

Gaining insight into the role of serine 282 in *B. napus* FAE1 condensing enzyme

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Abstract To gain some insight whether there is an absolute requirement for the serine 282 to yield a functional fatty acid elongase 1 condensing enzyme we have introduced point mutations in the *FAE1* coding sequence which led to the substitution of serine 282 with several aliphatic or aromatic amino acids. The mutated *FAE1* polypeptides were expressed in yeast. Gas chromatography analyses of the fatty acid methyl esters from yeast lysates and fatty acid elongase activity assays demonstrated that there is not an absolute requirement for serine at position 282 to yield a functional FAE1 condensing enzyme.

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1. Introduction

Fatty acid elongase 1 (FAE1) is a seed-specific condensing enzyme, 3-ketoacyl-CoA synthase, which catalyzes the first enzymatic reaction in very long chain monounsaturated fatty acid (VLCMFA) biosynthesis in high erucic acid Brassicaceae. This enzyme is a part of the fatty acid elongase complex located in cytosol and bound to endoplasmic reticulum membranes. Following the condensation of malonyl-CoA with a long chain acyl-CoA primer catalyzed by FAE1, three subsequent enzymatic reactions catalyzed by 3-ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydratase and enoyl-CoA reductase are crucial for very long chain fatty acid (VLCFA) biosynthesis [1,2]. It seems that only the FAE1 enzyme is unique in the VLCMFA biosynthesis pathway, being the rate-limiting step in the accumulation of VLCMFAs, while the other three enzymes of the complex do not appear to play a regulatory role in this pathway [3–5].

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Abbreviations: FAE1, fatty acid elongase 1; *FAE1*, fatty acid elongation 1; LEA, low erucic acid; VLCMFA, very long chain monounsaturated fatty acid; VLCFA, very long chain fatty acid; FAME, fatty acid methyl esters

Genes and coding sequences for FAE1s from different Brassicaceae were isolated [6–15] and manipulated extensively by expression in homologous species as well as in heterologous host systems [3–5,16–18]. However, our knowledge about the mechanisms and kinetic properties of FAE1 condensing enzymes is limited mainly due to limitations resulting from their membrane-bound nature. Currently, there is only one report on extensively purified membrane-bound condensing enzyme, the FAE1 from jojoba, and its characterization was limited to the substrate specificity [19].

We have shown that seed-specific expression of *Arabidopsis thaliana* FAE1 in high erucic acid *Brassica napus* cv. Hero has led to increased proportions of erucic acid (up to 11%) and total VLCFAs (up to 10%) in the oil, while the seed-specific expression of *A. thaliana* FAE1 in low erucic acid (LEA) canola cv. Westar complemented the mutation and partially restored the capacity to biosynthesize VLCMFAs in the developing seed [4,5].

Han et al. [18] expressed *A. thaliana* and *B. napus* FAE1 genes in yeast cells and concluded that in addition to oleic acid (18:1 $\Delta 9$), both elongases are able to elongate the palmitic acid to produce a 16:1 $\Delta 9$ acyl chain. Their results indicated that the *A. thaliana* FAE1 more efficiently utilizes 18:1-CoA ($\Delta 9$ and $\Delta 11$) primers to produce the respective isoforms of eicosenoic acid (20:1 $\Delta 11$ and $\Delta 13$) while the *B. napus* FAE1 favors 20:1 ($\Delta 11$ and $\Delta 13$) acyl substrates to make the respective docosenoic acid isoforms (22:1 $\Delta 13$ and $\Delta 15$). The expression of FAE1 in yeast showed that the heterologous condensing enzyme, together with the two endogenous reductases and the dehydratase, formed a functional fatty acid elongase complex that catalyzed the elongation of long chain fatty acid substrates into VLCFA products.

Recently, using a domain-swapping approach, Blacklock and Jaworski [20] produced chimeric *A. thaliana* and *B. napus* FAE1 polypeptides and investigated structural domains and residues responsible for substrate specificities of these condensing enzymes. They have shown that the N-terminal region excluding the transmembrane domains is involved in determining the substrate specificity in FAE1 condensing enzymes.

LEA rapeseed and canola (LEA and low glucosinolate rapeseed) cultivars were developed through traditional breeding and selection to meet the requirement for a high quality edible vegetable oil [21]. Several groups have focused their research on elucidating the mutation(s) involved in the loss of FAE1 condensing enzyme activity in LEA *B. napus* cultivars [18,22]. Recently, we have provided the experimental evidence that the

LEA trait in LEA and canola *B. napus* cultivars can be attributed to a single amino acid substitution in the FAE1 condensing enzyme which prevents the biosynthesis of eicosenoic and erucic acids. We have shown that the activity of a non-functional FAE1 of *B. napus* could be restored by substituting Phe282 by serine which is found in equivalent positions of all known FAE1 condensing enzymes [23,24].

As a continuation of our work we have focused on determining whether serine 282 in functional FAE1 condensing enzyme is a catalytic residue. To test our hypothesis which centers on hydrophathy of the segment containing the adjacent Asn283 and possible significance of maintaining a theoretical hydrophilic transition point in the sequence at Ser282 we used a site-directed mutagenesis approach to generate several mutated polypeptides, substituting serine 282 with either aliphatic or aromatic amino acids [25]. Here we report and discuss the results of analyses of mutated FAE1 polypeptides by heterologous expression in yeast.

2. Materials and methods

2.1. Site-directed mutagenesis

To introduce point mutations into the *FAE1* coding region, we used a QuikChange[®] site-directed mutagenesis kit (Stratagene). We designed the following oligonucleotide primer pairs:

SDT-1 (5'-TGTTGGTGGGGCCGCTATTTGCTC**ACCAACA**-AG-3') and **SDT-2** (5'-CTTGTG**GT**GAGCAAAATAGCGGCC-CACCAACA-3');

SDY-1 (5'-TGTTGGTGGGGCCGCTATTTGCTC**TACAACA**-AG-3') and **SDY-2** (5'-CTTGTG**T**AGAGCAAAATAGCGGCC-CCCAACAACA-3')

SDW-1 (5'-TGTTGGTGGGGCCGCTATTTGCTC**TGGAACA**-AG-3') and **SDW-2** (5'-CTTGT**TCC**AGAGCAAAATAGCGGCC-CCCAACAACA-3')

SDN-1 (5'-TGTTGGTGGGGCCGCTATTTGCTC**ACAACA**-AG-3') and **SDN-2** (5'-CTTGTG**TTG**AGAGCAAAATAGCGGCC-CACCAACA-3')

SDQ-1 (5'-TGTTGGTGGGGCCGCTATTTGCTC**CAGCAAC**-AAG-3') and **SDQ-2** (5'-CTTGT**TC**TGGAGCAAAATAGCGGCC-CCCAACAACA-3')

SDC-1 (5'-TGTTGGTGGGGCCGCTATTTGCTC**TGCAACA**-AG-3') and **SDC-2** (5'-CTTGTG**C**AGAGCAAAATAGCGGCC-CCCAACAACA-3')

SDG-1 (5'-TGTTGGTGGGGCCGCTATTTGCTC**CGCAACA**-AG-3') and **SDG-2** (5'-CTTGTG**CCG**AGAGCAAAATAGCGGCC-CACCAACA-3')

SDA-1 (5'-TGTTGGTGGGGCCGCTATTTGCTC**GCCAACA**-AG-3') and **SDA-2** (5'-CTTGTG**GCG**AGAGCAAAATAGCGGCC-CACCAACA-3')

SDV-1 (5'-TGTTGGTGGGGCCGCTATTTGCTC**GTCAACA**-AG-3') and **SDV-2** (5'-CTTGTG**ACG**AGAGCAAAATAGCGGCC-CACCAACA-3')

and **SDI-1** (5'-TGTTGGTGGGGCCGCTATTTGCTC**ATCAA**-CAAG-3') and **SDI-2** (5'-CTTGTG**ATG**AGAGCAAAATAGCGGCC-CCCAACAACA-3')

containing the desired mutations (in bold, underlined). Primers were complementary to opposite strands of pYES2.1/V5-His-TOPO bearing the *FAE1* gene. During the polymerase chain reaction, primers were extended with *PfuTurbo* DNA polymerase. This polymerase replicated both strands with high fidelity using the mutated oligonucleotide primers. Following temperature cycling, the product was treated with *DpnI* endonuclease (target sequence 5'-Gm⁶ATC-3') which is specific for methylated and hemimethylated DNA, to digest the parental DNA template and select the mutated DNA.

2.2. Heterologous expression of mutated FAE1 polypeptides in yeast

The mutated *FAE1* DNA sequences were cloned into the yeast expression vector pYES2.1/V5-His-TOPO (Invitrogen) downstream of the galactose-inducible promoter (*GALI*). All products were analyzed by sequence analyses using external primers *GALI* Forward primer (Invitrogen), V5 C-terminus Reverse primer (Invitrogen) and

internal primers for the *FAE1* sequence (*FAE1*-FW 5'-CGGCA-CGTGCGATGACTC-3' and *FAE1*-RV 5'-TCCTTCCTTTGCTT-CTATGTATG-3'). Yeast cells (line Inv Sc1, Invitrogen) were transformed with pYES2.1/V5-His-TOPO constructs bearing different mutated FAE1 polypeptides, using the *Saccharomyces cerevisiae* Easy-Comp[®] Transformation Kit (Invitrogen). As a control in our expression experiments, yeast cells were transformed with pYES2.1/V5-His-TOPO plasmid only. Transformants were selected by growth on synthetic complete medium lacking uracil (SC-ura) supplemented with 2% glucose. The colonies were transferred into liquid SC-ura medium with 2% glucose and grown at 28°C overnight. For expression studies the overnight cultures were used to inoculate 25 ml of SC-ura supplemented with 2% galactose to give an initial D_{600} of 0.2. The cultures were subsequently grown overnight at 28°C to D_{600} of 1.4 and used for biochemical analyses.

2.3. Fatty acid analyses and enzyme assays

The yeast cultures were grown overnight and the cells were spun to form a pellet. Cell pellets were saponified in 2 ml methanolic-KOH (10% KOH, 5% H₂O in methanol) for 2 h at 80°C. After saponification, samples were cooled on ice, 1 ml water and 2 ml hexane were added and non-saponifiable material was removed. The remaining aqueous phase was acidified with 6 N HCl. Free fatty acids were extracted into hexane, the solvent was removed under a stream of N₂, and free fatty acids were transmethyated in 2 ml of 1% H₂SO₄ in methanol for 1 h at 60°C. Fatty acid methyl esters (FAME) were extracted into hexane, the solvent was removed under a N₂ stream and the residue was dissolved in hexane for gas chromatography (GC) analysis under the conditions described previously [26].

Fatty acid elongase activity of the yeast homogenates was assayed essentially as described by Katavic et al. [5]. The assay mixture consisted of 80 mM HEPES-NaOH, pH 7.2; 1 mM ATP; 1 mM CoA-SH; 0.5 mM NADH; 0.5 mM NADPH; 2 mM MgCl₂; 1 mM malonyl-CoA; 18 μM [1-¹⁴C]oleoyl-CoA (0.37 GBq/mol) in a final volume of 500 μl. The reaction was started by the addition of 0.5 mg of protein and incubated at 30°C for 1 h. Reactions were stopped by adding 3 ml of 100 g/l KOH in methanol and the mixtures were heated at 80°C for 1 h to saponify acyl lipids and acyl CoAs. The tubes were cooled on ice and two 2-ml hexane washes were performed to remove non-saponifiable material. These hexane washes were discarded, and 1 ml water was added to the reaction mixtures. This mixture was then acidified by adding 650 μl concentrated 12 M HCl, extracted twice with 2 ml hexane, the hexane extracts combined and dried under N₂. Samples were transmethyated with 3 M methanolic HCl at 80°C for 1 h. 2 ml of water was added, samples were extracted twice with 1 ml hexane, dried under N₂, taken up in 110 μl of acetonitrile and quantified by radio-HPLC as described previously [27].

2.4. Preparation of yeast homogenates

Yeast homogenates were prepared essentially according to Ghanevati and Jaworski [28]. Cells were harvested and washed with 10 ml of ice-cold isolation buffer consisting of 80 mM HEPES-NaOH, pH 7.2, 5 mM EGTA, 5 mM EDTA, 10 mM KCl, 320 mM sucrose, 2 mM dithiothreitol, pelleted and resuspended in 500 μl of isolation buffer. Cells were broken using three 60-s pulses with a Mini-Beadbeater[®] (Biospec product, Bartlesville, OK, USA) using 0.5-mm glass beads. The homogenate was collected and briefly centrifuged to remove unbroken cells. Protein concentration was determined using the Bradford method [29].

3. Results and discussion

3.1. Hydrophathy analysis of functional FAE1 condensing enzyme

The Kyte–Doolittle hydrophilicity plot of functional FAE1 condensing enzyme in different Brassicaceae shows two highly hydrophobic N-terminal domains (Fig. 1). These domains were extensively analyzed by Ghanevati and Jaworski who reported that in FAE1 condensing enzymes, two transmembrane helices are most likely spanning the N-terminal region, one from amino acid 9 to 36 and the other one from amino

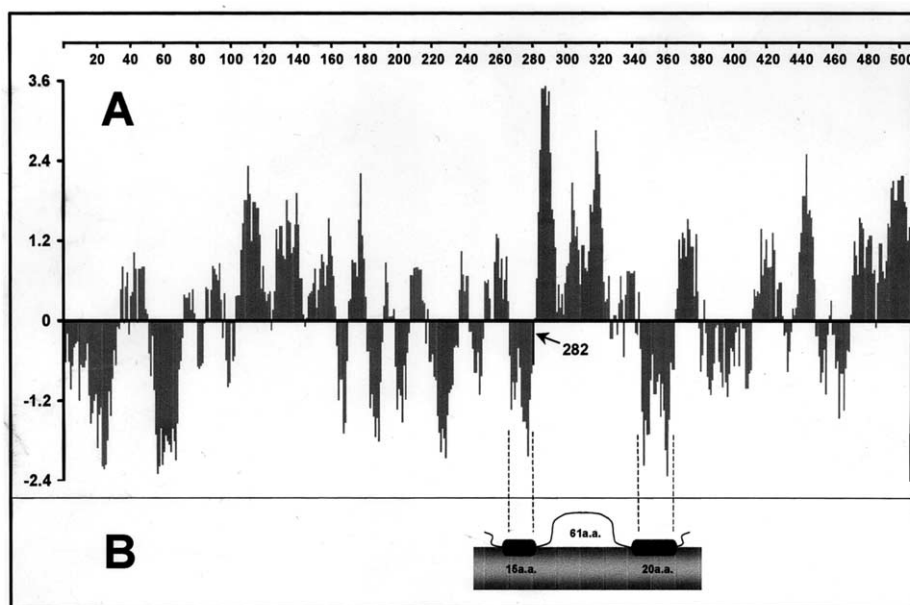


Fig. 1. Hydropathy analyses of FAE1 protein. A: Hydrophilicity plot (Kyte–Doolittle) for functional *B. napus* FAE1 polypeptide. The analyses were performed using Protean, Lasergene Biocomputing Software for Windows (DNASar, Madison, WI, USA) with the Residues to Average set to default value 9. B: Proposed model for catalytic domain spanning from valine 267 to glycine 365. We predict that two hydrophobic regions, one of 15 and the other one of 20 amino acids, could be membrane-associated domains with a hydrophilic loop in between. Serine 282 ends the hydrophobic (α -helix) domain (indicated by an arrow).

acid 48 to 76. They also pinpointed the active site residues cysteine 223, histidine 391 and asparagine 424 [28,30]. We propose the model of a catalytic domain spanning from valine 267 to glycine 365. We predict that two hydrophobic regions, one of 15 residues (from valine 267 to serine 282) and the other one of 20 amino acid residues (from threonine 345 to glycine 365), could be membrane-associated domains with a hydrophilic loop between them. The serine 282 ends the hydrophobic (α -helix) domain.

Previously, we have shown that the presence of hydrophobic phenylalanine 282 in the non-functional FAE1 condensing enzyme instead of the hydrophilic serine 282 in the functional FAE1 induces dramatic changes in the hydrophilicity values for amino acids in the transition region, from a highly hydrophobic domain around residue 278 to a highly hydrophilic domain around residue 286, shifting the asparagine 283 from the hydrophilic loop in the functional condensing enzyme to a hydrophobic domain in the non-functional FAE1 enzyme [24]. Based on these findings we hypothesized that

substitution of serine 282 with any amino acid that induces the shift of asparagine 283 from the hydrophilic loop to the hydrophobic domain will abolish FAE1 enzyme activity, while the substitution with any amino acid residue that does not induce this hydrophobic shift will result in an active FAE1 peptide (Table 1).

3.2. Expression of mutated FAE1 peptides in yeast and GC analyses

To test our hypothesis we introduced specific point mutations into the FAE1 nucleotide sequence resulting in the substitution of serine 282 with either threonine, asparagine, glutamine, cysteine, glycine, alanine, valine, isoleucine, tyrosine or tryptophan. We cloned the mutated FAE1 polypeptides into a yeast expression vector (pYES2.1/V5-His-TOPO) and expressed them in yeast cells. The results from yeast FAME analyses by GC revealed that the substitution of serine 282 with either asparagine, glutamine, valine, isoleucine, tyrosine or tryptophan resulted in complete loss of enzyme activity

Table 1

Hydrophilicity values for amino acids in the transition region from highly hydrophobic to highly hydrophilic protein domain in FAE1 polypeptide

Predicted	WTF (–)	SDS (+)	SDT (+)	SDN (+)	SDQ (+)	SDC (–)	SDG (–)	SDA (–)	SDV (–)	SDI (–)	SDY (+)	SDW (+)
280 L	–1.19	–0.79	–0.80	–0.49	–0.49	–1.16	–0.83	–1.08	–1.34	–1.38	–0.73	–0.79
281 L	–1.06	–0.66	–0.67	–0.36	–0.36	–1.02	–0.70	–0.94	–1.21	–1.24	–0.60	–0.64
282	–0.81	–0.41	–0.42	–0.11	–0.11	–0.78	–0.46	–0.70	–0.97	–1.00	–0.36	–0.40
283 N	–0.22	0.78	0.17	0.48	0.48	–0.19	–0.13	–0.11	–0.38	–0.41	0.23	0.19
284 K	0.79	1.18	1.17	1.48	1.48	0.81	1.13	0.89	0.62	0.58	1.23	1.19
285 P	1.70	2.10	2.09	2.40	2.40	1.73	2.06	1.81	1.54	1.51	2.16	2.11

Hydrophilicity values are determined using Kyte–Doolittle hydropathy analyses performed with Protean, Lasergene Biocomputing Software for Windows (DNASar, Madison, WI, USA) with the Residues to Average set to default value 9. Based on hydrophilicity values we hypothesized and predicted that substitution of serine 282 with an amino acid that can induce a shift of asparagine 283 from the hydrophilic loop into the hydrophobic domain will result in a non-functional (–) polypeptide and substitution with an amino acid residue that will not induce such a shift will result in a functional (+) FAE1 polypeptide.

WTF is Westar wild-type with phenylalanine 282.

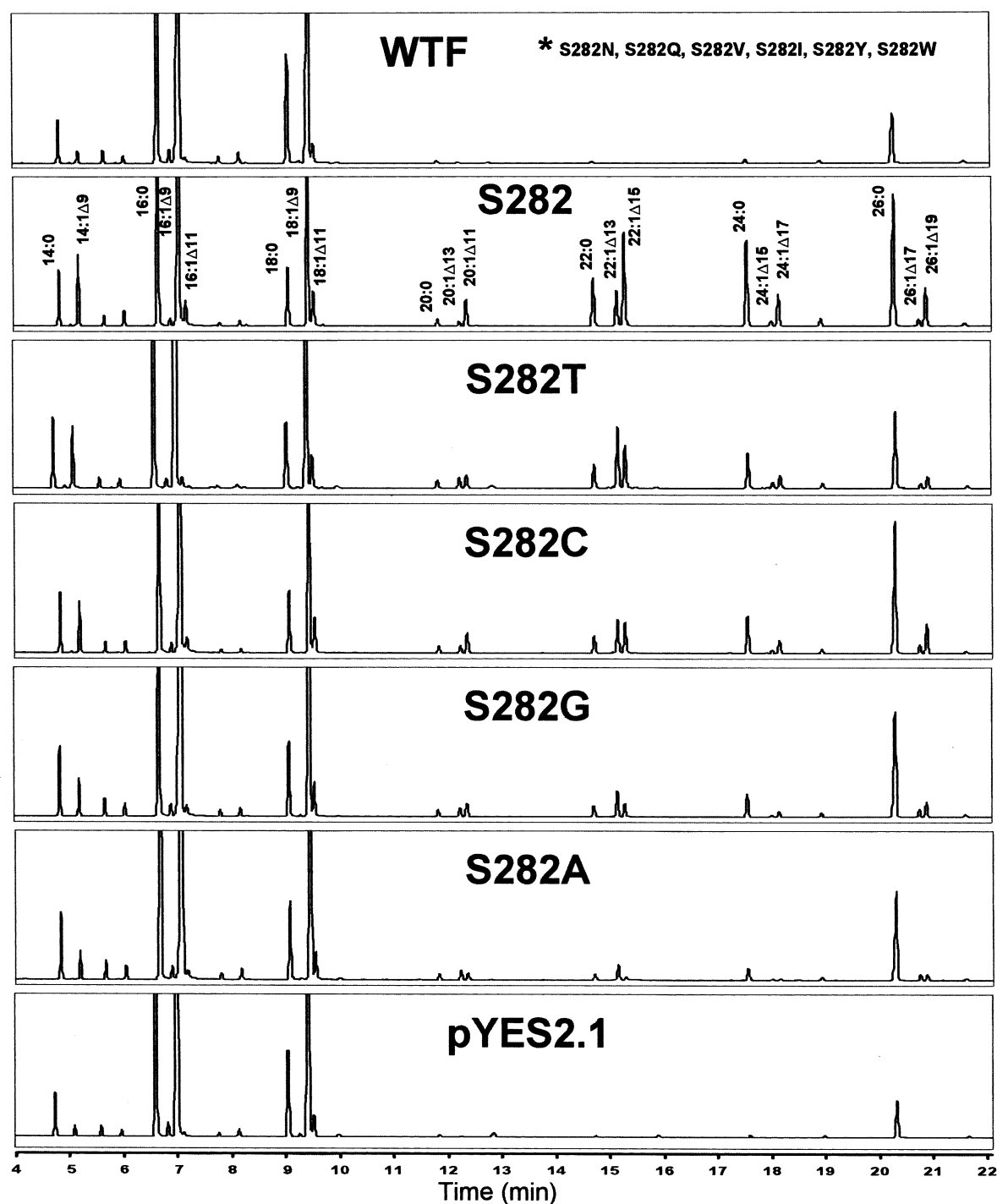


Fig. 2. GC chromatographs showing fatty acid profiles of yeast cells transformed with chimeric *FAE1* gene. FAME were prepared from yeast lysates expressing non-functional *FAE1* from LEA *B. napus* cv. Westar (WSF), functional *FAE1* with serine 282 (S282) and mutated *FAE1* peptides S282Y, S282W, S282N, S282Q, S282V, S282I, S282T, S282C, S282G and S282A. As a negative control we used pYES2.1/V5-His-TOPO plasmid (pYES2.1). *FAME profiles for S282Y, S282W, S282N, S282Q, S282V and S282I were all similar to WSF.

(Fig. 2). Based on our hypothesis, only the amino acids threonine, valine and isoleucine affected the *FAE1* activity the way we predicted (Fig. 3). The finding that substitution of serine with threonine resulted in an active enzyme was not surprising because the only functional plant fatty acid condensing enzyme reported to date that does not have serine at position 282 is a putative 3-ketoacyl-CoA synthase from the monocot *Sorghum bicolor* (broom corn) that contains threonine 282

[31]. It appears that size and/or electron density of the amino acid residue is involved in the deactivation of the *FAE1* enzyme.

3.3. Elongase activity in yeast cells upon expression of mutated *FAE1* clones

To determine elongase activity in yeast cells expressing different mutated *FAE1* clones we performed elongase activity

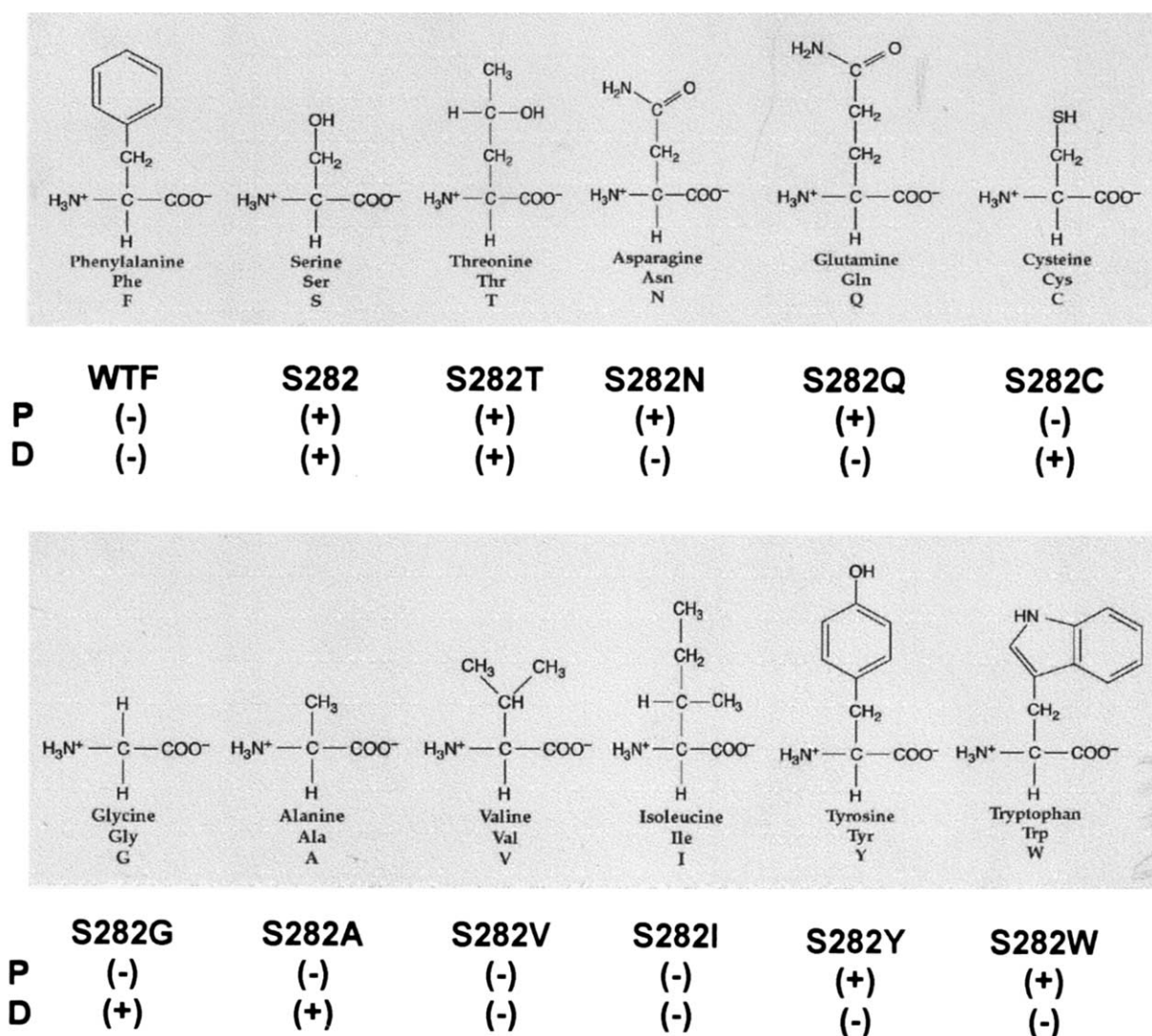


Fig. 3. Schematic presentation of amino acid residues used in site-directed substitution experiments to produce mutated FAE1 polypeptides. Structural formulas of amino acids. Substitutions that resulted in functional enzyme (+) and those that resulted in non-functional polypeptide (–) predicted based on our hypothesis (P) and detected, based on experimental data (D).

assays using yeast homogenates prepared from induced yeast cells and radiolabeled 18:1-CoA and malonyl-CoA as substrates. The yeast cell homogenates expressing mutated FAE1 with glycine 282 showed, on average, slightly higher total 20:1 and 22:1 elongase activity ($25.2 \pm 1.5 + 14.6 \pm 1.0$ pmol/min/mg protein) than cells expressing the FAE1 enzyme with serine 282 ($51.4 \pm 2.5 + 12.0 \pm 0.1$ pmol/min/mg protein). Substitution of serine with threonine 282 resulted in slightly lower enzyme activity ($45.3 \pm 3.9 + 10.7 \pm 1.0$ pmol/min/mg protein), substitution of alanine 282 led to significantly lower enzyme activity ($33.0 \pm 1.2 + 4.8 \pm 1.7$ pmol/min/mg protein) while, surprisingly, yeast cells expressing mutated FAE1 polypeptide with cysteine 282 showed a total enzyme activity ($1\text{-}z^{14}\text{C-}20:1+22:1$ production) of $56.2 \pm 0.5 + 18.0 \pm 0.7$ pmol/min/mg protein which was consistently higher than in yeast cells expressing the naturally occurring FAE1 enzymes with serine 282. Results are reported as the elongation products produced in reactions (pmol/min/mg protein) and are the mean $\pm$ S.D. of three determinations (Fig. 4).

4. Conclusions

In our effort to elucidate the role of serine 282 in functional FAE1 condensing enzyme and determine whether it is crucial for enzyme activity we have produced several mutated FAE1 polypeptides by substituting the amino acid residue serine with neutral, polar or non-polar aliphatic and aromatic amino acids and analyzed their function upon expression in yeast cells. Our analyses have shown that substitution S282G resulted in an enzyme with slightly higher total 20:1+22:1 elongase activity, which could be due to the relatively small size of its side chain. It is possible that a very small amino acid allows for proper folding of the protein molecule. Substitutions with the larger aromatic amino acids tyrosine and tryptophan or with aliphatic amino acids with longer (isoleucine) or branched side chains (asparagine, glutamine, valine) resulted in a non-functional enzyme. Substitutions S282T or S282A resulted in slightly or significantly lower enzyme activity, respectively. We expected the mutated FAE1 polypeptide

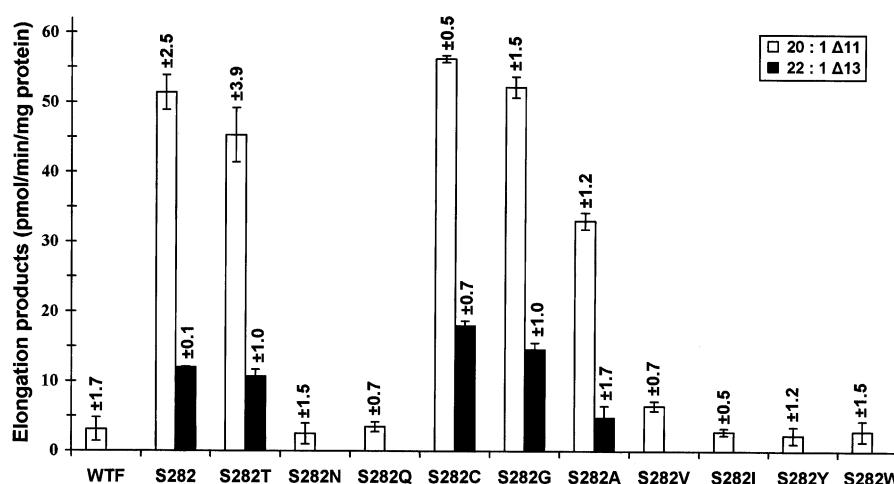


Fig. 4. Elongase activity assayed in lysates from yeast cells upon expression of FAE1 condensing enzyme. Lysates were prepared from yeast cells expressing non-functional FAE1 from LEA *B. napus* cv. Westar (WSF), functional FAE1 with serine 282 (S282) and mutated FAE1 peptides S282T, S282N, S282Q, S282C, S282G, S282A, S282V, S282I, S282Y and S282W. As a negative control we used pYES2.1/V5-His-TOPO plasmid (pYES2.1). Protein samples were incubated at 30°C with shaking at 100 rpm for 60 min with 18 μM [14 C]oleoyl-CoA (0.37 GBq/mol) and 1 mM malonyl-CoA in the presence of 1 mM ATP, 1 mM CoA-SH, 0.5 mM NADH, 0.5 mM NADPH and 2 mM $MgCl_2$ in a final volume of 500 μl. After incubation, reaction mixtures were saponified, transmethyalted and analyzed by HPLC equipped with a flow-through scintillation counter. Results are reported as the elongation products produced in reaction (pmol/min/mg protein) and are the mean $\pm$ S.D. of three determinations.

with threonine 282 to show activity similar to serine 282 because both enzymes are present in nature (although the genomic DNA clone encoding for putative 3-ketoacyl condensing enzyme with threonine 282 was thus far isolated from only one plant species). However, our results indicate that the FAE1 with threonine 282 has lower enzyme activity than FAE1 enzyme with a serine amino acid residue at the same position.

Surprisingly, the mutated FAE1 clone S282C showed consistently higher condensing enzyme activity than compared to the naturally occurring serine 282.

Overall, our results demonstrate that serine 282 in the FAE1 is not crucial for enzyme function because substitution with several small neutral either polar or non-polar aliphatic amino acid residues resulted in active enzymes.

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