–c), which was an atypical HLH protein (Figure 1d). In ectopic overexpression experiment, we showed that GhAIF3 was a negative regulator of cell elongation (Figure S1a–d), while *GhPRE1A* overexpression promoted fibre elongation (Zhao *et al.*, 2018). Since atypical HLH proteins do not have the ability to bind to DNA sequences, GhPRE1A and GhAIF3 may jointly affect the transcriptional activity of other transcription factors that will ultimately regulate fibre elongation. Subsequently, GhAIF3 was used as bait to screen the fibre Y2H library, and we identified PAGODA 1 SUPPRESSOR 1 (GhPAS1, Figure 1d), which acted as a typical bHLH transcription factor with the strong transcriptional activation activity (Wu *et al.*, 2021). With the Y2H,

BiFC and Co-IP experiments, we also verified that GhAIF3 interacted with GhPAS1 (Figure 1a–c), and GhPAS1 did not interact with GhPRE1A (Figure S2a).

To further investigate the role of *GhPAS1* in fibre elongation, we used the *35 S* promoter to drive *GhPAS1* overexpression and obtained the stably-inherited transgenic lines. It is important to note that the *GhPAS1*_OE fibres were longer than the WT fibres during the elongation and secondary wall thickening stages (Figure 1e and Figure S2b). Compared to the mature WT fibres, the length of *GhPAS1*_OE1 and *GhPAS1*_OE2 fibres increased by 10.84% and 14.23%, respectively (Figure 1f,g). Our previous studies showed that *GhPAS1* positively correlates to BR signals (Wu *et al.*, 2021). To better understand the role of *GhPAS1* in BR regulation for fibre elongation, we used ovule culture assays to explore BL and BRZ (a specific BR biosynthesis inhibitor) affection on fibre elongation. BL significantly promoted fibre elongation, while BRZ inhibited fibre elongation (Figure 1h,i). Under BRZ treatment, the length of *GhPAS1*_OE1, *GhPAS1*_OE2 and WT fibres decreased by 14.0%, 16.6% and 40.9% compared with the mock treatment, respectively, which indicated that *GhPAS1* overexpression reduced the sensitivity to BRZ (Figure 1h,i). Silencing *GhPRE1s* by double-stranded (ds) RNA-mediated interference technology significantly reduced fibre length (Zhao *et al.*, 2018). Additionally, when *GhPAS1* was introduced into the background of *dsGhPRE1* through hybridization, it also increased fibre length by 7.5% compared to the *dsGhPRE1* fibres (Figure S2c,d), which suggested that *GhPAS1* may act downstream of *GhPRE1A* to regulate fibre elongation. Further, ectopic overexpression of *GhPRE1A* partially restored the dwarf phenotype of the *Arabidopsis* BR weak receptor mutant *bri1_5*, just like *GhPAS1* (Figure S1e–h). Together, *GhPAS1* and *GhPRE1A* were involved in BR regulation of fibre elongation.

Because GhPAS1 has the ability to bind to DNA sequence in the basic region, we performed DAP-seq and identified 3671 target genes that should be directly regulated by GhPAS1 (Table S1). We found that they were mainly enriched in cell elongation, as well as in cell wall, cytoskeleton and phytohormones (Figure S3). One group of genes, *Expansins*, promote cell growth by loosening plant cell wall, and *GhEXPA8* has been shown to promote fibre elongation (Bajwa *et al.*, 2015). The target genes contain six *GhEXPs*, of which, *GhEXPA8* has the highest expression level (Figure 1j). Subsequently, our EMSA experiment verified that GhPAS1 is bound to the E-box region of the *GhEXPA8* promoter (Figure 1k). The DLR assay then indicated that GhPAS1 was also bound to the *GhEXPA8* promoter and generated a strong LUC fluorescence. However, it did not bind to the mutant *GhEXPA8* promoter (Figure 1l and Figure S4a). qRT-PCR results confirmed
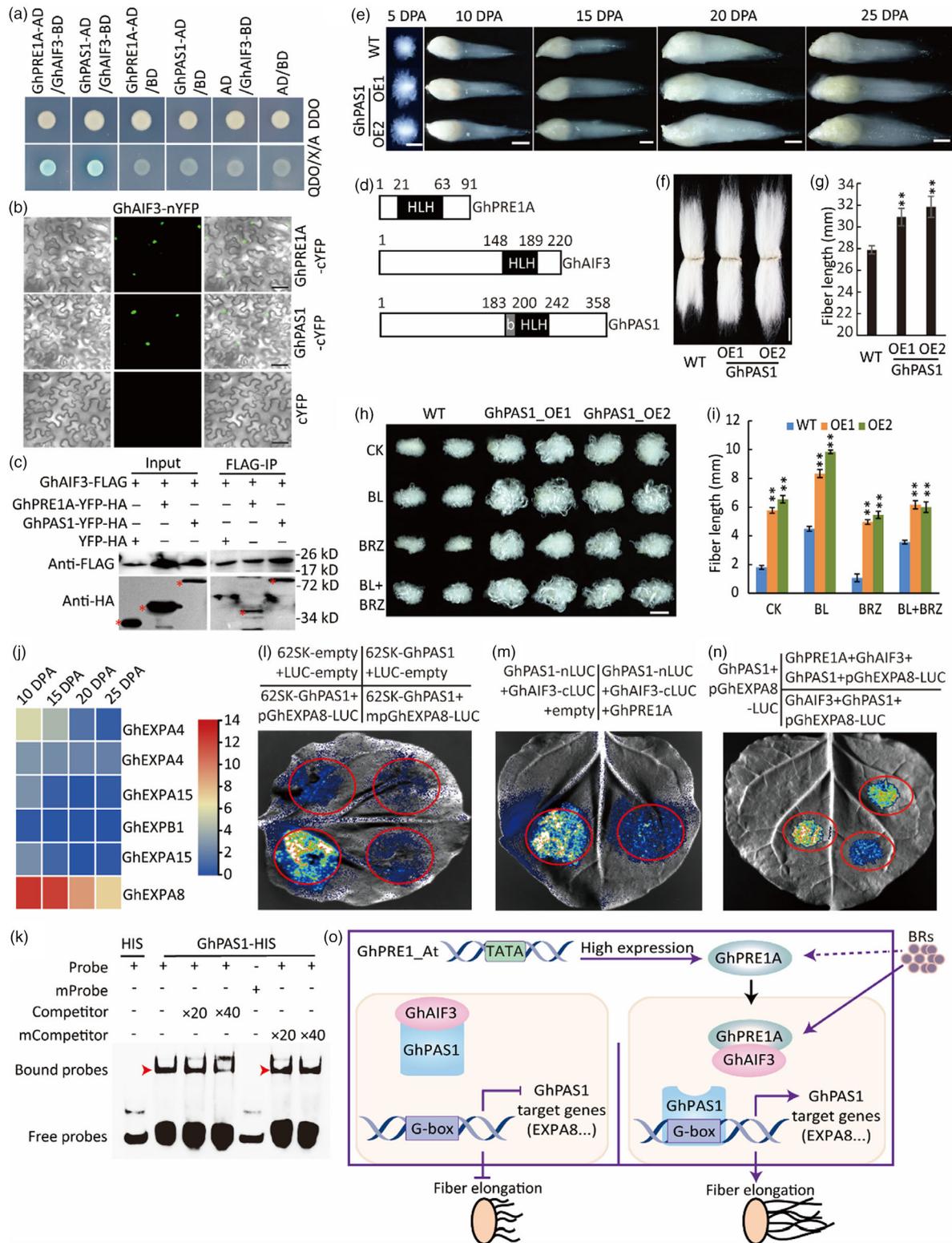
Figure 1 *GhPRE1A* promotes cotton fibre elongation by activating the DNA-binding bHLH factor *GhPAS1*. (a–c) Y2H (a), BiFC (b) and Co-IP (c) assays showed that GhPRE1A and GhPAS1 interact with GhAIF3, respectively. Bars, 50 μm. (d) Structural diagram of GhPRE1A, GhAIF3 and GhPAS1 proteins. The black, grey and white frames represent the HLH region, the basic region and the non-conserved region, respectively. (e) 5, 10, 15, 20 and 25 DPA fibres of WT and *GhPAS1* transgenic lines. Bars, 5 mm. (f–g) Mature fibres (f) and fibre length (g) of WT and *GhPAS1* transgenic lines. Bar, 1 cm. (**$P < 0.01$, Student's *t*-test). (h) Ovules were cultured in BT liquid medium for 12 days. Each treatment was treated with 0.1 μM BL, 10 μM BRZ, 0.1 μM BL and 10 μM BRZ, respectively. Bar, 5 mm. (i) Fibre length in the ovule culture assay. (**$P < 0.01$, Student's *t*-test). (j) Heat map of *GhEXPs* expression at the different stages of fibre development. (k–l) EMSA (k) and DLR (l) assays of GhPAS1 binding to the *GhEXPA8* promoter. (m) Co-expression of *GhPRE1A*, *GhPAS1-* nLUC and *GhAIF3*-cLUC in tobacco leaves. (n) Co-expression of *GhPRE1A*, *GhAIF3*, *GhPAS1* and p*GhEXPA8*-LUC in tobacco leaves. (o) Schematic model.

that *GhEXPA8* expression was significantly induced in *GhPAS1* overexpression lines (Figure S2e).

In the LCI assay, the co-expression of *GhPAS1*-nLUC and *GhAIF3*-cLUC resulted in a strong LUC fluorescence (Figure 1m and Figure S4b), which confirmed their interaction. However, the LUC fluorescence was significantly suppressed when *GhPRE1A* was co-expressed with *GhPAS1*-nLUC and *GhAIF3*-cLUC (Figure 1m and Figure S4b). Together, GhPRE1A interfered with the interaction between GhAIF3 and GhPAS1. We further explored whether GhPRE1A and GhAIF3 affect the transcriptional activation activity of GhPAS1 by driving the *GhEXPA8* promoter with the *LUC* gene expression as a reporter. The LUC fluorescence intensity decreased to the basal level when *GhAIF3* was co-expressed with *GhPAS1* and p*GhEXPA8*-LUC, which indicated that GhAIF3 inhibited the transcriptional activation activity of GhPAS1 (Figure 1n and Figure S4c). However, when *GhPRE1A* was co-expressed with *GhAIF3*, *GhPAS1* and p*GhEXPA8*-LUC, the strong LUC fluorescence was detected again (Figure 1n and Figure S4c). Therefore, GhPRE1A seems to alleviate the inhibitory effect of GhAIF3 on the activity of GhPAS1.

In this study, we revealed that the GhPRE1A-GhAIF3-GhPAS1 module regulates fibre elongation downstream of the BR signalling pathway (Figure 1o). Specifically, GhAIF3 interacts with GhPAS1 to form a heterodimer that inhibits the transcriptional activation activity of GhPAS1 on target genes, such as *GhEXPA8*. Additionally, GhPRE1A competitively binds to GhAIF3 and releases GhPAS1, which then reactivates the expression of downstream target genes and promotes fibre elongation. GhPRE1A and GhPAS1 are positive regulators of the BR signalling pathway. Together, our results suggest that the GhPRE1A-GhAIF3-GhPAS1 module could be located downstream of the BR signalling pathway in order to regulate fibre elongation. Ultimately, our work sheds new light on the regulatory mechanism of single-cell cotton fibre elongation and provides a theoretical basis for the genetic improvement of cotton fibre.

Acknowledgements

Conflict of interest

The authors declare no conflicts of interest.

Authors contributions

Z.R.Y. and F.G.L. supervised the experiments. H.H.W., L.Q.F., M.Z.G., L.L., L.S.L., L.Y.H., L.Z. and G.Q. performed the experiments and analysed the data. L.L.L., and J.Z. contributed the materials. H.H.W. and Z.R.Y. wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Heterologous overexpression of *GhPAS1*, *GhPRE1A* and *GhAIF3* in *Arabidopsis*.
Figure S2 The results of Y2H, fibre length and qRT-PCR analysis.
Figure S3 DAP-seq results.
Figure S4 The density values of LUC fluorescence intensity.
Table S1 Target genes of GhPAS1 identified by DAP-seq.