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A New Cotton SDR Family Gene Encodes a Polypeptide Possessing Aldehyde Reductase and 3-Ketoacyl-CoA Reductase Activities

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Abstract—To understand regulatory mechanisms of cotton fiber development, microarray analysis has been performed for upland cotton (*Gossypium hirsutum*). Based on this, a cDNA (*GhKCR3*) encoding a polypeptide belonging to short-chain alcohol dehydrogenase/reductase family was isolated and cloned. It contains an open reading frame of 987 bp encoding a polypeptide of 328 amino acid residues. Following its overexpression in bacterial cells, the purified recombinant protein specifically uses NADPH to reduce a variety of short-chain aldehydes. A fragment between Gly180 and Gly191 was found to be essential for its catalytic activity. Though the *GhKCR3* gene shares low sequence similarities to the ortholog of *Saccharomyces cerevisiae YBR159w* that encodes 3-ketoacyl-CoA reductase (KCR) catalyzing the second step of fatty acid elongation, it was surprisingly able to complement the yeast *ybr159wΔ* mutant. Gas chromatography–mass spectrometry analysis showed that very long-chain fatty acids, especially C26:0, were produced in the *ybr159wΔ* mutant cells expressing *GhKCR3*. Applying palmitoyl-CoA and malonyl-CoA as substrates, GhKCR3 showed KCR activity *in vitro*. Quantitative real time-PCR analysis indicated *GhKCR3* transcripts accumulated in rapidly elongating fibers, roots, and stems. Our results suggest that GhKCR3 is probably a novel KCR contributing to very long-chain fatty acid biosynthesis in plants.

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Key words: *Gossypium hirsutum*, short-chain alcohol dehydrogenase/reductase family, 3-ketoacyl-CoA reductase, fatty acid elongation, very long-chain fatty acids

The allotetraploid *Gossypium hirsutum* (upland cotton) represents over 95% of the annual cotton production in the world [1]. Through microarray transcriptome profiling of cotton UniESTs, a number of cDNAs were found to be preferentially expressed during fiber development [2-4]. When these genes were mapped to different metabolic pathways, fatty acid elongation was revealed to be one of the most upregulated biochemical pathways [3]. Further, very long-chain fatty acids (VLCFAs) were found to stimulate fiber elongation via activation of ethylene biosynthesis [5].

In plants, most VLCFAs are synthesized by the endoplasmic reticulum fatty acid elongation system consisting of four stepwise reactions [6]. The gene *YBR159w* encoding 3-ketoacyl-CoA reductase (KCR) catalyzing reduction of 3-ketoacyl-CoA to 3-hydroxyacyl-CoA was first identified and characterized in *Saccharomyces cerevisiae* [7, 8]. Ybr159p is a member of the short-chain alcohol dehydrogenase/reductase family (SDR), which constitutes a large protein family and exhibits a wide variety of substrate specificity for steroids, retinoids, prostaglandins, sugars, alcohols, and other small molecules [9]. Disruption of *ScYBR159w* gene (in *ybr159wΔ* mutant cells) resulted in significantly reduced VLCFA synthesis and accumulation of dihydrosphignosine, phytosphingosine, and ceramides [8]. The viability of *ybr159wΔ* mutant is due to the presence of the *AYR1* gene encoding 1-acyldihydroxyacetone-phosphate reductase [10], which is responsible for residual KCR activity [8]. Seven plant KCRs have been identified including maize GL8 [11, 12] and two isoforms each from *Arabidopsis thaliana* [7, 13], *Brassica napus* [14], and cotton [15].

Abbreviations: IPTG, isopropyl-β-D-1-thiogalactopyranoside; KCR, 3-ketoacyl-CoA reductase; MBP, maltose-binding protein; SDR family, short-chain alcohol dehydrogenase/reductase family; VLCFA, very long-chain fatty acid.

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Interestingly, applying stable isotope labeling to study *in vivo* metabolism of plant cells, a cytosolic VLCFA biosynthesis pathway was found that uses cytosolic acetyl-CoA as a starting substrate [16].

In this study we cloned a cDNA (*GhKCR3* gene) encoding a polypeptide belonging to the SDR family. Though the gene shares low sequence similarities to *YBR159w* gene orthologs from all known species, it was able to complement yeast *ybr159wΔ* mutant, but not *ayr1Δ* mutant, suggesting that it is a novel KCR involved in plant VLCFA biosynthesis.

MATERIALS AND METHODS

Plant material. Upland cotton (*Gossypium hirsutum* L. cv. Xuzhou 142) and *fl* mutant [17] cotton was grown in a fully automated greenhouse. Since the *fl* mutant has no fiber cells growing on the ovules, it is often used as a control for identification of genes expressed preferentially in fiber [18]. Wild-type cotton ovules at 10 days post anthesis (dpa), leaves, flowers, stems, roots, 10-dpa *fl* ovules, and wild-type cotton fibers stripped from 10-dpa ovules were harvested immediately and frozen in liquid nitrogen before RNA preparation.

Cloning, expression, and purification of GhKCR3 in *Escherichia coli*. Cotton *KCR3* gene was amplified from 10-dpa fiber cDNA by PCR using the following primers: 5′-CGCCATATGATGTGGATTTTTGGATG-GAAGGGAC-3′ (F1), 5′-CGCGGATCCCTACTTGG-GATTGGTCAAGGTGCAG-3′ (R1). The PCR product was digested with *Nde*I/*BamH*I and ligated into the expression vector pMAL-C2X (New England Biolabs, USA). For GhKCR3Δ-pMAL construct, the following overlapping primers, 5′-GTGTAGTATCCTCCCTCC-CTGTACGCAATCCG-3′ (R2) and 5′-CAGGGAGGG-AGGATACTACACTTGGTATGCTTACGG-3′ (F2), were designed. Two fragments were obtained by PCR using primers F1 and R2, F2 and R1. The full-length GhKCR3-pMAL was generated by PCR with F1 and R1 using the above two fragments as the templates. The constructs were sequenced and transformed into *E. coli* Rosetta strain. Transformed cells were grown to $A_{600} = 0.6$ in Luria–Bertani (LB) medium supplemented with 0.2% (w/v) glucose and 100 μg/ml ampicillin. Following isopropyl-β-D-1-thiogalactopyranoside (IPTG) (0.8 mM final concentration) induction, the cells were harvested. The pellet was resuspended and sonicated in 20 mM Tris-HCl (pH 7.4), 0.2 M NaCl. The cell lysates were centrifuged at 20,000*g* for 20 min at 4°C. The supernatant was used for purification of MBP-GhKCR3 using amylose resin as described by the manufacturer (New England Biolabs). The purified proteins were analyzed by SDS-PAGE and used for activity assay.

Aldehyde reductase activity assay. Aliphatic aldehydes with different carbon length were purchased from J&K Chemical Ltd. (China) for the reductase assay. The reaction was carried out in a sealed quartz cuvette with 0.5 ml buffer (100 mM potassium phosphate, pH 7.2, 200 mM NaCl, 1 mM dithiothreitol, 2 mg/ml bovine serum albumin, and 1% glycerol) containing 150 μM NADPH and 60 μM substrate. The reaction was initiated by addition of 10 μg MBP-GhKCR3 and run until a steady-rate was achieved for about 1 min. The reaction was monitored by measuring the decreasing of NADPH absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}\cdot cm^{-1}$). Kinetic constants were calculated with CurveExpert 1.38 (Hixson, USA), using the Michaelis–Menten equation for curve fitting. The same system was applied for the determination of reductase activity with NADH. The dehydrogenase activity was assayed using $NADP^+$ or NAD^+ as the cofactor and nonanol as the substrate.

Functional complementation of *GhKCR3* in yeast *ybr159wΔ* mutant cells. The *S. cerevisiae* haploid strain BY4742 *ybr159w* (MATa; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YBR159w::kanMX4) and BY4742 *ary1* (MATa; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; ayr1::kanMX4) were ordered from EUROSCAPF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). The plasmid pYTV-*GhKCR3* was constructed according to the gateway cloning method [15]. The *ybr159wΔ* or *ary1Δ* mutant cells transformed with pYTV-*GhKCR3* plasmid were cultured in SC-Ura medium containing 2% glucose. Expression of *GhKCR3* gene was induced by 2% galactose. The growth rates were determined by spotting assay on SC-Ura plates. The wild-type BY4742 and mutant cells transformed with the empty vector pYTV were prepared as controls.

GC-MS analysis of VLCFAs. Yeast cells were harvested when the A_{600} of the culture reached 0.8. The cells were washed with sterile distilled water. Fatty acids were extracted and converted to methyl esters according to the method described by Cahoon and Lynch [19]. The resulting methyl esters were separated on a DB-225MS column on the Agilent 6890N GC system coupled to an HP5973 mass detector. Fatty acid C17:0 was added before extraction as an internal standard for monitoring sample recovery and quantification.

VLCFA elongase activity assay. VLCFA elongase activity was measured in a volume of 200 μl containing 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 150 μM Triton X-100, 1 mM NADPH, 1 mM NADH, 10 mM β-mercaptoethanol, 40 μM palmitoyl-CoA, and 60 μM 2-^{14}C-labeled malonyl-CoA (0.05 μCi/ml) at 37°C. The reaction was initiated by the addition of 0.25 mg of microsomal proteins. Protein concentration was determined by the Lowry method using bovine serum albumin as the standard. At various times (0, 5, and 30 min) the reactions were stopped by adding 100 μl 75% KOH and 200 μl methanol, saponified at 70°C for 1 h, and acidified by adding 400 μl of 5 N HCl and 200 μl ethanol. Fatty acids were recovered by three extractions with 1 ml hexane each. The extracts were dried under nitrogen and separat-

ed by silica gel TLC using hexane–diethyl ether–acetic acid (30 : 70 : 1) as the developing solvent. Relative positions of fatty acids on TLC were determined by comparing the mobility with authentic standards as described [7].

RNA extraction and QRT-PCR. Total RNA was extracted from wild-type cotton ovules, fibers, flowers, leaves, stems, roots, and *fl* ovules [3, 18]. Cotton cDNA was reverse-transcribed from 5 μg of total RNA. QRT-PCR was performed with *GhKCR3* specific primers: 5′-GCAACTCCTTTCATGTTATCTCAA-3′ and 5′-GTA-ATCTCCACCTCTTCTTCCTTT-3′. Using independent RNA samples, the reactions were run in triplicate with the SYBR green PCR kit (Applied Biosystems, USA) in a DNA Engine Opticon-Continuous Fluorescence Detection System (MJ Research, USA). The cotton ubiquitin gene *UBQ7* was used as internal control in each reaction.

RESULTS AND DISCUSSION

The cotton cDNA named *GhKCR3* encodes a polypeptide belonging to the SDR family. The full-length cDNA (*GhKCR3*) contains a 948 bp open reading frame encoding a polypeptide of 316 amino acids with a predicted molecular mass of 34.5 kDa. It was submitted to GenBank with accession number AY902468. Analysis of GhKCR3 amino acid sequence reveals that it contains a Rossmann fold (coenzyme-binding domain), GXXXGXG, and a catalytically important Ser-Tyr-Lys triad (Fig. 1) characteristic of the SDR family [9]. By sequence comparison, most homologs of GhKCR3 were identified from PDB (Fig. 1). The fragment between residues Gly180 and Gly191 was revealed to be an extended loop structure for GhKCR3 (Fig. 1).

GhKCR3 reduces a variety of short-chain aldehydes in a NADPH-dependent manner. To characterize GhKCR3

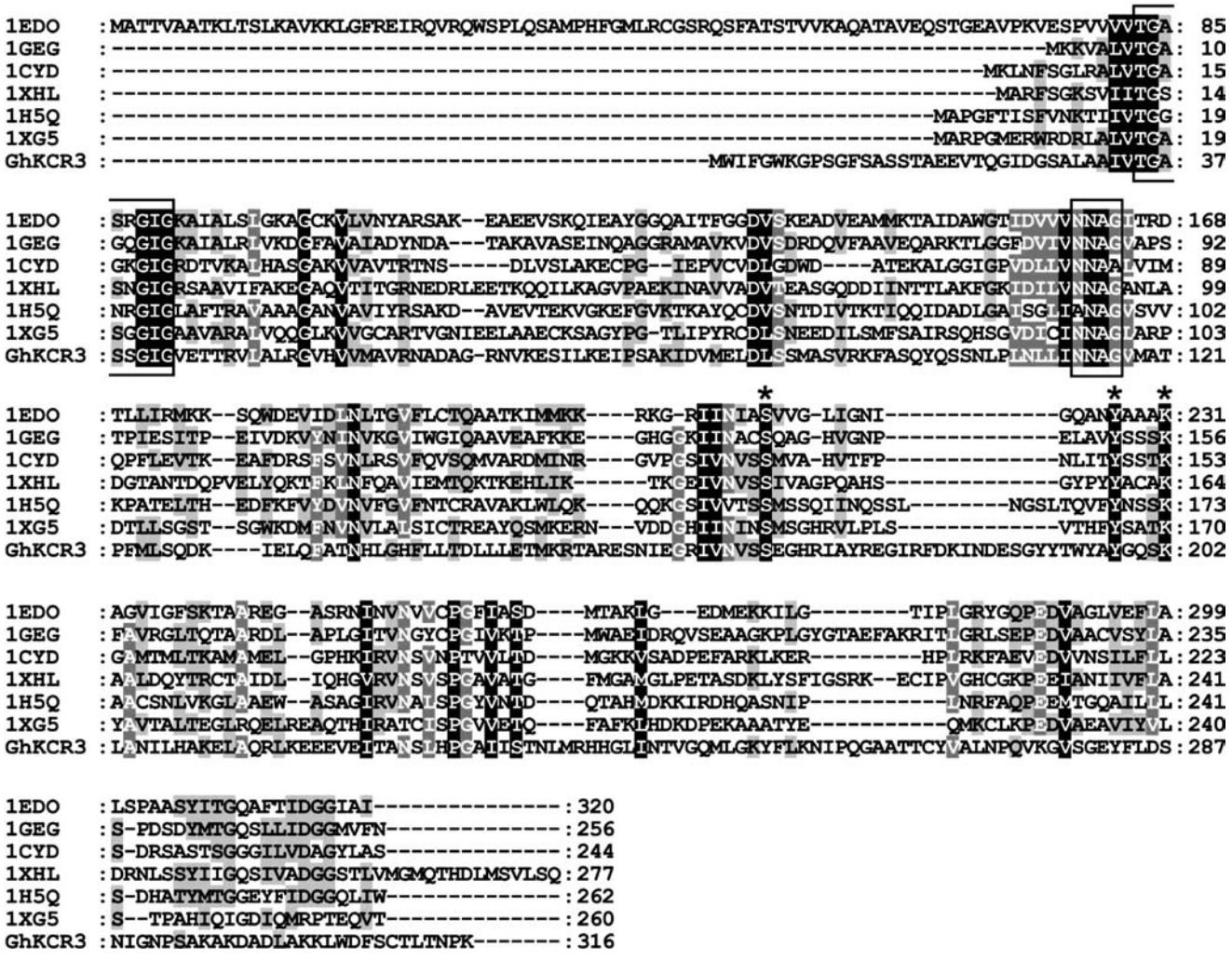

Fig. 1. Amino acid sequence alignment of GhKCR3 with most of the homologs identified from PDB with the following annotations: 1EDO (β-keto-ACP reductase), 1GEG (acetoin reductase), 1CYD (carbonyl reductase), 1XHL (short-chain dehydrogenase/reductase), 1H5Q (mannitol dehydrogenase), and 1XG5 (human putative dehydrogenase). Conserved motifs of the SDR family are highlighted with boxes (TGXXXGXG and NNAG motifs). The catalytically important S-Y-K triad is presented with asterisks.

and study the role of the identified extended loop, full-length and the truncated version of the gene were expressed as fusion protein with maltose-binding protein (MBP) in *E. coli* and induced by IPTG. The purified fusion protein was analyzed by SDS-PAGE (Fig. 2a). The molecular masses of the recombinant wild-type protein and the mutated protein were about 74 kDa, which agreed with their theoretical masses of 34.5 and 33.2 kDa, respectively, plus 40 kDa of MBP. Enzyme activity was measured by the substrate-dependent oxidation of NADPH as determined by a decrease in absorbance at 340 nm. The enzyme activity of GhKCR3 increased from propanal to tridecanal (Fig. 2b). The activity of the mutated GhKCR3 towards the same substrates was below detection limit, implying that the fragment between Gly180 and Gly191 is essential for the enzyme activity. Nonanal was selected for detailed study. The apparent K_m values for NADPH and nonanal were 12 and 26 μM, and their k_{cat} values were 17 and 12 min^{-1}, respectively (Fig. 2c). The enzyme was inactive with the aldehydes tested in the presence of NADH, indicating that it is NADPH-dependent. No reduction of $NADP^+$ or NAD^+ was detected in the presence of nonanol, suggesting the enzyme is aldehyde reductase, but not alcohol dehydrogenase.

GhKCR3 was able to rescue the yeast *ybr159wΔ* mutant, resulting in restoration of fatty acid elongation activity. Sequence alignment of GhKCR3 with *S. cerevisiae* protein sequences indicated ScYbr159p to be a homolog. As shown in Fig. 3a, *S. cerevisiae* haploid *ybr159wΔ* and *ayr1Δ* mutants were growing slowly on Sc-Ura plates. When *GhKCR3* was overexpressed in the mutant cells, wild-type growth rate was rescued in *ybr159wΔ* mutant but not in *ayr1Δ* mutant, suggesting that *GhKCR3* was able to complement the genetic deficiency caused by the disruption of the *YBR159w* gene.

Subcellular localization of GhKCR3 in yeast cells was studied. The insoluble membranous fractions (pellet) of endoplasmic reticulum were prepared from the wild-type yeast cells BY4742 transformed with either empty vector pYTV or pYTV-*GhKCR3* and further analyzed by immunoblotting using anti-His_6 and anti-ScKar2p,

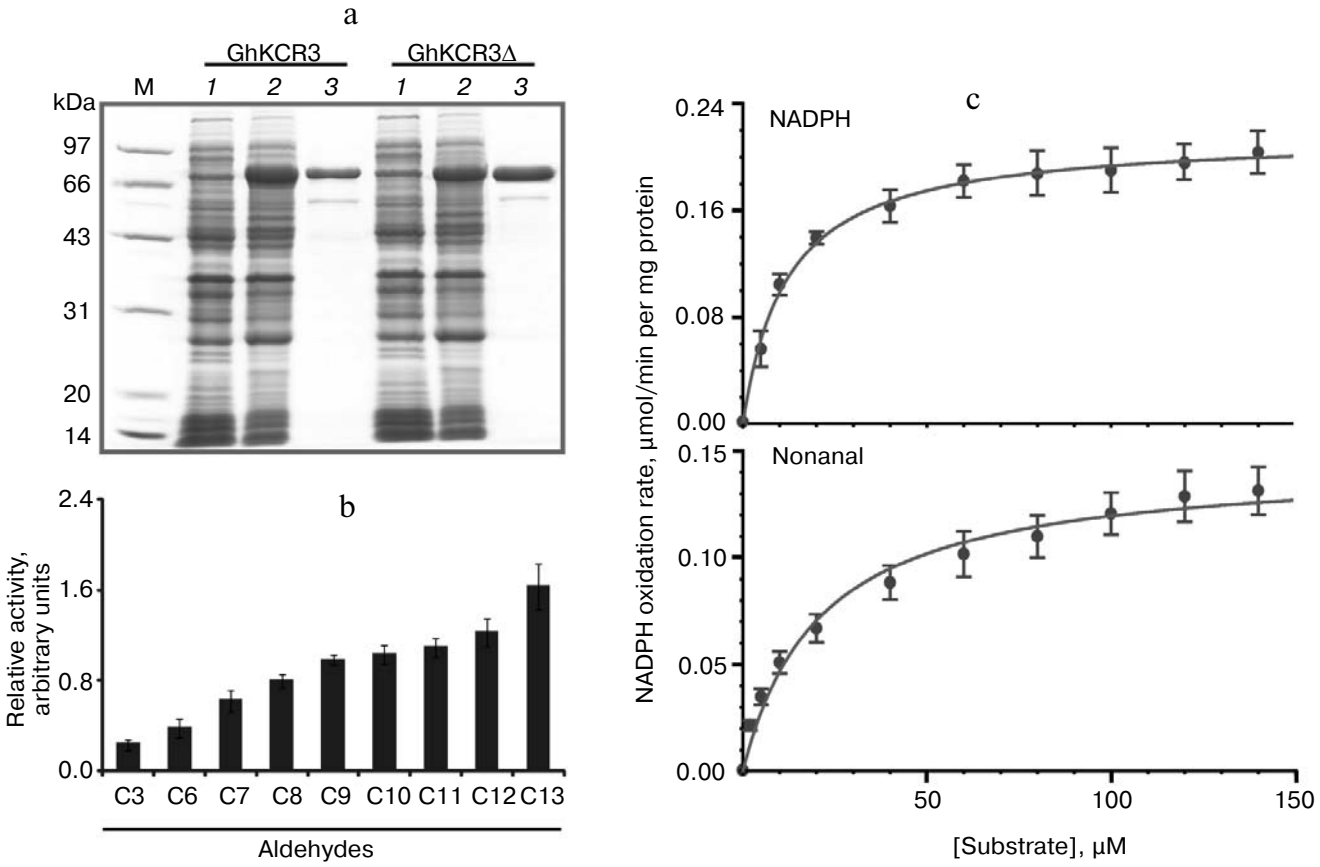

Fig. 2. Characterization of recombinant GhKCR3. a) SDS-PAGE of recombinant GhKCR3 and its truncated version GhKCR3Δ purified from *E. coli*. Lanes: *1*) total protein from uninduced cells; *2*) total protein from cells induced by IPTG; *3*) purified recombinant GhKCR3 or GhKCR3Δ; M, molecular mass standards. b) Aldehyde reductase assays with C3 to C13 aliphatic aldehydes. c) Dependence of aldehyde reductase activity of GhKCR3 on concentrations of NADPH and nonanal. Results are the mean ± SD of triplicate determinations from independent samples.

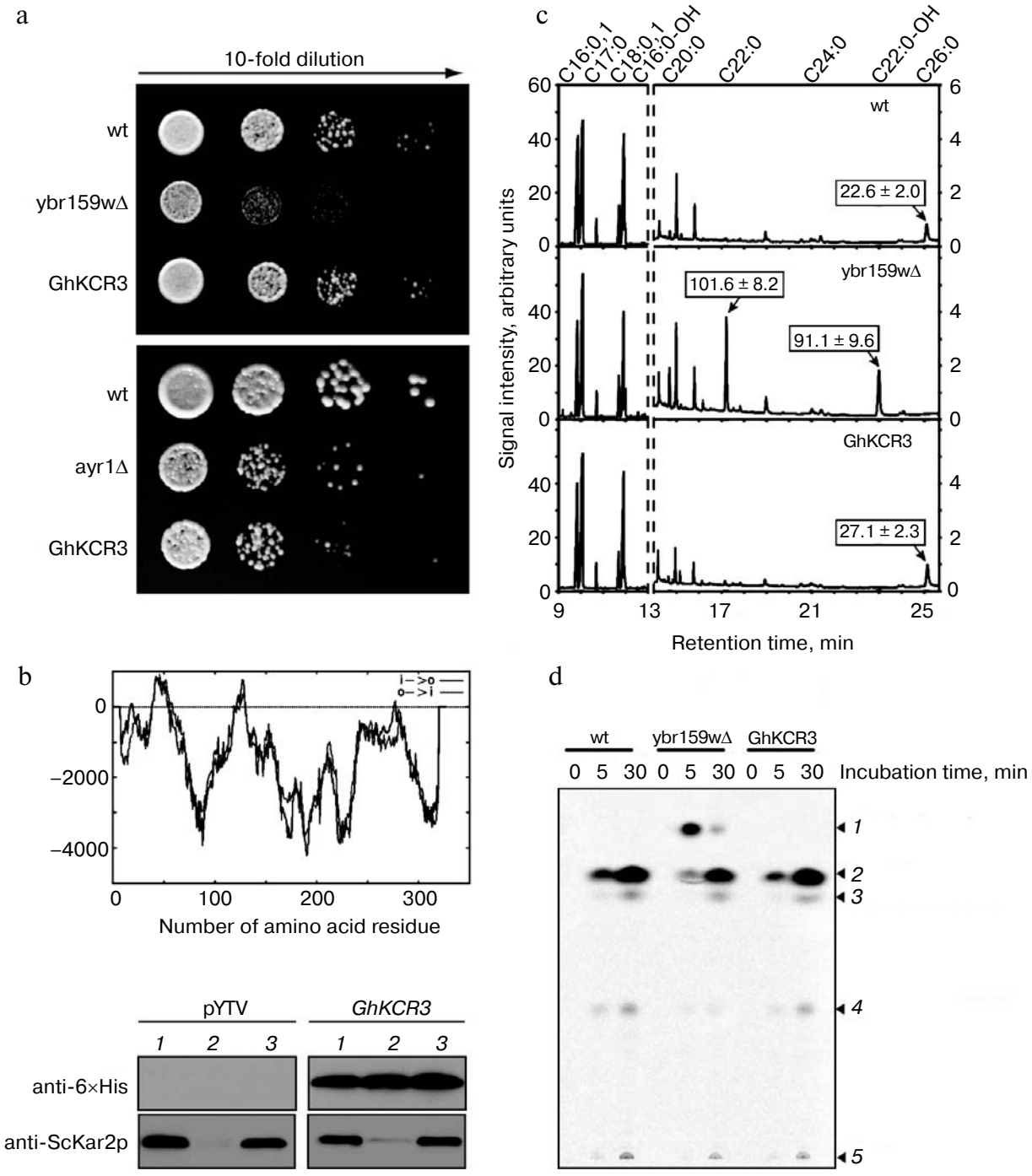

Fig. 3. Complementation of yeast *ybr159wΔ* mutant by *GhKCR3*. a) The growth rate of yeasts on Sc-Ura plates. wt, wild type; *ybr159wΔ* and *ayr1Δ*, mutants; GhKCR3, mutant expressing GhKCR3 protein. b) Membrane-spanning topology for GhKCR3 (upper part) was plotted with the TMpredict program. For Western blot analysis (lower part), antibodies against His_6 sequence of hybrid recombinant protein GhKCR3 (anti-6×His) and against marker protein of endoplasmic reticulum membranes (anti-ScKar2p) were used. Left (pYTV), proteins extracted from wild-type (wt) yeast cells transformed with an empty vector pYTV. Right (GhKCR3), proteins extracted from *ybr159wΔ* mutant cells transformed with pYTV-*GhKCR3*. *1*) Cell lysate; *2*) supernatant; *3*) pellet. c) GC-MS analyses of fatty acids extracted from wild-type, *ybr159wΔ*, and *ybr159wΔ* complemented by *GhKCR3*. Numbers in rectangles are values of corresponding peak areas. d) *In vitro* assay of fatty acid elongase. For TLC analysis, fatty acyl-CoA thioesters were extracted and hydrolyzed to free fatty acids. *1*) 3-Ketostearate; *2*) stearate; *3*) trans-2-stearate; *4*) 3-hydroxystearate; *5*) origin.

respectively. GhKCR3 protein was detected in both pellet and supernatant fractions, whereas the endoplasmic reticulum marker ScKar2p [20] was present only in the pellet (Fig. 3b). Gas chromatography–mass spectrometry (GC–MS) analysis indicated that the *ybr159wΔ* cells accumulated VLCFAs (C22:0 and C22:0-OH), and the mutant cells rescued by *GhKCR3* restored biosynthesis of C26:0, similar to the profile of the wild-type yeast (Fig. 3c).

To address how GhKCR3 affects fatty acid elongation in the *ybr159wΔ* cells, yeast microsomes were prepared and assayed for the elongase activity *in vitro* as previously described [15]. As expected, accumulation of 3-ketostearate and a smaller amount of the fully elongated product, stearate, was detected in the reactions catalyzed by the *ybr159wΔ* microsomes, whereas 3-ketostearate was not detectable and stearate increased in the reaction catalyzed by *GhKCR3* complementing *ybr159wΔ* microsomes (Fig. 3d). When both NADPH and NADH were omitted, the reactions were stopped at the intermediate 3-ketostearate [15]. In the absence of NADPH but with NADH, no obvious differences in the amount of intermediates and the final products generated using extracts from *ybr159wΔ* mutant transformed with or without *GhKCR3* were observed (data not shown), indicating that the reduction catalyzed by KCR was NADPH-dependent. The data demonstrated that GhKCR3 was functionally expressed as a 3-ketoacyl-CoA reductase in yeast cells, similar to the properties of GhKCR1 and GhKCR2 [15] as well as AtKCR1 [13]. However, GhKCR3 shares low amino acid sequence similarities to the orthologs of ScYbr159p. It shared 14% sequence identity to that of ScYbr159p, 10-14% to GhKCR1 and GhKCR2, 8-11% to AtKCR1 and AtKCR2, BnKCR1 and BnKCR2, ZmGL8a and ZmGL8b, and 9-11% to MmKCR and HsKCR (data not shown).

The neighbor-joining bootstrap tree was constructed using plant and mammalian ScYbr159p orthologs, bacterial and plant 3-ketoacyl-ACP reductases (FASII), yeast Ayr1p, mouse short-chain aldehyde reductase (MmSCALD), bacterial fatty aldehyde reductase (MaFALDR), PsTic32 homologs, and fatty acyl-CoA reductase (AtCER4) (Fig. 4). Both MmSCALD and MaFALDR are NADPH-dependent short-chain aldehyde reductases [21, 22]. GhKCR3 was shown to be distinct from other characterized KCRs and SCALD, and did not show fatty acyl-CoA reductase activity towards palmitoyl-CoA (data not shown). Its *Arabidopsis* homolog At4g11410 was not expressed in seeds, leaves, and roots and its function is not clear [23]. At4g23430 and At4g23420 were found to be homologs of pea Tic32 acting as an important components in chloroplast import machinery, but the molecular substrate of Tic32 is still unknown [23]. Whether GhKCR3 is directly associated with the fatty acid elongation system located in endoplasmic reticulum is still an intriguing question, because only part of the recombinant GhKCR3 was cosedimented in fractions of endoplasmic reticulum (Fig. 3b) and no endoplasmic reticulum-targeting signal sequence was present in the amino sequence of GhKCR3 (Fig. 1). GhKCR3 does not possess 3-ketoacyl-ACP reductase activity (data not shown), excluding the possibility that GhKCR3 participates in plastid *de novo* fatty acid synthesis pathway. GhKCR3 is possibly involved in a cytosolic fatty acid elongation system, which has been proposed in developing seeds of *Brassica napus* [16], but this hypothesis requires further investigation.

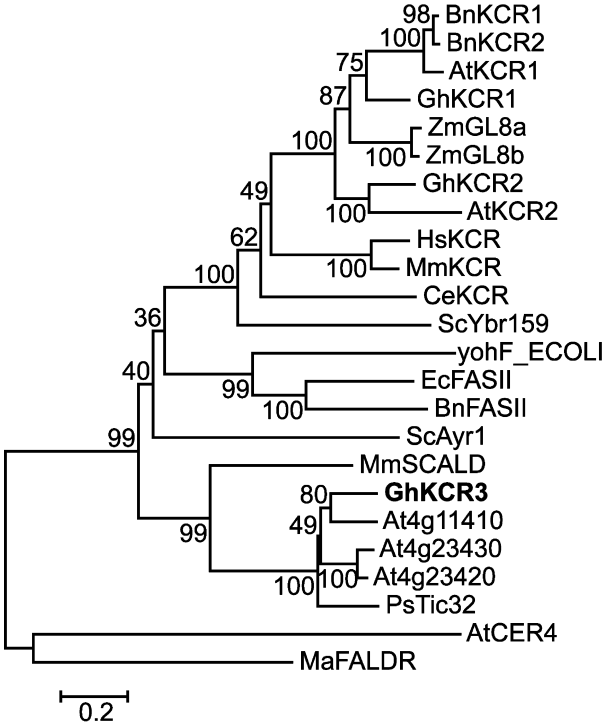

Fig. 4. Analysis of GhKCR3 by phylogenetic comparison. A neighbor-joining bootstrap tree was constructed from the sequence alignment of ScYbr159p orthologs, 3-ketoacyl-ACP reductases (FASII), yeast Ayr1p, short-chain aldehyde reductase (mouse SCALD and *M. aquaeolei* FALDR), PsTic32 homologs, and *Arabidopsis* CER4. The scale represents 0.2 changes per site. Sequences used here can be found in GenBank/EMBL database under accession numbers: AY902468 (GhKCR3), AY902466 and AY902467 (GhKCR1 and -2), AAO43448 and AAO43449 (BnKCR1 and -2), NY143811 and NM102292 (AtKCR1 and -2), AF302098 and AF527771 (GL8a and -b), AY557868 (ScYbr159), NP_498386 (CeKCR), AAP36605 (HsKCR), NM_00829 (MmKCR), CAQ32542 (yohF_ECOLI), NP_415611 (EcFASII), Q93X62 (BnFASII), AAZ22503 (ScAyr1), AAL79910 (MmSCALD), YP_959486 (MaFALDR), AAS38575 (PsTic32), NP_567936 (AtCER4).

***GhKCR3* was expressed in cotton tissues.** The expression levels of *GhKCR3* in various cotton tissues were quantified by quantitative real time-PCR (QRT-PCR). *GhKCR3* was expressed in all of the cotton tissues tested, including fast elongating fibers, ovules, roots, stems, leaves, and flowers (Fig. 5). The double mutant of maize paralogs *gl8a* and *gl8b* was found to be lethal during seed development [24], and the loss of *Arabidopsis KCR1* resulted in embryo lethality [13], indicating VLCFAs are essential for embryo development. Based on the above reasoning that *GhKCR3* was possibly involved in VLCFA biosynthesis, *GhKCR3* might serve as a ubiquitous component of a cytosolic fatty acid elongation system.

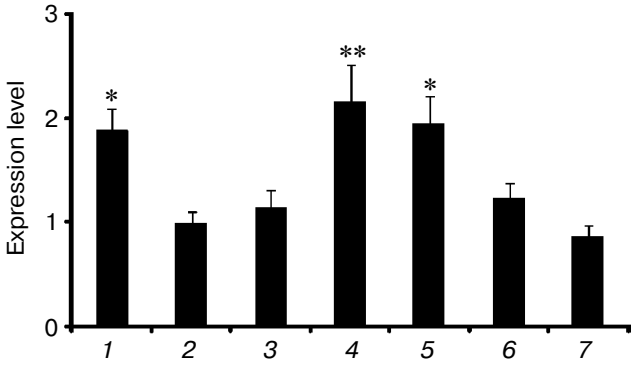

Fig. 5. Quantitative real-time PCR analysis of the transcript levels of *GhKCR3* expressed in variable cotton tissues. Total RNA samples prepared from wild-type 10-dpa cotton fibers [10wt(F)], ovules [10wt(O)], *fl* mutant cotton ovules (10fl), and variable cor QRT-PCR analysis. The fold-increase of *GhKCR3* expression was shown to be relative to 10-dpa wt ovules (arbitrarily set to 1). *1*) 10wt(F); *2*) 10wt(O); *3*) 10fl; *4*) root; *5*) stem; *6*) leaf; *7*) flower. Statistical significance was determined using one-way ANOVA software combined with Tukey's test throughout this figure. * $p < 0.01$; ** $p < 0.001$.

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