Temperature-sensitive Post-translational Regulation of Plant Omega-3 Fatty-acid Desaturases Is Mediated by the Endoplasmic Reticulum-associated Degradation Pathway*§

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Changes in ambient temperature represent a major physiological challenge to membranes of poikilothermic organisms. In plants, the endoplasmic reticulum (ER)-localized omega-3 fatty-acid desaturases (Fad3) increase the production of polyunsaturated fatty acids at cooler temperatures, but the FAD3 genes themselves are typically not up-regulated during this adaptive response. Here, we expressed two closely related plant FAD3 genes in yeast cells and found that their enzymes produced significantly different amounts of omega-3 fatty acids and that these differences correlated to differences in rates of protein turnover. Domain-swapping and mutagenesis experiments revealed that each protein contained a degradation signal in its N terminus and that the charge density of a PEST-like sequence within this region was largely responsible for the differences in rates of protein turnover. The half-life of each Fad3 protein was increased at cooler temperatures, and protein degradation required specific components of the ER-associated degradation pathway, including the Cdc48 adaptor proteins Doa1, Shp1, and Ufd2. Expression of the Fad3 proteins in tobacco cells incubated with the proteasomal inhibitor MG132 further confirmed that they were degraded via the proteasomal pathway in plants. Collectively, these findings indicate that Fad3 protein abundance is regulated by a combination of cis-acting degradation signals and the ubiquitin-proteasome pathway and that modulation of Fad3 protein amounts in response to temperature may represent one mechanism of homeoviscous adaptation in plants.

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3 The abbreviations used are: PUFA, polyunsaturated fatty acid; BF3, Brassica napus fatty acid omega-3 desaturase; BY-2, bright yellow-2; DPM5, dolichol-phosphate mannose synthase; ER, endoplasmic reticulum; ERAD, ER-associated degradation; Fad, fatty-acid desaturase; GFP, green fluorescent protein; mGFP, monomeric green fluorescent protein; MS, Murashige-Skoog; ORF, open reading frame; RFP, red fluorescent protein; SCD, stearoyl-CoA desaturase; SGal, synthetic galactose; ss, signal sequence; TMD, transmembrane domain; UPS, ubiquitin-proteasome system; WT, wild type.

4 The abbreviations used are: PUFA, polyunsaturated fatty acid; BF3, Brassica napus fatty acid omega-3 desaturase; BY-2, bright yellow-2; DPM5, dolichol-phosphate mannose synthase; ER, endoplasmic reticulum; ERAD, ER-associated degradation; Fad, fatty-acid desaturase; GFP, green fluorescent protein; mGFP, monomeric green fluorescent protein; MS, Murashige-Skoog; ORF, open reading frame; RFP, red fluorescent protein; SCD, stearoyl-CoA desaturase; SGal, synthetic galactose; ss, signal sequence; TMD, transmembrane domain; UPS, ubiquitin-proteasome system; WT, wild type.
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PUFAs including Fad6, which converts oleic to linoleic acid, and Fad7 and Fad8, each of which produces \( \alpha \)-linolenic acid (6). The majority of PUFAs in green tissues are synthesized by the Fad6 and Fad7/Fad8 enzymes in chloroplasts, whereas the PUFAs in non-green tissues such as roots are produced primarily by the Fad2 and Fad3 enzymes in the ER (6).

Although the desaturases present in the ER and chloroplast compartments share a number of structural features, including the presence of multiple transmembrane domains (TMDs) and several conserved histidine-rich regions presumed to coordinate iron atoms at the enzyme active site, they differ functionally in terms of their affinity for fatty acid substrates bound to different types of glycerolipids, primarily phosphatidylcholine in the ER, and galactolipids in plastids (7). Furthermore, both ER and chloroplast desaturases have been shown to be important for temperature adaptation in plants (8–11), and analyses of mutant plants disrupted in one or more of these enzymes have revealed that there may be significant exchange of glycerolipid species between the two compartments during temperature adaptation (12). As such, the development of an integrated picture for understanding how plants respond to temperature stress will depend, in part, on understanding how Fad enzymes are regulated in both the ER and chloroplast compartments.

To date, the majority of evidence suggests that Fad activity is regulated primarily at the post-transcriptional level (13). For instance, PUFA content in plant cells generally increases as temperature decreases, but amounts of mRNA for associated FAD genes often do not change appreciably at cooler temperatures. Furthermore, expression of either FAD2 genes in yeast cells (14, 15) or FAD7/FAD8 chimeric genes in transgenic Arabidopsis (16) reveals that Fad2 and Fad8 enzymes are short-lived proteins for which abundance is modulated by temperature. As such, one of the mechanisms for Fad regulation in plant cells appears to involve the modulation of Fad protein half-life in response to temperature. Although the details of Fad3 post-translational regulation are less clear, Horiguchi et al. (17) examined wheat root tips derived from plants that were cultivated at either 30 or 10 °C and found that Fad3 protein amount increases ~7.5-fold at cooler temperatures, and \( \alpha \)-linolenic acid content increases by 2-fold, but mRNA content increases only slightly. Although this increase in Fad3 protein could be explained in part by an enhanced association of polyribosomes with FAD3 mRNA at cooler temperatures, neither the half-life of Fad3 proteins nor any potential changes in the rate of protein degradation were measured.

Previously we expressed the FAD3 gene of Brassica napus (BF3) in yeast cells cultured at 30, 20, or 10 °C and observed results similar to those detected for Fad3 in wheat root tips, including a doubling in production of \( \alpha \)-linolenic acid, no change in the amount of mRNA, and an approximate 8.5-fold increase in BF3 protein steady-state amount (18). The similarities in the findings from these two studies (17, 18) indicate that yeast cells could serve as a powerful model system for gaining insight into the mechanisms of Fad3 post-transcriptional control. More recently, we identified another FAD3 gene from the tung tree (Vernicia fordii) (TF3) in which enzyme activity, like that of BF3 (18), is increased in yeast cells when cultivated at cooler temperatures; but the amount of \( \alpha \)-linolenic acid produced by this enzyme is significantly higher than that of BF3, suggesting that these enzymes are differentially regulated in yeast (19). Here we have provided a detailed examination of the post-translational regulation of TF3 genes expressed in either yeast or plant cells, showing that both Fad3 proteins are rapidly degraded in either cell type, that their degradation is dependent on the ubiquitin-proteasome degradation pathway, and that the half-life of the proteins expressed in yeast cells is modulated by changes in temperature. Furthermore, we demonstrate that both proteins possess a PEST-like degradation signal in their N terminus, which in yeast is responsible for mediating their rapid protein turnover, and that specific components of the ER-associated degradation (ERAD) pathway are required for their protein degradation. The implications for understanding the homeoviscous adaptation of plants are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—DIFCO brand yeast medium was purchased from Fisher Scientific. Synthetic complete amino acid supplement dropout mix lacking uracil was from Bufferad (Lake Bluff, IL). Restriction enzymes, ligases, calf intestinal alkaline phosphatase, and DNA ladders were from New England Biolabs (Beverly, MA). RNA molecular weight markers were from Roche Applied Science. TOPO cloning kits and chemically competent cells were from Invitrogen. Unless otherwise noted, all other chemicals were purchased from either Sigma-Aldrich or Fisher Scientific.

**Recombinant DNA Procedures**—GenBank accession numbers for the FAD3 genes used in this study are: B. napus, L01418.1; tung (V. fordii) AF047172.2; soybean, BAB18135.2, Arabidopsis thaliana, NP_180559; Betula pendula, AAN17504, AAN17503, and AAN17502; and Capsicum annuum, AAF27933. All general cloning procedures were performed as described by Sambrook et al. (20), and the integrity of DNA sequences modified by PCR or other mutagenic procedures was confirmed by sequencing. Full-length open reading frames (ORFs) for both BF3 and TF3 were subcloned previously into the high copy, galactose-inducible, yeast expression vector, pYes2.1 (Invitrogen), with and without an N-terminal c-Myc epitope tag (18, 19). These plasmids are called pYes2.1-TF3, pYes2.1-MycTF3, pYes2.1-BF3, and pYes2.1-MycBF3. Chimeric FAD3 sequences, with their N-terminal coding sequences swapped, were generated by overlap extension PCR (21) using plasmids pYes2.1-MycTF3 and pYes2.1-MycBF3 as templates. Specifically, the N-terminal coding region of TF3, from the beginning of the Myc tag to a conserved aspartic acid residue just upstream of the first predicted TMD (residue 65), was amplified using the appropriate forward and reverse primers, the latter of which contained an “overhang” region corresponding to 20 nucleotides of the BF3 sequence (beginning at the first codon of the predicted TMD, isoleucine 60). Descriptions of the primer sequences used in this cloning procedure as well as those described below are available upon request. A corresponding region of the BF3 ORF (from the beginning of the first TMD to the 3’-end) was amplified via PCR, and the resulting two sets of products were gel-purified; then the fragments coding for the N terminus of Myc-TF3 were fused to the
3′-end of BF3 using an additional PCR. The full-length chimeric ORF was then gel-purified and subcloned into pYES2.1, yielding p′YES2.1-Myc-T/B. Likewise a chimeric ORF that featured the N-terminal coding sequence of BF3 fused to TF3 was generated by first amplifying the 5′ coding sequence of Myc-BF3. The resulting fragments were fused using PCR, and then the full-length product was subcloned into pYES2.1 yielding p′YES2.1-Myc-B/T.

Low copy expression plasmids were constructed by digesting pYES2.1-MycTF3, pYES2.1-MycBF3, pYES2.1-Myc-T/B, and pYES2.1-Myc-B/T with SpeI, which removed the endogenous GAL1 promoter and a portion of the multicloning site. The desired DNA fragments were gel-purified and ligated to generate each respective low copy, pYC2-FAD3-based vector. Point mutations, deletions, and insertions were generated in the coding sequences of both BF3 and TF3 ORFs using the Stratagene QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA).

Binary vectors for expressing monomeric green fluorescent protein (mGFP), PEST-mGFP, BF3-mGFP, or TF3-mGFP and ss-RFP-HDEL in tobacco cells (see Fig. 8A for combinations) were constructed using the pSAT and pRCS2 vectors available from Chung et al. (22). Specifically, the ss-RFP-HDEL ORF (encoding the red fluorescent protein (RFP) fused to the N-terminal Arabidopsis chitinase signal sequence and the C-terminal HDEL ER retrieval signal) was amplified from pRTL2-ss-RFP-HDEL using the sticky-end PCR technique (24). Annealed PCR products were ligated into NcoI-XbaI-digested pSAT2, yielding pSAT2-ss-RFP-HDEL. The fusion gene cassette was then excised from this plasmid with I-PpoI and Ascl and ligated into similarly digested pRCS2, yielding pRCS2-ss-RFP-HDEL. The ORF for mGFP was PCR-amplified from pUC18-NcoI-mGFP (25) and then digested with NcoI and ligated into similarly digested pSAT4, yielding pSAT4-NcoI-mGFP. A PEST sequence was then added to the 5′-end of the mGFP ORF in pSAT4-NcoI-mGFP by amplifying (via PCR with pENTR/D-CDKB2;1 Pro-NT as template) sequences encoding for the N-terminal 75 amino acid residues of Arabidopsis B2-type cyclin-dependent kinase (CDKB2;1; GenBankTM accession number NP_177780.1) including a PEST motif (26). The resulting PCR products were digested with NcoI and ligated into NcoI-digested pSAT4-NcoI-mGFP, yielding pSAT4-PEST-mGFP. The fusion gene cassettes for both pSAT4-NcoI-mGFP and pSAT4-PEST-mGFP were then excised with I-SceI and ligated individually into I-SceI-digested pRCS2-ss-RFP-HDEL yielding pRCS2-ss-RFP-mGFP + ss-RFP-HDEL and pRCS2-PEST-mGFP + ss-RFP-HDEL, respectively. To construct pRCS2-TF3FAD3-mGFP + ss-RFP-HDEL and pRCS2-BnFAD3-mGFP + ss-RFP-HDEL, a second version of pRCS2-mGFP + RFP-HDEL encoding mGFP without a start methionine codon and with the codon for valine 2 being in-frame with a BamHI site was generated by amplifying the mGFP ORF via PCR, digesting the resulting PCR products with BamHI and XbaI, and then ligating these into the same sites in pSAT4, yielding pSAT4-mGFP-BamHI. Next, the TF3 and BF3 ORFs were amplified using sticky-end PCR and then ligated individually into NcoI-BamHI-digested pSAT4-mGFP-BamHI in order to fuse the FAD3 ORFs (minus their respective stop codons) in-frame with the mGFP ORF, yielding pSAT4-TF3FAD3-mGFP and pSAT4-BnFAD3-mGFP, respectively. The I-SceI cassettes were then removed from these plasmids and transferred into the I-SceI site of pRCS2 ss-RFP-HDEL to form pRCS2-TF3FAD3-mGFP + ss-RFP-HDEL and pRCS2-BnFAD3-mGFP + ss-RFP-HDEL.

Protein Immunoblotting and Half-life Analysis in Yeast Cells—Protein steady-state amounts and half-life measurements were determined using the method of Braun et al. (29) as modified by O’Quin et al. (30). Briefly, the protein half-life was determined by culturing yeast cells in SGal medium to induce the expression of the FAD3 genes; then transcription and translation were terminated by the addition of dextrose and cycloheximide to final concentrations of 2% (w/v) dextrose, 0.67% (w/v) yeast nitrogen base without amino acids, and the appropriate auxotrophic supplements minus uracil) at 30 °C and 300 rpm in an orbital shaker. Then a portion of the culture was harvested by centrifugation, resuspended in synthetic galactose (SGal) medium (similar to SD, with 2% (w/v) galactose replacing dextrose), and cultured for another 20–24 h at 30 °C, 300 rpm, or for 40–42 h at 20 °C, 300 rpm. For RNA blotting experiments, yeast cells were harvested from SGal cultures at $A_{600}$ ~ 1.0. The fatty acid content of the yeast cells was determined by gas chromatography as described previously (19).

Immunoprecipitation—Yeast cells were grown in SGal-Ura medium to early log phase and then cultivated for another 6 h in

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Yeast Strains and Culture Conditions—Saccharomyces cerevisiae strain MMY011α (MATα ade 2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 OLE1) (27) was used in all studies, except where indicated. Other strains included BY4742 (MATα his3Δ1 leu2Δ0 ura3Δ0) (28) and mutants of this parental line that harbored single gene disruptions in CUE1, DER1, DOA1, NPLA, SHPI, OTUI, UBRI, and UFD2 (all purchased from Invitrogen). Yeast cells were cultured overnight in liquid synthetic dextrose (SD) medium (2%) (w/v) dextrose, 0.67% (w/v) yeast nitrogen base without amino acids, and the appropriate auxotrophic supplements minus uracil) at 30 °C and 300 rpm in an orbital shaker. Then a portion of the culture was harvested by centrifugation, resuspended in synthetic galactose (SGal) medium (similar to SD, with 2% (w/v) galactose replacing dextrose), and cultured for another 20–24 h at 30 °C, 300 rpm, or for 40–42 h at 20 °C, 300 rpm. For RNA blotting experiments, yeast cells were harvested from SGal cultures at $A_{600}$ ~ 1.0. The fatty acid content of the yeast cells was determined by gas chromatography as described previously (19).

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Immunoprecipitation—Yeast cells were grown in SGal-Ura medium to early log phase and then cultivated for another 6 h in
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the presence or absence of 50 μM MG132. Twenty-five A600
units of cells were harvested by centrifugation, washed once
with ice-cold 1× phosphate-buffered saline, and then subjected
to alkaline lysis as described by Hiller et al. (33). Cell debris was
cleared by centrifugation, and then lysates were diluted 1:20
and incubated with protein G-Sepharose. Samples were centri-
fuged, and the clarified supernatant was subjected to immu-
noprecipitation using anti-Myc antibodies followed by pro-
tein G-Sepharose incubation. Protein complexes were
collected by centrifugation and analyzed by SDS-PAGE and
immunoblotting.

RNA Blotting—Yeast RNA was isolated and analyzed as
described by O’Quin et al. (30). A 154-bp digoxigenin-labeled
probe that hybridized to the 3′-untranslated region of mRNAs
expressed from high copy pYes2.1-based plasmids was gener-
ated using the PCR-digoxigenin probe synthesis kit (Roche
Applied Science) with pYes2.1 as the template DNA. A second,
286-bp probe capable of binding the 3′-untranslated region of
mRNAs produced from low copy pYC2/CT-based plasmids
was generated using pYC2/CT as the template. Additional
probes for binding URA3 and actin (ACT1) were generated
using yeast genomic DNA as the template. Anti-digoxigenin-
alkaline phosphatase (Roche Applied Science) and CDP-Star
substrate (Roche Applied Science) were used for chemilumi-
nescence-based mRNA detection.

Microarray Analysis—Differential display microarray anal-
ysis was performed essentially as described (34, 35). Briefly, dye-
conjugated cDNA probes were synthesized from 10 μg of total
RNA isolated from yeast cells cultured at 30 or 20 °C; then equal
portions of differentially labeled (Cy3 or Cy5) probes were
mixed and applied to a microarray containing all of the available
S. cerevisiae ORFs (manufactured by the Microarray Center,
Ontario Cancer Institute, Toronto, Canada). Microarrays were
washed extensively prior to dual color digital imaging. For data
analysis, images of the microarrays were acquired with an Axon
GenePix 4000A scanner and analyzed using GenePix Pro ver-
sion 3.0 software (Molecular Devices, Sunnyvale, CA). Grids
were aligned and adjusted to ensure optimal spot recogni-
tion. Individual spots were quantified visually, with poor
quality or absent spots discarded. The raw data for replicate
slides were then imported into the software package
GeneSpring (version 4.0.1, Agilent Technologies, Santa
Clara, CA) and normalized such that the median of all Cy5/
Cy3 background-subtracted, fluorescence intensity ratios
were equal to 1. Differentially expressed genes were identi-
fied as those showing at least 2-fold up- or down-regulation
at 20 °C. The entire experiment, including an independent
set of RNA preparations, was replicated, and only those
genes showing an approximate 2-fold or greater change in
both experiments are reported.

Analysis of Protein Degradation in Plant Cells—Culturing of
tobacco (Nicotiana tabacum cv. Bright Yellow-2 (BY-2)) sus-
pension-cultured cells in standard Murashige-Skoog (MS)
medium and transient co-transformation of cells with 4 μg of
plasmid DNA using a PDS1000 biolistic particle delivery system
(Bio-Rad Laboratories) were performed as described previously
(36). For localization experiments, cells were resuspended in
transformation buffer (MS medium plus 0.25 M sorbitol and
0.25 M mannitol), spread onto Whatman filter paper prewetted
with transformation buffer, biolistically bombarded, and then
incubated for ~3–4 h to allow for expression and sorting of
expressed proteins. Thereafter, cells were fixed in formalde-
hyde, sometimes incubated with 4′,6-diamidino-2-phenylindole
(Sigma-Aldrich), and then viewed using a Zeiss Axioskop 2
MOT epifluorescence microscope (Carl Zeiss Inc., Thorn-
wood, NY) with a Zeiss 63× Plan Apochromat oil immersion
objective. Image capture was performed using a Retiga 1300
charge-coupled device camera (Qimaging, Burnaby, Canada)
and OpenLab 5.0 (Improvement Inc., Lexington, MA). Figure
compositions for Fig. 8 were generated using Adobe Photoshop
CS (Adobe Systems Inc., Toronto, Canada).

Monitoring of the degradation of transiently expressed pro-
teins in BY-2 cells was based in part on methods described for
other fluorescent protein-based degradation assays developed
for mammalian cultured cells (37–39). Briefly, BY-2 cells were
bombarded biolistically as described above except that: (i) cells
were incubated in MS medium rather than transformation
buffer (to reduce any potential cellular damage from plasmoly-
sis); and (ii) 30 min after bombardment, cells were transferred
from Whatman paper, resuspended in MS medium in a culture
flask, and then incubated under standard conditions for main-
taining BY-2 cell cultures (i.e. 25 °C in the dark on an orbital
shaker). Approximately 3 h after being transferred, the cells
were treated with either 100 μM cycloheximide or 100 μM each
cycloheximide and MG132. Aliquots of cells were then col-
clected at various time points, fixed in formaldehyde, and viewed
using epifluorescence microscopy (see above) with identical
image acquisition settings (e.g. gain, offset, and exposure) used
for each cell analyzed. The mean fluorescence intensity of GFP
and RFP in co-transformed cells was calculated by defining
the boundary of each cell using the region-of-interest func-
tion in OpenLab software. The fluorescence intensities of 10
more selected co-transformed cells were then recorded for
each time point, i.e. only cells that possessed “normal”
(nontransformed cell-like) ER morphology and a peak pixel
fluorescence intensity below the saturation intensity thresh-
old of 4095 were analyzed. The raw data were then normal-
ized such that the median GFP/RFP ratios at t = 0 were equal
to 1. The results shown are representative of at least three
independent experiments.

RESULTS

Tung and Brassica FAD3 Genes Encode Short-lived Pro-
teins That Exhibit Differences in Enzyme Activity and Protein
Half-life—The yeast S. cerevisiae is a well characterized,
robust model system that is routinely used to gain insight
into the molecular details that underpin a wide range of cel-
lar processes in plants and animals, including protein tar-
getting, organelle biogenesis, enzyme regulation, and lipid
metabolism (40–44). Here, we also utilized yeast cells to
examine the fundamental aspects of the temperature-depen-
dent, post-translational regulation of Fad3 enzymes. Toward
this end, TF3 and BF3 were both tagged with the c-Myc
peptide epitope sequence at their N termini to allow a direct
comparison of the protein products on Western blots. The
Myc epitope tag was placed at the N terminus rather than the
C terminus because the addition of polypeptide tags to the N terminus of Fad3 does not interfere with their enzyme activity, assembly into ER membranes, or retrieval from post-ER compartments (45). Cultivation of Myc-tagged TF3- or BF3-transformed yeast cells in the presence of linoleic acid, the substrate of Fad3 enzymes, resulted in the synthesis of transformed yeast cells in the presence of linoleic acid, the polyunsaturated fatty acid enzyme products. Genes encoding either Myc epitope-tagged TF3 or BF3 were expressed in yeast cells cultured in the presence of linoleic acid. Then the cells were processed for either immunoblotting or GC. The white bars represent the percentage of α-linolenic acid present in yeast lipids (reported as the percent of total cellular fatty acids), which is a measure of Fad3 enzyme activity under steady-state conditions. The black bars represent Fad3 protein amounts normalized to the amount of TF3 protein. Shown at the bottom are representative immunoblots of protein extracts probed with either α-Myc antibody, which recognizes both TF3 and BF3 proteins, or α-DPMS antibody, which recognizes the endogenous yeast ER membrane protein DPMS. B, measurement of protein half-life in yeast cells through expression shutoff experiments. Transcription and translation were halted at t = 0 by the addition of dextrose (which inhibits the galactose-inducible promoter utilized on the FAD3 expression plasmid, pYes2.1) and cycloheximide, respectively. Aliquots were removed over time and processed for immunoblotting. Shown on the left are representative blots, and the graph on the right shows the average and standard deviation of protein amounts from three independent experiments (normalized to respective protein amounts at t = 0).

To determine whether the diverged N-terminal sequences of TF3 and BF3 might be responsible for their differences in protein steady-state amount and/or half-lives, the N termini of the proteins (i.e. from the beginning of the N-terminally appended Myc tag to the first residue of the first TMD) were swapped (see “Experimental Procedures” for details). The resulting chimeric proteins therefore had either the N terminus of BF3 replaced with the corresponding TF3 sequence (Fig. 2B, T/BF3) or, conversely, the N terminus of TF3 replaced with the corresponding BF3 sequence (B/TF3). Expression of both native and chimeric Fad3 proteins in yeast cells followed by quantitative Western blotting revealed that the addition of the TF3 N-terminal sequence to BF3 (T/BF3) significantly increased the amount of protein to a level nearly identical to that of TF3 (Fig. 2, B and C). On the other hand, the addition of the BF3 N-terminal sequence to TF3 (B/TF3) decreased the steady-state amount, with protein abundance similar to that of BF3 (Fig. 2, B and C). Furthermore, measurement of protein half-lives for both native and chimeric Fad3 proteins in yeast cells demonstrated that the differences in steady-state amounts of protein correlated with changes in the rates of protein degradation, with BF3 and the B/TF3 having similarly short half-lives and TF3 and T/BF3 having longer half-lives (Fig. 2, B and D; note that separate line graphs are shown for the chimeras T/BF3 and B/TF3 in Fig. 2D and that, for the purpose of comparison, both line graphs include also the curves of native TF3 and BF3). These results indicate that the N-terminal sequences of the Fad3 proteins contain information that influences protein half-life, thus contributing to the observed differences in their steady-state abundance.

As controls for the above mentioned experiments, we also determined the half-life of native BF3 without an appended Myc tag (ΔMyc-BF3) by using an antibody raised to a peptide sequence within the N terminus of the protein (i.e. residues 9–28; italicized in Fig. 2A). As shown in Fig. 2, B and D, the half-life of ΔMyc-BF3 was similar to that of BF3, indicating that absence of linoleic acid. As such, the Fad3 enzyme is still expressed in yeast cells but is unable to modify native fatty acid, and thus membrane lipid, composition.

The N Termini of Tung and Brassica FAD3 Contain a Degradation Signal That Confers a Short Half-life to the Desaturase Proteins—The differences in the protein steady-state amount, product formation, and protein half-life of TF3 and BF3 (Fig. 1) were somewhat surprising given their high degree of amino acid sequence similarity (70% identical; 88% similar), particularly in the regions near their three histidine-rich, active site boxes (Fig. 2A). On the other hand, these two enzymes possess significant differences in the sequences at their N termini upstream of the first predicted TMD (Fig. 2A, position marked by an arrow).
the epitope tag had no effect on protein degradation. We also determined whether the overexpression of Fad3 proteins in yeast cells might contribute to their rapid degradation, as their genes were expressed using a high copy (2 \( \mu \)) expression vector (pYES2.1). Transfer of the BF3 and TF3 ORFs to a low copy CEN/ARS-based vector (pYC2/CT), however, yielded no differences in either their protein half-lives or relative steady-state amounts in comparison with their high copy expressed counterparts (data not shown). Because the immunodetection of Fad3 proteins was more robust when the \( FAD3 \) genes were expressed from high copy vectors, these were used in the remaining experiments (except where indicated).

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**Figure A**

| Protein | Steady-state (% of TF3) | Half-life (h) |
|---------|--------------------------|---------------|
| TF3     | 100.0                    | 11.37         |
| BF3     | 33.4 ± 20.6              | 3.30          |
| T/BF3   | 93.6 ± 6.8               | 12.18         |
| B/TF3   | 28.2 ± 23.5              | 5.50          |
| ΔMyc-BF3| n.d.                     | 2.89          |
| DPMS    | n.d.                     | 31.12         |

**Figure B**

- **Figure C**
  - α-Myc
  - α-DPMS

**Figure D**

- WT + T/BF3
- WT + B/TF3
- WT + ΔMyc-BF3
RNA Blotting Experiments Reveal That the Amount of FAD3 mRNAs Is Variable and Does Not Correlate with Steady-state Amounts of Fad3 Protein—Because the protein steady-state amount is influenced, at least in part, by mRNA levels, we next investigated whether there were any differences in mRNA steady-state amounts or half-lives that might contribute to the observed differences in Fad3 (and chimeric Fad3) protein abundance. Although RNA blotting experiments revealed that there were indeed several differences in FAD3 mRNA amounts (supplemental Fig. S1), the amounts of mRNA did not correlate with the observed amounts of protein. For instance, the highest amount of mRNA was observed for the BF3 gene, which produces the smallest amount of protein in yeast cells. Measurement of RNA half-lives using transcriptional shutoff experiments also demonstrated that the FAD3 mRNAs were degraded at similar rates, although the BF3 and B/TF3 mRNAs were apparently degraded at a slightly faster rate than TF3 and T/BF3 (supplemental Fig. S1). Taken together, these data revealed that there was no obvious correlation between mRNA and protein amounts in yeast cells. Rather, the steady-state amount of each protein was more closely correlated with protein half-life, indicating that the accumulation of Fad3 proteins in yeast cells is determined primarily at the post-translational level.

Charge Density within the N-terminal Sequence of the Fad3 Proteins Influences Protein Turnover Rate—To identify determinants within the N-terminal region of the BF3 and TF3 proteins that potentially contribute to the regulation of their protein half-lives, the N-terminal sequences of these and a number of other plant Fad3 proteins were aligned and compared to identify specific amino acids, motifs, or physicochemical characteristics that were conspicuously conserved or diverged (Fig. 3A). This comparison revealed that the Fad3 N termini had two distinct regions: (i) a more conserved domain just upstream of the first TMD that was enriched in basic amino acids (lysines and arginines) and (ii) a domain closer to the N terminus that was more variable in terms of its length and composition but was generally enriched in acidic amino acids. The net charge in this latter variable region is shown to the right in Fig. 3A. Overall, this comparison revealed two obvious differences between the TF3 and BF3 N termini that might contribute to the observed differences in their protein half-lives. First, the BF3 protein contains a lysine residue in the conserved region that is changed to an asparagine in the tung protein (Fig. 3A). Second, the TF3 protein has significantly more acidic amino acids, mainly glutamic acid residues, near the juncture between the variable and conserved domains (Fig. 3A, solid arrows in variable acidic region). This juncture is also interesting in that it contains a predicted PEST sequence (Fig. 3A, underlined), a cis-acting degradation signal involved in the turnover of many different cellular proteins (46).

To determine whether the observed differences in charged amino acids between the TF3 and BF3 N termini contributed to differences in their protein half-life, we initially changed the conserved lysine residue of BF3 to an asparagine residue (i.e. similar to the residue found at the same position in TF3). This single amino acid substitution (BF3ΔK/N1) significantly increased both the steady-state amount and the protein half-life in comparison with native BF3 (Fig. 3, B and C). Substitution of a different lysine residue within the N-terminal region of BF3 also increased the steady-state protein amount (Fig. 3B, BF3ΔK/N2). Similarly, a second modification aimed at increasing the number of acidic amino acids, similar to that observed in TF3, also increased BF3 protein abundance (Fig. 3B, BF3+EEEE). Collectively, these results demonstrate that changes to the number and type of charged residues (lysine or glutamic acid) within the N terminus of BF3 significantly influence protein abundance.

Similar results were obtained with corresponding mutagenesis studies of TF3. For instance, replacement of the asparagine residue in TF3 with a lysine (similar to the residue found at the same position in BF3) decreased protein stability (Fig. 3D, TF3ΔN/K), whereas substitution of a different lysine residue with asparagine increased protein stability (Fig. 3D, TF3ΔK/N). In addition, deletion of either three or four glutamic acid residues to make the TF3 sequence more similar to that of BF3 significantly destabilized the protein (Fig. 3D, TF3-EEE and TF3-EEEE). Taken together, these results indicate that charge density within the N-terminal regions of Fad3 proteins plays a critical role in determining their steady-state protein amount and that this amount can be modulated by changing the number and/or type of charged residues present in this Fad3 protein sequence.

The Half-life of Fad3 Proteins Is Significantly Increased at Cooler Growth Temperatures—We demonstrated previously that both BF3 and TF3 enzymes produce significantly more PUFAs when yeast cells are cultivated at 20 °C (18, 19) and that the steady-state amount of BF3 protein increases at this cooler temperature (18). Thus, to characterize the potential differ-
Post-translational Regulation of Fad3 Enzymes

A

|       | N-terminal variable, acidic region | Conserved region | 1st TMD | Net charge in variable region |
|-------|-----------------------------------|-----------------|---------|-----------------------------|
| BF3   | MVVMDQRSNVDSDGKREDFQPEAQPPIKGDRAAAKPIKIKRCWQK3PLQDSMYVRD- | - | 1 | -1 |
| TF3   | MQQQYKTPFLNLGVNGFARKKEKKEDLDENPNFYDN- | - | 3 | -5 |
| SOY   | MVVMDQSVAGQNGGFIQYTVDPPLPPPIKIANRAAIKPICHTNLTLRSLYVVRD- | - | 2 | -1 |
| ATHAL | MVVMDQRTNVDAGDGRKFEFDQAPPPPIKIANRAAIKPICHVRK5LMSYSYVRD- | - | 2 | -5 |
| BET-1 | MKFQVPDEDEEMNAGGGFLQTVDPPLPPPIKIANRAAIKPICHKNPSLSTYVVRD- | - | 0 | 0 |
| CAP   | (51) ALVRMSPAR1LQTVQEREDLREKDQFDPQAPPLPPPIKIANRAAIKPICHVRK5LMSYSYVRD- | - | 5 | 5 |
| BSET-2 | (66) USAPFRPVSQPVSNRENVNGVDDQGQQPPPLPPPIKIANRAAIKPICHVRK5LMSYSYVRD- | - | 5 | 5 |
| BSET-3 | (70) MVVMDQVSEEEERENNGVFQEEETFDPPAGPPFNPNLONRAAIKPICHVRK5LMSYSYVRD- | - | 5 | 5 |

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C

FIGURE 3. The charge density within the N-terminal PEST sequences of Fad3 proteins influences protein stability. A, alignment of the N termini of Fad3 protein sequences from B. napus (BF3), tung (TF3), soybean (SOY), A. thaliana (ATHAL), B. pendula (BET-1, BET-2, and BET-3), and C. annuum (CAP). The alignment reveals a region of high conservation shared among the Fad3 sequences (highlighted in blue) located just upstream of the first TMD; this region is flanked by a variable N-terminal region that is enriched in acidic amino acids. Acidic residues (Glu and Asp) are highlighted in red, and basic residues (Lys and Arg) are highlighted in blue. The net charge within the N-terminal variable region (including up to 28 residues upstream of the conserved region) is shown on the right. The down arrows represent obvious differences in charge density in TF3 and BF3 amino acid sequences in the variable region, and the large arrowhead points to a position in the conserved region that is occupied by lysine in most Fad3 sequences but is changed to asparagine in the TF3 sequence. B. shown also is the predicted secondary structure of the BF3 amino sequence including elements of random coil (C), type-I β-turn (T), α-helix (H), and β-strand (S). Also conserved among the sequences is a weakly predicted PEST degradation sequence (underlined), identified using the Emboss epestfind program. B. substitution of lysine residues with asparagines or introduction of additional negatively charged amino acids into the BF3 protein both increase protein stability. Bottom panel, shows the native BF3 N-terminal sequence on top (BF3) followed by sequences with specific mutations (highlighted in bold italics). The arrows point to positions of obvious differences in charge density between the TF3 and BF3 polypeptide sequences (refers to panel A). The bar graph shows protein steady-state amounts of each construct normalized to the abundance of the parental BF3 protein. C. protein half-life analysis demonstrating that substitution of a single lysine residue with asparagine in the N terminus of Brassica Fad3 (BF3ΔK/N1) substantially increases protein half-life. D, similar types of modifications to the charge density in the TF3 N terminus modulate protein stability. This panel is labeled as described in B (except that protein abundance is normalized to TF3).

ences in post-transcriptional regulation, we compared directly the steady-state amounts and protein half-lives of TF3 and BF3 in yeast cells cultivated at 20 °C (compared with those cultured at 30 °C used in all of the experiments described above). Similar to previously reported results (18), the amount of BF3 protein increased significantly at cooler temperatures (~5-fold; see Fig. 5B as an example). The amount of TF3 protein, on the other hand, increased less than 2-fold at 20 °C (data not shown). As a result, the steady-state levels of BF3 and TF3 proteins were nearly identical at 20 °C (Fig. 4, A and B). Furthermore, the measurement of protein degradation rates (Fig. 4C) revealed that the half-life of both proteins was increased at 20 °C in comparison with the half-life of the same proteins at 30 °C, but the -fold change was greater for BF3 (~3.2-fold) than for TF3 (only 1.4-fold) (Fig. 4A). Interestingly, the N-terminal chimeric proteins, T/BF3 and B/TF3, showed similar -fold changes in degradation rates and protein steady-state amounts as compared with the native protein used as the source of the N-terminal polypeptide sequence. For instance, T/BF3 exhibited a 1.7-fold increase in half-life at cooler temperatures, which is similar to that of TF3 (1.4-fold), whereas B/TF3 (2.7-fold) was more similar to BF3 (3.2-fold) (Fig. 4C). Taken together, these data demonstrate that: (i) the stability of both BF3 and TF3 proteins is increased at 20 °C compared with 30 °C; (ii) the change in protein half-life is most significant for the BF3 enzyme; and (iii) the -fold change in protein half-life is determined, at least in part, by information contained within the N terminus of each protein.

The Yeast DOA1 Gene Is Essential for Temperature-sensitive Modulation of Fad3 Protein Half-life—To gain insight to the temperature-dependent mechanisms involved in regulating the half-life of Fad3 proteins in yeast cells, an RNA microarray experiment was conducted to characterize the global changes in gene expression when yeast cells were shifted from 30 to 20 °C. The rationale for these experiments was that yeast genes involved in temperature-sensitive degradation of Fad3 proteins
of proteins from the ER and their subsequent delivery to the proteasome for degradation (52–56).

To investigate the potential role of UBR1, UFD2, and/or DOA1 in Fad3 regulation, yeast strains disrupted individually in each gene were transformed with plasmids expressing either BF3 or TF3; then cells were cultivated at 30 or 20 °C, and protein amounts were determined by quantitative Western blotting. As a control, BF3 was expressed also in wild-type (WT) yeast cells cultivated at 30 or 20 °C, which showed the expected increase in BF3 steady-state protein abundance when cells were cultured at cooler temperatures (Fig. 5B) (18). A similar, but smaller -fold change in BF3 protein abundance was observed in the Δubr1 and Δufd2 strains, although it is noteworthy to mention that the amount of BF3 protein was increased by about 2-fold in the Δufd2 strain at 30 °C compared with the WT control (Fig. 5B). Expression of BF3 in the Δdoa1 strain, however, resulted

(e.g. protease-related genes) might themselves be regulated by temperature, with higher levels of expression at 30 °C contributing to more rapid degradation of Fad3 proteins and lower levels of expression at 20 °C promoting Fad3 protein stabilization.

Although a comparison of global changes in yeast gene expression did not reveal any specific protease-encoding genes that were down-regulated at cooler temperatures (data not shown), a number of genes associated with the ubiquitin-proteasome system (UPS) were modulated at 20 °C (Fig. 5A). For instance, genes encoding two different deubiquitinating enzymes (UBP16 and UBP8) showed increased expression at 20 °C, which could conceivably promote Fad3 stabilization by removal of polyubiquitin tags. However, because UBP16 and UBP8 encode enzymes associated with mitochondrial and nuclear functions, respectively (47, 48), and because Fad3 enzymes are located in the ER, these two genes were not investigated further. Similarly, UBI4 and SGT1 displayed decreased expression at 20 °C (Fig. 5A) but were not investigated further because they encode polyubiquitin, which is involved in many different cellular processes and a ubiquitin-ligase-associated protein specific to kinetochore assembly, respectively (49, 50). On the other hand, the down-regulation of all three of the remaining genes, UBR1, UFD2, and DOA1, was potentially relevant to the control of Fad3 protein half-life, because UBR1 encodes the main recognition component (E3 ligase) of the N-terminal end rule degron pathway (51), and UFD2 and DOA1 each encode proteins that modulate the activity of Cdc48, the primary chaperonin-type molecule involved in the extraction in a significant increase (>6-fold) in the amount of BF3 protein at 30 °C (Fig. 5B), and measurement of protein half-life revealed that the protein was significantly stabilized at this higher temperature (Fig. 5C). Notably, the rates of BF3 degradation in Δdoa1 cells were nearly identical at 30 and 20 °C, and the half-lives were similar to the half-life of BF3 expressed in WT yeast cells grown at 20 °C. Similar changes in protein abundance and half-life were observed when the TF3 protein was expressed in Δubr1, Δufd2, and Δdoa1 mutant strains (Fig. 6 and data not shown). Collectively, the RNA microarray data and mutant analyses suggested that DOA1 plays an important role in the temperature-dependent regulation of Fad3 by promoting its rapid degradation at 30 °C. Moreover, a reduction in DOA1 gene expression (and thus likely Doa1 activity) at 20 °C appeared to stabilize Fad3 proteins at cooler temperatures.

The Yeast SHP1 Gene Is Also Required for Rapid Degradation of Fad3 Proteins—To identify other components of the UPS potentially involved in degradation of the Fad3 proteins, we examined a number of additional yeast strain mutants disrupted in key aspects of the ubiquitin-dependent ERAD pathway. For comparative purposes, the Δubr1, Δufd2, and Δdoa1 strains were also included in this analysis. Thus, collectively, the mutants analyzed included several major substrate-recruiting (Npl4 and Shp1) and substrate-processing (Ufd2, Doa1, and Otu1) cofactors of Cdc48 (52), a putative channel-forming protein (Der1) involved in retrotranslocation of some proteins from the ER (57), and an ER membrane protein (Cue1) involved in recruitment of an E2 ubiquitin-conjugating enzyme utilized by several membrane-bound ubiquitin-ligases (58).
Degradation of Fad3 Proteins Requires Proteasomal Activity — To determine directly whether proteasomal function was required for Fad3 degradation in yeast cells, TF3 and BF3 were expressed in yeast cells cultured at 30 °C in the presence or absence of the proteasomal inhibitor MG132 (59) followed by quantitative Western blotting. As shown in Fig. 7A, the inclusion of MG132 increased the steady-state amount of each protein by ~25%, whereas the amount of endogenous DPMS protein in the same samples was unaffected. Furthermore, measurement of protein degradation rates revealed an effect of MG132 on TF3 and BF3 protein half-lives (Fig. 8B), indicating that Fad3 protein degradation was directly dependent on proteasomal activity.

To determine whether (poly)-ubiquitinated Fad3 proteins could be detected in yeast cells, we immunoprecipitated BF3 and TF3 proteins from cell lysates using anti-Myc antibodies and then blotted the immunoprecipitates with either anti-Myc or anti-ubiquitin. Fig. 7C shows a Ponceau-stained membrane illustrating the total protein used to program the immunoprecipitation reactions (left side of membrane) as well as proteins recovered after immunoprecipitation (right side of membrane). Subsequent Western blotting of the immunoprecipitated samples with anti-Myc antibodies (Fig. 7D) revealed the presence of Myc-tagged BF3 protein (i.e. ~40 kDa, position marked by an arrow) as well as several other higher and lower molecular mass cross-reacting proteins (positions marked by arrowheads). Notably, the abundance of these proteins was increased by treatment of yeast cells with MG132 (Fig. 7D). Moreover, Western blotting of these same immunoprecipitation samples with anti-ubiquitin antibodies revealed the presence of several higher molecular mass immunoreactive proteins (greater than 40 kDa) that were not present in samples obtained from yeast cells transformed with empty plasmid (pYes2.1) as a control (Fig. 7E, positions marked by arrowheads; note again that the intensity of these bands also increased with MG132 treatment). The lower molecular mass immunoreactive proteins (i.e. less than 40 kDa) detected using the anti-Myc antibody (Fig. 7D) presumably were not detected using the anti-ubiquitin antibody, because
ubiquitin is generally removed (i.e. recycled) from protein substrates prior to proteasomal degradation (Fig. 7, compare panels D and E) (60). Results similar to those presented in Fig. 7, C–E, for BF3 were obtained for TF3 (data not shown).

In summary, the data presented in Fig. 7 indicate that both the steady-state amounts and half-lives of TF3 and BF3 proteins increased in response to MG132 treatment and, thus, that proteasomal activity is required for mediating Fad3 protein half-life. This premise was further supported by immunoprecipitation experiments demonstrating that Fad3 proteins are ubiquitinated under in vivo steady-state conditions.

**Fad3 Proteins Are Rapidly Degraded in Plant Cells by a Proteasome-dependent Mechanism**—The results presented above demonstrated that Fad3 proteins exhibited a relatively short half-life when expressed in yeast cells and that their degradation was mediated by the ubiquitin-proteasome pathway. To begin to determine whether a similar mechanism regulates Fad3 protein abundance in plants, we developed a dual fluorescence protein reporter-based assay in tobacco BY-2 suspension-cultured cells as a means of monitoring the degradation rates of transiently expressed proteins. Specifically, GFP was employed as a reporter molecule to which various passenger protein sequences were fused, and then the resulting transgenes were introduced (via biolistic bombardment) into BY-2 cells, which serve as a model plant cell system (61–63). Thereafter, cycloheximide was added to block new protein synthesis, and GFP fluorescence decay was monitored (via microscopy) over time. To overcome any inherent cell-to-cell variability in fluorescence intensity (i.e. because of differences in levels of transgene expression), cells were also co-transformed with an RFP fusion construct expressed from the same plasmid as GFP, thus allowing RFP to serve as an internal control. The ratio of GFP-to-RFP fluorescence detected in BY-2 cells, therefore, served as a useful measure of GFP protein abundance/degradation between individual cells independent of differences in transgene number and expression.

Four dual gene expression constructs were used in this assay (Fig. 8A), including ss-RFP-HDEL (encoding RFP fused to an N-terminal ER signal sequence and a C-terminal HDEL ER retrieval motif) paired with either of the control proteins, GFP or PEST-GFP (where GFP was appended at its N terminus to a PEST-containing sequence known to confer, via the UPS, a shorter half-life to expressed proteins (26)), or with BF3-GFP or TF3-GFP. Transient co-expression of GFP + ss-RFP-HDEL (Fig. 8B, upper panels) or PEST-GFP + ss-RFP-HDEL (lower panels) followed by epifluorescence microscopy confirmed that both sets of control proteins targeted to their expected subcellular locations in BY-2 cells, namely the ER (ss-RFP-HDEL), the cytosol and nucleus (GFP), and primarily the nucleus (PEST-GFP).

To monitor protein degradation rates, cycloheximide was added 3 h after the transformation event, and then GFP and RFP fluorescence was measured over time. Because GFP and RFP are known to have long half-lives (≥24 h) (64, 65), it was expected that their ratios of fluorescence intensity should change little during the cycloheximide chase period (Fig. 8C). The addition of the PEST sequence, however, should increase the rate of GFP degradation, leading to a reduction in the GFP/RFP ratio over time. Moreover, the addition of MG132 would be expected to decrease PEST-GFP degradation rates, thereby increasing the ratio of GFP/RFP fluorescence (Fig. 8C).

As shown in Fig. 8D, measurements of actual fluorescence ratios for these control proteins in BY-2 cells revealed degradation curves that were similar to the theoretical expectations, with the major difference observed at the early time points. For instance, analysis of the GFP + ss-RFP-HDEL curve showed a large decrease in the ratio of fluorescence between the 0 and 4 h time points, after which the ratio subsequently leveled off (indicating that the two proteins were degraded at similar rates). Although the reason for this decrease at early time points is unknown, it is possible that differences in the rates of GFP and RFP protein folding/chromophore formation contribute to this
process, i.e. GFP folds approximately five times faster than RFP (66, 67). Also, as observed in Fig. 8D, fusion of a PEST sequence to GFP significantly decreased the ratio of GFP/RFP fluorescence over time, whereas inclusion of MG132 consistently increased the ratio of GFP/RFP over time. These latter data indicate that, similar to the results presented for yeast cells, Fad3 protein degradation appears to be mediated by the UPS in plant cells.

**DISCUSSION**

Fatty-acid desaturases play an essential role in plant lipid metabolism by synthesizing the PUFAs that comprise the major components of cellular membranes. Modulation of Fad activity, and thus alteration of the content and ratios of PUFA, is therefore considered important for maintaining overall membrane fluidity, including in response to changes in ambient temperatures. Here we have demonstrated that two differentially regulated plant Fad3 enzymes are short-lived proteins when expressed in either yeast or plant cells and that the stability of these enzymes is significantly increased when yeast cells are cultured at cooler temperatures. We demonstrated also that this temperature-sensitive protein turnover of Fad3 enzymes in yeast is regulated by cis-acting degradation signals within their N termini. Furthermore, the degradation of Fad3 proteins in either yeast or plant cells is sensitive to inclusion of MG132, revealing that these proteins are degraded by the UPS in each cell type and suggesting conservation of the underlying mechanism. Overall, the modulation of the amount of Fad3 protein in response to temperature provides a mechanism for the rapid adjustment of PUFA content in cellular membranes, and thus similar regulation may contribute to the process of homeoviscous adaptation in plants. It is also now apparent that, as discussed below, there are both similarities and differences in the regulatory mechanisms of Fads located in various subcellular compartments, as well as several conserved and plant-specific features of Fad regulation in eukaryotic cells, in general.
Identification of an N-terminal Degradation Signal in Fad3 Proteins—Expression of native and chimeric Fad3 proteins in yeast cells demonstrated that their N-terminal region contains a PEST-like degradation signal and that the charge density within this region influences Fad3 protein turnover rates (Figs. 2 and 3). Notably, a similar degradation signal has been identified in the ER-localized stearoyl-CoA desaturase (SCD) of mammalian cells (69). That is, like Fad3 (Fig. 3C), lysine residues were shown to be important for determining SCD half-life, with substitution of a single lysine residue within an N-terminal sequence resulting in a significant increase in protein half-life (70). The ER-localized Fad2 enzymes of plants have been also shown to contain degradation signals within their N termini (15). Unlike Fad3, however, the plastidial Fad8 enzyme has been shown to contain a distinct degradation signal within the last 44 amino acids of the protein sequence (16), with the difference in positioning of these degradation signals in Fad8 and Fad3 possibly being a reflection of unique aspects of their protein biogenesis. For instance, Fad3 proteins lack a cleavable N-terminal signal sequence and are instead co-translationally inserted into ER-membranes via a signal-anchor-dependent mechanism (45). Fad8 proteins, on the other hand, contain a cleavable, N-terminal transit peptide required for their import into plastids (16). Thus, these differences in cellular targeting pathways (and differences in the presence or absence of a cleavable N-terminal targeting sequence) may have placed evolutionary con-

FIGURE 8. Fad3 proteins are short-lived when expressed in plant cells and degraded by a proteasome-dependent mechanism. A, dual gene expression vectors used to monitor protein degradation rates in tobacco BY-2 cells. Each construct includes ss-RFP-HDEL (serving as an internal fluorescence control and driven by the nopaline synthase (NOS) promoter) paired with GFP, PEST-GFP, BF3-GFP, or TF3-GFP (driven by the cauliflower mosaic virus 35S (35S) promoter). B, representative epifluorescence images of cells transformed with either GFP + ss-RFP-HDEL (upper panels) or PEST-GFP + ss-RFP-RFP (lower panels), as well as the 4’,6-diamidino-2-phenylindole (DAPI) staining of these cells. C, model depicting the in vivo dual fluorescence protein reporter-based degradation assay including schematic illustrations of the fluorescence patterns and intensities for GFP + ss-RFP-HDEL (upper cells) or PEST-GFP + ss-RFP-HDEL (lower cells) in co-transformed BY-2 cells following the shutoff (at t = 0) of protein translation by the addition of cycloheximide and the corresponding theoretical curves of the GFP/RFP fluorescence ratios for these proteins over time. D, actual fluorescence values, demonstrating that the GFP protein alone is relatively stable over the cycloheximide chase period (4–24 h), whereas the PEST-GFP protein is rapidly degraded during the same time period. Note also that the addition of MG132 to the cell culture increases the ratio values, as would be expected for stabilization of the PEST-GFP protein in the presence of this proteasomal inhibitor. E, epifluorescence images of cells transformed with either BF3-GFP + ss-RFP-HDEL or TF3 + ss-RFP-HDEL, showing the expected ER localization of each protein. F, degradation curves of BF3-GFP (left graph) and TF3-GFP (right graph), demonstrating rapid degradation of the Fad3-GFP proteins and an increase in fluorescence ratio when MG132 is included in the experiment.
strains on the positioning of the degradation signal with respect to the mature protein.

**Identification of Specific Components of the ERAD Pathway in Yeast Cells That Mediate Fad3 Turnover**—Previous analyses implicated the ERAD pathway in the degradation of mammalian SCD (71), plant Fad2 (15), and yeast SCD (29), and here we have demonstrated that Fad3 proteins are also degraded by a UPS-dependent process in both yeast (Fig. 7) and plant (Fig. 8) cells. Specifically, we showed, using yeast cells and a combination of differential display microarray analysis and expression of Fad3 in various yeast mutant backgrounds, that **DOA1**, **SHP1**, and **UFD2** genes are all involved in Fad3 post-translational regulation. The proteins encoded by these genes are well known to play multiple roles within the UPS of yeast cells, including, among other things, binding to and modulating the activity of Cdc48, the main chaperone of the ERAD pathway (reviewed in Ref. 52).

Of course, although it now remains to be determined whether orthologs of **DOA1**, **UFD2**, and/or **SHP1** function in a similar manner to regulate Fad3 protein abundance in plant cells, several lines of evidence, albeit indirect, support this premise. For instance, a previous study using native or mutant versions of the ER membrane protein Mlo from barley demonstrated that when expressed in either plant, yeast, or human cells, their turnover is mediated by similar ERAD-like degradation pathways, suggesting that the ERAD pathway in eukaryotic organisms is well conserved (72). It has been shown also that most of the UPS-related genes in yeast have at least one or more putative homologs in plants (73), including *Arabidopsis*, wherein a recent genome-wide analysis revealed that its UPS is actually several times larger than that in other model organisms such as yeast, flies, nematodes, mice, and humans (74). Indeed, searches of the NCBI protein database using yeast Doa1, Ufd2, and Shp1 as queries confirmed that there are putative homologs to each of these proteins in *Arabidopsis*, as well as in other diverse plant species (supplemental Figs. S2–S4). Furthermore, the *Arabidopsis* protein identified with the highest degree of sequence similarity to Shp1 (*i.e.* Pux4) (supplemental Fig. S4) is encoded by a gene that is part of at least a 15-member gene family (PLIX) in *Arabidopsis* (77). Notably, this family also includes PLIX1, which encodes a protein that, similar to the yeast and mammalian Shp1 proteins, binds to and modulates the activity of Cdc48 (52, 75, 76). Studies aimed at determining whether *Arabidopsis* PLIX4 and PLIX1 and/or other members of the PLIX gene family, as well as putative *Arabidopsis* DOA1 and UFD2 genes, are involved in Fad3 regulation are currently being pursued in our laboratories through a combination of reverse genetic studies and expression of epitope-tagged Fad3 in mutant transgenic plants.

**Temperature-sensitive Regulation of Fad3 Protein Half-life**—The identification of both a cis-acting degradation signal at the N terminus of Fad3 and at least one factor in yeast cells (*i.e.* Doa1) in which expression is down-regulated in response to temperature raises questions about how temperature-dependent regulation of Fad3 is actually implemented. For instance, are there multiple, specific components of the ERAD pathway for which activity is modulated by temperature, thereby changing how the degradation machinery interacts with Fad3 (and other temperature-sensitive proteins) within the cell? Alternatively, or perhaps additionally, does the N-terminal degradation signal in Fad3 serve as a temperature-sensitive degron that alters the susceptibility of the protein to the degradation machinery at various temperatures?

Although both of these scenarios are likely involved, there is, as already mentioned, only limited information on the functionality of the ERAD pathway in plant cells; and despite the UPS being known to regulate many aspects of plant growth and development including response to abiotic stress such as cold temperature (74, 77), even less is known regarding how the ERAD pathway in plants might be modulated by temperature. However, the properties of a temperature-sensitive degron in the Fad3 N terminus can readily be proposed on the basis of our current understanding of degrons and PEST sequences in general. A PEST sequence is a signal present on a protein that confers rapid protein degradation, usually via the UPS (46). The signal may be located in any part of the protein sequence and is classically defined as a sequence of at least 12 amino acids that contains at least one proline (P), one glutamic or aspartic acid (E or D), and one serine or threonine (S or T). Furthermore, a PEST sequence, in general, is associated with a protein region of marginal thermodynamic stability that may be fully destabilized by regulated changes in protein structure (46). As such, there are two types of PEST sequence, one that is constitutively active (*i.e.* always exposed) and another that is regulated (46). For instance, the phytochrome protein has a half-life of over 100 h in the dark, but in the light the protein absorbs light energy and undergoes a conformational change that exposes a PEST sequence, resulting in the rapid degradation of the protein with a half-life of approximately 1 h (46).

Each of the Fad3 proteins presented in Fig. 3A contains a predicted PEST signal in the N-terminal sequence, with the polypeptide sequence in this region enriched in glutamic acid residues and also containing a stretch of amino acids predicted to form a turn-type structure followed by an α-helix. Interestingly, these features are generally similar to the characteristics of the regulated PEST sequence found in the papillomavirus E2 protein (78). The PEST sequence in this protein is located in a marginally stable region that includes a stretch of glutamic acid residues followed by a turn region and a short α-helix (79). Phosphorylation of a serine residue within the turn region destabilizes the protein structure, thereby exposing the PEST signal and rendering the E2 protein susceptible to rapid degradation (78, 79). Although it is unknown whether the serine residue in the predicted turn region of some Fad3 proteins (Fig. 3A) is phosphorylated, the stability of secondary structures, in general, is known to be temperature-sensitive (80). Therefore, the simplest model for a temperature-sensitive degron in the Fad3 sequence is one that consists of a marginally stable PEST sequence in which the surrounding secondary structure (or structures) is sensitive to changes in ambient temperature. For instance, an elevation of temperature would promote local unfolding, thereby exposing the PEST sequence and rendering the Fad3 protein susceptible to rapid degradation. This model will be the focus of future structural studies using synthetic peptides that represent native (as well as mutant) versions of the Fad3 N termini.
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