

# Addition of an N-terminal epitope tag significantly increases the activity of plant fatty acid desaturases expressed in yeast cells

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**Abstract** *Saccharomyces cerevisiae* shows great potential for development of bioreactor systems geared toward the production of high-value lipids such as polyunsaturated omega-3 fatty acids, the yields of which are largely dependent on the activity of ectopically expressed enzymes. Here, we show that the addition of an N-terminal epitope tag sequence (either Myc or hemagglutinin) to oleate desaturase (FAD2) or omega-3 linoleate desaturase (FAD3) enzymes from plants, which catalyze consecutive reactions in the production of long chain omega-3 fatty acids, significantly increases their activity up to fourfold when expressed in yeast cells. Quantitative protein blotting using an antibody specific for native FAD2 revealed that the steady-state amount of the epitope-tagged FAD2 protein

was also approximately fourfold higher than that of its untagged counterpart, demonstrating a direct relationship between the epitope tag-induced increase in enzyme amount and fatty acid product formation. Protein half-life and RNA blotting experiments indicated that the half-lives and mRNA content of the tagged and untagged FAD2 proteins were essentially the same, suggesting that the epitope tags increased protein abundance by improving translational efficiency. Taken together, these results indicate that the addition of an epitope tag sequence to a plant fatty acid desaturase (FAD) not only provides a useful means for protein immunodetection using highly specific, commercially available antibodies, but that it also significantly increases FAD activity and the production of polyunsaturated fatty acids in yeast cells.

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## Introduction

*Saccharomyces cerevisiae* is an excellent eukaryotic model organism that has been widely used for the development of metabolic engineering strategies aimed at producing high-value metabolites and therapeutic proteins (Porro et al. 2005; Dumas et al. 2006; Branduardi et al. 2008). Although *S. cerevisiae* is not considered an “oleaginous” microorganism (i.e., it does not naturally accumulate >20% of its dry mass as oil), it shows great potential for production of desirable lipid compounds such as sterols, steroid hormones, and polyunsaturated fatty acids (Veen and Lang 2004; Hoffman et al. 2008). Toward this end, a significant amount of information is available regarding the genes and enzymes involved in lipid metabolism in these yeast cells

(Czabany et al. 2007), and a variety of mutants and growth conditions have been identified that induce the accumulation of large amounts of cellular oil (Dyer et al. 2002a; Kamisaka et al. 2006; Kamisaka et al. 2007). Furthermore, *S. cerevisiae* cells are able to take up a variety of structurally diverse fatty acids from their growth media and incorporate them to fairly high levels in their own lipids (Walenga and Lands 1975), demonstrating the high capacity for these cells to serve as bioreactors for production of a diverse array of lipids (Dyer et al. 2002a; Veen and Lang 2004).

*S. cerevisiae* has a simple cellular fatty acid composition that is dominated by only four fatty acids, including palmitic (16:0), palmitoleic (16:1), stearic (18:0), and oleic (18:1) acids, and, combined with the absence of any polyunsaturated fatty acids in this yeast, it is an outstanding model system for studying the structure/activity relationships of ectopically expressed fatty acid desaturases (FADs) from other evolutionarily diverse organisms. Yeast cells have been also used routinely as a model system for understanding the cellular properties and regulation of FAD enzymes, such as the characterization of their intracellular targeting signals and post-translational regulation by temperature (Dyer et al. 2001; McCartney et al. 2004; Tang et al. 2005). The functional properties of many plant desaturases, including the microsomal oleate desaturase (FAD2) and omega-3 linoleate desaturase (FAD3) enzymes, have been characterized in yeast cells (Reed et al. 2000; Broadwater et al. 2002), and cultivation of these transgenic yeast in the presence of a variety of FAD fatty acid substrates has yielded a wealth of information about their substrate–product relationships. Notably, FAD2 and FAD3 enzymes catalyze the production of linoleic and linolenic acids, respectively, each of which is nutritionally important in the human diet. Certain derivatives of linoleic and linolenic acid, such as arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, are high-value fatty acids that are traditionally obtained through dietary sources such as fish oil, and these fatty acids are essential for human brain activity, neonatal development, and proper functioning of the visual system (Uauy and Dangour 2006). Decreasing availability of fish oils due to increased demand and declining fish stocks, however, has generated a need to develop alternative sources of these important fatty acids (Dyer et al. 2008).

While most of the above-mentioned studies with FADs were conducted primarily to gain insight to how these enzymes function in their own endogenous environments (i.e., in plants), they have also helped to establish a significant knowledge base about how they operate in yeast cells, and coupling this understanding with certain lipid-promoting conditions and/or genetic mutants in *S. cerevisiae* has created an excellent opportunity for the development

and evaluation of robust lipid metabolic engineering strategies in this organism (Veen and Lang 2004). The yield of desired fatty acids in any lipid metabolic engineering system is determined in part by both the total amount of lipid within the cells, as well as the percentage of the desired fatty acid relative to other fatty acids within the total lipid pool. As such, metabolic engineering strategies that aim to produce high amounts of polyunsaturated fatty acids are critically dependent on the activity of enzymes such as FAD2 and FAD3 to shift the cellular fatty acid composition in favor of polyunsaturated fatty acids.

Here, we describe a modification to plant FAD sequences that promotes a substantial increase in their enzyme activity when expressed in yeast cells. Specifically, we show that addition of either a Myc or hemagglutinin (HA) epitope tag sequence to the N termini of either *Arabidopsis thaliana* FAD2, tung (*Vernicia fordii*) FAD2, or rapeseed (*Brassica napus*) FAD3 significantly increases the amount of fatty acid products formed by each enzyme in *S. cerevisiae*. Characterization of the molecular mechanisms by which these epitope tags influence FAD enzyme activity revealed that the increase in fatty acid product formation was closely correlated with an increase in the steady-state amount of tagged FAD protein, but not with tagged FAD protein half-life nor mRNA levels. Overall, the elevation of activity of plant FADs via an appended N-terminal epitope tag should increase not only the efficacy of using yeast cells to investigate the structure/function relationships of these enzymes but also promote the development of yeast-based bioreactor systems for the production of high-value lipid compounds.

## Materials and methods

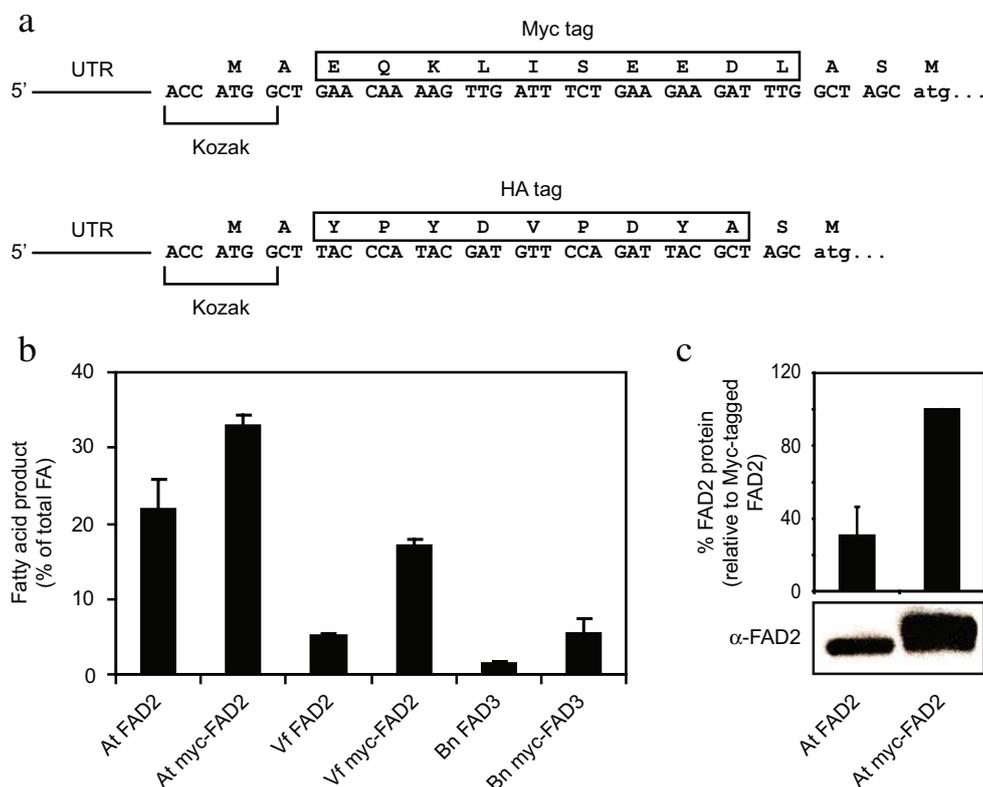
**Plasmid constructions** Myc- and HA-epitope-tagged versions of *A. thaliana* FAD2 (Genbank number L26296), tung (*V. fordii*) FAD2 (AF525534), and *B. napus* FAD3 (L01418) were constructed previously for studies aimed at characterizing the functionality and subcellular localization of the enzymes in yeast and plant cells (Dyer and Mullen 2001; Dyer et al. 2002a, b; McCartney et al. 2004). The epitope tag sequences were generated using synthetic oligonucleotides that included codons that were optimized for expression in yeast cells. The open reading frames (ORFs) of native and epitope-tagged sequences were subsequently amplified (via the polymerase chain reaction [PCR]) using primers that added a Kozak sequence (ACCATGG; Kozak 1987) at the methionine initiation codon (underlined), and PCR fragments were then directly subcloned into the yeast expression vector pYes2.1 (Invitrogen, Carlsbad, CA, USA), a high-copy (2  $\mu$ m) plasmid that contains a galactose-inducible gene promoter and a

uracil prototrophic selectable marker (see Fig. 1a for Kozak and epitope tag sequences used in this study). DNA sequences of all FAD ORFs in pYes2.1 were determined by automated sequencing to ensure the fidelity of the PCR and cloning process.

To create low-copy versions of the *Arabidopsis* FAD2 expression plasmids, the vectors pYes2.1-AtFAD2, pYes2.1-AtmycFAD2, and pYes2.1-AtHAFAD2 were each digested with *SpeI* and *XbaI*, which cut just upstream of the pYes2.1 GAL1 promoter and downstream of the stop codon for each of the respective *Arabidopsis* FAD2 ORFs. The vector pYC2/CT (CEN/ARS, URA; Invitrogen) was digested with *SpeI*, which cut the plasmid both upstream of the GAL1 promoter and also within the multiple cloning site, just upstream of the transcription terminator. The DNA fragments in each restriction digest were then separated on

agarose gels, and the fragments of interest were gel purified and ligated to generate pYC2-AtFAD2, pYC2-AtmycFAD2, and pYC-AtHAFAD2.

**Yeast strains and growth conditions** The *S. cerevisiae* strain MMYO11 $\alpha$  (MAT $\alpha$  ade 2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 OLE<sup>+</sup>; McCammon et al. 1990) was used in all studies. Wild-type yeast cells were maintained on YPD media (1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] dextrose) solidified with 2% (w/v) agar. Plasmids encoding various FADs were transfected into yeast cells using the lithium acetate method of Gietz and Woods (1994), and transformants were selected and maintained on synthetic dextrose (SD) plates (2% [w/v] dextrose, 0.67% [w/v] yeast nitrogen base without amino acids, 2% [w/v] agar) containing appropriate amino acid supplements without uracil.



**Fig. 1** Expression of native (untagged) and epitope-tagged plant FADs in yeast cells using a high-copy, galactose-inducible expression vector (pYes2.1). **a** Diagrams illustrating the epitope tag sequences used in this study. Shown are the DNA sequences corresponding to the (sense) mRNA strand and encoded Myc and HA-epitope amino acid sequences. Each mRNA includes a common 5' UTR derived from the pYes2.1 expression vector followed by a Kozak translation initiation sequence (ACCATGG; Kozak 1987), an alanine codon linker, and then the Myc or HA-epitope tag sequence (boxed, Fritze and Anderson 2000). The translation start codon corresponding to the native FAD sequences is shown in lower case (atg). All constructs that lacked an epitope tag possessed a Kozak translation initiation sequence at their start codon. **b** Gas chromatography analysis of yeast fatty acid

composition showing the accumulation of fatty acid products (i.e., linoleic acid for FAD2 and linolenic for FAD3) synthesized by native or Myc-epitope-tagged versions of *Arabidopsis* (At) FAD2, *V. fordii* (Vf) FAD2, and *B. napus* (Bn) FAD3. Note that yeast cells expressing FAD3 enzymes were cultivated in media containing the FAD3 enzyme substrate, linoleic acid. Bars represent the average and standard deviation of three independent experiments. **c** Protein immunoblot analysis of yeast cells expressing native or Myc-epitope-tagged *Arabidopsis* (At) FAD2, probed with a native anti-FAD2 antibody. Shown are the representative immunoblot and the corresponding bar graph illustrating the average and standard deviation of relative protein amounts determined from quantitative densitometry of three independent experiments

Single colonies were inoculated into liquid SD-Ura starter cultures and grown overnight in an incubator shaker at 30 °C, 300 rpm. Optical density was measured at 600 nm ( $OD_{600}$ ), and an appropriate volume of the overnight culture was harvested by centrifugation, and the cell pellet was resuspended in fresh synthetic galactose (SGal)-Ura media (2% [w/v] galactose instead of dextrose) to give a final  $OD_{600}$  of 0.25. For cultures of yeast cells expressing the *B. napus* FAD3 protein, linoleic acid (Sigma-Aldrich, St. Louis, MO, USA) was added to the growth media at 0.1% (v/v). Cell cultures were incubated for an additional 20–22 h at 30 °C, 300 rpm to late log-stationary phase. For RNA isolations (see below), yeast cells were harvested at mid-log phase (~1.0  $OD_{600}$ ).

**Measurement of yeast fatty acid composition** Yeast cells from a 50-ml overnight culture were harvested by centrifugation and washed once in 0.5% (v/v) tween 20 and once with deionized water. Cells were then converted to spheroplasts by enzymatic digestion of cell walls using Zymolyase-20T (ICN, Costa Mesa, CA, USA) as described (Dyer et al. 2002a), and then lipids were extracted using a modified method of Bligh and Dyer (1959). Briefly, spheroplasts were resuspended in 800  $\mu$ l of water to osmotically lyse the cells, then 3 ml of chloroform/methanol (1:2 [v/v]) was added, and the samples were vortexed vigorously for 30 s. Samples were allowed to stand for 5 min at room temperature with occasional vortexing, then centrifuged at 4,000 rpm, 30 °C for 5 min to pellet the denatured proteins and cellular debris. The supernatant was recovered and transferred to a glass tube, and the pellet was washed with 1 ml of chloroform. After centrifugation as described above, the chloroform wash was combined with the previous supernatant. One milliliter of 0.88% (w/v) KCl was added to the samples to promote phase separation, and after centrifugation at 2,000 rpm, 30 °C for 5 min, the lower chloroform layer containing the lipid was transferred to Teflon-capped vials. Lipid concentration was estimated gravimetrically by transferring 0.3 ml of chloroform extract to pre-weighed vials and evaporating the chloroform under a stream of nitrogen. A volume representing 1 mg of lipid was transferred to screw-capped tubes and evaporated under nitrogen to dryness, and the lipid residue was resuspended in sodium methoxide. Fatty acid methyl esters were prepared using sodium methoxide and separated and identified by gas chromatography and flame ionization detection, as described previously (Dyer et al. 2002b).

**Determination of steady-state protein amount and protein half-life by protein immunoblotting** To determine the steady-state amount of FAD proteins, yeast cells were harvested and lysed according to a modified method described by Braun et al. (2002). Briefly, a volume

representing 1.0  $OD_{600}$  units (for cells containing the high-copy pYes2.1-based plasmids) or 2.0  $OD_{600}$  units (for pYC2/CT-based, low-copy plasmids) of cell culture was harvested by centrifugation, and the cells were then resuspended in 1 ml of ice-cold sterile water; 150  $\mu$ l of 7.5% (v/v)  $\beta$ -mercaptoethanol in 1.85 M NaOH was added, the samples were vortexed and incubated on ice for 15 min. Then, 150  $\mu$ l of 55% (w/v) trichloroacetic acid (TCA) was added, and the samples were vortexed and incubated on ice for another 10 min. Samples were then centrifuged 10 min at 4 °C, and pellets were resuspended in 100  $\mu$ l of 1 $\times$  LDS Sample Buffer (Invitrogen) containing 48% (w/v) urea by vigorous vortexing for 15 min at 37 °C. Samples were centrifuged for 10 min at 14,000 rpm, and the supernatant was transferred to a fresh tube and stored at -20 °C until SDS-PAGE. Samples were incubated for 5 min at 37 °C prior to loading the gels; boiling lysate samples prior to SDS-PAGE was avoided since this causes plant desaturases to precipitate from solution (O'Quin and Dyer, unpublished data).

For protein half-life analysis, cells were cultured to mid-log phase (~1.0  $OD_{600}$ ), then transcription and translation of the FAD genes were halted by the addition of 2% (w/v) dextrose and 0.5 mg/ml cycloheximide (final concentrations), respectively. A volume representing 2.0  $OD_{600}$  units of cells was removed, and the cultures returned to an incubator/shaker. Aliquots of the same OD were also removed at time=1 h, 2, 4, and 8 h. Each aliquot was immediately processed up until the point of adding the TCA as described above, and samples were kept at this step until all remaining samples had been collected. Lysates were then prepared and analyzed by SDS-PAGE as described above.

Proteins were resolved in 10% (w/v) acrylamide Nupage gels (Invitrogen) using MagicMark XP (Invitrogen) as standards and then transferred to Immuno-Blot PVDF membranes (0.4  $\mu$ m; Bio-Rad, Hercules, CA, USA) using a Bio-Rad Trans-Blot Cell (Bio-Rad), according to the companies' protocols for buffers and conditions. Protein immunoblotting was performed as described by Towbin et al. (1979). Affinity-purified primary antibodies for *Arabidopsis* FAD2 were generated in rabbits by Bethyl Laboratories (Montgomery, TX, USA) using a synthetic peptide corresponding to an amino-terminal portion of the FAD2 protein sequence (amino acid residues 9-28, -VPTS SKKSETDTTKRVPCEK-); anti-*Arabidopsis* FAD2 IgGs were used at a dilution of 1:5,000. Sources and dilutions of other antibodies used in this study were as follows: donkey anti-rabbit IgGs linked to horseradish peroxidase (HRP; Amersham Biosciences, Piscataway, NJ; 1:10,000); mouse anti-Myc polyclonal antibodies (Covance, Richmond, CA, USA; 1:1,000); goat anti-mouse IgGs, HRP (BioSource International, Camarillo, CA, USA; 1:10,000); mouse anti-

dolichol phosphate mannose synthase (DPMS) monoclonal antibodies (Molecular Probes, Eugene, OR, USA; 1:100). Protein immunodetection was performed with the ECL Plus Western Blotting Detection System from Amersham Biosciences according to company protocols. Visualization of immunoreactive proteins was performed using a FUJI LAS-1000 (Fujifilm, Stamford, CT, USA), and quantification of band intensities was performed using Image Gauge software (Fujifilm).

**RNA extraction and RNA blotting** Total RNA was extracted from yeast cells using the hot acidic phenol method of Collart and Oliviero (1993). The concentrations and purities of the extracted RNA were determined spectrophotometrically by measuring at OD<sub>260</sub> and OD<sub>280</sub>. RNA samples were separated on denaturing agarose gels using the NorthernMax kit (Ambion, Austin, TX, USA), then RNA was transferred to Nytran SuPerCharge membranes using a Turboblotter (Schleicher & Schuell, Keene, NH, USA) and hybridized using a digoxigenin (DIG)-labeled probe (PCR-DIG probe synthesis Kit, Roche, Nutley, NJ, USA) that bound to the 3'-untranslated region (UTR) of FAD mRNAs expressed from pYC2/CT (nucleotides 726-1,048). Anti-DIG-alkaline phosphatase Fab fragments solution (Roche) was used as the antibody in the northern blotting and CDP-Star (Roche), and chemiluminescence was detected, captured, and quantified using a FUJI LAS-1000.

## Results

**Addition of the Myc epitope tag to FAD2 and FAD3 increases their enzyme activity and steady-state protein abundance in yeast cells** To investigate the effects of epitope tagging on plant desaturase activity, a single copy of the Myc epitope peptide sequence (-EQKLISEEDL-; Fritze and Anderson 2000) was added to the N termini of *Arabidopsis* FAD2, tung FAD2, and *B. napus* FAD3 proteins using standard recombinant DNA techniques (Fig. 1a). Thereafter, both the native (untagged) and modified (tagged) proteins were expressed in yeast cells using the high-copy, galactose-inducible vector pYes2.1. Enzyme activity was evaluated by extracting total cellular lipids from late-log, stationary phase cell cultures and determining their fatty acid composition by gas chromatography. Cells expressing the native or tagged FAD3 proteins were cultivated also in the presence of linoleic acid, which is readily taken up into yeast and serves as a substrate for the FAD3 enzyme (Reed et al. 2000; Dyer et al. 2001). As shown in Fig. 1b, the amount of fatty acid product formed by each Myc-epitope-tagged enzyme (i.e., linoleic acid for FAD2 and linolenic for FAD3) was

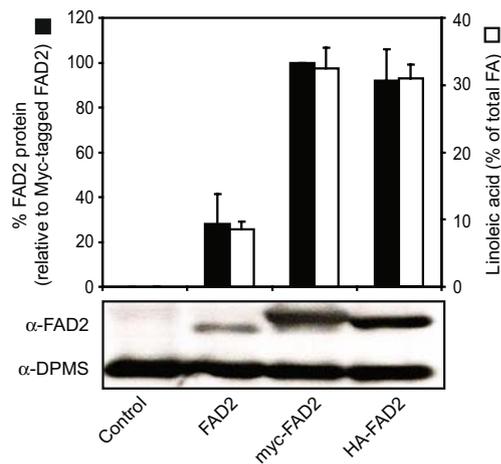
significantly higher than the amount generated by the corresponding untagged, native enzymes.

To begin to characterize the mechanism(s) involved in this upregulation of enzyme activity for the tagged proteins, an antibody was raised that recognized both the native and epitope-tagged versions of the *Arabidopsis* FAD2 protein. Immunoblot analysis of proteins extracted from yeast cells expressing either native or epitope-tagged *Arabidopsis* FAD2 revealed that the steady-state amount of Myc-tagged FAD2 protein was approximately threefold higher than that of its untagged counterpart (Fig. 1c). These results demonstrate a direct correlation between increased epitope-tagged FAD protein amounts and increased fatty acid product formation (i.e., FAD enzyme activity) in yeast cells.

**Effects of plasmid copy number and epitope tag sequence on FAD2 enzyme activity and steady-state protein abundance** Since multi-copy expression plasmids such as pYes2.1 are known to be variable in terms of copy number per cell and that this might contribute to artifacts associated with protein overexpression, the *Arabidopsis* FAD2 sequence was transferred to a single copy (CEN/ARS) vector (i.e., pYC2/CT) to obtain a more sensitive measure of the effects of the appended N-terminal epitope tag on FAD protein abundance and fatty acid product accumulation. The Myc epitope tag was also replaced with the HA-epitope peptide sequence (-YPYDVEDYA-; Fritze and Anderson 2000; refer to Fig. 1a) to investigate whether the increase in FAD activity and fatty acid product amount was due to any specific amino acids within the Myc epitope tag sequence.

As shown in Fig. 2, immunoblot analysis (with anti-FAD2 antibodies) of proteins derived from yeast cells expressing the various FAD2 sequences in low-copy plasmids revealed a dramatic increase (nearly fourfold) in both Myc- and HA-tagged FAD2 protein amounts, i.e., in comparison to the amount of native (untagged) FAD2 protein. Moreover, these changes in protein abundance in cells expressing epitope-tagged versus native FAD2 proteins were mirrored closely by a similar fold increase in linoleic acid product accumulation (Fig. 2). Taken together, these results indicate that the observed effects of the epitope tag on FAD activity and fatty acid product accumulation are not due to overexpression artifact(s) or any sequence-specific features of the Myc epitope tag.

**Effects of epitope tags on FAD2 protein half-life and mRNA content** Previous analyses of soybean FAD2 enzymes expressed in yeast cells demonstrated that they are regulated, at least in part, by a post-translational mechanism that involves rapid protein turnover (Tang et al. 2005). To determine if the increase in steady-state protein amount of the *Arabidopsis* epitope-tagged FAD2 proteins observed here might be also due to an increase in protein half-life, the



**Fig. 2** Enzyme activity and quantitative immunoblot analysis of native (untagged) and Myc- and HA-epitope-tagged *Arabidopsis* FAD2 proteins expressed in yeast cells. Native, Myc-, or HA-tagged *Arabidopsis* FAD2 genes were expressed from a low-copy, galactose-inducible vector, and then yeast cells were harvested, their lipid content determined by gas chromatography, and relative FAD2 protein amounts were determined by immunoblotting with anti-FAD2 antibodies. The lower panel shows a representative immunoblot, which includes the immunodetection of the endogenous endoplasmic reticulum membrane protein dolichol phosphate mannose synthase (DPMS; Orlean 1990), serving as a loading control. The bar graph illustrates both quantitative analysis of FAD2 steady-state protein levels (relative to Myc-epitope-tagged FAD2) as well as the amount of FAD2 fatty acid product (i.e., linoleic acid) present in each yeast strain

rates of protein degradation of both Myc- or HA-epitope-tagged *Arabidopsis* FAD2 were determined and compared to the half-life of the native, untagged enzyme. For protein half-life studies, the transcription and translation of the introduced FAD genes were suppressed by addition to the growth media of glucose and cycloheximide, respectively (Braun et al. 2002), and then aliquots of cell culture were removed over time, and total protein lysates were analyzed by SDS-PAGE and immunoblotting (with anti-FAD2 antibodies). As summarized in Fig. 3a, quantitative analysis of the protein immunoblots (see Fig. 3b and c) demonstrated that the half-life of untagged FAD2 protein was 5.44 h, while the half-life of Myc- and HA-tagged FAD2 was 4.74 and 7.40 h, respectively. Statistical analysis of the experimental replicates used to calculate these protein half-life values indicated that they were not statistically different ( $p$  value=0.7424,  $n=5$ ). Thus, the fourfold increase in steady-state protein abundance observed for either Myc- or HA-tagged FAD2 (Fig. 2) cannot be explained by a significant increase in their protein half-lives relative to that for native (untagged) FAD2.

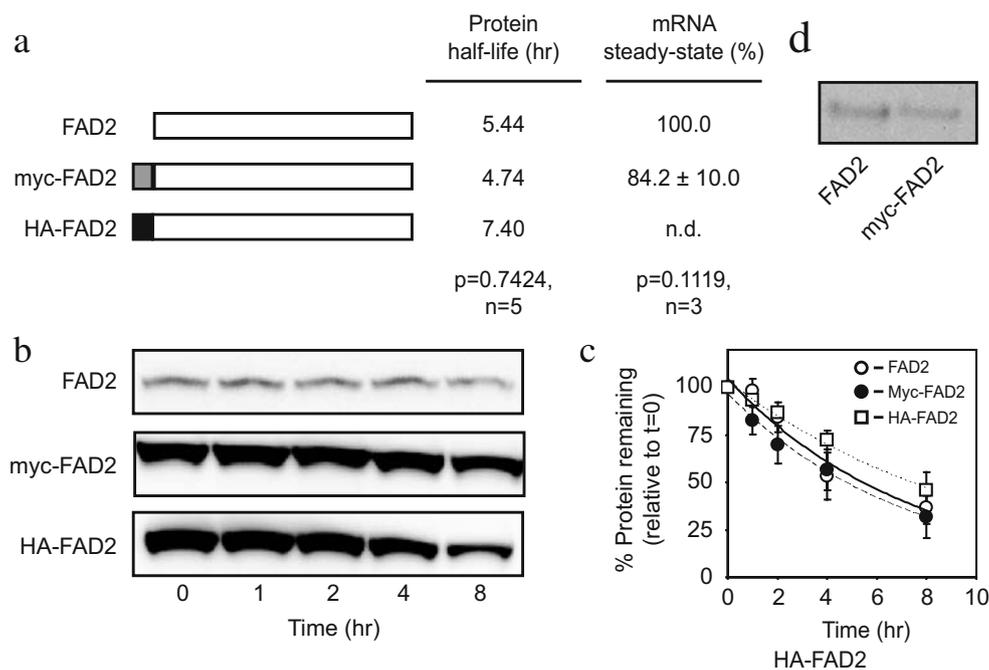
To determine if the observed differences in steady-state amount of FAD2 protein might be due instead to differences in the quantities of mRNA encoding tagged or untagged proteins, RNA gel blotting experiments were

performed using a DIG-labeled probe that recognized the 3' UTR region found in all of the FAD genes expressed from the pYC2/CT plasmid (see “Methods and materials” for details). As summarized in Fig. 3a, quantitative analysis of the RNA immunoblot revealed no significant differences in the steady-state amounts of mRNA encoding native or Myc-epitope-tagged FAD2 (Fig. 3d). Taken together, the results indicate that the increase in epitope-tagged FAD2 steady-state protein amounts and the subsequent increase in enzyme activity are not due to enhanced protein stability or changes in mRNA content due to the epitope tag, but rather is likely due to a tag-specific increase in translational efficiency of the mRNA. The resulting increase in protein steady-state abundance is directly correlated to an increase in the percentage of fatty acid products in the yeast cells.

## Discussion

Here, we demonstrate that addition of either the Myc- or HA-epitope tag sequence to the N termini of several plant FAD proteins significantly increases their enzyme activity when expressed in yeast cells (Fig. 1b). We chose to add these peptide epitope tags to the N termini (Fig. 1a) rather than to internal or C-terminal regions of these proteins in order to minimize any potential negative effects on FAD structure/activity relationships and/or subcellular targeting. For instance, it is well established that FADs possess three internal histidine-rich boxes that coordinate two iron atoms at their active site (Shanklin et al. 1994). Plant FAD2 and FAD3 enzymes are also known to be anchored in the ER membrane by four internal transmembrane-spanning regions and then retained in the ER by C-terminal peptide retrieval sequences (McCartney et al. 2004), that if deleted, completely abolish their enzyme activity (McCartney et al. 2004). Furthermore, addition of the Myc tag to the C-terminus of the structurally related FAD8 protein, which is an omega-3 desaturase located in the plastids of plant cells, also results in deactivation of the enzyme (Matsuda et al. 2005). Consequently, the most logical choice for placement of an immunoreactive epitope tag sequence to the FAD2 and FAD3 enzymes examined in this study was at their N termini.

To begin to investigate the underlying mechanism(s) responsible for the increase in enzyme activity of epitope-tagged FADs in yeast cells, we examined protein steady-state abundance of *Arabidopsis* FAD2 by raising an antibody that recognized both the native and epitope-tagged versions of the FAD2 enzyme. Immunoblot analysis demonstrated that the steady-state abundance of the epitope-tagged FAD2 proteins increased significantly in comparison to the unmodified protein, and the increase in protein abundance was accompanied by an increase in the



**Fig. 3** Determination of protein half-life and mRNA steady-state levels for native (untagged) and differentially epitope-tagged *Arabidopsis* FAD2 proteins expressed in yeast cells. Native, Myc-, or HA-tagged *Arabidopsis* FAD2 genes were expressed from a low-copy, galactose-inducible vector (pYC2/CT), and cells were cultured to mid-log phase in galactose media. **a** Line drawings representing each FAD2 construct and a summary of the results of their protein half-life and mRNA levels, the results of which are shown in **b–d**. Protein half-lives (**b** and **c**) were determined by quantitative protein immunoblotting following addition of glucose and cycloheximide to the growth media at  $t=0$  to suppress transcription and translation of the FAD genes, respectively, and removal of aliquots at  $t=0, 1, 2, 4,$  and  $8$  h. Proteins were extracted and resolved by SDS-PAGE, and subsequent immuno-

blotting of membranes was carried out using anti-FAD2 antibodies. A representative series of protein immunoblots is shown in **b**, and **c** shows a quantitative analysis of native, Myc-, or HA-tagged *Arabidopsis* FAD2 protein decay (data points represent the average and standard deviations obtained from five independent experiments). Protein half-lives were determined by fitting these data with a single exponential equation and solving for 50% of the protein remaining.  $R^2$  values for the curves ranged from 0.96 to 0.99. For RNA gel blot analysis (**d**), cells were harvested at mid-log phase, mRNA was extracted and resolved on denaturing agarose gels and transferred to membranes, and then membranes were incubated with a DIG-labeled probe that recognized the same sequence in the 3' UTR of each FAD2 mRNA

percentage of fatty acid products in yeast cells (Fig. 2). These results demonstrate that there were no sequence-specific features of the epitope tag(s) that contributed to the increased activity of the FAD2 enzymes (since the Myc and HA sequences are entirely different; Fig. 1a), but rather there must be some other shared quality of the peptide epitopes (or their encoded sequences) that contributed to an increase in protein steady-state amounts.

The steady-state amount of a protein within cells is known to be determined by the combination of several factors, including mRNA abundance, the rate of protein translation, and the rate of protein degradation. Here, we demonstrated that addition of an epitope tag to *Arabidopsis* FAD2 did not affect mRNA levels or the rate of protein degradation (Fig. 3), and therefore, the increase in FAD2 protein steady-state amounts likely was due a priori to an increase in the rate of mRNA translation. Although we did not test this possibility directly, there are several precedents in the literature demonstrating that the optimization of approximately the first 15–30 codons of the coding region

of an mRNA species can significantly improve the translation and, hence, accumulation of the encoded ectopically expressed protein (Batard et al. 2000; Hehn et al. 2002; Flis et al. 2005). Furthermore, all of the plant FAD ORFs (either the native or epitope-tagged sequences) were cloned into the yeast expression vectors such that there were no differences in their 5' UTR sequences. That is, all of the FAD 5' UTR sequences in this study were identical since they were derived entirely from the yeast expression vector pYC2/CT, and thus, each plant FAD ORF began with a similar Kozak translation initiation sequence (Kozak 1987) at their start codon (Fig. 1a). Therefore, the differences observed in steady-state amount of native (untagged) and epitope-tagged FAD2 protein amounts are not likely due to their shared 5' UTR regions, but rather the codon-optimized (for *S. cerevisiae*) sequences present within the epitope tag sequences.

Another mechanism that can be used to increase the amount of FAD protein (and subsequent fatty acid production formation) in yeast cells is to culture the yeast

at cooler growth temperatures, which can significantly increase protein steady-state abundance by increasing protein half-life (Tang et al. 2005). The drawback to using this approach in a commercial bioreactor system, however, is that the doubling time of yeast cells is significantly longer at cooler growth temperatures, resulting in much longer times for biomass (and product) accumulation. In addition, certain plant FADs may not be susceptible to temperature-induced changes in enzyme activity (Kajiwara et al. 1996). Therefore, epitope tagging represents a powerful new method that can be used to increase the activity of plant FADs in *S. cerevisiae* under normal growth temperatures, thereby increasing the ratio of polyunsaturated fatty acids in yeast lipids. Coupling these lipid modifying enzymes with conditions that promote significant accumulation of total oil in yeast cells could provide a robust platform for producing high-value oils. Furthermore, since *S. cerevisiae* is “Generally Regarded as Safe” (Verstrepen et al. 2006), it is conceivable that the value-added oils produced in these yeast cells could be used directly in food and feed applications.

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## References

- Batard Y, Hehn A, Nedelkina S, Schalk M, Pallett K, Schaller H, Werck-Reichhart D (2000) Increasing expression of P450 and P450-reductase proteins from monocots in heterologous systems. *Arch Biochem Biophys* 379:161–169
- Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917
- Branduardi P, Smeraldi C, Porro D (2008) Metabolically engineered yeasts: ‘potential’ industrial applications. *J Mol Microbiol Biotechnol* 15:31–40
- Braun S, Matuschewski K, Rape M, Thoms S, Jentsch S (2002) Role of the ubiquitin-selective CDC48<sup>UFD1/NPL4</sup> chaperone (segregase) in ERAD of OLE1 and other substrates. *EMBO J* 21:615–621
- Broadwater JA, Whittle E, Shanklin J (2002) Desaturation and hydroxylation. Residues 148 and 324 of Arabidopsis FAD2, in addition to substrate chain length, exert a major influence in partitioning of catalytic specificity. *J Biol Chem* 277:15613–15620
- Collart MA, Oliviero S (1993) Preparation of yeast RNA. In: Ausubel FM, Brent R, Kingston RE et al (eds) *Current protocols in molecular biology*. Greene, New York, pp 13.12.1–13.12.5
- Czabany T, Athenstaedt K, Daum G (2007) Synthesis, storage and degradation of neutral lipids in yeast. *Biochim Biophys Acta* 1771:299–309
- Dyer JM, Mullen RT (2001) Immunocytological localization of two plant fatty acid desaturases in the endoplasmic reticulum. *FEBS Lett* 494:44–47
- Dyer JM, Chapital DC, Cary JW, Pepperman AB (2001) Chilling-sensitive, post-transcriptional regulation of a plant fatty acid desaturase expressed in yeast. *Biochem Biophys Res Commun* 282:1019–1025
- Dyer JM, Chapital DC, Kuan JW, Mullen RT, Pepperman AB (2002a) Metabolic engineering of *Saccharomyces cerevisiae* for production of novel lipid compounds. *Appl Microbiol Biotechnol* 59:224–230
- Dyer JM, Chapital DC, Kuan J-CW, Mullen RT, Turner C, McKeon TA, Pepperman AB (2002b) Molecular analysis of a bifunctional fatty acid conjugase/desaturase from tung. Implications for the evolution of plant fatty acid diversity. *Plant Physiol* 130:2027–2038
- Dyer JM, Stymne S, Green AG, Carlsson AS (2008) High-value oils from plants. *Plant J* 54:640–655
- Dumas B, Brocard-Masson C, Assemet-Lebrun K, Achstetter T (2006) Hydrocortisone made in yeast: Metabolic engineering turns a unicellular microorganism into a drug-synthesizing factory. *Biotechnol J* 1:299–307
- Flis K, Hinzpeter A, Edelman A, Kurlandzka A (2005) The functioning of mammalian CIC-2 chloride channel in *Saccharomyces cerevisiae* cells requires an increased level of Kha1p. *Biochem J* 390:655–664
- Fritze CE, Anderson TR (2000) Epitope tagging: general method for tracking recombinant proteins. *Methods Enzymol.* 327:3–16
- Gietz RD, Woods RA (1994) High efficiency transformation in yeast. In: Johnston JA (ed) *Molecular genetics of yeast: practical approaches*. Oxford University Press, New York, pp 121–134
- Hehn A, Morant M, Werck-Reichhart D (2002) Partial recoding of P450 and P450 reductase cDNAs for improved expression in yeast and plants. *Methods Enzymol* 357:343–351
- Hoffman M, Wagner M, Abbadi A, Fulda M, Feussner I (2008) Metabolic engineering of  $\omega$ 3-very long chain polyunsaturated fatty acid production by an exclusively acyl-CoA-dependent pathway. *J Biol Chem* 283:22352–22362
- Kamisaka Y, Noda N, Tomita N, Kimura K, Kodaki T, Hosaka K (2006) Identification of genes affecting lipid content using transposon mutagenesis in *Saccharomyces cerevisiae*. *Biosci Biotechnol Biochem* 70:646–653
- Kamisaka Y, Tomita N, Kimura K, Kainou K, Uemura H (2007) DGA1 (diacylglycerol acyltransferase gene) overexpression and leucine biosynthesis significantly increase lipid accumulation in the  $\Delta$ snf2 disruptant of *Saccharomyces cerevisiae*. *Biochem J* 408:61–68
- Kajiwara S, Shirai A, Fujii T, Toguri T, Nakamura K, Ohtaguchi K (1996) Polyunsaturated fatty acid biosynthesis in *Saccharomyces cerevisiae*: expression of ethanol tolerance and the FAD2 gene from *Arabidopsis thaliana*. *Appl Environ Microbiol* 62:4309–4313
- Kozak M (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* 15:8125–8148
- Matsuda O, Sakamoto H, Hashimoto T, Iba K (2005) A temperature-sensitive mechanism that regulates post-translational stability of a plastidial  $\omega$ -3 fatty acid desaturase (FAD8) in *Arabidopsis* leaf tissues. *J Biol Chem* 280:3597–3604
- McCammon MT, Veenhuis M, Trapp SB, Goodman JM (1990) Association of glyoxylate and beta-oxidation enzymes with peroxisomes of *Saccharomyces cerevisiae*. *J Bacteriol* 172:5816–5827
- McCartney AW, Dyer JM, Dhanoa PK, Kim PK, Andrews DW, McNew JA, Mullen RT (2004) Membrane-bound fatty acid desaturases are inserted co-translationally into the ER and contain different ER retrieval motifs at their carboxy termini. *Plant J* 37:156–173
- Orlean P (1990) Dolichol phosphate mannose synthase is required in vivo for glycosyl phosphatidylinositol membrane anchoring. *O*

- mannosylation, and N glycosylation of protein in *Saccharomyces cerevisiae*. *Mol Cell Biol* 10:5796–805
- Porro D, Sauer M, Branduardi P, Mattanovich D (2005) Recombinant protein production in yeasts. *Mol Biotechnol* 31(3):245–259
- Reed DW, Schäfer UA, Covello PS (2000) Characterization of the *Brassica napus* extraplastidial linoleate desaturase by expression in *Saccharomyces cerevisiae*. *Plant Physiol* 122:715–720
- Shanklin J, Whittle E, Fox BG (1994) Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. *Biochemistry* 33:12787–12794
- Tang GQ, Novitzky WP, Carol Griffin H, Huber SC, Dewey RE (2005) Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation. *Plant J* 44:433–446
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76:4350–4354
- Uauy R, Dangour AD (2006) Nutrition in brain development and aging: role of essential fatty acids. *Nutr Rev* 64:24–33
- Veen M, Lang C (2004) Production of lipid compounds in the yeast *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 63:635–646
- Verstrepen KJ, Chambers PJ, Pretorius IS (2006) The development of superior yeast strains for the food and beverage industries: challenges, opportunities, and potential benefits. In: Querol A, Fleet GH (eds) *The yeast handbook: yeasts in food and beverages*. Springer, Berlin, pp 399–444
- Walenga RW, Lands WE (1975) Effectiveness of various unsaturated fatty acids in supporting growth and respiration in *Saccharomyces cerevisiae*. *J Biol Chem* 250:9121–9129