Genome-wide identification of cyclophilin genes in *Gossypium hirsutum* and functional characterization of a CYP with antifungal activity against *Verticillium dahliae*

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Abstract

**Background:** Cyclophilins (CYPs), belonging to the peptidyl prolyl cis/trans isomerase (PPIase) superfamily, play important roles during plant responses to biotic and abiotic stresses.

**Results:** Here, a total of 79 CYPs were identified in the genome of *Gossypium hirsutum*. Of which, 65 GhCYPs only contained one cyclophilin type PPIase domain, others 14 GhCYPs contain additional domains. A number of cis-acting elements related to phytohormone signaling were predicted in the upstream of GhCYPs ORF. The expression analysis revealed that GhCYPs were induced in response to cold, hot, salt, PEG and *Verticillium dahliae* infection. In addition, the functional importance of GhCYP-3 in *Verticillium* wilt resistance was also presented in this study. GhCYP-3 showed both cytoplasmic and nuclear localization. Overexpression of GhCYP-3 in *Arabidopsis* significantly improved *Verticillium* wilt resistance of the plants. Recombinant GhCYP-3 displayed PPIase activity and evident inhibitory effects on *V. dahliae* in vitro. Moreover, the extracts from GhCYP-3 transgenic *Arabidopsis* displayed significantly inhibit activity to conidia germinating and hyphal growth of *V. dahliae*.

**Conclusions:** Our study identified the family members of cotton CYP genes using bioinformatics tools. Differential expression patterns of GhCYPs under various abiotic stress and *V. dahliae* infection conditions provide a comprehensive understanding of the biological functions of candidate genes. Moreover, GhCYP-3 involved in the resistance of cotton to *V. dahliae* infection presumably through antifungal activity.

**Keywords:** Cyclophilin, Cotton, Resistance, *Verticillium dahliae*, Antifungal activity

Background

The allotetraploid *Gossypium hirsutum* L., the most important fiber crop, is cultivated worldwide because of its high yield [1]. Environmental stresses, such as cold, drought, heat, salinity, various pests and pathogens, threaten cotton growth, yield and fiber quality. For example, an area of about 300 million hectares of cotton is subject to *Verticillium* wilt and the economic loss is more than RMB 12 billion every year in China. *Verticillium* wilt, the most serious disease to influence cotton production in China, caused by soil-borne fungus *Verticillium dahliae*. At present, few resistant varieties have been cultivated by traditional cross-breeding in *G. hirsutum*, which contributes 95% of the total cotton yield in the world [2]. Genetic engineering using plant resistance genes is becoming an alternative to improve cotton resistance to *V. dahliae*.

Cyclophilins (CYPs), possessing peptidyl prolyl cis/trans isomerase (PPIase) activity, are classified in the immunophilin family of proteins [3]. CYPs play important roles in various biological processes, including transcription regulation [4], protein folding [5], signal transduction [6] and...
ROS (reactive oxygen species) regulation [7]. With the availability of whole genome sequencing, the identification and characterization of plant CYPs are carried out mainly in Arabidopsis thaliana (29 AtCYPs) [8], Oryza sativa (27 OsCYPs) [9] and Glycine max (62 GmCYPs) [10]. The majority of studies reveal the involvement of plant CYPs mostly in different types of abiotic stress. For example, Arabidopsis CYPs showed evidence of response to wounding [11]. Rice OsCYP19-4 showed over 10-fold up-regulation in response to cold. Overexpressing of OsCYP19-4 could enhance rice plants cold-resistance with significantly increased tiller and spike numbers, and consequently enhanced grain weight [12]. Transgenic plants overexpressing OsCYP21-4 exhibited increased tolerance to salinity and hydrogen peroxide treatment [13]. Ectopic expression of pigeon pea (Cajanus cajan L.) CYP, CcCYP in Arabidopsis exhibited high-level tolerance against drought, salinity and extreme temperatures [14]. Against biotic stress, especially against pathogen infection, only several plant CYPs have been studied in plant-pathogen system. Pepper cyclophilin (CACYP1) gene expression increased in response to Xanthomonas campestris pv. vesicatoria and Colletotrichum gloeosporioides [15]. Fungal infection with Fusarium solani f. sp. pumilii increased the level of Solanum tuberosum CYP gene StCyP mRNA in tubers [16]. The expression of V. vinifera VviCyP was highly induced by Plasmopara viticola [17]. In cotton, a cyclophilin-like gene GhCYP1 was cloned from G. hirsutum. Overexpression of GhCYP1 in transgenic tobacco plants conferred higher tolerance to salt stress and Pseudomonas syringae pv. tabaci infection compared with control plants [18].

In 2015, the genome of G. hirsutum L. acc. Texas Marker-1 (TM-1) was sequenced, more than 70,000 protein-coding genes were predicted (NAU version 1.1) [19, 20]. Recently, an improved de novo–assembled genome for G. hirsutum L. acc. TM-1 were generated (NAU version 2.1) [21]. The genome-sequencing project facilitates the survey of all CYP genes in cotton. In the present study, the CYP gene family members in G. hirsutum and their expression patterns under various abiotic stresses and on V. dahliae infection were systematically investigated. Furthermore, the function of GhCYP-3 was analyzed to reveal its role in cotton resistance to V. dahliae infection. Our study will enlighten the novel insights into the function of CYP genes in plant against multivariate stress responses in the future and provide more candidate genes for resistance breeding in cotton.

**Results**

**Up to 79 CYPs were identified in the genome of G. hirsutum TM-1**

A local BLASTP search was performed with the Arabidopsis CYP proteins as query, which resulted in 79 CYP candidates from G. hirsutum NAU version 1.1, 74 CYPs from G. hirsutum JGI version 1.0 and 78 CYPs from G. hirsutum NAU version 2.1 (Table 1). These candidates were submitted to Pfam to confirm the existence of cyclophilin type PPIase domain (CLD, PF00160) and named GhCYP-1 to GhCYP-79. The characteristics of the individual CYP, including CDS length, protein length, molecular weight, and isoelectric point (pl) were presented in Table 1. The protein length varied from 69 amino acid (aa) residues (GhCYP-70) to 801 aa (GhCYP-55). The molecular weight ranged from 7.5 kDa (GhCYP-70) to 90.5 kDa (GhCYP-55), and the pl values ranged from 4.6 to 12.0. Most of the GhCYPs were expected to be in the cytoplasm. Also, some GhCYPs exhibited chloroplast, mitochondrial, nuclear and extracellular localization. Of the 79 GhCYPs, 65 only contained one CLD domain, but the remaining 14 GhCYPs contain additional domains, including tetratricopeptide-like repeats (TPR, PF00515, PF07719, PF13181, PF13414), Zinc finger (zf-CCHC, PF00098), RNA recognition motif (RRM, PF00076) and WD40 (PF00400) (Fig. 1).

**Cis-elements potentially related to hormonal signal for GhCYPs**

Here we surveyed the presence of cis-elements potentially related to the hormonal signal, in the -2 kb 5′ flanking region upstream to the start codon of these GhCYPs. In total six types of hormones related cis-elements in the promoters were predicted (Fig. 2). Of these GhCYPs, 66 GhCYPs had ethylene (ET) responsive element (ERE), 35 GhCYPs contained salicylic acid (SA) responsive element (TCA-element), 47 GhCYPs harbored abscisic acid (ABA) responsive element (ABRE), 38 GhCYPs possessed gibberellin (GA) responsive element (P-box; TATC-box), 47 GhCYPs contained methyl Jasmonate (MeJA) responsive element (CGTCA-motif), and 26 possessed auxin responsive element (TGA-box; AuxRR-core). In total, 217 cis-elements related to ET, 150 cis-elements related to MeJA, 132 cis-elements related to ABA, 57 cis-elements related to GA, 43 cis-elements related to SA and 34 cis-elements related to auxin were identified in all GhCYPs (Fig. 2). The enrichment of hormone-responsive cis-elements in the upstream of these GhCYPs suggests that they are likely to be involved in plant responses to various hormone signal pathways.

**Expression patterns of GhCYPs under various abiotic stresses**

Expression profiles of GhCYPs were examined in roots of cotton plants under four different abiotic stress conditions using high-throughput RNA-seq data (Additional file 2: Table S2). The transcripts with low Fragments Per Kilobase of exon per Million fragments mapped (FPKM) were probably false assembly. Therefore, in this study GhCYPs with FPKM >10 and present in at least two
| Gene Name | Gene ID (NAU1) | CDS (bp) | Protein (aa) | MW (kDa) | pI | Subcellular location | Gene ID (JGI/NAU2) Identity (%) (JGI/NAU2) | Identity (%) (JGI/NAU2) |
|-----------|----------------|----------|--------------|----------|----|----------------------|---------------------------------------------|--------------------------|
| GhCYP-1   | Gh_A01G0027    | 675      | 224          | 24.5     | 7.3 | Cyto                 | Gohir.A01G003000/GH_A01G0029                | 100/100                  |
| GhCYP-2   | Gh_A01G0031    | 516      | 171          | 18.2     | 7.9 | Cyto                 | Gohir.A01G003400/GH_A01G0034                | 99/99                     |
| GhCYP-3   | Gh_A01G1361    | 522      | 173          | 18.2     | 8.5 | Cyto                 | Gohir.A01G164200/GH_A01G1763               | 100/100                  |
| GhCYP-4   | Gh_A01G1747    | 495      | 164          | 18.0     | 8.2 | Cyto/Mito/Chlo       | Gohir.A01G204600/GH_A01G2217               | 100/100                  |
| GhCYP-5   | Gh_A02G0528    | 708      | 235          | 26.5     | 9.8 | Mito                 | Gohir.A02G054200/GH_A02G0571               | 100/100                  |
| GhCYP-6   | Gh_A02G1526    | 1116     | 371          | 41.9     | 6.6 | Cyto                 | No found/GH_A02G1850                       | -100                     |
| GhCYP-7   | Gh_A03G0499    | 966      | 321          | 35.0     | 8.7 | Chlo                 | Gohir.A03G057400/GH_A03G0669               | 100/100                  |
| GhCYP-8   | Gh_A03G0865    | 648      | 216          | 24.5     | 6.1 | Chlo                 | Gohir.A03G099700/GH_A03G1148               | 100/100                  |
| GhCYP-9   | Gh_A03G1688    | 1221     | 406          | 45.9     | 8.8 | Cyto                 | Gohir.A03G191300/GH_A03G2153               | 100/100                  |
| GhCYP-10  | Gh_A04G1046    | 522      | 173          | 18.5     | 10.1| Cyto                 | No found/GH_A04G1474                       | -100                     |
| GhCYP-11  | Gh_A04G1047    | 522      | 173          | 18.3     | 8.2 | Cyto                 | No found/GH_A04G1475                       | -100                     |
| GhCYP-12  | Gh_A05G0642    | 1026     | 341          | 37.6     | 8.6 | Cyto                 | Gohir.A05G078500/GH_A05G0800               | 74/73                    |
| GhCYP-13  | Gh_A05G3461    | 567      | 188          | 20.4     | 8.2 | Cyto                 | No found/GH_A05G4196                       | -100                     |
| GhCYP-14  | Gh_A05G4019    | 1836     | 611          | 70.5     | 6.2 | Nucl                 | Gohir.A05G003300/GH_A05G0027               | 100/100                  |
| GhCYP-15  | Gh_A06G0418    | 870      | 289          | 31.9     | 6.9 | Extra                | Gohir.A06G049800/GH_A06G0538               | 100/100                  |
| GhCYP-16  | Gh_A06G0767    | 1494     | 497          | 56.1     | 8.7 | Nucl                 | Gohir.A06G085500/GH_A06G0934               | 100/100                  |
| GhCYP-17  | Gh_A07G0324    | 2373     | 790          | 89.3     | 12.0| Nucl                 | Gohir.A07G038000/GH_A07G0439               | 100/99                   |
| GhCYP-18  | Gh_A07G0325    | 2388     | 795          | 89.9     | 11.6| Nucl                 | Gohir.A07G038100/GH_A07G0440               | 93/93                    |
| GhCYP-19  | Gh_A07G0986    | 1866     | 621          | 70.0     | 7.0 | Cyto                 | Gohir.A07G108500/GH_A07G11190             | 100/100                  |
| GhCYP-20  | Gh_A07G2012    | 1359     | 452          | 50.0     | 4.9 | Chlo                 | Gohir.A07G219400/GH_A07G2484              | 100/99                   |
| GhCYP-21  | Gh_A08G0354    | 711      | 236          | 26.9     | 9.2 | Mito                 | Gohir.A08G040200/GH_A08G0443              | 100/100                  |
| GhCYP-22  | Gh_A08G1077    | 855      | 284          | 31.0     | 8.2 | Extra/PM/Chlo        | Gohir.A08G122000/GH_A08G1462              | 88/97                    |
| GhCYP-23  | Gh_A08G1194    | 483      | 160          | 17.4     | 8.5 | Cyto                 | Gohir.A08G133100/GH_A08G1597              | 100/100                  |
| GhCYP-24  | Gh_A08G1470    | 1032     | 343          | 38.0     | 5.4 | Extra                | Gohir.A08G162600/GH_A08G1886              | 100/100                  |
| GhCYP-25  | Gh_A08G1670    | 1986     | 661          | 72.8     | 10.7| Nucl                 | Gohir.A08G187500/GH_A08G2136              | 94/94                    |
| GhCYP-26  | Gh_A09G0254    | 1308     | 435          | 47.9     | 4.6 | Cyto                 | Gohir.A09G026100/GH_A09G0300              | 100/100                  |
| GhCYP-27  | Gh_A09G0853    | 624      | 207          | 22.2     | 9.4 | Cyto/Mito            | Gohir.A09G090200/GH_A09G1082              | 96/96                    |
| GhCYP-28  | Gh_A09G1765    | 1032     | 343          | 37.9     | 6.0 | Extra                | Gohir.A09G197200/GH_A09G2134              | 100/100                  |
Table 1  The list of the putative CYP genes identified in *G. hirsutum* (Continued)

| Gene Name | Gene ID (NAU1) | CDS (bp) | Protein (aa) | MW (kDa) | pl | Subcellular location | Gene ID (JGI/NAU2) | Identity (%) (JGI/NAU2) |
|-----------|----------------|----------|--------------|----------|----|----------------------|---------------------|------------------------|
| GhCYP-29  | Gh_A10G0832    | 1089     | 362          | 40.3     | 6.7 | Cyto                 | Gohir.A10G092800/GH_A10G0930 | 97/96                  |
| GhCYP-30  | Gh_A10G1682    | 576      | 191          | 20.7     | 8.2 | Cyto                 | Gohir.A10G187900/GH_A10G2053 | 100/100                |
| GhCYP-31  | Gh_A10G1687    | 609      | 202          | 22.1     | 8.6 | Cyto                 | Gohir.A10G188300/GH_A10G2060 | 90/89                  |
| GhCYP-32  | Gh_A10G2121    | 666      | 221          | 23.9     | 9.4 | Cyto                 | Gohir.A10G236300/GH_A10G2615 | 100/100                |
| GhCYP-33  | Gh_A11G0678    | 996      | 331          | 37.2     | 5.3 | Extra                | Gohir.A11G074800/GH_A11G0773 | 82/82                  |
| GhCYP-34  | Gh_A11G0987    | 705      | 234          | 26.8     | 8.9 | Mitto/Nuc            | Gohir.A11G108500/GH_A11G1131 | 100/100                |
| GhCYP-35  | Gh_A12G0709    | 1791     | 596          | 65.3     | 8.2 | Mitto/Nuc            | Gohir.A12G077300/GH_A12G0891 | 100/100                |
| GhCYP-36  | Gh_A12G2539    | 765      | 254          | 28.6     | 5.9 | Cyto                 | Gohir.A12G082500/GH_A12G0712 | 100/100                |
| GhCYP-37  | Gh_A13G0333    | 1200     | 399          | 44.6     | 6.4 | Cyto                 | No found/GH_A13G0375 | -/100                  |
| GhCYP-38  | Gh_A13G0846    | 525      | 174          | 18.8     | 7.8 | Cyto                 | Gohir.A13G103900/GH_A13G1226 | 100/100                |
| GhCYP-39  | Gh_D01G0026    | 675      | 224          | 24.6     | 7.3 | Cyto                 | Gohir.D01G002600/GH_D01G0028 | 100/100                |
| GhCYP-40  | Gh_D01G0030    | 516      | 171          | 18.2     | 7.4 | Cyto                 | Gohir.D01G003100/GH_D01G0033 | 99/99                  |
| GhCYP-41  | Gh_D01G0206    | 780      | 259          | 28.1     | 10.3| Chlo                 | Gohir.D01G020500/GH_D01G0221 | 100/100                |
| GhCYP-42  | Gh_D01G1605    | 522      | 173          | 18.3     | 8.5 | Cyto                 | Gohir.D01G156100/GH_D01G1877 | 100/100                |
| GhCYP-43  | Gh_D02G0593    | 705      | 234          | 26.3     | 9.8 | Mitto                | Gohir.D02G059200/GH_D02G0586 | 100/100                |
| GhCYP-44  | Gh_D02G1247    | 750      | 249          | 28.4     | 8.1 | Chlo                 | Gohir.D02G124600/GH_D02G1353 | 98/100                 |
| GhCYP-45  | Gh_D02G2108    | 1233     | 410          | 46.4     | 8.6 | Cyto                 | Gohir.D02G212200/GH_D02G2325 | 100/99                 |
| GhCYP-46  | Gh_D03G0186    | 1086     | 361          | 40.6     | 5.5 | Cyto                 | Gohir.D03G020200/GH_D03G0207 | 99/99                  |
| GhCYP-47  | Gh_D03G1033    | 966      | 321          | 35.0     | 8.2 | Chlo                 | Gohir.D03G108800/GH_D03G1247 | 100/100                |
| GhCYP-48  | Gh_D04G1620    | 522      | 173          | 18.5     | 9.6 | Cyto                 | Gohir.D04G164700/GH_D04G1813 | 100/100                |
| GhCYP-49  | Gh_D04G1621    | 522      | 173          | 18.3     | 8.2 | Cyto                 | Gohir.D04G164800/GH_D04G1814 | 100/100                |
| GhCYP-50  | Gh_D04G1937    | 570      | 189          | 20.5     | 8.2 | Cyto                 | Gohir.D04G163000/GH_D04G1813 | 100/100                |
| GhCYP-51  | Gh_D05G0033    | 1836     | 611          | 70.6     | 6.0 | Nucl                 | Gohir.D05G003700/GH_D05G0030 | 100/100                |
| GhCYP-52  | Gh_D06G0456    | 870      | 289          | 31.9     | 7.3 | Extra                | Gohir.D06G049100/GH_D06G0504 | 100/100                |
| GhCYP-53  | Gh_D06G2331    | 1491     | 496          | 55.9     | 8.1 | Nucl                 | Gohir.D06G084800/GH_D06G0925 | 100/100                |
| GhCYP-54  | Gh_D07G0381    | 2367     | 788          | 89.4     | 12.0 | Nucl                 | Gohir.D07G042100/GH_D07G0441 | 100/99                 |
| GhCYP-55  | Gh_D07G0382    | 2406     | 801          | 90.5     | 11.5 | Nucl                 | Gohir.D07G042200/GH_D07G0442 | 98/97                  |
samples were identified as potentially expressed transcripts. We focused on the significantly differentially expressed genes (fold change [FC] > 2 or FC < 0.5) in the various stresses. GhCYPs that showed significant differential expression at least one treatment time were listed in Fig. 3. In the cold treatment, 14 GhCYPs were commonly down-regulated significantly apart from GhCYP-49 at 1 hct (hours after cold treated) and 3 hct and GhCYP-52 at 6 hct, which showed up-regulated significantly. At 1 hht (hours after hot treated), 22 of 26
Fig. 1 Schematic representation of multi-domain GhCYPs. CLD, cyclophilin-like-domain; RRM, RNA recognition motif; zf-CCHC, CCHC-type zinc finger protein; WD, tryptophan-aspartate repeat; TPR, tetratricopeptide repeat.

Fig. 2 Potential cis-elements in a 2 kb 5' flanking region upstream from the start codon of each GhCYP involved in phytohormone signaling. Potential cis-elements related to hormonal signal for GhCYPs are shown as colored bar at the bottom. The total number of cis-elements related to ET, MeJA, ABA, GA, SA, and Auxin was shown in brackets.
GhCYPs are response to hot treatment. Of these, only the expression level of GhCYP-49 and GhCYP-61 was up-regulated significantly. At 3 hht, 6 hht and 12 hht, only 12 GhCYPs exhibited differential expression pattern. Of these, up to 11 genes were up-regulated. Most GhCYP genes were found to be down-regulated under the condition of salt treatment apart from GhCYP-3, GhCYP-24, GhCYP-38, GhCYP-42, GhCYP-49, GhCYP-57 and GhCYP-61. After PEG (polyethylene glycol) treatment, only 8 GhCYPs displayed differential expression pattern, of which, GhCYP-3, GhCYP-42, GhCYP-49 and GhCYP-73 were up-regulated, GhCYP-41, GhCYP-47
and GhCYP-75 were down-regulated. Only the expression of GhCYP-38 showed be down-regulated at 3 hpt (hours after PEG treated) and 6 hpt, and then be upregulated at 12 hpt. These expression patterns suggest that CYPs undertake multiple functions to help the cotton counter various complex environmental challenges.

Expression profiles of GhCYPs under the stress of V. dahliae

To gain a better understanding of the roles of CYP family genes in the cotton resistance against V. dahliae, the expression profiles were obtained by RNA-Seq. A total of 30 GhCYP genes showed differential expression at least one time point (hpi, hours post inoculation), including 19 upregulated genes and 11 downregulated genes (Fig. 4). Notably, levels of GhCYP-10, GhCYP-22, GhCYP-48 and GhCYP-59 were up-regulated at all five treatment time points. Additionally, the expression of GhCYP-11, GhCYP-27, GhCYP-37, GhCYP-45 and GhCYP-74 was down-regulated in most time points. These results revealed that GhCYPs were associated with the interaction between cotton and V. dahliae.

GhCYP-3 contains conserved amino acid residues and has PPlase activity

GhCYP-3, cloned from G. hirsutum cv. JM20, contains a single cyclophilin domain, 173 amino acid residues with a calculated molecular mass of 18.2 kDa and a pl of 8.34. Alignment with previously characterized Arabidopsis and human CYP (AtCYP19-1 and hCypA) revealed that GhCYP-3 contains seven conserved amino acid residues that critically affect PPlase activity (Fig. 5a). GhCYP-3 was found to be expressed in root, stem and leaf of unchallenged cotton plants with V. dahliae (Fig. 5b). Translation fusion of GhCYP-3 with GFP was constructed under the control of 35S promoter, and then transiently expressed in onion epidermal cells. Fluorescent imaging of the GhCYP-3-GFP bombarded onion epidermal cells showed both cytoplasm and nuclear localization (Fig. 5c).

The GhCYP-3 ORF was cloned into pET-32a at SacI and BgIII sites, thus the recombinant plasmid was constructed (Fig. 6a). SDS-PAGE and western blot analysis revealed that the fusion TrxA-6×His-S-tag-6×His (THS, 20.4 kDa; empty vector as control) and TrxA-6×His-S-tag-GhCYP3 (THS-CYP, 34.6 kDa) proteins were highly expressed in the E. coli BL21(DE3) at 37°C with 1 mM IPTG for 4 h (Fig. 6b). The recombinant THS-CYP protein was purified using the nickel-affinity. Further, we measured the PPlase activity of the recombinant protein by a coupled assay using synthetic peptide synthetic peptide succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. The isomerization of the peptide substrate was observed in the presence of recombinant GhCYP-3, showing OD values higher than that of the blank, a negative control (Fig. 6c). These results indicated that GhCYP-3 has PPlase activity in vitro.

GhCYP-3 expression is upregulated in response to V. dahliae invasion

To analyze the expression pattern of GhCYP-3 in three cotton cultivars with different degrees of resistance to V. dahliae, the roots of two-week-old cotton seedlings were sampled at 0, 4, 8, 12, 24 and 48 hpi. The qRT-PCR results revealed that the transcriptional levels of GhCYP-3, compared with 0 hpi, were significantly upregulated in two-resistant cultivars Pima90-53 and JM-20 at 4 hpi. However, at the same inoculation time, GhCYP-3 in susceptible Han-208 was strongly downregulated and reached the highest expression level until 24 hpi (Fig 7). These results suggested that GhCYP-3 is upregulated and earlier involved in the cotton interaction with V. dahliae in resistant than in susceptible cultivars.

Overexpression of GhCYP-3 in Arabidopsis improves plant Verticillium wilt resistance

To further evaluate whether the GhCYP-3 functions in plant resistance to Verticillium wilt, we designed primers to amplify the ORF of GhCYP-3 into the plant expression vector pBI121 (Fig. 8a), which will make GhCYP-3 overexpression in transgenic Arabidopsis plants. By selection on kanamycin-containing medium (Fig. 8b), ten potential T1 transgenic lines were generated. After PCR-verification of gene insertion (Fig. 8c), two lines of the T3 generation displaying GhCYP-3 overexpression (Fig. 8d) were selected for analysis of PPlase activity and Verticillium wilt resistance.

Under unstressed conditions (treated with H2O), no significant PPlase activity was enhanced in two GhCYP3-expressing transgenic lines compared to the WT (wild type). However, these two transgenic lines, infected with V. dahliae for 3 d, showed increased PPlase activity of 0.51±0.03 (L1) and 0.45±0.03 nmol·s⁻¹·mg⁻¹ protein (L2) compared to 0.35±0.03 nmol·s⁻¹·mg⁻¹ protein observed in the WT (Fig. 8e).

In response to V. dahliae infection, two independent overexpressing lines displayed less wilting and smaller degree of leaf etiolation in comparison with WT plants at 15 dpi (Fig. 9a). Disease evaluation further indicated that the disease indices of transgenic lines were significantly lower than that of the WT (Fig. 9b). Furthermore, we examined the colonization in stems of infected WT and transgenic plants by isolation and cultivation of V. dahliae on PDA (potato dextrose agar) for 8 days. As a result, less fungal colonies came out from transgenic plants comparing to WT (Fig. 9c and d). These fungi recovery assay indicated that GhCYP-3 has a specialized effect of growth inhibition on V. dahliae.
GhCYP-3 exhibited obvious inhibitory effects on V. dahliae.

We further assessed the inhibitory effects of GhCYP-3 on V. dahliae. As showed in Fig. 10a, distinct inhibition zones formed around the disc containing recombinant GhCYP-3. Additionally, V. dahliae spores were inoculated with plant extracts from WT and transgenic lines and then be spread on PDA plates. After 48 h, extracts from all the plants significantly reduced the number of colonies compared to the H2O (as control). However, the number of fungal colonies on plates contain transgenes extracts was significantly less than the WT (Fig. 10b and c). These results indicated that GhCYP-3 can efficiently inhibit conidia germinating and hyphae growth of V. dahliae in vitro.

Discussion

CYP genes family has been systematically analyzed in several plants, such as Arabidopsis [8], rice [22] and soybean [10]. Recently, 78 CYP genes were also identified in G. hirsutum L. acc. TM-1 from Illumina paired-end genomic sequencing (NAU version 1.1) by Chen et al. [23]. Now, NAU version 2.1, highly accurate reference grade genome assemblies and annotations for G. hirsutum, was generated [21]. A fairly large number of gaps and erroneous assemblies were successfully filled and corrected in new NAU version 2.1 [21]. Therefore, in the present study we identified a total of 79 CYP genes by integrating NAU version 1.1, JGI database and NAU version 2.1 (Table 1). Compared to the previous report from Chen et al., the supplementary three CYPs (GhCYP-49, GhCYP-
60 and GhCYP-70) were identified in this study. Besides, two CYPs (Gh_Sca140771G01 and Gh_A12G1281), undiscernable in JGI database and NAU version 2.1, were removed. It thus made the candidate CYP genes in <em>G. hirsutum</em> more reliable.

All 79 CYPs in <em>G. hirsutum</em> have conservative PPIase domain (CLD). Of which, 14 GhCYPs are multi-domain proteins (Fig. 1). In addition to the CLD, GhCYP-14 and GhCYP-51 contain RRM and zf-CCHC. The RRM domain was found in proteins involved in RNA processing where it mediates binding to various RNAs to execute both housekeeping functions and regulatory mechanisms [24]. Proteins that contain zinc fingers typically interact with DNA and RNA, and serve primarily to alter the binding specificity of a particular protein. In addition, GhCYP-14 and GhCYP-51 were predicted to be localized in the nuclear (Table 1). Therefore, these two CYPs possibly mediate ribosomal association while the CLD catalyzes peptidyl prolyl cis-trans isomerization of nascent polypeptides. GhCYP-19 and GhCYP-56 contain WD40 domain that generally serve as a rigid scaffold for protein interactions [25]. Other 10 multi-domain GhCYPs contain TPR domain, which mainly act as interactive scaffolds in the formation of protein complexes and regulators of RNA metabolism involved in the immune response [26, 27].

Regulation of gene expression via specific cis-regulatory elements in the promoter regions has evolved as a major adaptive mechanism to respond to environmental stress in plants [28]. Phytohormones are critical to the regulation of plant development and defense [29]. Thus, the analysis of the putative cis-regulatory elements relating to hormone helps to advance our understanding of GhCYPs involving stress tolerance in cotton. Six hormones (ET, MeJA, ABA, GA, SA and Auxin) responsive regulatory elements were detected in the potential promoter regions of GhCYPs (Fig. 2), indicating that GhCYPs involve different hormone-mediated signaling pathways.

In 2010, we isolated 203 ESTs from a cDNA library using suppression subtractive hybridization (SSH) with a resistant upland cotton cultivar Jimian20 induced with <i>V. dahliae</i>. Of which, an EST encoded a partial polypeptide with homology to CYP [30]. This is the first report...
that CYP is involved in the interaction between cotton and phytopathogen. In 2011, *GhCyp1* was cloned from *G. hirsutum* cv. Zhongmian 35. Overexpression of *GhCyp1* in transgenic tobacco plants conferred higher tolerance to salt stress and *P. syringae pv. tabaci* infection compared with control plants [18]. Here the RNA-seq expression analysis of the cyclophilin gene families for *G. hirsutum* revealed that many of the genes potentially play important roles in various stress response (Figs. 3 and 4). So far as we know, almost all of the reported CYP genes were involved in plant stress reactions by their up-regulation expression, such as *OsCYP19-4* and *OsCYP21-4* from rice against cold [12] and salt [13], *CcCYP* from pigeon pea against drought, salinity and extreme temperatures [14], CyPs from *Arabidopsis* against wounding [11], and several cyclophilin genes in response to phytopathogen infection [15–17]. Therefore, significantly higher expression of CYP genes in cotton, such as *GhCYP-49* and *GhCYP-52* to cold, *GhCYP-3*, *GhCYP-24*, *GhCYP-42*, *GhCYP-49* *GhCYP-57* and *GhCYP-61* to salt, *GhCYP-3*, *GhCYP-24*, *GhCYP-27*, *GhCYP-38*, *GhCYP-42*, *GhCYP-49* and *GhCYP-61* to hot, *GhCYP-3*, *GhCYP-42*, *GhCYP-49* and *GhCYP-73* to PEG, and

**Fig. 6** PPlase activity assays of recombinant GhCYP-3 in vitro. **a**, GhCYP-3 with SacI and BgIII sites was amplified by PCR and cloned into the prokaryotic expression vector pET-32a. **b**, Expression and purification of recombinant GhCYP-3 in *Escherichia coli*. Recombinant GhCYP-3 expression was induced with 1 mM IPTG for 3 h. The resulting proteins were separated by 10% SDS-PAGE and analyzed by western blot using His antibody. 34.6 kDa Recombinant TrxA-6xHis-S-tag-GhCYP-3 (THS-CYP) protein (arrows) was purified on nickel-NTA agarose columns. M, marker; Lane 1-2, empty vector pET-32a without IPTG; Lane 3-4, pET-32a- GhCYP-3 without IPTG; Lane 5, empty vector pET-32a with IPTG; Lane 6, pET-32a- GhCYP-3 with IPTG. **c**, A protease-coupled assay was used to measure PPlase activity of recombinant GhCYP-3. The prolyl cis-trans isomerization of the tetrapeptide substrate (Suc-Ala-Phe-Pro-Phe-2,4-difluoroanilide) was reflected by an increase in absorbance at 390 nm. The curves represent isomerization of the Suc-AFPF-pNA substrate over the course of 350 s in the absence of GhCYP-3 (Blank) and in the presence of 200 nM recombinant GhCYP-3 protein. Values represent the mean of three biological replicates.

**Fig. 7** Expression patterns of *GhCYP-3* in three cotton cultivars with different degrees of resistance to *V. dahliae* Linxi2-1. The expression level of *GhCYP-3* in roots was normalized to Ct value for *GhUBQ14*. Data represent mean ± SE of three determinations. Significant differences at *p* = 0.05 (*) and *p* = 0.001 (**), based on Dunnett’s multiple comparison tests.
GhCYP-3, GhCYP-5, GhCYP-10, GhCYP-17, GhCYP-18, GhCYP-20, GhCYP-22, GhCYP-23, GhCYP-28, GhCYP-30, GhCYP-33, GhCYP-46, GhCYP-48, GhCYP-51, GhCYP-54, GhCYP-59, GhCYP-62, GhCYP-64 and GhCYP-67 to *V. dahliae* suggests likely functional importance under these stress conditions. However, further functional studies are required to unravel the precise role of these candidates during cotton response to biotic and abiotic stress conditions.

GhCYP-3 contained the above-mentioned EST sequence related to *V. dahliae*-infection and showed 98% similarity in amino acid sequences with GhCyp1. Thus, GhCYP-3 potentially plays an important role in regulating cotton immune response. The results of expression (Fig. 7) and overexpression analysis (Fig. 9) further supposed that *GhCYP-3* was involved in the cotton defence to *V. dahliae*. Plant CYPs could locate in multiple cell organelles, such as ER [31], chloroplast [9], Golgi [13], cytoplasm and nucleus [32, 33]. Interestingly, the GhCYP3-GFP fusion protein was localized in the cell cytoplasm as well as the cell nucleus in onion epidermal cells (Fig. 5c). Nuclear localization was proposed to play a possible role in the regulation of gene expression [33]. GmCYP1 was as a “helper” that activates the enzymatic activity of a *Phytophthora sojae* RXLR effector Avr3b in a PPlase activity-dependent manner [34]. Furthermore, GmCYP1 was demonstrated to interact with the isoflavonoid regulators GmMYB176 and 14-3-3 protein, suggesting that it participates in isoflavonoids metabolism and plays role in defense [33]. AtCYP57 was proved to be involved in the *A. thaliana* response to *P. syringae* infection by influencing callose accumulation and *PAD4* (peptidyl arginine deiminase type 4) expression, which interacts with EDS1 (enhanced disease susceptibility 1) to provide basal immune response in plants. Nucleus location makes authors inferred that AtCYP57 could directly regulate the translation of defence genes [32]. Therefore, we inferred that GhCYP-3 play resistance function, may like AtCYP57 and GmCYP1, in the cell nucleus by directly interacting with some transcription factor to regulate the translation of defence genes, which lead to the production of antimicrobial metabolites.

Alternatively, GhCYP-3 was also located in the cytoplasm, indicating that it needs to play some extra roles, presumably including direct antifungal activity. Antifungal activities of CYPs have been reported from some plants including ginseng [35], Chinese cabbage [36], chickpea [37] and black-eyed pea [38]. In our study,

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**Fig. 8** Genetic transformations of *Arabidopsis* with *GhCYP-3* and identification of transgenic lines. **a**, Schematic representation of pBI121-GhCYP-3 vector. **b**, Kanamycin resistance screening of positive seedlings. **c**, PCR analysis of genomic DNA from Kanamycin resistant lines. **d**, Semi-qPCR confirmed expression of *GhCYP-3* in transgenic lines. **e**, PPlase activity in wild-type (WT) and two transgenic lines. Data represent mean ± SE (*n* > 20) from three independent experiments. Asterisks indicate significant differences between WT and transgenic lines (Sidak’s multiple comparisons test; ns= no significant, *P* < 0.05, **P** < 0.01). M, marker; L1 and L2, transgenic *Arabidopsis* lines; P, plasmid of pBI121-GhCYP-3 as positive control.
recombinant GhCYP-3 displayed evident inhibitory effects on *V. dahliae* on the plate (Fig. 10a). In a state of nature, the concentration of CYP in plant is hardly so high in the plate. Otherwise, there was no definite evidence to suggest that CYP is involved in plant resistance to pathogens by direct antifungal activity in vivo at present. However, we do not rule it out, because the extracts from *GhCYP-3* transgenic *Arabidopsis* displayed significantly inhibit activity to conidia germinating and hyphae growth of *V. dahliae* (Fig. 10b). Many characterized antifungal proteins active on the fungal cell wall, plasma membrane and or intracellular targets [39]. For example, Buforin 2 from the stomach tissue of *Bufo bufo gargarizans* can translocate through the plasma membrane and exhibits antifungal activity upon interaction with fungal DNA and RNA [40]. During the infection process, *V. dahliae* forms penetration peg and specialized fungus-host interface to secret secretory effector proteins [41]. This is probably a chance for antifungal proteins including GhCYP-3 produced by host plant encountering and entering into the cytoplasm of *V. dahliae*. Additionally, we identified 10 putative CYPs (VdCYPs) in *V. dahliae* strain VdLs.17 genome with very high sequence similarity with GhCYP-3 (Additional file 3: Table S3). These mean that GhCYP-3 may replace VdCYPs and carry out the same biological function in the cytoplasm of *V. dahliae*. Thus, we inferred that the antifungal activity of GhCYP-3 has been shown to be due to the effect on the normal function of VdCYPs, which is essential for the development of *V. dahliae*. Nevertheless, further research is needed to confirm this bold deduction.

**Conclusions**

This is the first systematic analysis of CYP family genes in cotton aiming to help clarifying the gene sequence characteristics and expression patterns. The putative cis-regulatory elements predication and expression divergence
suggested that the GhCYP genes are involved in multiple phytohormone regulation pathways and responses to various abiotic stress and V. dahliae infection. These results will provide potential clues for the selection of candidate genes for further in-depth study on the functional characterization. Furthermore, GhCYP-3 showed both cytoplasmic and nuclear localization. Heterologous overexpression of GhCYP-3 in Arabidopsis significantly improved Verticillium wilt resistance of the plants. Recombinant GhCYP-3 and the extracts from GhCYP-3 transgenic Arabidopsis displayed significantly inhibit activity to V. dahliae. These results indicated that GhCYP-3 was associated with resistance of cotton to V. dahliae infection presumably through antifungal activity, and it will offer an important candidate gene for Verticillium wilt tolerance in cotton molecular breeding.

Methods
Identification and characterization of CYP family genes in G. hirsutum
Genome assemblies of G. hirsutum TM-1 from Nanjing Agricultural University (NAU version 1.1 and version 2.1) and JGI (version 1.0) were retrieved from the CottonFGD website (https://cottonfgd.org/). Arabidopsis CYPs, accessed from TAIR website (https://www.Arabidopsis.org/) were used as query to identify putative CYPs in G. hirsutum genomes by local BLAST using BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). The identified GhCYPs were further verified through the Pfam database (http://pfam.xfam.org). The molecular weight (MW) and isoelectric point (pI) of each protein were calculated using ExPASy program (http://www.expasy.org). The signal peptide was predicted with the program of SignalP 4.1. The amino acid sequences were aligned with DNAMAN software (Vers. 7; Lynnon Corporation, Quebec, Canada), using default parameters. CELLO v2.5 (http://cello.life.nctu.edu.tw/) was used for the subcellular localization prediction of GhCYPs. The putative cis-acting elements in the promoter regions were predicted using NAU version 2.1 database with the Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Genome-wide expression analysis of GhCYPs
A genome-wide expression analysis of the cotton CYP genes in various abiotic stresses and V. dahliae infection was performed using high-through RNA sequence data, which was downloaded from NCBI databases (SRP044705)
and extracted from our RNA-seq data [42]. Genes with FPKM ≥ 10 were used for further expression analysis.

**Plant materials and V. dahliae strains**
The cotton seeds *G. hirsutum* cv. Ji Mian 20 (JM20), Han208, CCR18 and *G. barbadense* cv. Pima90-53 were preserved at the North China Key Laboratory for Crop Germplasm Resources of Education Ministry, Hebei Agricultural University, Baoding, China. Cotton seedlings were grown in commercial sterilized soil at 28°C /25°C (day/night) temperatures with a 16-h-light/8-h-dark regime. *A. thaliana* was grown in pots containing vermiculite soil with temperature at 23°C day and 20°C night, under a 16/8 h photoperiod. *V. dahliae* strain Linxi2-1 was isolated from a symptomatic upland cotton plants growing in agricultural fields in Linxi county, Hebei Province, China [43]. These highly aggressive defoliating *V. dahliae* strains were maintained on PDA. The conidial suspension was prepared according to previous description [44] and adjusted to 10^7 spores per milliliter and 10^8 spores per milliliter with distilled water was used to the inoculation of cotton and *Arabidopsis*, respectively. The plant was infected with *V. dahliae* soil drench method [44]. 10 mL of the conidial suspension was directly injected with a needle without piercing into the bottom of each pot. Seedlings received sterile water in the same manner were used as control.

**Gene cloning and subcellular localization**
Total RNA was extracted from leaf tissues of JM20 with an RNA plant plus reagent (TIANGEN Biotech, China). First-strand cDNA was synthesized from an aliquot of 1 μg of total RNA with a PrimeScript™ RT Reagent Kit and gDNA Eraser (TaKaRa, China). *GhCYP-3* was amplified with primers CYP-F1 and CYP-R1 (Additional file 1: Table S1), designed based on the sequences of *Gh_A01G1361* (*G. hirsutum* L. acc. TM-1) [20]. A *GhCYP-3-GFP* fusion construct under the control of the 35S promoter was generated by cloning the ORF into the *SalI* and *BamHI* sites of the binary vector pCamE. The vector expressing GFP alone served as control. Protein subcellular localization in onion (*Allium cepa*) epidermal cells was determined according to the protocol of Yang (2015) [44].

**qRT-PCR and semi-quantitative PCR**
Total RNA and cDNA were prepared by the method described above. The qRT-PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the qPCR kit for SYBR Green (TaKaRa, China). qPCR conditions consisted of one cycle of 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 58 °C, and 20 s at 72 °C. The cotton ubiquitin 14 (UBQ14) gene served as an internal standard [45]. Fold-changes in expression were calculated via the 2^−ΔCt method. Semi-quantitative RT-PCR was done with an Applied Biosystems® 2720 Thermal Cycler with *Arabidopsis AtACT2* (AT3G18780) as the internal standard [46]. The resulting products were resolved on a 1.5% agarose gel. All primers are listed in Additional file 1: Table S1 Three biological and three technical replicates were analyzed for all quantitative experiments.

**Generation and evaluation resistance of transgenic Arabidopsis**
*GhCYP-3* was cloned into pBl121 vector at the *XbaI* and *SacI* sites by PCR with primers CYP-X and CYP-S (Additional file 1: Table S1). The chimeric construct was introduced into *Agrobacterium tumefaciens* strain GV3101 for *Arabidopsis* transformation using the floral dip method [47]. Putative transformants were selected on MS (Murashige and Skoog) medium containing 50 mg L⁻¹ kanamycin, and then be further verified by PCR for gene insertion and semi-quantitative RT-PCR for gene expression. Independent T₃ transgenic lines were used to produce the T₅ generations, which were randomly chosen as representative lines and subjected to analysis. Disease severity of *Arabidopsis* plants caused by *V. dahliae* was assessed according to symptoms manifested on the leaves. The disease index (DI) was calculated as previously described [48]. Fungal recovery assay was also used to evaluate the resistance of the plant through assessment of Verticillium colonization recovered from stem sections according to the method of Fradin (2009) [49]. In each treatment, 18 individual plants were used and all the experiments were repeated thrice.

**Purification and PPIase activity assay of recombinant protein**
The *GhCYP-3* ORF was cloned into expression vector pET-32a (+) (Novagen, Darmstadt, Germany) with forward primer CYP-Bg-F and reverse primer CYP-Sa-R (Additional file 1: Table S1) which will introduce the *BglII* and *SacI* site into the 5′ and 3′ end of the ORF, respectively. To induce the expression of *GhCYP-3* protein with a His-tag in *E. coli* BL21(DE3) (TransGen Biotech, Beijing, China), a final concentration of 1.0 mmol L⁻¹ isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the culture when the OD₆₀₀ value reached 0.4–0.6, and the culture was allowed to continue growing for 4–6 h before harvesting. The proteins were separated on a SDS–PAGE gel and detected by western blot using anti-His-Tag mouse monoclonal antibody (1:5000; CW Biootech, Beijing, China). The recombinant protein was purified using a 6×His-Tagged Protein Purification Kit (CW Biotech, Beijing, China). The PPlase activity of the recombinant protein was assayed in vitro using the tetrapeptide substrate Suc-AAPF-pNA (N-succinyl-Ala-Leu-Pro-Phe-p-nitroanilide; Sigma-Aldrich, Ontario, Canada)
in a Shimadzu UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan) as described by Yoon (2016) [12]. Three biological and two technical replicates were analyzed for all measurement.

Assay of antifungal activity
The antifungal activity of the recombinant protein was tested against V. dahliae using filter paper disc diffusion method. V. dahliae spores were uniformly spread on the PDA medium plates and then cultured for 60 h at 28°C. Sterilized blank paper discs of 6 mm diameters, impregnated with tested protein, were placed on the surface of PDA medium previously spread with V. dahliae. The plates were inoculated at 25°C for 10 d. The antifungal activity of extracts from Arabidopsis plants transformed with GhCYP-3 was performed as described previously [50] with the following modifications. Conidial suspension adjusted to a density of 10^5 conidia ml\(^{-1}\), and germinated on PDA overnight at 25 °C prior to assay. Total homogenates (5 g) from ten Arabidopsis plants were prepared by directly grinding plant leaves into a fine powder in liquid nitrogen with no buffer added. Subsequently, extracts were collected by centrifugation at 10000 g for 10 min at 25 °C. Conidial suspensions (25 μl) were mixed with 225 μl of extract, and incubated for 1 h at 25°C. The mixture (50 μl) were spread onto PDA plates and incubated at 25 °C for 48 h and fungal colonies enumerated. All experiments were repeated three times independently.

Statistical analysis
All experiments were performed at least three times for each determination. Statistical analysis was performed using GraphPad Prism® 6 software (Graph Pad, San Diego, CA, USA). Unless otherwise indicated, data were evaluated by using analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test.

Additional files

**Additional file 1:** Table S1. Primers list (DOCX 15 kb)

**Additional file 2:** Table S2. The transcripts of GhCYPs with FPKM in response to abiotic stress (XLSX 22 kb)

**Additional file 3:** Table S3. Putative CYPs identified in V. dahliae strain VdLs17 (DOCX 13 kb)

Abbreviations
ABA: Abscisic acid; ABRE: Abscisic acid responsive element; CLD: Cyclophilin type PPIase domain; CYP: Cyclophilin; DI: Disease index; EDS1: Enhanced disease susceptibility 1; ERE: Ethylene responsive element; ET: Ethylene; FC: Fold change; FPKM: Fragments per kilobase of exon per million transcripts per kilobase of transcript per million mapped; GA: Gibberellin; hct: Hours after cold treated; hht: Hours after hot treated; hps: Hours after post inoculation; hpt: Hours after PEG treated; MeJA: Methyl jasmonate; MS: Murashige and Skoog; PAD4: Peptidyl arginine deiminase type 4; PDA: Potato dextrose agar; PEG: Polyethylene glycol; pl: Isoelectric point; PPIase: Peptidyl prolyl cis-trans isomerase; ROS: Reactive oxygen species; RMRT: RNA recognition motif; SA: Salicylic acid; TPR: Tetratricopeptide-like repeats; WT: Wild type

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Authors’ contributions
JY, XW and ZM designed the experiments. JY carried out the study and wrote the manuscript. GW and LJ performed the qRT-PCR and evaluated the resistance of transgenic Arabidopsis to V. dahliae. LH and CZ purified the recombinant protein and measured its antifungal activity. HK and YZ carried out the data analysis. All the authors have read and approved the publication of the manuscript.

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Availability of data and materials
The data generated or analyzed during the current study are included in this published article and its supplemental data files and available from the corresponding author on reasonable request.

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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