Cloning and functional characterization of two cDNAs encoding NADPH-dependent 3-ketoacyl-CoA reductase from developing cotton fibers

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ABSTRACT

Genes encoding enzymes involved in biosynthesis of very long chain fatty acids were significantly up-regulated during early cotton fiber development. Two cDNAs, *GhKCR1* and *GhKCR2* encoding putative cotton 3-ketoacyl-CoA reductases that catalyze the second step in fatty acid elongation, were isolated from developing cotton fibers. *GhKCR1* and 2 contain open reading frames of 963 bp and 924 bp encoding proteins of 320 and 307 amino acid residues, respectively. Quantitative RT-PCR analysis showed that both these genes were highly preferentially expressed during the cotton fiber elongation period with much lower levels recovered from roots, stems and leaves. *GhKCR1* and 2 showed 30%-32% identity to *Saccharomyces cerevisiae* Ybr159p at the deduced amino acid level. These cotton cDNAs were cloned and expressed in yeast haploid *ybr159wΔ* mutant that was deficient in 3-ketoacyl-CoA reductase activity. Wild-type growth rate was restored in *ybr159wΔ* cells that expressed either *GhKCR1* or 2. Further analysis showed that *GhKCR1* and 2 were co-sedimented within the membranous pellet fraction after high-speed centrifugation, similar to the yeast endoplasmic reticulum marker ScKar2p. Both *GhKCR(s)* showed NADPH-dependent 3-ketoacyl-CoA reductase activity in an *in vitro* assay system using palmitoyl-CoA and malonyl-CoA as substrates. Our results suggest that *GhKCR1* and 2 are functional orthologues of ScYbr159p.

Keywords: very-long-chain fatty acids, endoplasmic reticulum, fatty acid elongation system, 3-ketoacyl-CoA reductase, *Gossypium hirsutum*, short-chain alcohol dehydrogenase/reductase protein family.

INTRODUCTION

Cotton fibers are seed trichomes of ovule epidermis that have significant economical importance in textile production. Fiber properties such as length, strength, uniformity and yarn spinning efficiency are no longer entirely satisfactory predictors as our society progresses forward. Instead, surface friction forces that resulted directly from the chemical constituents of fiber cells play an important role in modern costume industry [1]. As derivatives of very long chain fatty acids (VLCFA, fatty

acids >C18), waxes are major components of cotton fiber cuticle [2] and display inverse relationships with micronaire, a measure of fiber linear density and fineness [3]. Recently, it has been reported that an ABC transporter from *Arabidopsis thaliana* mediated transport of lipids out of epidermal cells to the plant surface for biosynthesis of waxy cuticle [4].

Biosynthesis of fatty acids is a primary metabolic event representing a vital aspect for every cell of all organisms to survive [5]. Plants synthesize fatty acids within the plastids, and use a discrete and highly conserved group of dissociated enzymes named fatty acid synthase system type 2 (FASII) [6]. This system, similarly to the eukaryotic mitochondrial FAS [7-10], differs from the mammalian cytosolic fatty acid synthase system type 1 (FASI) where all reactions are carried out by a single multifunctional polypeptide [11]. Fatty acids synthesized within plastid are

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exported to endoplasmic reticulum (ER) for elongation, resulting in the formation of VLCFAs that are essential cellular precursors of waxes, seed storage lipids and sphingolipids [5]. Biosynthesis of VLCFA in plants occurs on the cytosolic face of the ER membrane and is carried out by a membrane-bound fatty acid elongation system (FAE). The first reaction involves condensation of malonyl-CoA with a long chain acyl substrate producing a 3-ketoacyl-CoA, catalyzed by 3-ketoacyl-CoA synthase (KCS). Subsequent reactions include reduction to 3-hydroxyacyl-CoA catalyzed by 3-ketoacyl-CoA reductase (KCR) and dehydration to an enoyl-CoA by 3-hydroxyacyl-CoA dehydratase, followed by a second reduction catalyzed by *trans*-2-enoyl-CoA reductase to form the elongated acyl-CoA [12]. A number of plant KCS(s) that are all specific to saturated and monounsaturated fatty acids have been identified and partially characterized [2]. An orthologue of *Saccharomyces cerevisiae* *trans*-2-enoyl-CoA reductase was found in *A. thaliana* [13]. To date, 3-hydroxyacyl-CoA dehydratase has not been identified from any sources.

3-Ketoacyl-CoA reductase encoded by *S. cerevisiae* *YBR159w* catalyzes the second important step of VLCFAs biosynthesis [14, 15] and is a member of the short-chain alcohol dehydrogenase reductase family (SDR) [16]. Disruption of *ScYBR159w* (*ybr159wΔ*) resulted in significantly reduced VLCFA synthesis and accumulation of dihydrosphingosine, phytosphingosine and ceramides [15]. Four plant KCRs including maize *YBR159* (GL8) [17, 18], *A. thaliana* *YBR159* [14] and two isoforms of *Brassica napus* [19] were found to be orthologues of yeast *Ybr159p*. The *GL8* mutant maize displayed defects in the biosynthesis of the cuticular waxes deposited on the outer epidermis, rendering the plant a characteristic “glossy” phenotype [17]. In this study, we identified two cDNAs from developing cotton fiber cells. We characterized these cotton proteins by functional complementation of a yeast mutant, *ybr159wΔ*. Results obtained from biochemical assays indicated that the cotton gene products possess NADPH dependent 3-ketoacyl-CoA reductase activities that are important for VLCFAs biosynthesis.

MATERIALS AND METHODS

Library screen, sequencing and phylogenetic analyses

Complementary DNA library was constructed by using total RNA extracted from 10 dpa cotton (*Gossypium hirsutum* L.cv. Xuzhou 142) fiber cells [20]. The library was screened using the *AtYBR159w* [14] coding sequence as a probe at low stringency as previously described [21]. Putative full-length *GhKCR1* and *GhKCR2* cDNAs were obtained by sequencing all colonies that hybridized to the probe from both ends.

QRT-PCR analysis of *GhKCR(s)* expression patterns

Real time quantitative RT-PCR (QRT-PCR) was carried out us-

ing the SYBR green PCR kit (Applied Biosystems) in a DNA Engine Opticon-Continuous Fluorescence Detection System (MJ Research). Following PCR primers were used: *GhKCR1*, 5'-CACTTTGGGT-TCTTTATCACTCTT-3' (forward, F) and 5'-TTTATCTTCT-TCACGCCTTCAT-3' (reverse, R); *GhKCR2*, 5'-TATCCTTTCT-CACCCGCTCC-3' (F) and 5'-CACCCTTCCTC CTCCACCTC-3' (R). Each pair of primers produced a single DNA fragment of appropriate size in any stages of fiber development, different cotton tissue and mutant cotton ovules. All QRT-PCRs were carried out with following parameters: initiation with a 10 min denaturation at 95°C, followed by 42 cycles of amplification with 10 sec denaturation at 94°C, 20 sec annealing according to the melting temperature of each pair of primers, 20-30 sec extension at 72°C, then read the plate for fluorescence data collection at 78-80°C. After a final extension at 72°C for 5-10 min, melting curve was performed from 65°C to 95°C (1 sec hold per 0.2°C increasing) to check the specificity of the amplified product. Samples were analyzed in triplicates using independent RNA samples and were quantified by the comparative cycle threshold method [22].

Functional complementation of yeast *ybr159wΔ* mutant strain by cotton enzymes

ORFs of *GhKCR1* and *GhKCR2* were amplified using the following primers: 5'-CACCACAAAATGGAAGCCTGCTTCTTC-GATACT-3' (F1) and 5'-TTCCTTCTTCTAGAACTTTTCAT-3' (R1); 5'-CACCACAAAATGCAGCCCACATGGTTGTTAGC-3' (F2) and 5'-CGCAAGGACCTCTGCTCGCC TTCG-3' (R2). The sequence “CACC” at the beginning of each forward primer is required for cloning into pENTR TOPO® vector (Invitrogen) and the sequence “ACAAA” is used to enhance gene expression in yeast. The constructed vectors, pENTR TOPO::*GhKCR1* and pENTR TOPO::*GhKCR2* were verified by sequence analysis. The genes were transferred from pENTR TOPO to yeast expression vector pYTV [23] via LR reaction, using homologous sequences present on both vectors, resulting in pYTV::*GhKCR1* and pYTV::*GhKCR2*. *Gal1* promoter was fused at the N-terminus and 3× FLAG, 6× His and 2× IgG were present to facilitate detection of the fusion protein [23].

The *S. cerevisiae* haploid strain BY4741 *ybr159wΔ* (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *ybr159w::kanMX4*, EUROSCARF) was grown on YPD (1% [wt/v] yeast extract, 2% [wt/v] peptone, and 2% [wt/v] D-glucose) supplemented with 300 μg/ml of Geneticine. pYTV::*GhKCR(s)* were transformed into *ybr159wΔ* mutant cells. Wild-type strain BY4741 and *ybr159wΔ* mutant cells were transformed with plain pYTV separately and were used as controls. The transformants were selected on synthetic complete medium lacking uracil (Sc-Ura) plates. The growth rates of wild-type BY4741 transformed with pYTV, *ybr159wΔ* mutant transformed with pYTV, and those transformed with pYTV::*GhKCR(s)* for overexpression in *ybr159wΔ* were examined on synthetic complete medium lacking tryptophan (Sc-Trp) containing 2% [wt/v] galactose and 0.05% [wt/v] D-glucose.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from yeast cells transformed by pYTV::*GhKCR(s)* using RNeasy Mini Kit (Qiagen) and cDNA was synthesized from 5 μg total RNA using the SUPERScript™ first-strand synthesis system for RT-PCR (Invitrogen). RT-PCR primers were the same as described in the previous section for ORF cloning. Yeast actin gene, *ACT1* (Accession Number NP_116614), was used as internal control in parallel reactions.

Preparation of ER extracts from yeast cells

Yeast cells transformed by pYTV or pYTV::GhKCR(s) were grown to exponential phase ($2-4 \times 10^6$ cells/ml) in Sc-Ura medium containing 2% (wt/v) galactose and 0.05% (wt/v) D-glucose at 30°C. The cells were harvested by centrifuging for 15 min at 1300 g, 4°C. The cell pellets were washed with sterile H₂O, and suspended in ice-cold lysis buffer containing 50 mM Tris, pH 7.5, 1 mM EGTA, 0.5 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 μ g/ml pepstatin A. The cells were disrupted with glass beads and centrifuged for 15 min at 15000 g, 4°C to remove the cell debris. The supernatants were centrifuged for 90 min at 85,000 g in a Sorval Ti70 rotor at 4°C to generate pellet (P85) and supernatant (S85) fractions. P85 was suspended in lysis buffer and used for elongase assay. The protein concentrations were determined by the method of Lowry *et al* [24], using bovine serum albumin as the standard. Equivalent amount of total lysate, P85 and S85 were precipitated by adding trichloroacetic acid (TCA) to 10% final concentration and processed for immunoblotting.

Immunoblotting

Equivalent amount of total lysate, high speed supernatant (S85) and membrane pellet (P85) were separated on 12% SDS-PAGE and electro-transferred to PVDF Hybond^p membrane (Amersham Biosciences). Recombinant GhKCR(s) were detected by using mouse monoclonal antibody against His-tag as the primary antibody (Invitrogen) and affinity-purified goat anti-mouse IgG conjugated with horseradish peroxidase as the second antibody. *S. cerevisiae* ER marker protein Kar2p [25] was detected by using polyclonal rabbit antibody against ScKar2p [26], a gift from M. Rose, as the primary antibody and affinity-purified goat anti-rabbit IgG conjugated with horseradish peroxidase as the second antibody (Bio-Rad Laboratories). The recognized epitopes were detected by using the ECL Western blotting detection system (Amersham Biosciences).

Elongase assays of cotton 3-ketoacyl-CoA reductases over-expressed in yeast cells

Palmitoyl-CoA was used as acyl-CoA acceptor in all reactions. The elongation assays were divided into two groups. One was supplemented with both NADPH and NADH [15], and another was supplemented only with NADH. The elongation assays contained 50 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 0.15 mM Triton X-100, 1 mM NADPH, 1 mM NADH, 10 mM β -mercaptoethanol, 40 μ M palmitoyl-CoA, 60 μ M [2-¹⁴C] malonyl-CoA (6.5 dpm/pmol, PerkinElmer Life Sciences) in a final reaction volume of 0.2 ml. To initiate the elongation reaction, 0.05 mg ER protein extracted from yeast cells was added. The reaction was incubated at 30°C and continued for the indicated time. The reactions were stopped by adding 0.1 ml of 75% KOH (w/v) and 0.2 ml of ethanol, saponified at 70°C for 1 h, and then acidified by adding 0.4 ml of 5 N HCl with 0.2 ml of ethanol. Fatty acids were collected using 1 ml of hexane. The extractions were dried under nitrogen, and separated by thin-layer chromatography (TLC) using hexane/diethyl ether/acetic acid (30:70:1). The TLC plates were exposed to a PhosphorImager screen, the resulting image was analyzed, and the lipids were quantified using a Bio-Imaging Analyzer with 2D Image master software (Amersham Biosciences).

Hydropathy analysis

Hydropathy plot for each GhKCR gene was produced according to the method described previously [27].

RESULTS

Identification of two cotton 3-ketoacyl-CoA reductases

A total of 19 colonies were obtained as a result of library screen with 15 found to encode for GhKCR1 and 4 for GhKCR2 after sequencing of the full-length insert. GhKCR1 possessed a 963 bp ORF that encoded a protein of 320 amino acids with a predicted molecular mass of 36 kD. GhKCR2 contained a 924 bp ORF that encoded a protein of 307 amino acids with a predicted molecular mass of 34 kD. We submitted GhKCR1 and 2 to GenBank with accession numbers AY902466 and AY902467, respectively. As shown in Fig. 1, GhKCR1 shared 68% sequence identity to that of AtYBR159 and Bn-KCR2, 66.7% to BnKCR1, 65% to ZmYBR159, 30% to ScYbr159p, 39% to human HsKCR and 41% to mouse MuKCR. GhKCR2 shared 59% total sequence identity with that of GhKCR1 and showed similar or slightly lower amino acid identities to the above-mentioned enzymes. All these proteins belong to SDR family [16] characterized by the presence of Rossmann fold (the nucleotide binding site) and a triad of catalytically important and highly conserved Ser-Tyr-Lys residues. A canonical dilysine ER retention signal is present in the C-terminus of GhKCR1 but not in GhKCR2. These results suggested that GhKCR1 and 2 might be the orthologues of AtYBR159.

GhKCR(s) showed fiber-preferential expression patterns

Relative transcript levels of GhKCR(s) from 3, 5, 10, 15, 20 dpa wild-type cotton ovules together with their fibers (wt) were compared with either those of 0 dpa wt ovules or 10 dpa ovules of a fuzzless-lintless mutant (*fl*) by QRT-PCR analysis (Fig. 2). GhKCR1 transcripts increased about 30-fold and that of GhKCR2 increased about 20-fold only 5 d after flowering, whereas, very low levels of transcripts were observed in roots, stems and leaves for either gene (Fig. 2). Both GhKCRs were suppressed significantly in ovules of the *fl* mutant as well (Fig. 2).

Functional complementation of yeast mutant ybr159 Δ cells by the cotton enzymes

As seen in Fig. 3A, without transformation of GhKCR(s), *S. cerevisiae* haploid ybr159 Δ mutant cells grew very slowly on Sc-Trp⁻ plate. Wild-type growth rate was resumed in mutant cells expressing either GhKCR1 or 2 suggesting that heterologous expression of GhKCR(s) was able to complement the genetic deficiency. In agreement with the plate assay, ybr159 Δ mutant cells transformed by pYTV::GhKCR2 performed similarly with that of wild-type whereas cells expressing GhKCR1 grew even better than the wild-type (Fig. 3B). RT-PCR analysis of ybr159 Δ mutant cells transformed by either pYTV::GhKCR1 or

Fig. 1 Amino acid sequence alignment of GhKCR1 and GhKCR2. Amino acid sequence alignment of GhKCR1 (GenbankTM accession no. AY902466) and GhKCR2 (GenbankTM accession no. AY902467) with orthologues from *B. napus* (GenbankTM accession no. AAO43448 and AAO43449), *A. thaliana* (GenbankTM accession no. NP_564905), maize (GenbankTM accession no. AAB82767), *S. cerevisiae* (GenbankTM accession no. AAS56194), humans (GenbankTM accession no. AAP36605) and mouse (GenbankTM accession no. NM_00829). Black shading indicates strictly conserved residues, whereas gray shading marks regions of less strict conservation. An active triad of highly conserved S-Y-K residues is marked with an asterisk and canonical dilysine ER retention motif is indicated (▼).

pYTV::GhKCR2 showed that all the transgenes were actively transcribed during the log phase of yeast culture (Fig. 3C). Our data suggested that the genetic complementation phenomenon was related to the biochemical functions of individual GhKCR(s).

Subcellular localization of GhKCR proteins expressed in yeast

Insoluble membranous fractions (P85) were separated from the high speed supernatant (S85) by differential centrifugation using total proteins extracted from *ybr159wΔ*

Fig. 2 Both *GhKCR* genes are highly preferentially expressed in fast-elongating wild-type cotton fibers, but not in roots, stems, leaves and mutant cotton ovules. 0, +3, +5, +10, +15, +20 and +10 fl indicate that total RNA samples prepared from 0, 3, 5, 10, 15, 20 dpa wide-type or 10 dpa fl mutant cotton ovules were used as the template for QRT-PCR analysis. Error bars indicate means $\pm$ SE obtained from three independent experiments. Cotton ubiquitin gene, *UBQ7* (GenbankTM accession no. AY189972), was included as the template control. Fold increase or decrease was calculated using data obtained from mRNA samples prepared from ovules harvested around the day of anthesis (0 dpa).

cells transformed either with pYTV or pYTV::*GhKCR*(s). Equivalent amount of total lysate, P85 and S85 were separated on SDS-PAGE and analyzed by immunoblotting using anti-His and anti-ScKar2p separately. Both GhKCR1 (Fig. 4A) and GhKCR2 (Fig. 4B) were detected mainly in P85 fraction in a similar manner with that of ER marker ScKar2p [25, 26]. These data agreed with predictions of hydropathy plots since both GhKCR1 and 2 showed at least one membrane-spanning domain at their N-termini (Fig. 4). In the total lysate (TL) and P85 fractions prepared from *ybr159wΔ* cells without being transformed by the *GhKCR*(s), only ScKar2p was detected (Fig. 4).

Fig. 3 Yeast *ybr159wΔ* mutant cells can be functionally complemented by overexpression of individual *GhKCR*(s). (A) Wild-type rate of cell division was obtained in *ybr159wΔ* mutant cells that overexpressed either *GhKCR1* or *GhKCR2*. All cells were inoculated from overnight culture and were grown in SC-Trp, 2% Galactose liquid media to an OD_{600} of 0.5 before being plated out on SC-Trp solid media in a series of 10-fold dilutions with sterile H_2O . Cells were then incubated at 30°C for 2 days before being examined with naked eyes. (B) Growth curves of individual yeast strains cultured in SC-Trp, 2% Galactose liquid media up to 50 h at 30°C. Cell numbers were determined by using a haemocytometer. Error bars indicate means $\pm$ SE from three independent cultures. (C) RT-PCR analysis of yeast cell lines expressing different *GhKCR*(s) harvested at the indicated time after liquid culture. We included only one actin gene in the figure as the template control since it was expressed in a very similar manner in all strains.

Fig. 4 GhKCRs expressed in yeast were associated with their ER fractions. Membrane-spanning topology for each GhKCR was plotted according to White and Wimley [27] and was placed in the upper panel of each subset labeled (A) and (B) representing analysis of GhKCR1 and 2, respectively. Western blotting results were reported in the lower panel of each subset. Data on the left were obtained by using protein extracted from *ybr159wΔ* mutant cells transformed by the plain vector pYTV and data on the right were obtained by using protein extracted from *ybr159wΔ* mutant cells transformed by pYTV::GhKCR1 and pYTV::GhKCR2 accordingly.

a significant amount of 3-ketostearate with a concomitant reduction in 3-hydroxystearate as well as the final elongated product, stearate (Fig. 5). Overexpression of GhKCR1 and 2 separately in *ybr159wΔ* mutant cells in the presence of NADH and NADPH resulted in enhanced

production of 3-hydroxystearate, *trans*-2-stearate and stearate (Fig. 5B, Tab. 1) in the assay. In the absence of NADPH as a cofactor, however, no obvious differences in the amount of intermediates and the final product generated using extract from *ybr159wΔ* mutant with or without being transformed by GhKCR(s) were observed (Fig. 5C, Tab.1). When both NADH and NADPH were omitted, the reactions were stopped at the intermediate of 3-keto-stearate, indicating that the reduction step was cofactor-dependent (Fig. 5D). These data suggested that the both GhKCR(s) are NADPH-dependent 3-ketoacyl-CoA reductases.

DISCUSSION

In this report, we isolated two cDNAs, GhKCR1 and 2 from developing cotton fiber cells, and characterized their functions both in genetic as well as biochemical terms. The proteins were found to restore the growth rate of *S. cerevisiae ybr159wΔ* mutant to that of wild-type cells in a genetic complementation assay (Fig. 3). Recombinant GhKCR(s) displayed 3-ketoacyl-CoA reductase activities in an *in vitro* reaction that used palmitoyl-CoA as the substrate in the presence of malonyl-CoA (Fig. 5). Further studies showed that recombinant GhKCR1 and 2 were co-localized with the ER marker protein ScKar2p in *S. cerevisiae* mutant *ybr159wΔ* cells (Fig. 4), indicating that these cotton enzymes function as microsomal 3-ketoacyl-CoA reductases.

In *S. cerevisiae*, Ybr159p was shown to constitute majority of the KCR activity since extracts from *ybr159wΔ* mutant cells leads to a significant accumulation of 3-ketostearate in the elongase assay (Fig. 5B). Using extracts from both wild-type and mutant cells that overexpressed GhKCR(s), 3-ketostearate was not accumulated. Instead, production of 3-hydroxystearate and stearate was enhanced. Similar levels of 3-hydroxy-fatty acyl intermediates were found using extracts from mutant cells and those that overexpressed GhKCR(s) when NADPH was removed in the assay system (Fig. 5C) suggesting that GhKCR(s) were NADPH-dependent 3-ketoacyl-CoA reductases. NADPH-dependent 3-ketoreductase activities were previously detected from rat liver [28]. The cofactor requirement for the mammalian microsomal KCR protein has not been settled yet [29, 30]. The plant 3-ketoacyl-[acyl carrier protein] reductase involved in plastid fatty acid synthesis was NADPH-dependent [31]. NADPH binding was found to induce a conformational change that put three conserved amino acid residues into the active site to facilitate catalytic function [32].

In maize and *Arabidopsis*, only one gene homologous to yeast YBR159w was identified [14, 17]. Recently, two highly homologous BnKCR(s) from *Brassica* genomes were

Fig. 5 GhKCRs function as NADPH-dependent 3-ketoacyl-CoA reductase in *ybr159wΔ* mutant cells. **(A)** Schematic drawings of reactions from palmitoyl-CoA to stearoyl-CoA. **(B)** *ybr159wΔ* mutant yeast cells transformed with *GhKCR1*, 2 or 3 metabolized palmitoyl-CoA at the same rate as wild-type cells in the presence of both NADH and NADPH. **(C)** When NADPH was not included in the experiment, all cells metabolized palmitoyl-CoA very slowly in the same way as that of *ybr159wΔ* mutant. **(D)** 3-ketostearate was accumulated similarly in all cell lines if both NADPH and NADH were not added in the assay. All reactions were initiated by adding 50 μ g of proteins extracted from wild-type (wt), *ybr159wΔ* or *ybr159wΔ* mutant cells expressing *GhKCR1* or 2 (KCR1 or 2 respectively). For TLC analysis, the fatty acyl-CoA thioesters were extracted as free fatty acid. The assays were stopped at the indicated time and the fatty acids were extracted and separated by TLC. Positions labeled as 1, 2, 3, 4 and O denote 3-ketostearate, 3-hydroxystearate, *trans*-2-stearate, stearate and the origin of TLC respectively. Relative positions were determined by comparing the mobility with authentic standards as described [14].

identified [19] and they are homologues of AtYBR159. Three independent lines of evidence suggest that *GhKCR1* and 2 cDNAs reported in the current work might represent two different *KCR* genes: (i) The GhKCRs show different expression patterns during various stages of cotton fiber development and also in different cotton tissue (Fig. 2). The expression profiles of the both genes are in accordance with our previous findings that several *GhKCS* genes encoding putative 3-ketoacyl-CoA synthases [33] and cotton genes encoding lipid transfer proteins [34, 35] were highly expressed at the same period. Our data indicate that long chain fatty acid biosynthesis and supply may be in high demand during the fast cell elongation period. (ii)

GhKCR1 and 2 show significant sequence similarity to ScYbr159p and share 59% amino acid identity with each other. (iii) Although GhKCR2 protein is recovered from the microsomal fractions in the same way as GhKCR1, it is different from GhKCR1 since it lacks a predicted ER retention signal (Fig. 4).

In conclusion, we isolated two cDNAs encoding 3-ketoacyl-CoA reductases from developing cotton fibers. Genetic complementation and biochemical characterization of these cotton genes in yeast cells demonstrated that they could function as NADPH-dependent 3-ketoacyl-CoA reductases. Both theoretical and experimental analysis strongly suggested that GhKCRs are involved in ER-asso-

Tab. 1 Relative amount of 3-hydroxystearate, *trans*-2-stearate, and stearate of palmitate elongation catalyzed by GhKCR(s) in the presence or absence of NADPH. The values represent the relative amount of different intermediates at 30 min in relation to that measured in the yeast mutant cells transformed with plain vector that was arbitrarily defined as 1.

Products	Steps	Sample					
		wt		GhKCR1		GhKCR2	
		+	–	+	–	+	–
3-Ketostearate	1		0.56 ± 0.09		0.85 ± 0.1		0.85 ± 0.08
3-Hydroxystearate	2	1.3 ± 0.1	1.6 ± 0.12	1.7 ± 0.11	0.95 ± 0.08	1.5 ± 0.09	1.00 ± 0.08
<i>Trans</i> -2-stearate	3	1.2 ± 0.09	1.9 ± 0.1	2.5 ± 0.2	1.00 ± 0.09	1.7 ± 0.11	1.10 ± 0.09
Stearate	4	1.3 ± 0.09	1.9 ± 0.14	2.3 ± 0.15	1.00 ± 0.03	1.8 ± 0.13	1.00 ± 0.09

+, with both NADH and NADPH; –, with only NADH.

ciated very long chain fatty acid elongation. Our work indicates that the VLCFA elongation pathway is upregulated during cotton fiber development and is similar to that found in yeast cells. Whether *GhKCR*(s) are essential for the elongation of VLCFAs in cotton need further investigation.

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