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GENE NOTE

Functional characterization of the *Arabidopsis thaliana* orthologue of Tsc13p, the enoyl reductase of the yeast microsomal fatty acid elongating system

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Abstract

The protein encoded by the *Arabidopsis At3g55360* gene was selected as a candidate for the enoyl reductase of the microsomal elongase system based on its homology to the Tsc13p protein of *S. cerevisiae*. The studies presented here demonstrate that heterologous expression of *At3g55360* functionally complements the temperature-sensitive phenotype of a yeast *tsc13* mutant that is deficient in enoyl reductase activity. Furthermore, AtTSC13 is shown to interact physically with the Elo2p and Elo3p components of the yeast elongase complex. *At3g55360* apparently encodes the sole enoyl reductase activity associated with microsomal fatty acid elongation in *Arabidopsis*. Consistent with this conclusion, *AtTSC13* is ubiquitously expressed in *Arabidopsis*.

Key words: *Arabidopsis*, elongase, enoyl-CoA reductase, fatty acid elongation, very long chain fatty acids, VLCFAs.

Microsomal fatty acid elongation generates the very long chain fatty acids (VLCFAs) that are critical components of several classes of lipids. Higher plants accumulate VLCFAs as components of triacylglycerols, cuticular waxes and sphingolipids. Plant VLCFAs are either saturated or monounsaturated, whereas animals accumulate polyunsaturated VLCFAs. $C_{16/18}$ substrate fatty acids are lengthened by the sequential addition of C_2 units in four successive biochemical reactions: condensation of malonyl-CoA with the acyl-CoA substrate, 3-ketoacyl-CoA reduction, 3-hydroxyacyl-CoA dehydration, and, finally, enoyl-CoA reduction. The elongating enzymes are organized in a complex referred to as the elongase (Cinti *et al*., 1992).

Genes encoding components of the elongase have been identified from a number of different species. In *Arabidopsis*, mutations in the *FATTY ACID ELONGATION* (*FAE1*) gene result in reduced levels of very long chain fatty acids in seed (James and Dooner, 1990; Kunst *et al*., 1992; Lemieux *et al*., 1990). Extensive characterization of *FAE1* and the related *KCS1* has confirmed that these genes encode condensing enzymes (Ghanevati and Jaworski, 2001; Roscoe *et al*., 2001; Todd *et al*., 1999). In higher plants the *FAE1*-like multigene family (there are 21 putative *KCS* genes, including *FAE1*, in the *Arabidopsis* genome) encodes enzymes responsible for the synthesis of a diverse range of VLCFAs (James *et al*., 1995). While the *FAE1*-like gene family appears to be unique to plants, the *ELO*-like genes, first identified in *Saccharomyces cerevisiae* (Oh *et al*., 1997), appear to encode a class of condensing enzymes that are present in fungi, mammals, and plants (Tvrdik et al., 2000).

Recently, genes encoding the 3-ketoreductase and enoyl-CoA reductase have been identified. The yeast *YBR159* gene was found to be involved in heterologous synthesis of polyunsaturated VLCFAs (Beaudoin *et al*., 2002), and biochemical characterization confirmed its function as the 3-ketoreductase (Han *et al*., 2002). The *Arabidopsis At1g67730* gene, which functionally complemented the yeast *ybr159*Δ mutant, showed homology to the maize gene *Glossy8* (G8), which was required for the normal synthesis of cuticular waxes (Xu *et al*., 2002). The *TSC13* gene encoding the enoyl-CoA reductase was identified in a genetic screen for mutants with defects in sphingolipid synthesis in yeast (Beeler et al., 1998). *TSC13* was shown biochemically to encode the enoyl-CoA reductase and to be a physical component of the microsomal elongase, interacting with the yeast Elo2/3 proteins (Kohlwein *et al*., 2001).

A number of presumptive orthologues of *TSC13* were identified from species including mammals, fungi (*S. pombe*), and *Arabidopsis* (Kohlwein *et al*., 2001). This work has now been extended to demonstrate that the *Arabidopsis* gene *At3g55360* (designated hereafter *AtTSC13*) functions as a microsomal enoyl-CoA reductase. The *AtTSC13* cDNA was PCR-amplified with the AT-Tsc13 up (5′-GGGCCC**CTCGAG**CAAGGTCACCGTCGTCTCC) and AT-Tsc13 down (5′-GGGCCC**CTCGAG**CTAAAGGAATGGAGGAAG) primer pair using a RIKEN *Arabidopsis* Full Length (RAFL) *AtTSC13* cDNA (from the RIKEN Bioresource Center) as template. The amplified fragment was restricted with *Xho*I (sites underlined and in bold), purified using the Qiagen PCR purification kit, and ligated into the *Sal*I site of pADH1, thereby generating a yeast plasmid constitutively expressing the HA-tagged AtTSC13 protein. Heterologous expression of the HA-tagged AtTSC13 protein in yeast rescued the ts-lethality of the *tsc13–1 elo3*Δ double mutant (Fig. 1A), indicating that *AtTSC13* was very likely to encode the functional orthologue of the yeast *TSC13* enoyl-CoA reductase.

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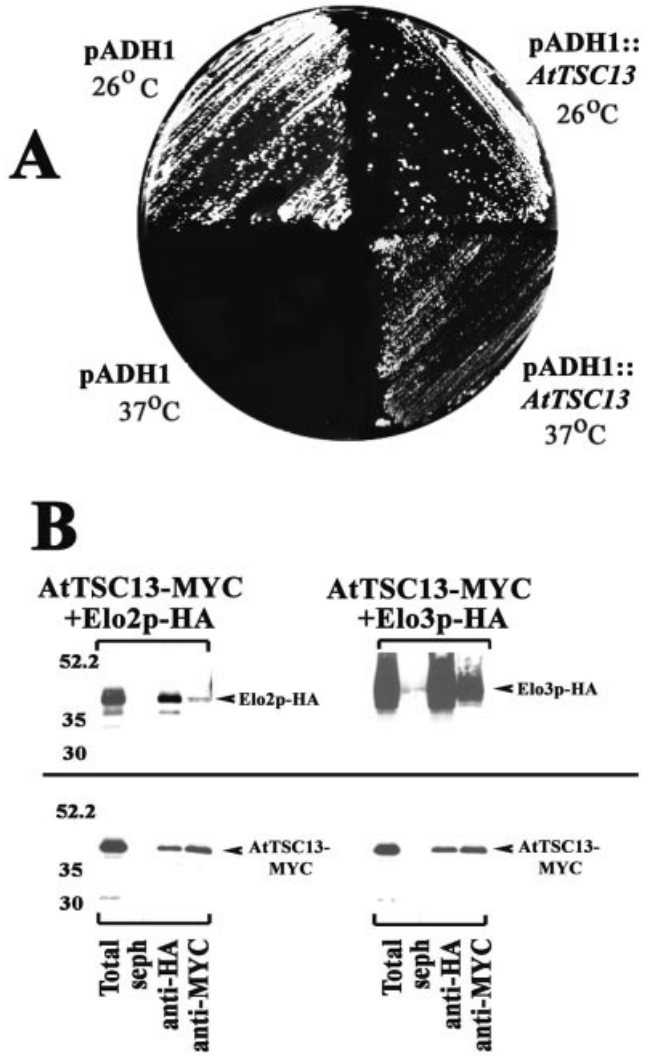

Fig. 1. AtTSC13 complements the ts-lethality of the *tsc13–1elo3*Δ mutant and the MYC-tagged *AtTSC13* coimmunoprecipitates with the Elo2p and Elo3p of the yeast elongase. (A) The *tsc13–1elo3*Δ mutant was transformed with the pADH1 plasmid (left half) or the plasmid with the *AtTSC13* cDNA fused to the ADH1 promoter in pADH1 (right half). Transformants were streaked onto YPD plates and incubated at 26 °C (top half) or 37 °C (bottom half) for 3 d prior to photographing. (B) Plasmids carrying a MYC-tagged allele of *AtTSC13* and an HA-tagged allele of *ELO2* (left panel) or *ELO3* (right panel) were co-transformed into the *tsc13–1elo3*Δ mutant. Microsomes were prepared, solubilized, and immunoprecipitated with unconjugated Sephadex beads (SEPH), or Sephadex beads conjugated with anti-HA or anti-MYC antibodies as described (Han *et al*., 2002). The immunoprecipitated proteins were separated by SDS-PAGE, immunoblotted, and detected with either anti-HA antibodies (top) or anti-MYC antibodies (bottom).

Further evidence was provided by the demonstration that AtTSC13 physically interacted with the ELO protein components of the yeast elongase. A triple-myc tag was introduced at the N-terminus of AtTSC13 by replacing the coding sequence of the *S. cerevisiae TSC13* gene with that of the *AtTSC13* gene in the MYC-TSC13-426 plasmid (Kohlwein *et al*., 2001). This was accomplished by introducing *Sal*I sites after the MYC tag and before the stop codon of the *S. cerevisiae TSC13* gene in MYC-TSC13-426 by QuikChange mutagenesis (Stratagene, La Jolla, California). The *Sal*I fragment carrying the yeast *TSC13* gene was deleted and replaced with a PCR-generated *Sal*I-ended fragment carrying the *AtTSC13* cDNA. The myc-tagged AtTSC13 was co-expressed in yeast cells with HA-epitope tagged forms of either Elo2p or Elo3p (Kohlwein *et al*., 2001). Microsomal fractions were solubilized, and elongase components were immunoprecipitated. Immunoprecipitation with the HA antibody not only pulled down the HA-tagged Elo proteins, but also the myc-tagged AtTSC13 (Fig. 1B). This is a clear demonstration that AtTSC13 is in physical proximity to the yeast Elo proteins. Based on this co-IP data and the rescue of the *tsc13–1elo3*Δ mutant, AtTSC13 is very likely to be the *Arabidopsis* enoyl reductase of the microsomal fatty acyl elongase.

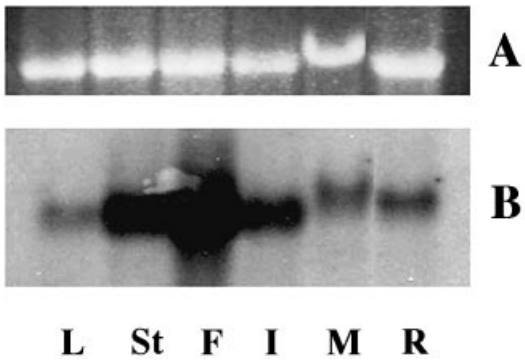

Fig. 2. The *AtTSC13* gene is ubiquitously expressed in *Arabidopsis* tissues. (A) RNA loadings (10 μg well^{-1}) visualized by ethidium bromide staining. (B) RNA hybridized with a ^{32}P-labelled *AtTSC13* cDNA probe. The blot was hybridized using phosphate buffer overnight at 65 °C, washed at high stringency, and exposed to film for 6 h. RNA was isolated from leaves (L), stems (St), flowers (F), immature siliques (I), mature siliques (M), and roots (R).

As outlined above, the microsomal fatty acid elongase synthesizes VLCFAs, which are incorporated into a number of different classes of lipids. In yeast, loss of Tsc13p activity is associated with a decrease in the levels of VLCFAs found in sphingolipids. Higher plants accumulate many different types of VLCFA-containing lipids and, as mentioned earlier, there is a large family of FAE1-like condensing enzymes. However, this proliferation of activities does not appear to be represented in the other components of the elongase; there are only two 3-ketoreductase/G8 orthologues present in *Arabidopsis* (At1g67730/G8 and At1g24470) and AtTSC13 represents the only candidate enoyl-CoA reductase. Thus it seems likely that AtTSC13 is the sole enoyl-reductase component of the microsomal elongase. The expression pattern of *AtTSC13* was analysed by northern blotting and it was determined that this transcript was present in all *Arabidopsis* tissues tested (leaves, stems, flowers, siliques, and roots) (Fig. 2). Upon prolonged exposure of the autoradiogram, the presence of transcripts in mature seeds (data not shown) was also identified. Consistent with these results, data in the Nottingham *Arabidopsis* Stock Centre's *Arabidopsis* Affymetrix database (http://ssbdjc2.nottingham.ac.uk/narrays/experimentbrowse.pl; http://www.cbs.umn.edu/arabidopsis/) on the expression of At3g55360 confirms the presence of this transcript in a wide range of *Arabidopsis* tissues, including seedlings, lateral and primary roots, pollen, shoots, petioles, auxiliary buds, and suspension-cultured cells. Taken together with the authors' northern blot data, this indicates the likelihood that *AtTSC13* is ubiquitously expressed in *Arabidopsis* tissues. Therefore, AtTSC13 is likely to participate in

the synthesis of most (if not all) VLCFAs. This would include the seed-specific VLCFAs found in storage triacylglcerols, the epidermal cuticular waxes and the sphingolipids.

In conclusion, the sole orthologue of the elongase-associated enoyl-CoA reductase present in the *Arabidopsis thaliana* genome has been identified. It is predicted that AtTSC13 is a ubiquitous component of the multiple distinct elongases including those that depend on the FAE1-like family of condensing enzymes as well as those that utilize the ELO-like family of (putative) condensing enzymes. As such, it is also predicted to be an essential gene in *Arabidopsis*.

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