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Post-translational Regulation of Plant Fatty Acid Desaturases as Expressed in Saccharomyces cerevisiae

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Post-translational Regulation of Plant Fatty Acid Desaturases
as Expressed in *Saccharomyces cerevisiae*

A Thesis

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by

Linda Bourassa

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Abstract

Differences have been shown in the steady-state accumulation and half-lives between *Brassica* FAD3 (BF3) and tung FAD3 (TF3) proteins expressed in yeast cells cultured at 30°C. TF3 has a greater steady-state accumulation and longer half-life than BF3. These differences are attributed to post-translational modification and have been shown to be controlled by an N-terminal element. I attempted to determine specific amino acids important for regulation, and further characterize the mechanism contributing to the differences. Through site-directed mutagenesis, it was shown that replacing lysine residues with asparagines in the BF3 and TF3 N-termini increased protein stability, while replacing an asparagine with lysine in the TF3 N-terminus decreased its stability. Furthermore, I showed that the TF3 polyglutamic region (six consecutive glutamic acid residues) is primarily responsible for the higher steady-state amount of TF3 in comparison to BF3. This negatively charged region likely acts as an electrostatic shield protecting the protein from degradation.

Introduction

Alternate energy sources

Abundant and inexpensive energy costs are key factors in economic stability and growth. Finite and dwindling petroleum-based energy supplies have a direct impact not only on the U.S. economy but also on the world market. Relying on decreasing natural petroleum-based energy supplies in the face of continued population growth is resulting in an economic picture that is increasingly susceptible to lower supply and higher demand. Petroleum-based products are not only used for electricity production and vehicle propulsion, but also for many industrial applications such as plastics, medicines, food items, solvents and lubricating oils.

Without additional energy sources, demand will eventually outpace supply. Alternate energy sources for electricity production could take the form of wind, water and nuclear, but renewable sources are also needed for chemical feedstocks. Ethanol is a heavily funded and politically popular potential renewable alternate energy source, although it is not without controversy. Ethanol is currently derived from the fermentation of corn starch or sugar cane starch, but diverting so much corn into ethanol production has resulted in much higher food prices, potential shortages, and less competitiveness on the world grain and meat markets. Future endeavors are heading toward ethanol production from the fermentation, or gasification, of left over plant biomass (mainly cellulose), and the ability to produce ethanol from waste plant biomass would confer a huge economic advantage to ethanol production where there currently is none. In the meantime, another increasingly emphasized alternate energy source is lipids. A lipid can be broadly defined as any fat-soluble (hydrophobic) biologically based molecule, but

the term lipid is more specifically used to refer to fatty acids and their derivatives. Fatty acids are carboxylic (organic), acids characterized by the presence of a carboxyl group.

Lipids as an energy source

Lipids hold increasing potential in fuel, industrial and food applications and can be obtained from naturally occurring vegetable oils or animal fats. Specialized processing of triglycerides from various vegetable oils can be used to produce a mix of fatty acid esters known as biodiesel which is now increasingly used as a substitute for diesel fuel. Fatty acids are also used in a wide variety of end-use industries (rubber, plastics, detergents, etc.) and make up the greatest proportion of plant-derived raw material currently consumed in the chemical industry. Another potentially very important use of fatty acids relates to food products. Efficient food production is becoming more important as the world's population increases and petroleum-based energy supplies decrease and become more expensive. Production and transportation costs are directly related to energy costs and all impact the bottom line consumer price. Renewable energy, chemical feedstock and efficient food production decisions are generally based on economic, return on investment, criteria. However, subsidies, mandates and tax incentives from the government also play a key role in shaping policies in these areas.

For plant oils to be economically valuable for almost any industrial application, they must be highly enriched in a single fatty acid that has double bonds or functional groups in specific positions. Some plants naturally produce highly enriched specialty oils, but these plant species often have limiting agronomic traits such as small seeds, low yields, toxicity, and/or limited geographical growing areas. Domesticated oilseed crops, on the other hand, yield several tons of oil per hectare, but their fatty acid compositions are generally not useful for direct industrial application. Vegetable oils must be extracted, purified and treated with chemicals before use in

most industrial applications, and each step of these processes requires additional time and money. In a free market economy, both time and money are critical considerations in sound business decisions. Therefore, genetic engineering of plants to produce oils with specific, desirable fatty acid compositions would significantly reduce production costs and make plant oils much more useful for industrial purposes. Furthermore, some industrial applications require that the oils have high oxidative stability (e.g., lubricants) or that they be readily oxidized (e.g. drying oils used in formulation of inks, dyes, coatings and resins). Such properties are typically absent from or lacking in plant vegetable oils (Jaworski and Cahoon, 2003). The production of oxidatively stable soybean oil without hydrogenation is of increasing importance because recent studies have linked the consumption of trans-fatty acids found in partially hydrogenated oils with an increased risk for coronary heart disease (Hu et al., 1997). Modification of the fatty acid composition of soybean seeds to yield lower levels of polyunsaturated fatty acids such as linolenic acid could improve oil stability and flavor, and eliminate the need for hydrogenation and associated trans-fatty acid production (Dutton et al., 1951; Lui and White, 1992). Conversely, soybean oil with greatly increased linolenic acid content would make it more valuable for specialty industrial purposes such as drying agents or production of films such as those found on linoleum.

Plant breeding programs have been used successfully to make dramatic changes in seed oil composition. One example is Canola which, through selective breeding, has higher oleate and lower polyunsaturates in comparison to its parental rapeseed oil. Breeding improvements for enhanced oil traits is still being researched, but molecular biology techniques offer an alternative and more direct method for modifying plant oil composition. Plant oil production through molecular biology involves gathering information on both the genetic makeup and biochemical

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processes involved in oil synthesis and storage. Knowledge of this "big picture" allows for understanding the pathways well enough that directed changes can be made. Directed modification of plant's oil production requires genetic engineering which is essentially, directly changing the plant's genetic makeup to strategically change its metabolic processes.

Genetic manipulation and metabolic engineering of plant seed oils would have significant nutritional and financial benefits. A thorough understanding of the enzymes and metabolic pathways that produce the oils is essential for strategically manipulating plant oil production in a directed and predictable manner. Knowledge of genes and genetics is not enough. A plant's genome is complete, encoding for all its proteins, but this information by itself gives no indication of the specifics involved in the process. The biochemical pathway relates to these specifics and includes factors such as when the relevant proteins and enzymes are transcribed and translated, post-translational modifications, the order in which steps occur, feedback regulation, required protein interactions, necessary cofactors, etc. Biochemical pathways are intrinsically complex and mapping them is no easy feat, but mapping the biochemical pathway is an essential first step in the metabolic engineering of specialty oil products.

One can not test out and map a biochemical pathway without having insight into the big picture. With regard to metabolic engineering of plant seed oils, this includes a general understanding of lipids. Lipids serve many functions in living organisms including energy storage, cell membrane structural components, and signaling molecules. One of the key roles of fatty acids in biological systems is membrane fluidity. Membranes surround all living cells and the cell membrane must be a dynamic structure if the cell is to grow and respond to environmental changes. Research shows that each membrane of the cell has a characteristic and distinct complement of lipid types and that, within a single membrane, each class of lipids has a

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distinct fatty acid composition (Browse et al., 1993). At the same time, composition of each membrane and lipid class is largely conserved throughout the plant kingdom. The diversity and its conservation throughout evolution imply that differences in lipid structure are important for membrane function (Ohlrogge and Browse, 1995). All microorganisms, plants and animals regulate the synthesis of unsaturated fatty acids during changing environmental conditions as well as in response to nutrients.

Fatty acids

Fatty acids are multiple linked hydrocarbons possessing a carboxyl (COOH) group at one end. Most fatty acids are straight-chain compounds with an even number of carbon atoms. Chain-lengths range from 2 to 80 but commonly are found in the 12 to 24 range. Fatty acids are broken down into four classes depending on their chain length. Chain lengths of 2 to 6 are called short-chain, 8 to 10 are called medium-chain, and 12 to 18 are called long-chain, and 20 to 24 are called very long-chain fatty acids. Chain length is an important factor in determining the physical and biological properties of fatty acids. Length is a crucial parameter in acyl chain contribution to membrane structure and stability. Solubility is decreased and melting point increased as fatty acyl chain length is increased. This effect results from the weak apolar interactions which determine how the membrane interacts with its environment. For example, restricted solubility of lipids in the surrounding aqueous milieu is governed by the tendency of acyl chains to remain in association with one another rather than to associate with the aqueous environment. Similarly, response to temperature fluctuations is modulated by the extent to which thermal influences cause acyl chains to dissociate from one another and assume a more randomized structure.

Fatty acids can be of two general types: saturated or unsaturated, although there can be additional functional groups such as hydroxyl, epoxy, conjugated, acetylenic, etc. which also play roles in their conformation and function. One of the key roles of fatty acids in any cell is to maintain membrane fluidity. Toward this end, a main strategy used by the cell to keep membranes fluid at physiological temperatures, is to alter the composition of membrane phospholipids by adjusting the ratio of saturated to unsaturated fatty acids. Saturated fatty acids have no carbon-carbon double bonds (they are "saturated" with hydrogen) while the unsaturated fatty acids typically have one to three double bonds. Unsaturated fatty acids have profound effects on the fluidity and function of biological membranes and unsaturated fatty acid homeostasis in many organisms is achieved by feedback regulation (Aguilar and de Mendoza, 2006). In most plant tissues, over 75% of the fatty acids are unsaturated.

Saturated fatty acids, being straight chains, tend to pack closely together and would result in a membrane that is relatively solid at low temperatures, seriously impacting membrane fluidity and cell survival. Where a more loosely packed membrane structure is advantageous, the rigidity of lengthy saturated acyl chains can be countered by acyl chains with double bonds. In unsaturated fatty acids, a carbon to carbon double bond is introduced by the desaturase reaction and results in a bend, or kink, in the carbon chain. Introducing a double bond of cis (same side) geometric configuration results in a bending of the chain with a change of approximately 30° from the linearity of the saturated chain. Double bonds also are non-rotating and restrict acyl chain movement. Resulting Van der Waals forces increase the distance between chains, thereby decreasing chain to chain interaction. Further, some charge concentration around the double bond increases polarity in the acyl chain. The extent to which double bonds cause bends or curved shapes within the biological membrane is not well understood. Physical and biochemical
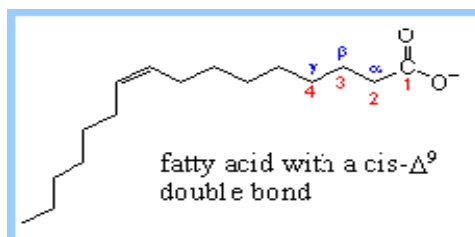
data, however, clearly show that unsaturated acids decrease membrane rigidity and melting point.

Multiple desaturase reactions can occur, each introducing an additional kink in the fatty acid chain. Because of the bends in the hydrocarbon backbone, the membrane bilayer or lipid body containing the fatty acid cannot pack into a regular structure, allowing the membrane to remain more fluid at low temperatures. Changing the ratio of saturated to unsaturated fatty acids is the main strategy employed by the cell to ensure membranes remain sufficiently fluid to maintain necessary cellular function at any temperature conducive to life. Increasing temperatures result in an increase in saturated (decrease in unsaturated) fatty acids, while decreasing temperatures result in an increase in the amount of unsaturated (decrease in saturated) fatty acids in cell membranes.

Within membrane phospholipids, acyl chain length and the number and position of double bonds markedly influence fluidity, permeability and stability of biological membranes. Since the physiological properties of unsaturated fatty acids largely depend on the length and position of double bonds, fatty acids are named using these parameters. The number of carbons and the number of double bonds are denoted by two numbers separated by a colon (e.g., 18:1). The number before the colon denotes the number of carbon atoms (normally even) and the number following the colon refers to the number of double bonds. Since bond position and orientation are also important contributors to the physiological properties of fatty acids, additional naming conventions are used. The delta (Δ) positions are carbons counted from the carboxyl end of the fatty acid, while the omega (ω) positions are counted back from the terminal carbon. The position of an individual double bond, or the specificity of an enzyme inserting it, is assigned using the delta (Δ) nomenclature. The delta nomenclature describes a bond position

relative to the carboxyl end of the acyl chain. An individual fatty acid within a family of structurally related acids is designated using the (n-) nomenclature. Here, the position of the first double bond from the methyl end is described. The (ω-) designation is also still widely used to designate the position of a double bond from the methyl end. For example, linoleic acid (18:2 ω-6), is an 18-carbon chain with 2 double bonds, and with the first double bond in the sixth position from the CH_3 end. Linoleic acid can also be described as $18:2\Delta^{9,12}$ to indicate that there are 2 double bonds located at the 9th and 12th carbon positions, counting from the carboxyl end of the fatty acid. In most naturally occurring fatty acids, the double bonds are in the cis-configuration (same side), and it is assumed double bonds are cis, and separated by a single methylene (CH_2) group, unless otherwise noted. For example, linoleic acid is described by $18:2cis\Delta^{9,12}$ to indicate both the position and orientation of the double bonds. A cartoon drawing of a fatty acid along with some examples with their common names are shown in Figure 1-1.

(A)

(C)

14:0	myristic acid
16:0	palmitic acid
18:0	stearic acid
$18:1cis\Delta^{9}$	oleic acid
$18:2cis\Delta^{9,12}$	linoleic acid
$18:3cis\Delta^{9,12,15}$	linolenic acid
$20:4cis\Delta^{5,8,11,14}$	arachidonic acid
$20:5cis\Delta^{5,8,11,14,17}$	eicosapentaenoic acid (an ω-3 fatty acid because of double bond 3C from methyl end)

(B)

$CH_3(CH_2)_{14}$-COO$^-$

Non-polar   polar

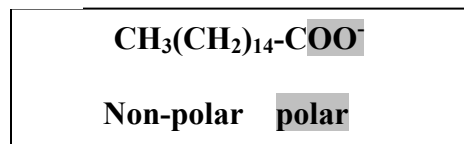Figure 1-1. Fatty acid identification. Panel A shows the general structure of a fatty acid. The example shown is oleic acid, which is a monounsaturated fatty acid with the cis double bond located at carbon 9. Panel B shows the charge distribution within the fatty acid that contributes to its amphipathicity. Panel C lists some common fatty acids, both saturated and unsaturated, and demonstrates naming conventions.

Fatty acid desaturases

Fatty acid desaturases (FADs) are the enzymes that catalyze the insertion of a double bond into fatty acids, resulting in more unsaturated forms. Two distinct families of fatty acid desaturases, soluble and membrane-bound, which do not seem to be evolutionarily related, have been identified. Soluble desaturases include plant plastidial stearoyl-ACP desaturases and cyanobacterial DesA proteins. In plants, the first double bond is introduced into fatty acids by the soluble stearoyl-ACP desaturase. Stearoyl-ACP desaturase is unique to the plant kingdom, as all other known (eukaryotic) desaturases are integral membrane proteins (Ohlrogge and Browse, 1995). Although no specific references could be located, it is likely that Cyanobacteria DesA is a soluble protein, since cyanobacteria are the evolutionary precursors to photosynthetic plants. *Euglena*, a protist with both animal-like (flagellum) and plant-like (chloroplasts) characteristics, also appears to have a soluble desaturase (Shanklin and Cahoon, 1998), further supporting the evolutionary lineage.

The second and more common desaturase family consists of membrane-bound enzymes. Integral membrane proteins are anchored in the membrane by hydrophobic stretches of amino acids referred to as a transmembrane-spanning domain, or TMD, and these proteins normally can only be separated from membranes using detergents, nonpolar solvents or denaturing agents. Consequently, investigation of these desaturases by traditional biochemical approaches has been limited because it is difficult to solubilize and purify them while retaining enzyme activity (Schmidt et al., 1994). Two members of this membrane-bound desaturase family that have been extensively studied and characterized include the endoplasmic reticulum (ER) localized stearoyl-CoA desaturases (SCDs) from yeast and mammals. Plants, like the other eukaryotes studied,

also have ER resident desaturases. Moreover, plants have an additional group of membrane-bound desaturases that are localized in plastids, or chloroplasts. Regardless of whether they are in the plastid or ER, all known plant integral membrane desaturases have basically the same general structure, and likewise, contribute to the highly unsaturated fatty acids that typically accumulate in plant cell membranes (Browse and Sommerville, 1991, Heinz, 1993). However, plant ER-bound enzymes, rather than plastid-localized enzymes, have been shown to be the major contributors to production of fatty acids that are destined for seed storage oils (Yasdav et al. 1993). Two important ER-bound desaturases found in plants include oleate desaturase (FAD2) and linoleate desaturase (FAD3). FAD2 introduces a second double bond into oleic acid (18:1 ω-9) to produce linoleic acid (18:2 ω-6), while FAD3 catalyzes a third double bond into linoleic acid to produce linolenic acid (18:3 ω-3).

Membrane-bound yeast and mammalian SCDs have also been shown to be structurally similar to their plant homologs, although mammalian SCDs primarily act on fatty acyl-CoA substrates while plant desaturases act on fatty acid substrates bound to glycerolipids (mainly phosphatidylcholine in the ER and galactolipids in the plastid). Mammalian SCDs have been extensively studied and characterized, and provide a good starting point toward understanding plant FAD regulation. SCD has been shown to be a key regulatory enzyme in unsaturated fatty acid biosynthesis. Biochemical and molecular characterizations of SCD have revealed the presence of two hydrophobic domains thought to span the lipid bilayer twice each, resulting in four putative transmembrane (TM) regions whose function is to anchor the desaturase in the microsomal membrane. SCD has also has been shown to contain three conserved histidine-box motifs containing eight histidine residues. Mutation of any of the eight conserved His residues

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results in a loss of desaturase activity suggesting that they play an essential role in determining

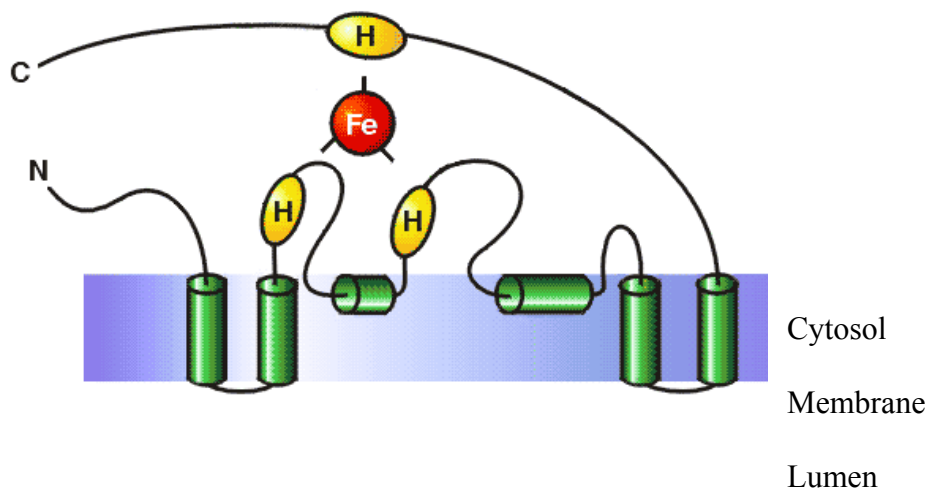catalytic activity (Shanklin et al., 1994).

Figure 1-2. Membrane-bound FAD structure. Topology model for desaturase protein structure, including positions of the membrane-spanning domains and orientation of the N and C termini, histidine boxes and di-iron active site. Drawing based on previous studies (Shanklin et al. 1994, Dyer and Mullen 2001) and obtained from Covello Lab, Plant Biotechnology Institute, Saskatoon, SK, Canada (http://cbr.pbi.nrc.ca/covellop/r-fattyacid.html)

Fatty acid desaturases selectively convert a single bond between two carbon atoms in a

fatty acyl chain by catalyzing highly selective, O_2-dependent, dehydrogenation reactions (Los

and Murata, 1998). This pathway is almost universal and is used by bacteria, yeasts, algae,

higher plants, protozoa and animals. SCD uses oxygen and electrons from the NADH-

cytochrome b_5 reductase and cytochrome b_5 microsomal electron transport chain to catalyze the

insertion of a cis double bond between carbons 9 and 10 ($\Delta 9$) of a spectrum of fatty acids.

Desaturases that introduce a double bond into the front end of fatty acids (between the $\Delta 1$ and $\Delta 9$

positions) all appear to have cytochrome b_5 fused to the N-terminus of the desaturase itself

(Napier et al., 1997). Yeast SCD has a cytochrome b_5 fusion, but it is at the C terminus.

Plant membrane-bound desaturases share numerous features with animal SCDs, such as

localization to, and anchorage in, the microsomal membrane, positioning of His-domains and

dependence on cytochrome b_5 and NADH as electron donors (Shanklin et al., 1994). Several

genes for various acyl-lipid desaturases have been cloned from both cyanobacteria and higher plants (Sakamoto et al., 1994, Napier et al., 1999, Los and Murata, 1998). These desaturases introduce double bonds at the $\Delta6$, $\Delta9$, $\Delta12$ and ω-3 positions of fatty acids.

Protein regulation

FADs are catalytic proteins, and proteins are known to be regulated in a number of different ways. Regulation occurring after the DNA is transcribed to form messenger RNA (mRNA) is known as post-transcriptional regulation. Regulation at this level ultimately controls the amount of protein that can be produced from an mRNA and some examples include alternative splicing, polyadenylation, capping, transport out of the nucleus, and mRNA stability. Alternate splicing can result in different proteins being translated from the same pre-mRNA. Polyadenylation, or the covalent linkage of a poly(A) tail to the 3' end of an mRNA molecule, increases mRNA lifespan by slowing down the degradation process. Capping the mRNA helps protect the 5' end of the mRNA from degradation. Slowing degradation either through polyadenylation or capping would potentially result in more protein being synthesized from the same mRNA, so mRNA stability is important in protein concentration. Capping also plays a role in ribosome binding, the key first step in protein translation and a selective mechanism has been suggested through which the number of ribosomes loaded onto particular mRNA molecules is increased. The number of ribosomes bound to the mRNA and the efficiency of translational initiation potentially changes the rate of mRNA translation into protein, with more bound ribosomes theoretically yielding faster protein synthesis. Any of these post-transcriptional mechanisms could result in more (or less) protein being translated from the same number of mRNA transcripts.

Another level of regulation occurs after the mRNA has been translated into protein, and is referred to as post-translational regulation. Post-translational regulation occurs at the protein level and includes such factors as protein targeting, stability, allostery, oligomerization, and covalent modification. Chemical modification of a protein after its translation can extend the range and function of the protein. Examples of chemical modification include attaching functional groups such as acetate, phosphate, lipids and carbohydrates. Attaching phosphates (phosphorylation) often activates or inactivates the enzyme, while attaching lipids and carbohydrates can affect localization and/or protein stability. Post-translational regulation may also include removing amino acids from the amino end of the protein or cutting the peptide chain in the middle, both of which have effects on the structure and function. Other structural changes, such as the covalent formation of disulfide bridges between amino acids, can also impact enzyme activity.

Proteins often carry localization signals that determine their ultimate destination within the cell. For instance, plant FADs are targeted to the ER by co-translational insertion into ER membranes, and retained in that location by distinct C-terminal ER retrieval signals (McCartney et al., 2004). In addition, proteins can also carry signals that determine their lifetime. Different proteins have demonstrated a wide range of half-lives within the cell, anywhere from a few minutes to many days. Protein half-life depends not only on the inherent stability of the protein's structure, but also on highly specialized cellular degradation machinery which often recognizes specific amino sequence and/or structural features. The shortest lived proteins are usually those that are important in controlling cellular processes, such as enzymes that catalyze rate determining steps in metabolic pathways, or proteins that regulate cell growth and division. Post-translational regulation is instrumental in this relatively instantaneous control of protein

level and metabolite amounts in cells, and protein degradation has been shown to be one of the main pathways by which real time adjustment of short lived proteins is accomplished. Rapid degradation of proteins makes it possible for the concentrations of their metabolites to be changed quickly in response to cell requirements or environmental stimuli.

Desaturase regulation/degradation

Fatty acid desaturases are critical in maintaining membrane fluidity, an essential cellular process. The importance of being able to adjust membrane fluidity rapidly is particularly important in poikilothermic organisms such as plants, yeast, reptiles and fish. Poikilothermic organisms have no internal body temperature control; their body temperature is environmentally driven, and they are often subject to external temperature changes varying as much as 20 degrees in a single day. Plants potentially have a greater need for rapid response than other poikilothermic organisms in that most plants are sessile and cannot escape their environmental conditions; they must adapt and persevere to survive in spite of changes in forecast. Plants can only react to environmental changes by adjusting their chemical composition, and so maintaining appropriate membrane fluidity requires rapid changes in fatty acid composition. Plants respond to cooler temperatures by increasing their polyunsaturated fatty acid content, which provides greater fluidity at lower temperatures. This gives significant credence to FAD2 and FAD3 being important control points for adjusting membrane fluidity, likely through rapid post-translational regulation. Yeast too could be subject to ambient temperature variations and would need a rapid response mechanism to adapt and survive. Even homeothermic organisms, ones with internal body temperature control such as mammals, often need to make rapid adjustments to maintain membrane fluidity, since nutritional intake affects membrane fatty acid composition. For example, a diet rich in saturated fat results in a high number of those fats being partitioned into

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biological membranes, so mechanisms must exist to rapidly adjust membrane fatty acid composition in order to maintain appropriate fluidity.

Cells must have the ability to rapidly adjust amounts of polyunsaturated fatty acids in their membranes, and maintaining a short half-life of the desaturases, possibly through ubiquitin-mediated proteolysis of the FAD enzymes, is one way to fine tune this process. Research with mammalian SCD has shown that the N-terminal segment (approximately 30 residues) of SCD constitutes a motif responsible for its rapid degradation (Mziaut et al., 2000). Ozols (1997) determined that mammalian SCD is degraded by a proteasome-independent pathway, and work by Mziaut et al. (2000) supported this contention. In contrast, Kato et al. (2006) found that SCD was rapidly degraded in a proteasome inhibitor-sensitive manner and concluded that the ubiquitin proteasome dependent ER-associated degradation (ERAD) pathway is also involved in constitutive SCD degradation. Yeast SCD (OLE1) is also a very short lived protein, and a variety of yeast mutants were used to show that its half-life was exquisitely regulated by the ubiquitin/proteasome system (Braun et al., 2002).

Although information on plant FAD regulation is more fragmentary and a mechanistic picture of the pathways has not been ascertained, it is entirely feasible that plant FADs are also regulated by degradation, and more specifically, by ubiquitin-mediated proteolysis. Ubiquitin is a small, highly conserved 76 amino acid 8500 KD protein. Proteins within the cell (either soluble or membrane-bound) may be marked for degradation by covalent attachment of one or more ubiquitin molecules. In an ATP requiring reaction, ubiquitin's terminal carboxyl group is conjugated, via a thioester bond, to ubiquitin-activating enzyme (E1). The ubiquitin is then transferred to a specific sulfhydryl group on one of numerous homologous proteins named ubiquitin-conjugating enzymes (E2s). Ubiquitin-protein ligase (E3) then transfers the activated

ubiquitin from E2 to a lysine amino group in the target protein, thereby forming an isopeptide bond. E3 therefore appears to play a key role in selecting the proteins to be degraded. However, the large number of E2s in a cell suggests that these proteins also function in target protein selection and some E2s are known to transfer ubiquitin directly to target proteins. Normally this ubiquitination process is repeated, and a series of ubiquitin molecules are added to the target protein in a process known as polyubiquitination.

Once ubiquitinated, the substrate is transferred to the proteasome for degradation. Although the process of removing the protein from the membrane and bringing it to the proteasome for degradation has not been completely characterized, much has been discovered over the last few years about the process. Schuberth and Buchberger (2005) proposed a model where Ubx2 (an integral ER membrane protein) coordinates the assembly of a highly efficient ERAD protein complex (Cdc48/Npl4/Ufd1) at the ER membrane. Although additional recruitment factors likely exist, Ubx2 has been shown to contribute significantly to the recruitment of Cdc48 and Ufd1 to the ER membrane. Cdc48 associates with the cofactors Ufd1-Npl4 to remove polyubiquitinated proteins from the ER membrane and move them to the cytosol for their subsequent degradation by the proteasome. The Cdc48 molecular chaperone is able to bind a number of cofactors resulting in a large multi-functional protein complex. An important role of the cofactors is to provide binding sites for the polyubiquitinated chains. One predicted Cdc-48 cofactor is Doa1 which, Mullally et al. (2006) suggested, mediates Cdc48 binding to ubiquitinated proteins. It has been shown that the Cdc48 complex is required for the removal of ubiquitinated substrates from the ER membrane before they can be brought to the proteasome for degradation, and the complex may play a key role in modulating the ubiquitin-proteolysis pathway by providing multiubiquitination of bound substrates (by the cofactor Ufd2), catalyzing

deubiquitination of proteins (by the cofactor Otu1), or releasing the substrates as monoubiquitinated proteins (by the cofactor Ufd2) (Rumpf and Jentsch, 2006). Carlson et al. (2006) proposed that Cdc48 (p97 in mammals) facilitates substrate presentation to the proteasome and increases the rate and efficiency of degradation of proteins that contain transmembrane domains (like those found in FADs).

The proteasome is a large hollow barrel-like complex, providing an enclosed cavity in which proteins can be degraded. The 26S proteasome is the most common form of the proteasome and is normally involved in ubiquitin-mediated degradation of proteins. Ubiquitinated proteins enter through openings at either end of a central core, and each end associates with a regulatory subunit that contains multiple ATPase active sites and ubiquitin binding sites. The regulatory subunit recognizes ubiquitinated proteins and transfers them to the catalytic core, consisting of four stacked rings, where they are degraded. As mentioned previously, numerous studies have shown mammalian and yeast FADs to be regulated, at least in part, by ubiquitin-dependent proteolysis and this characteristic could potentially be conserved in the plant desaturases as well.

We know that plant FADs are structurally and functionally similar to mammalian and yeast SCDs and that they are likely also subject to rapid post-translational regulation. Matsuda et al. (2005) examined plant plastid FAD7 and FAD8 in *Arabidopsis* leaf tissues. They found that despite having a high degree of structural relatedness, FAD7 and FAD8 activity are regulated differentially in response to temperature. Additionally, their results suggested that the FAD8 contained regulatory sequences required for temperature sensitive expression that are not present in FAD7. They further showed that the C-terminal region of FAD8 acts in an autoregulatory fashion to destabilize the protein at high temperature, causing a reduction in the amount of active

enzyme, in the absence of a concurrent decrease in its transcript level, and due at least in part to post-translational destabilization. No structural motifs or amino acid residues in the FAD8 C-terminal were speculated or identified as potentially responsible for temperature-sensitive regulation in FAD8.

Two other important ER resident plant FADs, FAD2 and FAD3, play key roles in increasing polyunsaturated fatty acids and have also demonstrated differential regulation with respect to temperature sensitivity. Tang et al. (2005) studied two isoforms of soybean oleate desaturase (FAD2-1A and FAD2-1B) that were nearly identical in protein sequence. When the proteins were expressed in yeast grown at 30°C, it was found that the *in vivo* decay of FAD2-1A was much faster than FAD2-1B, and that differences in the N-terminus of FAD2-1A appeared to influence the enzyme stability. Tang's experiments with proteasome inhibitor MG132, suggested that the 26S proteasome, and consequently ubiquitin-mediated degradation, was implicated in the temperature dependent regulation of FAD2-1A.

TaFAD3 expressed in wheat (*Triticum aestivum*) tips also showed temperature dependent regulation. Levels of TaFAD3 mRNA under different growth conditions were nearly the same, but protein levels were significantly greater in low temperature growth conditions (Horiguchi et al., 2000). They surmised that post-translational regulation could be achieved by modulation of translational efficiency (and/or stability of the protein) and demonstrated that TaFAD3 mRNA had enhanced association with ribosomes at cooler temperatures. Dyer et al. (2001) obtained similar results when expressing the rapeseed oil (*Brassica napus*) FAD3 gene in yeast. They noted no significant changes in FAD3 mRNA levels in response to temperature while there were significant changes in amounts of FAD3 protein and fatty acid accumulation, presumably due to regulation at the post-transcriptional or post-translational level. Dyer (2004) continued the

investigation of FAD3s in yeast cells by studying the activity of tung (*Aleurites fordii*) FAD3. Interestingly, despite 70% amino acid identity (88% similarity) between the *Brassica* and tung FAD3 protein sequences, there was an extraordinary difference in enzyme activity and linolenic acid accumulation when each FAD3 was expressed in yeast cells. Both tung and *Brassica* enzymes showed increased linolenic acid production at cooler growth temperatures, but levels synthesized by tung FAD3 were at least 10-fold higher than those of BF3 at each temperature.

Research focus

ER resident FAD3 from *Brassica napus* (BF3) and tung (TF3) was the main focus of the research in this thesis. Bryan (2004) previously showed that the steady-state amounts and half-lives of these two proteins are significantly different when expressed in yeast cultured at 30°C. At the 30°C growth temperature, TF3 has a significantly greater steady-state accumulation and longer half-life than BF3. (Note that while TF3 is more stable than BF3, TF3 is still considered to be a short-lived protein in comparison to most other ER resident membrane proteins.) At 20°C, the steady-state amount of each protein was increased, more so for BF3, and there were no longer any differences between the steady-state amount or half-life of the BF3 and TF3 proteins. Bryan (2004) swapped the N-terminal domains (up to the first transmembrane region including approximately the first 65 amino acids) between BF3 and TF3 and expressed the chimeric proteins in yeast cells cultured at 30°C. She found that placing the BF3 N-terminal domain on the TF3 protein (5'BF3) produced a protein with reduced steady-state amount and shorter half-life, comparable to the properties of native BF3 protein. Likewise, placing the TF3 N-terminal domain on the BF3 protein (5'TF3) resulted in a protein with an increased steady-state amount and longer half-life, comparable to native TF3.

Figure 1-3 shows the results of fusing the N-terminus of BF3 with the C-terminal portion of TF3 (5'BF3) and conversely, the N-terminal sequence of TF3 to the C-terminal portion of BF3 (5'TF3). Panel A shows the steady-state amounts of BF3, 5'TF3 and 5'BF3 proteins relative to native TF3 extracted from yeast grown at 30°C. In these experiments, western blots using the α-myc antibody detected a protein at approximately 40KD and another sample was run using α-DPMS as a loading control. Panel B shows representative western blots for half-life. Extractions were done on samples collected at 0 (steady-state), 1, 2, 4, and 8 hour time points after addition of the translational inhibitor cycloheximide. Panel C displays the half-life western blot results in graphical form, displaying data for both high copy and low copy plasmids.

Figure 1-4 shows the same experiments performed with cultures grown at 20°C. As with previous experiments, there were no significant differences in steady-state accumulation and half-life between BF3 and TF3 or either of the 5'TF3 or 5'BF3 chimerics. Cultures grown at 20°C showed a dramatic increase in the steady-state amount of BF3 and 5'BF3, but no corresponding change in the steady-state accumulation or half-life of TF3 and 5'TF3. Results clearly suggest that swapping N-termini between BF3 and TF3 proteins increases half-life for BF3 (5'TF3) and decreases half-life for TF3 (5'BF3), strongly indicating that the N-termini of these proteins play a significant role in their differential regulation when the proteins are expressed in yeast cells grown at 30°C. Studies with the chimerics once again confirmed that similar differences are not evident when cultures are grown at 20°C.
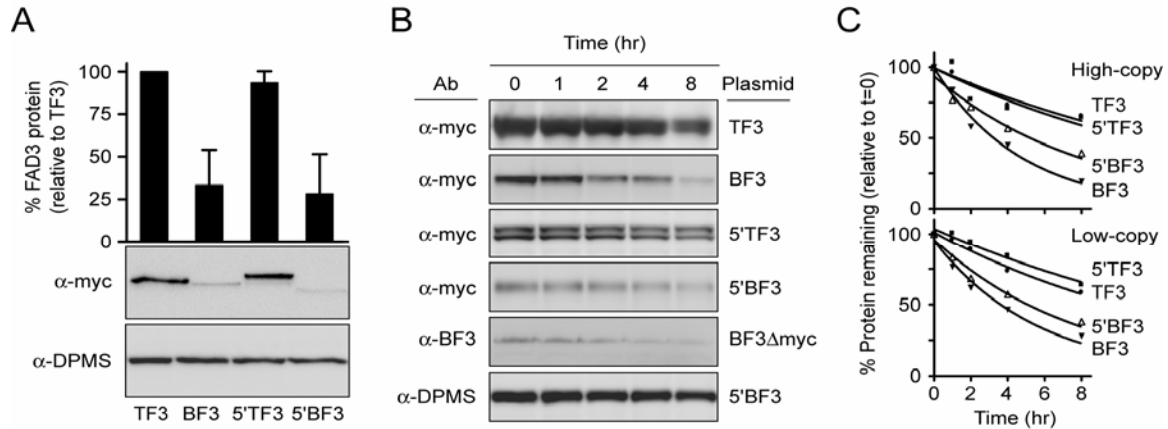
Figure 1-3. Protein accumulation at 30°C growth temperature. Western blot of protein extracted from TF3, BF3 and chimerics 5'TF3 (TF3 N-terminal fused to BF3), and 5'BF3 (BF3 N-terminal fused to TF3) at 30°C growth temperature. Data are expressed relative to TF3. (A) Representative western blot of steady-state accumulation probed with anti-myc antibody. Anti-DPMS blot provided loading control. Bar graph provides quantified data from at least 3 independent experiments showing averages and standard deviations. (B) Representative western blots of protein half-life probed with anti-myc antibody. (C) Line graphs of protein half-life probed with anti-myc antibody. Values represent averages obtained from at least 3 independent experiments, and lines generated by a single exponential best fit of the data. Graphs are shown for both high and low copy plasmids. (Bryan and Dyer, 2004).
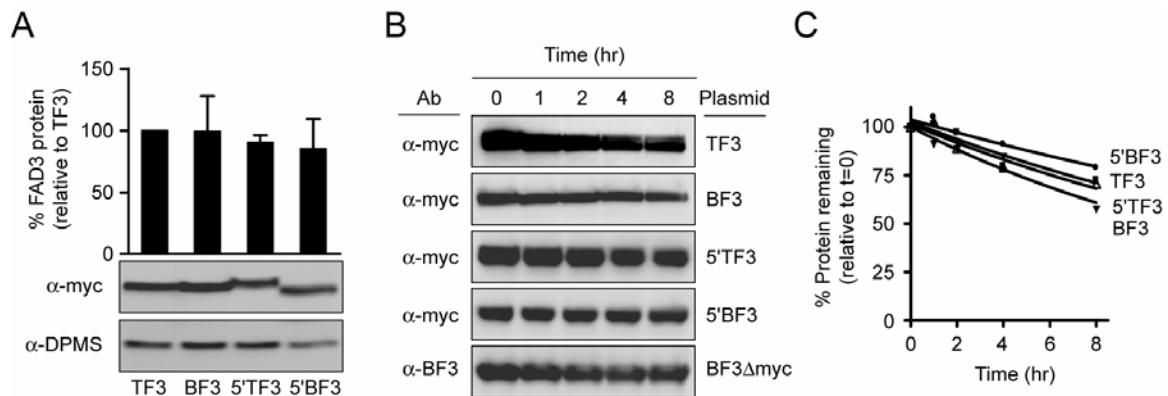
Figure 1-4. Protein accumulations at 20°C growth temperature. Western blot of protein extracted from TF3, BF3 and chimerics 5'TF3 (TF3 N-terminal), and 5'BF3 (BF3 N-terminal) at 20°C growth temperature. Data are expressed relative to TF3. (A) Western blot of steady-state accumulation probed with anti-myc antibody. Anti-DPMS blot provided loading control. Bar graph indicates quantified data with error bars indicating standard deviation. (B) Western blot of protein half-life probed with anti-myc antibody . (C) Line graph of protein half-life probed with anti-myc antibody. Graph shown for high copy plasmid. (Bryan and Dyer, 2004)

Experiments with the 5'BF3 and 5'TF3 chimerics pointed to the BF3 and TF3 N-termini

as the likely control points of their differential regulation when expressed in yeast cultures grown

at 30°C. The next logical step in evaluating the differential regulation of BF3 and TF3 was to

compare the BF3 and TF3 N-termini to determine which specific amino acid residues might be responsible. Bryan (2004) noted three major differences between the N-terminal domains of BF3 and TF3: length (TF3 is 6 residues longer); charge (TF3 has more acidic residues); and a conserved lysine (found in a number of plant FADs including BF3, but not found in TF3). The length and charge difference between BF3 and TF3 is due primarily to the fact that TF3 has a stretch of six glutamic acids and one aspartic acid in its N-terminal domain, whereas BF3 has only two glutamic acids in the corresponding location. Figure 1-5 shows the N-terminal amino acid sequence of nine plant FAD3s with the conserved lysine highlighted in BF3 and the polyglutamic region highlighted in TF3. Corresponding locations in the other protein of interest are also highlighted for comparison purposes.

```
Soybean              MVKDTKPLAYAANNGYQKEAFDPSAPPPFKIAEIRV
Soybean              MVQAQPLQHVGNFAFKEDQAYFDPSAPPPFKIANIRA
Mung Bean            MIQAQTLQHFGNGAREGDQSYFDPGAPPPFKIADIRA
Tobacco              MGSLGISEIYDKNSFNEMEFEFDPSAPPPFRLAEIRN
BF3                  MVVAMDQRSNVNGDSGARKEEGFDPSAQPPFKIGDIRA
Arabidopsis          MVVAMDQRTNVNGDPGAGDRKKEERFDPSAQPPFKIGDIRA
BET                  MKEPVLEEMENAGGFGNGFHGVVEKDDFDPSAPPPFKIAEIRA
TF3                  MKQQQYKDTPILNGVNGFHAKEEEEEDFDLSNPPPFNIGQIRA
Per         VSSGARLSKSGADGEVFDGQQQYYEGIGKRAADKFDPAAPPPFKIADIRA
```

Figure 1-5. N-Terminal sequence alignment of nine plant FAD3s. Seven of the nine have a lysine highlighted in BF3 and not found in TF3. TF3 has a stretch of six glutamic acids, also highlighted, not found in any of the other proteins. (Bryan, 2004)

Since FAD protein abundance and half-life in other organisms have been shown to be regulated to a large degree by ubiquitin-mediated degradation, differences in ubiquitination might play a key role in the significant differences between BF3 and TF3 protein accumulation when expressed in yeast grown at 30°C. Substrates are ubiquitinated at one or more lysine residues and some proteins have been shown to have preferential lysine binding/ubiquitination sites, while others seem to be relatively indiscriminant about which lysine residue is ubiquitinated. The conserved lysine residue in BF3, not found in TF3, could explain BF3's lesser protein accumulation and shorter half-life, since lysine ubiquitination is the first step in the

ubiquitin-mediated proteolysis pathway. To test this theory, Bryan (2004) mutated the conserved

lysine in BF3 to asparagine (BF3 K_N). Figure 1-6 details the results of these experiments.

Panel A shows the location of the conserved lysine residue denoted by ↓. Panel B are

western blots probed with α-myc showing the half-lives of the BF3 and BF3 (K_N) proteins.

BF3 was also probed with α-DPMS as a loading control. Samples were extracted at 0 (steady-

state amount), 1, 2, 4 and 8 hour time points. Panel C is a graphical representation of half-life

for BF3, BF3 K_N and TF3. Taken together, these results suggest that changing the conserved

lysine in BF3 to asparagine yields significantly more steady-state protein and a longer half-life of

the BF3 K_N protein as compared to BF3 wild type. Notably, when expressed in yeast cultured

at 30 °C, the half-life of BF3 K_N protein was not significantly different from the wild type TF3

protein (Figure 1-6, panel C).

A

```
                          ↓
Brassica             MVVAMDQRSNVNGDSGARKEEG**FDPSAQPPFKIGDIRA**
Arabidopsis      MVVAMDQRTNVNGDPGAGDRKKEER**FDPSAQPPFKIGDIRA**
Mung                    MIQAQTLQHFGNGAREGDQSY**FDPGAPPPFKIADIRA**
Per.      MAVSSGARLSKSGADGEVFDGQQQYEGIGKRAADK**FDPAAPPPFKIADIRA**
Soybean               MVKDTKPLAYAANNGYQQKGSS**FDPSAPPPFKIAEIRA**
Bet.             MKEPVLEEMENAGGFGNGFHGVVEKDD**FDPSAPPPFKIAEIRA**
Tung             MKQQQYKDTPILNGVNGFHAKEEEEEED**FDLSNPPPFNIGQIRA**
                                           **  .  ***:*.:***
```
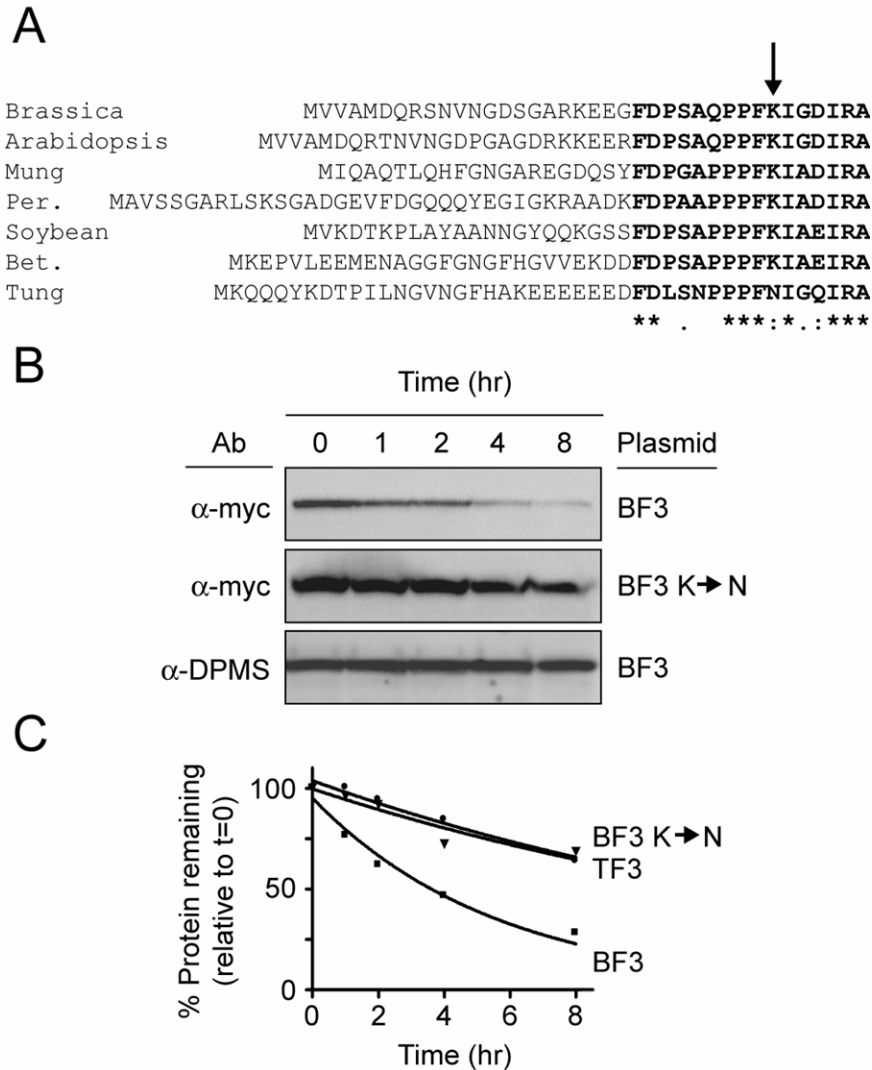
B

Figure 1-6. FAD3 expression of BF3 K→N mutant. Panel A indicates by ↓ a conserved lysine present in all FAD3s shown except tung FAD3, which has an asparagine (N) at this position. Panel B shows representative western blots of half-life of the native BF3 protein compared to a BF3 K→N mutant, where the conserved lysine has been changed to an asparagine. Panel C provides a graphical depiction of half-life data, where each data point represents the average value obtained from at least three independent experiments (Bryan and Dyer, 2004).

Bryan (2004) identified the FAD3 N-terminus as a critical element in controlling protein accumulation and half-life, and demonstrated that a largely conserved lysine plays an important role in determining BF3 half-life. Bryan's data also suggested that differences in steady-state accumulation and half-life between the BF3 and TF3 proteins were not due to mRNA amounts (data not shown), but occur post-transcriptionally and likely due to a combination of translational

efficiency and/or post-translational degradation. The data also showed that BF3 is degraded more quickly than TF3, and suggests their differential regulation is related to differences in ubiquitination and/or proteolysis. A number of similarities have already been identified between mammal and yeast SCD's and other plant FADs; it is highly likely another shared characteristic is regulation through rapid turnover by the proteasome, mediated through ubiquitin-dependent proteolysis.

A microarray analysis (data not shown) was completed by the Dyer lab to determine if there were any proteolysis-related genes that were downregulated at cooler temperatures, which could help explain why FAD3 protein half-life goes up at cooler temperatures. Microarray data was evaluated for proteolysis-related genes that were down regulated at 20°C when compared to 30°C. Several genes were detected including UFD2, UBR1, and DOA1, and BY4742 yeast knockouts of these genes were obtained. BY4742 knock-outs were also obtained for the genes CUE1 and NPL4, which were previously shown by Braun et al. (2002) to play a role in the Cdc48 chaperone portion of the ubiquitin-mediated proteolysis pathway. Mutation of the CUE1 and NPL4 genes resulted in an increase in endogenous yeast stearoyl CoA desaturase half-life. Thus, we were interested to see if these same genes, in addition to those whose expression was downregulated at cooler temperatures, had any effect on FAD3 protein half-life when expressed in yeast. As stated previously, many proteins interact to determine which proteins are targeted for ubiquitination and subsequent delivery to the proteasome. Expressing BF3 and TF3 in a background deficient in these proteolysis related genes, and consequently their respective protein products, might yield evidence to support ubiquitin-mediated degradation as the major mechanism in the rapid turnover and differential regulation of the BF3 and TF3 proteins.

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Research Objective

The purpose of my research program was to continue Bryan's (2004) work and further

elucidate which post-translational regulatory mechanisms are responsible for the differences in

steady-state amounts of the BF3 and TF3 proteins, when extracted from yeast cultures grown at

30°C, and to identify specific amino acids involved in this process. Bryan already showed that

the N-terminus in general, and specifically a largely conserved lysine, may be significant factors

in the differential regulation of the two proteins. My research was designed to determine if other

N-terminal amino acid residues play a role in the differential regulation and elucidate whether

the regulatory differences were related to ubiquitination and proteolysis. I hypothesized that

mutating the asparagine in TF3 to a lysine as found in BF3, and a number of other plant FAD3's,

would decrease the steady-state accumulation and half-life of TF3.

Bryan (2004) also observed that besides the conserved lysine, the major differences

between BF3 and TF3 N-termini were due to length and charge. The length and charge

differences between the N-terminals of the BF3 and TF3 proteins are primarily due to the four

additional glutamic acids residues found in TF3, but not in BF3. A preferential ubiquitination

site, proximal to an acidic region, was found in Rpn4 (Ju and Xie, 2006) which raised the

question as to whether a similar phenomenon was occurring in TF3. I surmised that the

polyglutamic region in TF3 may be a key variable in the regulatory differences between the BF3

and TF3 proteins, and that these differences are related to variations in ubiquitination and

proteolysis. If this polyglutamic region is an important factor in the differential regulation of

BF3 when compared to TF3, I hypothesized that reducing the number of glutamic acids in TF3

should result in a reduction of steady-state protein and half-life of TF3, and conversely, that

increasing the number of glutamic acids in BF3 should result in an increase of steady protein and

half-life. Additionally, if the differential regulation of the BF3 and TF3 proteins is related to ubiquitin-mediated degradation, additional insight should be gleaned from both immunoprecipitation experiments to detect ubiquitination, and expression of FAD3 in proteolysis-deficient yeast mutants.

Concisely, my research aims to further define the specific amino acids involved in the differential regulation of the BF3 and TF3 proteins, gain a better understanding of their degradation pathway, and determine whether ubiquitin-mediated proteolysis plays a significant role.

Materials and Methods

Sources

Primers and competent bacterial cells were from Invitrogen. Chemicals were from Sigma-Aldrich unless otherwise noted. Media was from Becton Dickinson (DIFCO). Restriction enzymes were from New England Biolabs. All protein related products including apparatus, buffers, membranes, standards, etc. were from Invitrogen unless otherwise noted.

Yeast strains

Saccharomyces cerevisiae strain BY4742 (MATα his3Δ1 leu2Δ0 ura3Δ0) was used in all studies (Brachmann et al, 1998). BY4742 variants used were wild-type, ΔCUE1, ΔDOA1, ΔNPL4, ΔUBR1 and ΔUFD2 (all purchased from Invitrogen). Parent yeast strains were maintained on YPD plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) solidified with 2% w/v agar.

Plasmid construction

Starting plasmids were constructed by Jami Bryan and obtained from the Dyer lab. Plasmids included *Brassica* pYES 2.1 myc BF3, *Aleurites* pYES 2.1 myc TF3 and an empty pYES2.1 vector for use as a negative control. A variety of mutagenized BF3 and TF3 sequences were created using the Quick Change site-directed mutagenesis protocol (Stratagene). A typical mutagenesis reaction included 25μL Pfu master mix (Stratagene), 4μL each primer (Invitrogen diluted to 5μM), 2μL (100ng) DNA and 15μL sterile ddH$_2$0 which were placed in a PCR tube. A PTC-200 thermocycler (MJ Research) was programmed to 95°C for 30 seconds, followed by 20 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 7 minutes to complete the mutagenesis.

PCR reactions were then transferred to new tubes and 10μL NEB buffer 4 (New England

Biolabs), 40μL ddH$_2$0 and 2μL Dpn1 were added. Samples were placed in a 37°C heating block

for 2.5 hours to digest parent DNA. Samples were cleaned and concentrated in Zymo columns

(Zymo Research) and resulting DNA was used to transform bacteria. Plasmids derived from

single bacterial colonies were subject to DNA sequencing to confirm that the desired mutation

was introduced and ensure no unintended changes were inadvertently made in the open reading

frame. A comprehensive list of all FAD3 mutations with specific amino acid changes indicated

is provided in Figure 2-1.

```
BF3                      MVVAMDQRSNVNGDSGARKEEGFDPSAQPPFKIGDIRA
BF3 K_N(1)               MVVAMDQRSNVNGDSGARKEEGFDPSAQPPFNIGDIRA
BF3 K_N(2)               MVVAMDQRSNVNGDSGARNEEGFDPSAQPPFKIGDIRA
BF3 K_N(2) 2_6E    MVVAMDQRSNVNGDSGARNEEEEEEGFDPSAQPPFKIGDIRA
BF3 K_N(1) 2_6E    MVVAMDQRSNVNGDSGARKEEEEEEGFDPSAQPPFNIGDIRA
BF3 2_6E           MVVAMDQRSNVNGDSGARKEEEEEEGFDPSAQPPFKIGDIRA


TF3                   MKQQQYKDTPILNGVNGFHAKEEEEEEEDFDLSNPPPFNIGQIRA
TF3 6_3E               MKQQQYKDTPILNGVNGFHAKEEEDFDLSNPPPFNIGQIRA
TF3 6_2E                MKQQQYKDTPILNGVNGFHAKEEDFDLSNPPPFNIGQIRA
TF3 N_K 6_2E           MKQQQYKDTPILNGVNGFHAKEEDFDLSNPPPFKIGQIRA
TF3 K_N(2) 6_2E         MKQQQYKDTPILNGVNGFHANEEDFDLSNPPPFNIGQIRA
TF3 K_N(2)        MKQQQYKDTPILNGVNGFHANEEEEEEEDFDLSNPPPFNIGQIRA
TF3 N_K          MKQQQYKDTPILNGVNGFHAKEEEEEEEDFDLSNPPPFKIGQIRA
TF3 LNN_PAK      MKQQQYKDTPILNGVNGFHAKEEEEEEEDFDPSAPPPFKIGQIRA
```

Figure 2-1 Comprehensive list of FAD3 mutants, with specific amino acid changes highlighted.

Mutations for changing charge density in FAD3s.

Plasmid pYES myc BF3 K_N (1) was constructed to determine if this largely conserved

lysine was a significant factor in the short half-life of the BF3 protein when expressed in yeast

grown at 30°C. Plasmid pYES myc BF3 K_N changed a single largely conserved lysine in the

BF3 N-terminal sequence to an asparagine, and was constructed using primers 5'GCGCACCAC

CGTTTAATATCGGAGATATAAGG and 5'CCTTATATCTCCGATATTAAACGGTGGTGC

GC.

Plasmid pYES myc BF3 K_N(2) was constructed to determine the impact mutating this lysine found in both BF3 and TF3. This lysine is located proximal to the glutamic acid region to an asparagine and was constructed using primers 5'CCGGTGCCCGGAATGAAGAAGGGTTT G and 5'CAAACCCTTCTTCATTCCGGGCACCGG.

Plasmid pYES myc TF3 N_K was constructed to determine if restoring this largely conserved lysine to TF3 would decrease its steady-state level and half-life when expressed in yeast grown at 30°C using primers 5'TAAGCAATCCTCCTCCATTCAAGATTGGTCAGATC CGAGCTGC and 5'GCAGCTCGGATCTGACCAATCTTGAATGGAGGAGGATTGCTTA.

Plasmid pYES myc TF3 LNN_PAK was constructed to determine the effect of replacing other amino acids proximal to the largely conserved lysine in an attempt to make this region of TF3 more like BF3. Plasmid pYES myc LNN_PAK incorporated two additional point mutations in addition to the asparagine to a lysine (TF3 N_K) including changing a leucine to a proline and an asparagine to an alanine. Plasmid LNN_PAK was constructed using primers 5'GAAGAAGA AGAGGATTTCGACCCAAGCGCACCTCCTCCATTCAAGATTGGT and 5'ACCAATCTTG AATGGAGGAGGTGCGCTTGGGTCGAAATCCTCTTCTTCTTC.

Plasmid pYES myc TF3 K_N(2) was constructed to see what impact mutating this lysine found in both BF3 and TF3 would have on protein accumulation. Plasmid pYES TF3 K_N(2) changed a single lysine located proximal to the glutamic acid region to an asparagine and was constructed using primers 5'GTTTTCATGCTAATGAAGAAGAAGAAG and 5'CTTCTTCTT CTTCATTAGCATGAAAAC.

Mutations for changing charge density and length in FAD3s.

Plasmid pYES myc BF3 2_6E was constructed to determine the impact of increasing the number of glutamic acids in BF3, making it more TF3-like. Plasmid pYES myc BF3 2_6E contained the insertion of four additional glutamic acids, increasing the number of glutamic acids from two to six and increasing both the charge density (more negative) and length of the protein. Plasmid pYES myc BF3 2_6E was constructed using primers 5'CCGGTGCCCGGAAGGAA GAAGAAGAAGAAGGGTTTGATCCAAGCGC and 5'GCGCTTGGATCAAACCCTT CTTCTTCTTCTTCTTCCTTCCGGGCACCGG. Several attempts were made to use the same primers with the pYES myc BF3 K_N(1) and pYES myc BF3 K_N(2) plasmids to create pYES myc BF3 K_N(1) 2_6E and pYES myc BF3 K_N(2) 2-6E plasmids respectively. Another set of primers, 5'CCGGTGCCCGGAAGGAAGAAGAAGAAGAAGAGGGG TTTGATCCAAGCGC and 5'GCGCTTGGATCAAACCCCTCTTCTTCTTCTTCTTCCTTCCGGGCACCGG were also tried, but proved unsuccessful. Additionally an attempt was made to introduce the K_N(1) and K_N(2) mutations into the pYES myc BF3 2_6E plasmid to create the pYES myc BF3 2_6E K_N(1) and pYES myc BF3 2_6E K_N(2) plasmids. This attempt also proved unsuccessful.

Plasmid pYES myc TF3 6_3E was inadvertently constructed due to improper primer design (aiming for 6_2E) and was not apparent until sequencing was completed. Like the plasmid pYES myc TF3 6_2E, it was constructed to determine the impact of decreasing the number of glutamic acids in TF3, making it more BF3-like. Plasmid pYES myc TF3 6_3E resulted from the deletion of three glutamic acids, decreasing the number of glutamic acids from six to three and decreasing both the charge density (less negative) and length of the protein. Plasmid pYES myc TF3 6_3E was constructed using primers 5'GGTTTTCATGCTAAAGAA GAAGAGGATTTCGACTTAAGC and 5'GCTTAAGTCGAAATCCTCTTCTTCTTTAGCA

TGAAAACC. The same primers were also used to introduce the 6_3E mutation into the pYES myc TF3 N_K mutant creating the pYES myc TF3 N_K 6_3E plasmid. Experiments were conducted with both the pYES myc TF3 6_3E and pYES myc N_K 6_3E plasmids before the primer design error was discovered.

Plasmid pYES myc TF3 6_2E was constructed to determine the impact of decreasing the number of glutamic acids in TF3, making it more BF3-like. Plasmid pYES myc TF3 6_2E resulted from the deletion of four glutamic acids, decreasing the number of glutamic acids from six to two and decreasing both the charge density and length of the protein. Plasmid pYES myc TF3 6_2E was constructed using primers 5'GGTTTTCATGCTAAAGAAGAAGATTTCGACT TAAGC and 5'GCTTAAGTCGAAATCTTCTTCTTTAGCATGAAAACC. The same primers were used in an attempt to introduce the 6_2E mutation into the pYES myc TF3 N_K and pYES myc TF3 K_N(2) plasmids to create pYES myc TF3 N_K 6_2E and pYES myc TF3 K_N(2) 6_2E plasmids respectively. Sequencing showed that these attempts were also not successful.

Functional complementation

Arabidopsis DOA1 ortholog (At3G18860) was obtained from the National Resources Centre for Plant Genomics (CNRGV) in Toulouse (cDNA clone BX823857). The AtDOA1 gene was PCR amplified out of the pCMV SPORT 6 vector using PfuUltra PCR protocol (Stratagene) and primers, 5'AACTCCCGGGCCACCATGGTCATGGATATCGATTTCAAC and 5'ACATACCCGGGTTACTGACGAATTACAAGATCGATATC. 25μL master mix (Stratagene), 2μL (100 ng) DNA, 4μL (5μM) primers, and 19μL ddH$_2$0 were placed in a PCR tube. The tube were placed in the PTC-200 thermocycler which was programmed to run at 95°C for 2 minutes, followed by 35 cycles of 95°C for 20 seconds, 68°C for 20 seconds and 72°C for 2.5 minutes, with a final step of 72°C for 3 minutes.

Loading dye was added to PCR product and run out on 0.8% agarose gel. The band specific to the gene was excised and cleaned and concentrated using Zymo Clean (Zymo Research) and eluted in 30μL ddH$_2$0. Since the Pfu polymerase produces a blunt ended product, an additional PCR reaction was performed to add a T to the 3' ends of the amplified gene in order to increase the probability of successfully ligating it into the pYes2.1 vector. 20μL of the amplified gene (insert), 5μL Ex-Taq buffer, 5μL dNTPs, 19μL ddH$_2$0 and 1μL Ex-taq polymerase were combined and the reaction was run at 72°C for 6 minutes in the PTC-200 thermocycler. After heating, the T-tailed insert was cleaned and concentrated using Zymo Clean columns (Zymo Research). Following cleaning, the insert was cloned into the pYES 2.1 vector (Invitrogen) using TOPO cloning procedures (Invitrogen).

Competent DH5α bacterial cells (Invitrogen) were transformed using the bacterial transformation procedures outlined below. Plasmid DNA was extracted using Wizard Plus Mini-preps (Promega). A restriction enzyme digest was completed using EcoRI and XbaI to determine if the gene was ligated correctly into the vector.

Bacterial transformation

2μL plasmid DNA (approximately 2 micrograms) was added to competent cells on ice and the mixture was incubated for 5 minutes, followed by heat shock at 42°C for 30 seconds in a heating block. The sample was then returned to ice and 250μL SOC medium was added. Then the culture was incubated with shaking for 1 hour at 37°C. 100μL aliquots were plated and incubated at 37°C overnight.

A single bacterial colony was pricked with a sterile toothpick and the toothpick was used to inoculate a 3mL LB-amp culture (100 micrograms per mL ampicillin). Cultures were shaken

at 37°C overnight. Cells were centrifuged at maximum speed for 30 seconds and DNA was extracted from the pellet using Wizard Plus Mini-preps (Promega).

Yeast transformation

The lithium acetate method of Geitz et al. 1995 was used to introduce plasmid DNA into yeast. A sterile loop was used to scrape a sample of yeast cells from fresh (<3 days old) YPD plate. Cells were resuspended in 1mL sterile H_2O by vortexing, then pelleted by centrifuging at maximum speed in a microfuge for 30 seconds. The supernatant was removed and the pellet was resuspended in 1mL 100mM lithium acetate. The tubes were then incubated for 5 minutes in 30°C heating block. Cells were pelleted by centrifugation as above for 30 seconds. Supernatant was removed and the following solutions were added in order: 240µL PEG 3350 (50% w/v), 36µL 1.0M lithium acetate, 25µL denatured salmon sperm DNA (2.0 mg/mL), 5µL plasmid DNA and 45µL sterile H_2O. Pellets were vortexed until completely suspended (at least one minute), then incubated at 42°C for 20 minutes. Cells were pelleted by centrifugation in a microfuge for 30 seconds and the pellets were resuspended in 80µL of sterile H_2O. The cells were spread onto SD-URA plates and the plates were incubated at 30°C for two days or left on the bench (at room temperature) for three days.

Yeast transformants were maintained on SD (2% w/v dextrose, 0.67% w/v yeast nitrogen base without amino acids and a 2g/L synthetic complete medium–uracil auxotrophic supplement; Bufferad) solidified with 2% w/v agar. Single colonies were selected using sterile toothpicks and inoculated into liquid SD-URA starter cultures which were grown overnight (20-23 hours) in an incubator/shaker at 30°C, 300 rpm. Culture cell growth was quantified by measuring the optical density (OD) at 600nm using a 401 spectrophotometer (Milton Roy). Yeast cells were then removed from the SD-URA and back-diluted into either 5 or 10 mL SGal-URA cultures at a

starting concentration of 0.25 OD/mL. These cultures were also grown overnight, and 1 OD of cells from each was used for protein extraction.

Protein extraction

Samples were centrifuged at maximum speed for 1 minute and pellets were placed on ice and resuspended in 1mL cold H_2O by vortexing. 150µL of 7.5% β-mercaptoethanol in 1.85M NaOH was added to each sample, followed by vortexing and incubation on ice for 15 minutes. 150µL of 55% trichloroacetic acid was added. Samples were again vortexed and allowed to incubate on ice for at least 10 additional minutes. Samples were pelleted by centrifugation 4°C at maximum speed for 10 minutes. 90µL modified LDS sample buffer (2.5mL 4X LDS sample buffer (Invitrogen), 4.8g urea, sterile H_2O to 9mL) was added to each pellet and pellets were resuspended by vortexing, followed by incubation at 37°C for 15 minutes. Samples were pelleted by centrifugation at room temperature at maximum speed for 5 minutes, then the supernatant was removed and stored at -20°C until use.

Half-life determination

500µL of 40% dextrose in ddH_2O and 50µL 10% cycloheximide in EtOH were added to 10mL of cell cultures to shut off transcription (inhibit galactose-inducible promoters) and translation, respectively. 1OD of cells was removed at time points 0, 1, 2, 4 and 8 where the numerals indicate the number of hours after the addition of the dextrose and cycloheximide. Cultures were returned to the shaker 30°C at 300rpm between samplings. 1 OD samples were processed immediately using the protein extraction procedure listed above up to the addition of the 55% trichloroacetic acid. The samples remained on ice at this stage until all time points had been collected. The remaining steps were performed immediately following collection of the last sample.

Proteasome Inhibitor

10mL S-Gal cultures were grown overnight in 50mL falcon tubes. In the morning, 5mL of each culture was transferred to a new 50mL falcon tube and 5uL (1mg/210μL) MG132 proteasome inhibitor was added to the new tube. Cultures were returned to the incubator shaker for an additional three hours. OD was measured and protein extracted according to protein extraction procedures.

Immunoprecipitation

NP-40 buffer was used for the lysis buffer. 250mL of buffer was made by dissolving 2.2g NaCl into 12.5mL Tris-HCl pH 8.0. 2.5mL Tergitol (NP-40) and sterile H_20 to 250mL (final volume) were added, and the buffer was stored at 4°C. One complete mini EDTA free protease inhibitor (Roche) per 10mL lysis buffer was added before use. In a 15mL falcon tube, 20 OD of yeast cells were suspended in 500μL cold NP-40 lysis buffer with protease inhibitor and placed on ice. Glass beads (Sigma, 1.25g) were added and tubes were vortexed for a period of 30 seconds, to break open the cells, and then returned to ice. Vortexing was repeated until the majority of cells were broken when examined by microscopy. Cells debris was removed by centrifugation and the supernatant was transferred to a 1.5mL centrifuge tube. 40μL Sepharose G (Zymed) was added to pre-clear the extracts and tubes were rocked at 4°C for 1 hour. The samples were centrifuged at 4°C at maximum speed for 10 minutes and the supernatants were transferred to new tubes. 4μL anti-myc 9E10 antibody (Covance) was added to each sample and tubes were rocked at 4°C for 1 hour. 1μL goat anti-mouse antibody (Zymed) was added and tubes were rocked for an additional 30 minutes. 80μL Sepharose G was added and tubes were rocked for 1 hour and 30 minutes. Tubes were spun at 4°C at maximum speed in a microfuge for 10 minutes. Supernatant was discarded and pellets were washed twice with 400μL 50% lysis

buffer. After the final wash, pellets were resuspended in 60μL modified LDS loading buffer and immunoprecipitation complexes were eluted by boiling for 10 minutes. The samples were centrifuged at room temperature for 5 minutes to pellet the sepharose. 20μL aliquots of the supernatant were removed and stored at -20°C for later use.

SDS-PAGE and Western blotting

20μL of protein extract with 2.5μL reducing agent (Invitrogen) was heated at 70°C for 10 minutes. 15μL aliquots of protein extract were loaded on NuPAGE 10% Bis-Tris gels along with 8μL of MagicMark XP protein standard (Invitrogen) and 15μL aliquot of negative control (pYES 2.1 empty plasmid). The inner chamber of a Novex Mini-Cell apparatus was filled with 200 mL NuPAGE MOPS 1X run buffer (Invitrogen) and 500μL anti-oxidant and the outer chamber contained only the 1X run buffer. Gels were run at 100V for approximately 2.5 hours.

Gels were transferred to polyvinylidene fluoride (PVDF) membranes overnight at 20V in a Trans Blot Cell (Biorad) using NuPAGE transfer buffer. Membranes were blocked in 2.5g powdered milk mixed into 40 mL 1X Tris-Buffered Saline with 1% Tween-20 (TBST) for 1 hour while rocking. Membranes were rinsed in TBST then incubated with either anti-myc 9E10 (1:1000), anti-ubiquitin (1:1000) or anti-myc 9E10 (1:1000) and anti-dolichol mannose phosphate synthase (DPMS) (1:100) anti-mouse primary antibodies for 1 hour. Membranes were washed using fresh TBST each time, for two 15 minute washes followed by three 5 minute washes. Membranes were incubated with goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (1:10,000) for 1 hour followed by another cycle of TBST washes as described above.

Blots were developed using ECL plus (Amersham Biosciences) and imaged with an LAS-3000 Imager (Fuji). Data quantification was completed using Multi-gauge computer software (Fuji).

Results

Lysine residues and protein stability

Bryan (2004) observed differences in BF3 and TF3 steady-state accumulation and half-life when expressed in yeast grown at 30°C, and attributed this effect to post-translational regulation. Likewise, (Matsuda et al., 2005) determined that the response of FAD8 enzymatic activity to temperature changes, within the physiological range, depends mainly on post-translational mechanisms. However, Matsuda found it impractical to identify single structural motifs or amino acid residues responsible for FAD8 temperature sensitivity. Consistent with the FAD8 results, studies comparing two isoforms of soybean microsomal omega-6-desaturase FAD2-1 proteins, FAD2-1A and FAD2-1B, showed very different patterns of *in vivo* decay when expressed in yeast cells grown at 30°C, while there was little difference in the decay patterns at 20°C growth temperature (Tang et al., 2005). Tang and coworkers concluded that the N-terminal region of FAD2-1A may be viewed as a temperature dependent destabilization domain. Tang's results with FAD2-1A and FAD2-1B parallel almost identically Bryan's (2004) findings for BF3 and TF3: BF3 and TF3 also show very different decay patterns when expressed in yeast cells grown at 30°C and little difference when cultures are grown at 20°C. Since BF3 has the same temperature-dependent characteristics as FAD2-1A, we suggest that the N-terminal region of BF3 can also be viewed as a temperature dependent destabilization domain.

Bryan (2004) constructed a mutant with the lysine to asparagine (K→N) point mutation in the N-terminal of the BF3 protein (BF3 K_N(1)). Analysis of numerous experimental trials indicated that the BF3 K_N(1) mutant had significantly increased steady-state accumulation and half-life compared to BF3 when yeast cells were cultured at 30°C (Figure 1-6). These results suggest that this largely conserved lysine plays an important role in protein instability and

support the hypothesis that ubiquitination is involved in FAD3 protein regulation. Our expectation was that the converse experiment, where the corresponding asparagine in TF3 is changed to lysine (TF3 N→K) would result in a less stable protein, and that we would see a reduction in the amount and half-life of this protein when compared to TF3.

Primers were designed to introduce this change, and site-directed mutagenesis was performed to change the targeted asparagine in TF3 to a lysine (TF3 N_K, Figure 3-2 panel A). Bacterial transformation, liquid cultures and minipreps were completed and sequencing confirmed that the intended mutation was successfully created. Yeast cells were transformed with the mutant plasmid and protein was extracted and analyzed by SDS-PAGE and western blots, using the anti-myc (α-myc) antibody to detect the in-frame epitope tag added at the N terminus of the FAD protein. All subsequent mutagenesis and other experimental trials were conducted in precisely the same manner unless otherwise noted.

In line with our expectations, western blotting results clearly indicate a downward trend in the steady-state protein accumulation of TF3 N_K as compared to TF3 (Figure 3-2 panel C). Ultimately though, the TF3 N→K mutation decreased protein accumulation only slightly. The same result was observed in five trials with little experimental variability and only a modest standard deviation. Experiments with the BF3 K_N(1) and TF3 N_K mutants confirm that this lysine residue, which is conserved amongst most FAD3s, plays an important role in conferring instability to proteins, but the TF3 N_K mutant did not produce the dramatic results that would be expected if this lysine was the only preferential ubiquitination site. I performed a multiple sequence alignment on the N-termini of twenty-four plant FADs. Of these, twenty-one contained a region corresponding to the BF3 conserved lysine. Figure 3-1 shows the results of the sequence alignment.

```
Tobacco2 (TC4005)              MGSLGISEIYDKNSFNEMEFEFDPSAPPPFRLAEIRNVIP
Wheat (TC269043)              MAPAMRPEQEASCKATEDHRSEFDAAKPPPFRIGDVRAAVP
Soybean2 (TC208804)          MVQAQPLQHVGNGA---GKEDQAYFDPSAPPPFKIANIRAAIP
BF3                           MVVAMDQRSNVNGDSGA---RKEEGFDPSAQPPFKIGDIRAAIP
Arab2 (TC285883)              MVVAMDQRTNVNGDPGAGDRKKEERFDPSAQPPFKIGDIRAAIP
Medicago (TC107097)          MAVKEPQTLQHVGNGDVVDAKKHQQNFDPSAPPPFKIAEIRAAIP
TF3                          MKQQQYKDTPILNGVNGFHAKEEEEEEDFDLSNPPPFNIGQIRAAIP
Cotton2 (TC74252)           MSPSMKETSLETMEKKGE--DVNGIMKKEQDKLFDPSEAPPFRINDIRAAIP
Poplar1 (TC47736)         MKESCLERSNKKDASNDT--VDGIDG--------EGDFDPSAPPPFKIAEIRAAIP
Sorghum (TC103702)        *GRVAVPATSTATATQGQE----------APA-----FDPAAAPPFGLADIRAAIP
Rice (TC337814)           *LRVAAPTSRVATVEEDDNEN-------NAPPPPCEDFDPGAAPPFGLADIRAAIP
Barley (TC140239)         *LRVSAPTRLASVLEEDKR----GLGGAEEEAGSSSAGFNPGAPPPFGLAEIRAAIP
Sunflower (TC20032)       *VKVSAPLRIPSIDQQDLDLDLERERISSLDVQEEEIFDAGAPPPFKLADIRAAIP
Arab1 (TC286492)          *LNVSTPLTTPIFEESPLEE---------D--NKQRFDPGAPPPFNLADIRAAIP
Lettuce (TC8768)          *LKVSAPVDVQFVEEDEKI--------NVGE--SE--FNPGAPPPFTLADIRAAIP
Cotton1 (TC60826)         *LNVSAPLKVTITSEEDKGKEESTTDGVNNG--EF---DPGAAPPFKLSDIKAAIP
Potato (TC143233)         *LRVSAPLRIQIAGEEEEQT-----TNN-GD--EF--FDPGASPPFKLSDIKAAIP
Tomato (TC169929)         *LRVSAPLRIQIAGEEEEQT-----TNN-GD--EF--FDPGAPPPFKLSDIKAAIP
Tobacco1 (TC3987)         *LRVSAPLRIQTVGEEEESER-----TNNSGG--EF--FDPGAPPPFKLSDIKAAIP
Poplar2 (TC67587)         *LNVAVPVSVPVCEEEDKERESVNGVNEQEG--GF--FDPGAPPPFKLSDIRAAIP
Aquilegia (TC14209)       *VKVSAPLKIASLDGEEESQKFNNGVVRVEE--EENEFDPGAPPPFGLADIRAAIP
```

Figure 3-1. N-terminal sequence alignment of 21 plant FADs. Highlighted residue is equivalent to conserved lysine in BF3. Note that only 11 out of the 21 proteins contain a lysine at this location. Entries starting with * indicate leading amino acids residues have been omitted to permit data to fit in available space.

The alignment showed that 11 of the 21 proteins contained a lysine in the location corresponding to the one found in BF3. If this trend continues throughout the plant kingdom, perhaps the lysine is not as well conserved as initially thought, and could help explain why results with the TF3 N_K mutant were not as dramatic as expected. On the other hand, the BF3 N-terminal contains one additional lysine residue and the TF3 N-terminal contains three more. Perhaps one of these others is also involved in ubiquitination and subsequent degradation.

Ju and Xie (2006) identified a preferential ubiquitination site in a protein called Rpn4. They determined that this preferential lysine was located next to an acidic domain, and that this motif constituted a portable degradation signal. In this case, the lysine (position 187) was not immediately adjacent to the acidic domain (position 211), but the general observation of a lysine residue proximal to an acidic region raised the issue as to whether the lysine in TF3 located immediately before the six glutamic acid residue run, might be the preferential lysine in TF3, and

thus plays the major role in the ubiquitination and degradation of the TF3 protein. BF3 does not have a polyglutamic stretch, but BF3 does have a lysine in a similar position to the one found in TF3, just upstream of a shorter, two residue acidic region. Note also that many FAD3s contain a region in the N-terminus that is enriched in acidic amino acids.

To investigate the potential role of this lysine in the BF3 and TF3 proteins, site-directed mutagenesis was performed to change this lysine to an asparagine in both proteins (BF3 K_N(2) and TF3 K_N(2)). Although the experimental replicates with the BF3 K_N(2) and TF3 K_N(2) mutants exhibited fairly high standard deviations (Figure 3-2 panel B), a general trend emerged indicating that when these lysines were replaced with asparagines, the stability of both proteins increased, suggesting that these lysines too play a role in determining FAD3 protein stability.

Therefore, lysine residues appear to play an important role in conferring instability to the BF3 and TF3 proteins, and this characteristic is likely conserved in other FAD3 proteins. From the BF3 K_N(1), BF3 K_N(2), TF3 K_N(2) and TF3 N_K mutants, we suggest that the largely conserved lysine in BF3, and lysine residues in general, play a role in the observed stability difference between the TF3 and BF3 proteins. TF3 has two additional lysine residues in its N-terminus, but there are no others in BF3's N-terminus, so it is apparent that the differential stability is not due solely to lysine residues; none of the lysine mutations in the BF3 N-terminus stabilized the protein to an extent that the steady-state amount of protein was statistically similar to TF3 run on the same gel. We surmised that other features in the N-terminal regions of these proteins must be involved in their differential regulation and decided to look to at the polyglutamic region found in TF3 in an attempt to identify additional potential regulatory motifs.

(A)

```
BF3                 MVVAMDQRSNVNGDSGARKEEGFDPSAQPPFKIGDIRA
BF3 K_N(1)          MVVAMDQRSNVNGDSGARKEEGFDPSAQPPFNIGDIRA
BF3 K_N(2)          MVVAMDQRSNVNGDSGARNEEGFDPSAQPPFKIGDIRA


TF3          MKQQQYKDTPILNGVNGFHAKEEEEEEDFDLSNPPPFNIGQIRA
TF3 N_K      MKQQQYKDTPILNGVNGFHAKEEEEEEDFDLSNPPPFKIGQIRA
TF3 K_N(2)   MKQQQYKDTPILNGVNGFHANEEEEEEDFDLSNPPPFNIGQIRA
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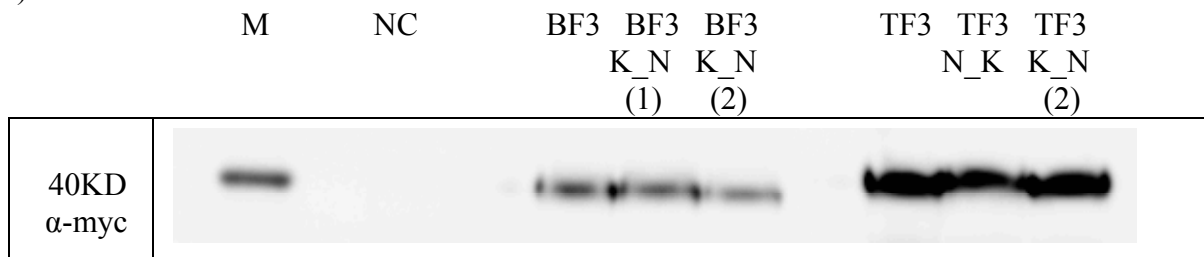
(B)
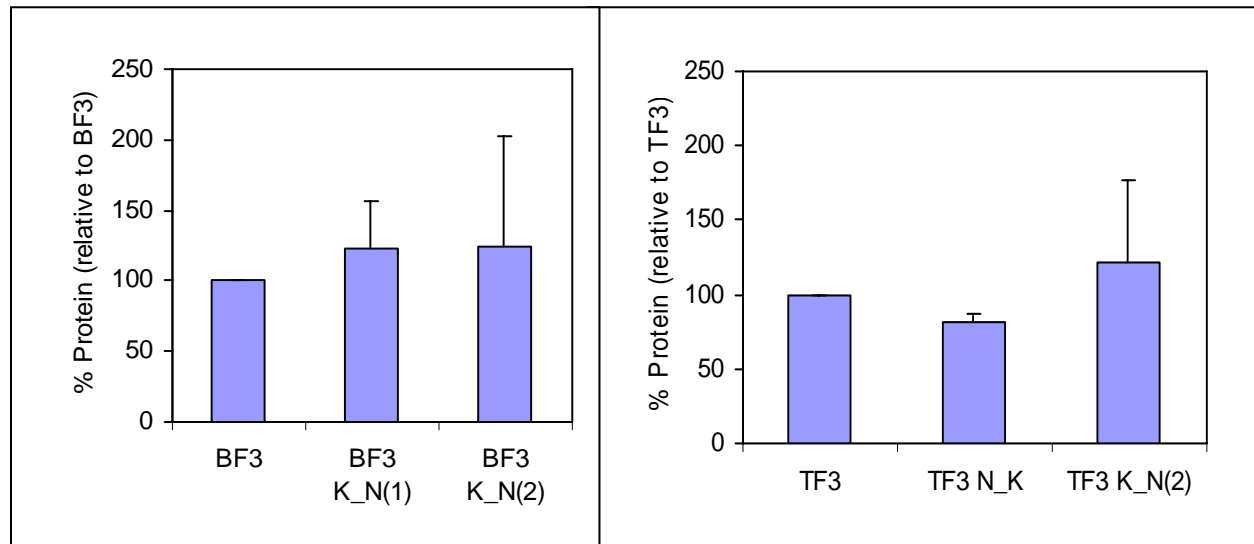
(C)

Figure 3-2. Lysine mutants. Panel A shows N-terminal amino acid sequences of BF3 and TF3 proteins with lysine mutations highlighted. Panel B is a graphical display of experimental results from at least three independent experiments. Although standard deviations are high, the trend shows that when lysine is replaced with asparagine, (BF3 K_N(1), BF3 K_N(2), TF3 K_N(2)), protein stability increased. Likewise when asparagine is replaced with lysine (TF3 N_K), protein stability decreased. Panel C is a representative western blot showing steady-state accumulation of proteins expressed from mutated genes as compared to native. M, protein standard marker and NC, negative control yeast lysate containing no myc-FAD3 proteins.

Acidic residues and protein stability

Ju and Xie (2006) made an argument for a portable degradation signal next to an acidic region in Rpn4. Since TF3 had a lysine followed by an unusually long run of six glutamic acids, the possibility existed that some of the same factors were pertinent to its regulation. The polyglutamic region found in TF3 and highlighted in Figure 3-3 panel A, is the major contributor to both the length and charge differences between BF3 and TF3. This extended negatively charged region could have many effects, including acting as an electrostatic shield around the proximal lysine and potentially pushing other binding proteins away. Moreover, the glutamic acid stretch could also influence protein folding in the local region near the lysine residues.

Site-directed mutagenesis was performed to increase the number of glutamic acids in BF3 from two to six (BF3 2_6E), and decrease the number of glutamic acids in TF3 from six to three (TF3 6_3E) and six to two (TF3 6_2E). Figure 3-3 panel A shows the N-terminal amino acid sequence of BF3, TF3 and the BF3 2_6E, TF3 6_3E and TF3 6_2E mutants. A representative western blot analysis of the various polyglutamic mutants compared to wild type protein when expressed in yeast cells is shown in Figure 3-3 panel C. The results clearly demonstrate that expansion of the polyglutamic acid track in BF3 leads to higher steady-state accumulation of this protein when compared to unaltered BF3. In these experiments, the α-myc antibodies detected a protein approximately 40KD in size in extracts from yeast cells containing wild type BF3 (lanes labeled BF3 #1, #2, #3). The same antibodies detected a slower migrating band in cells carrying the mutant 6 glutamic acid form of this protein (BF3 2_6E #1, #2, #3), consistent with the expected size and charge alterations of the mutated protein. Inspection and quantitation of these bands versus loading controls (α-DPMS, lower panel) demonstrate that the mutant BF3 2_6E protein is higher in abundance compared to the wild type BF3 form. Conversely, the shortened

polyglutamic acid track in the mutant TF3 protein (TF3 6_2E #1, #2) led to lower steady-state abundance of this protein relative to the wild type form (TF3 #1, #2) along with an altered electrophoretic mobility.

As seen in Figure 3-3 panel D, TF3 often runs as a doublet (two bands) on the gel, which might be due to some post-translational modification of some of the protein, but also could be an artifact of the electrophoresis system, since the observation is random and not easily repeatable. This second band does not appear in TF3 6_2E mutant suggesting that the polyglutamic region is an important factor as to whether this post-translational modification occurs. Taken together, these results indicate that the polyglutamic acid track found in TF3 is an integral component in its stability when expressed in yeast, with an increased number of acidic residues corresponding to increased protein stability. Numerous attempts were also made to insert four glutamic acid residues into the BF3 K_N(1) and BF3 K_N(2) and to delete four glutamic acids from the TF3 N_K and TF3 K_N(2) mutant backgrounds. These mutants were unsuccessful however, so combinations of both lysine and glutamic acid mutations could not be fully explored.
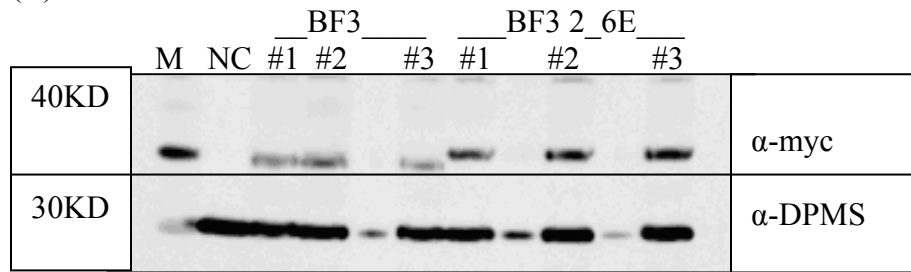
(A)

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BF3           MVVAMDQRSNVNGDSGARKEEGFDPSAQPPFKIGDIRA
BF3 2_6E      MVVAMDQRSNVNGDSGARKEEEEEEGFDPSAQPPFKIGDIRA

TF3           MKQQQYKDTPILNGVNGFHAKEEEEEEDFDLSNPPPFNIGQIRA
TF3 6_3E      MKQQQYKDTPILNGVNGFHAKEEEDFDLSNPPPFNIGQIRA
TF3 6_2E      MKQQQYKDTPILNGVNGFHAKEEDFDLSNPPPFNIGQIRA
```
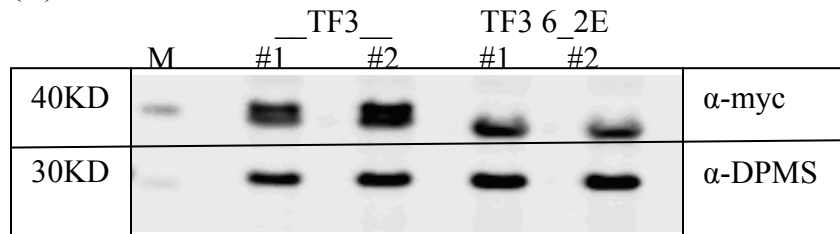
(B)
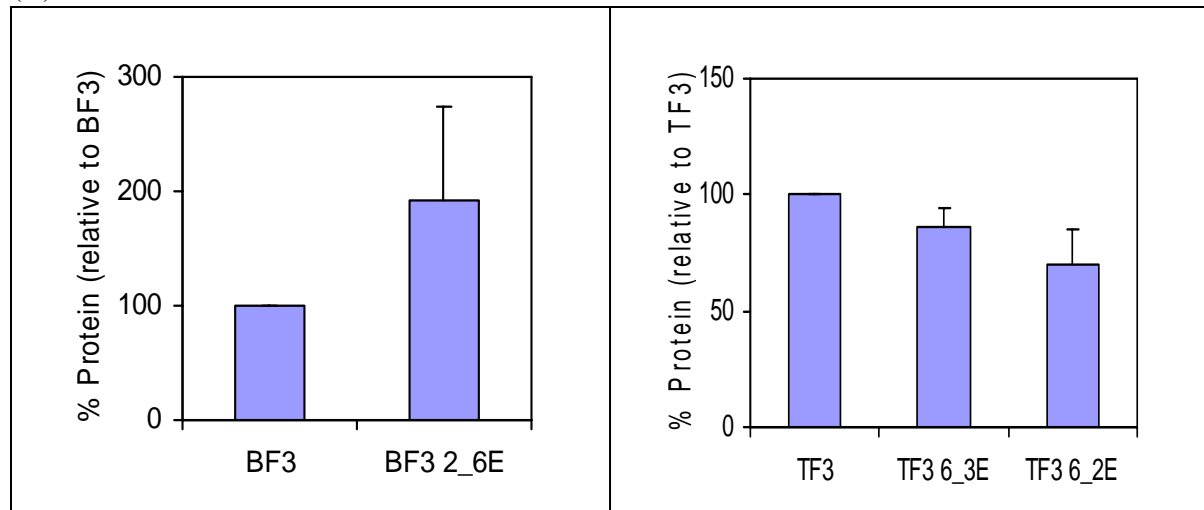
(C)

(D)

Figure 3-3. Glutamic acid mutants. Panel A shows N-terminal amino acid sequences of BF3 and TF3 proteins with glutamic acid mutations highlighted. Panel B is a graphical display of experimental results showing average and standard deviation of at least three independent experiments. Although standard deviation was high with the BF3 2_6E mutant, protein accumulation increased significantly with the addition of glutamic acids (BF3 2_6E) and a stepwise reduction is evident with the decrease in glutamic acids (TF3 6_3E and TF3 6_2E). Panel C and D are representative western blots showing proteins expressed from mutated genes as compared to native ones (#1, #2, etc., are independent replicates of the experiment). Panel C, the addition of four glutamic acids to BF3 (BF3 2_6E) showed increased protein accumulation and decreased electrophoretic mobility compared to the native protein. Panel D, decreasing the number of glutamic acids in TF3 (TF3 6_3E and TF3 6_2E), decreased its protein accumulation and increased its migration slightly.

Ubiquitination and protein degradation

Tang's work with two isoforms of soybean oleate desaturase, FAD2-1A and FAD2-1B, suggested that the 26S proteasome is involved in the temperature-dependent stability of the desaturase enzyme expressed in yeast cells (Tang et al., 2005). From Tang and other previous studies, and supported by our mutagenesis work with the lysines and polyglutamic region in the BF3 and TF3 proteins, we suggest that ubiquitin-mediated proteolysis could hold the key to their differential regulation. To further investigate this hypothesis, we used three different approaches: 1. Incubation of yeast cells expressing FAD3s in the presence of a proteasome inhibitor to determine effects on protein steady-state amount; 2. Immunoprecipitation of FAD3 proteins to determine if they are ubiquitinated; and 3. Expression of FAD3s in a variety of yeast mutants defective in the proteasomal pathway to determine effects on steady-state amount.

Proteasome inhibitor

Tang et al. (2005) showed that at 30°C, incubation of yeast cultures with 10μM MG132, a proteasome inhibitor, for 3 hours resulted in significantly enhanced accumulation of both FAD2-1A and FAD2-1B proteins, as compared to the amount of FAD2s in untreated cells. At the 20°C growth temperature, the presence of MG132 resulted in a modest increase in FAD2-1A, but no apparent change in the amount of FAD2-1B enzyme. These results suggest that the 26S proteasome pathway is likely involved in FAD2-1 protein degradation in yeast cells, and that the proteins are more susceptible to this mode of degradation at the elevated growth temperature. Since BF3 and TF3 appear to be similar to FAD2-1A and FAD2-1B in experiments conducted to date, a similar trial was done using MG132 with the BF3 and TF3 proteins. In this case however, addition of 10μM MG132 for 3 hours had no effect on protein expression. Figure 3-4

shows a representative western blot of protein expression from cultures with (+) or without (-)

the addition of the MG132 proteasome inhibitor. Protein amounts were identical for each FAD3

regardless of whether the inhibitor was added or not, suggesting that either FAD3 degradation is

not mediated by the proteasome, or experimental procedures were faulty. No positive controls

were included to demonstrate whether the proteasome inhibitor was able to permeate the yeast

cell membranes. Another report suggests that only certain yeast mutant strains are able to take

up MG132 from the growth media. Notably, the yeast strain employed in these studies was

different from that of Tang, which might explain the difference in sensitivity of FAD stability to
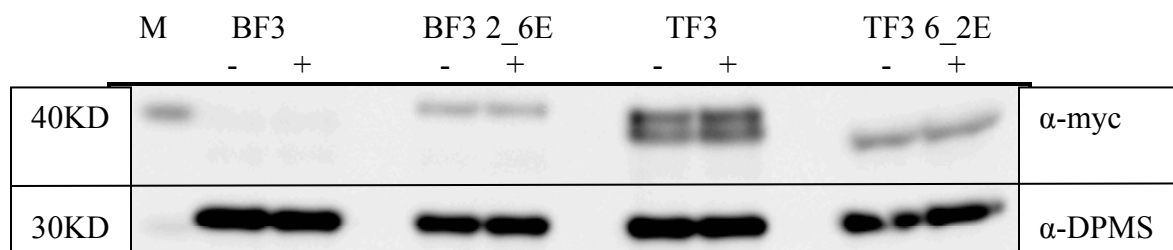
this inhibitor.

Figure 3-4. Proteasome inhibitor effects. MG132 proteasome inhibitor added to BF3, BF3 2_6E, TF3 and TF3 6_2E. Cultures without the inhibitor (-) versus cultures to which inhibitor was added (+). Blots were probed with α-myc for FAD3 detection and α-DPMS as a loading control. Results demonstrate that the proteasome inhibitor had no effect on protein accumulation in these experiments.

Immunoprecipitation

If differences in protein accumulation and half-life between BF3 and TF3 were due

primarily to protein stability and ubiquitin-mediated proteolysis, there could potentially be

differences in ubiquitination status of the two proteins. To determine if we could detect any

ubiquitination of BF3 and TF3, we conducted immunoprecipitation experiments, followed by

western blot analysis with α-ubiquitin antibodies. Yeast cells were lysed and

immunoprecipitated with the α-myc antibodies. Figure 3-5 shows a representative

immunoprecipitation western blot probed with the α-ubiquitin antibodies. In this experiment, the

α-ubiquitin antibodies did not detect any epitope tagged BF3 protein at approximately 40KD

(BF3 #1, #2, #3, #4). The same antibodies did detect a protein band at this size for TF3 (TF3 #1, #2). The results may indicate one of two things. First, the possibility exists that BF3 is not ubiquitinated, while TF3 is. Second, perhaps the BF3 protein is degraded so quickly that not enough protein is immunoprecipitated for the α-ubiquitin antibodies to detect. The band in the negative control lane (NC#2) at approximately 40KD is likely cross-reacting from saturated positive control (PC) lane. The blot is representative of numerous trials. A band of approximately 40 KD was always seen in TF3 samples, but usually absent or observed only occasionally (or very faintly) for BF3.

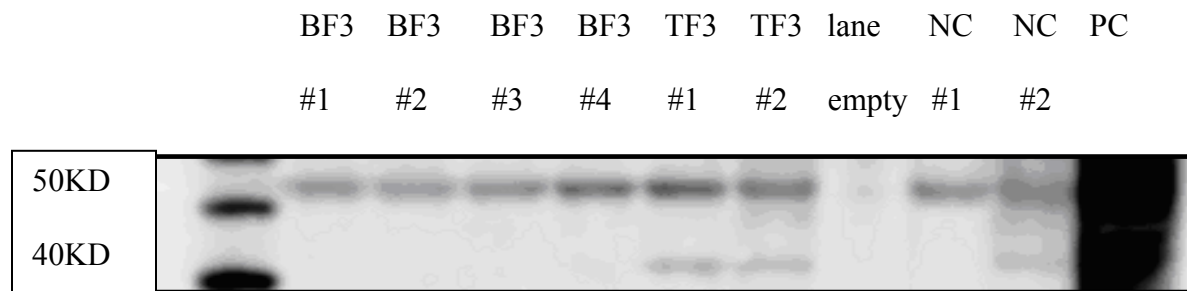| BF3 | BF3 | BF3 | BF3 | TF3 | TF3 | lane | NC | NC | PC |
| #1 | #2 | #3 | #4 | #1 | #2 | empty | #1 | #2 | |

Figure 3-5. Immunoprecipitation and ubiquitination detection. Representative western blot showing anti-ubiquitin detection of samples immunoprecipitated with anti-myc antibody. A whole-cell lysate was used as a positive control (PC) for ubiquitinated protein detection. Band of interest at approximately 40KD and interpreted relative to negative control (NC). Bands evident in NC#2 believed to come from PC inter-lane bleed-through due to overload of PC sample. Note no 40K bands are visible in the four BF3 samples and a 40K band is clearly evident in two TF3 samples. Bands at approximately 50KD are anti-myc heavy chain immunoglobulins.

FAD3 expression in yeast mutants

To further investigate our belief that the differences in FAD3 protein stability are related to ubiquitin-mediated proteolysis, we expressed the BF3 and TF3 proteins in a number of yeast mutants defective in proteolysis-related genes. If ubiquitin-mediated proteolysis is indeed involved in FAD3 turnover, the expectation would be that the BF3 and TF3 proteins would be stabilized at 30°C in a yeast system where proteolysis was defective. Comparisons were done with the BF3 and TF3 proteins expressed in BY4742 yeast mutants ΔCUE1, ΔDOA1, ΔNPL4, ΔUBR1 and ΔUFD2 cultured at 30°C. As mentioned before, DOA1, UBR1, and UFD2 genes were downregulated at 20°C, as determined by microarray analysis, and CUE1 and NPL4 were

previously shown to be involved in rapid degradation of the yeast SCD. Notably, disruption of CUE1 and NPL4 resulted in stabilization of the yeast SCD protein at 30°C. A representative western blot of BF3 and TF3 expressed in these various yeast mutants is shown in Figure 3-6. Panel A shows the steady-state amount of BF3 in relation to wild type yeast cells grown at 30°C. Inspection and quantification of these bands versus a DPMS loading control demonstrate that BF3 is destabilized in ΔCUE1 and ΔNPL4 and stabilized in ΔDOA1, ΔUBR1 and ΔUFD2. Notably, there were no effects on the steady-state amount of DPMS protein. Panel B shows TF3 protein accumulation in wild type yeast as compared to that expressed in the ΔCUE1, ΔDOA1, ΔNPL4, ΔUBR1 and ΔUFD2 at the same 30°C growth temperature. Like BF3, TF3 also appears to be destabilized in ΔCUE1 and ΔNPL4. In ΔUBR1 and ΔUFD2, TF3 protein accumulation seems to decrease slightly where BF3 accumulation appeared to increase in these same mutants. The most obvious effect, however, was with the ΔDOA1 strain, where TF3, like BF3, was significantly stabilized. These data suggest that the DOA1 gene plays an important role in destabilizing the FAD3 proteins at 30°C.

While the observed increases in FAD3 protein stability in ΔDOA1, ΔUBR1 and ΔUFD2 were somewhat expected, the reduction (or near absence) of FAD3 protein in the ΔCUE1 and ΔNPL4 strains was surprising, since these same mutants led to stabilization of yeast SCD. These results suggest that plant and yeast FADs might be regulated by different proteins of the ubiquitin-proteasomal pathway (described in greater detail in the Discussion section). Notably, however, the experiment with ΔCUE1 and ΔNPL4 mutant yeast strains was performed just once and should be repeated before any specific conclusions can be made.
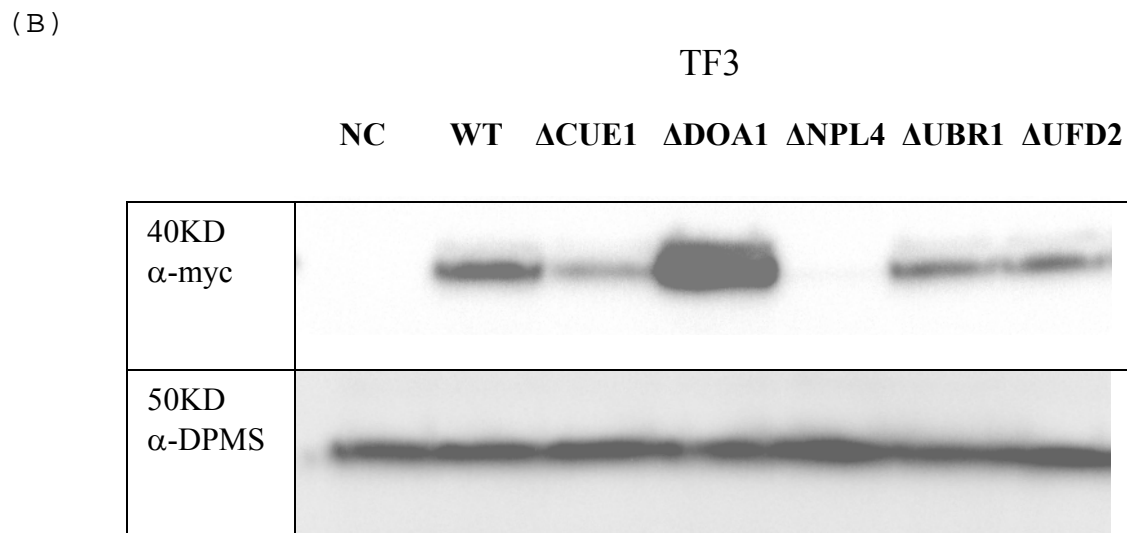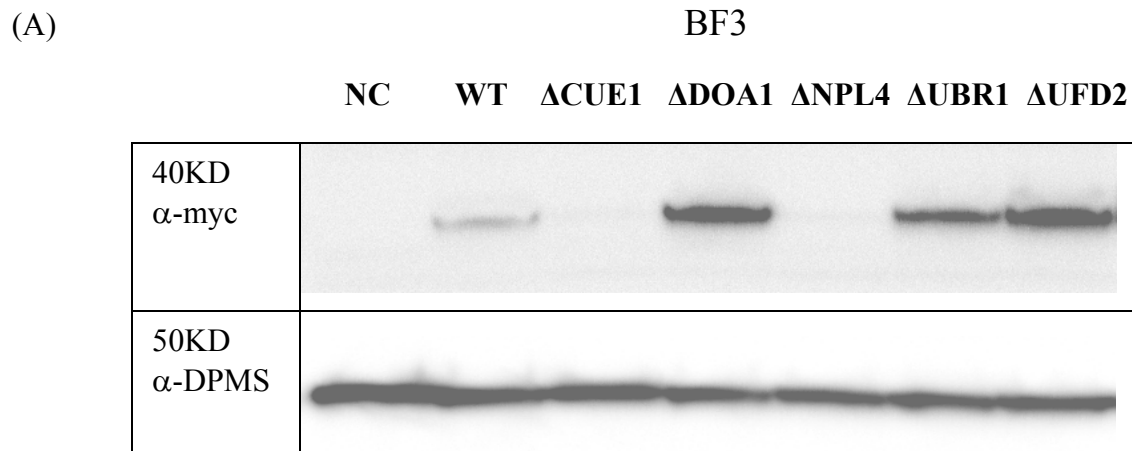
(A)

BF3

Figure 3-6. FAD3 expression in yeast mutants cultured at 30°C. FAD3 protein extracted from BY4742 WT yeast compared to mutant backgrounds ΔCUE1, ΔDOA1, ΔNPL4, ΔUBR1 and ΔUFD2. Representative western blots of steady-state accumulation probed with anti-myc antibody, including anti-DPMS blots as controls. (A) BF3 steady-state accumulation showing reduction of protein in ΔCUE1 and ΔNPL4, and increase in protein in ΔDOA1, ΔUBR1 and ΔUFD2. (B) TF3 steady-state accumulation shows similar decrease in ΔCUE1 and ΔNPL4 and increase in ΔDOA1. Unlike BF3, TF3 protein accumulation appeared to decrease slightly or remain the same in the ΔUBR1 and ΔUFD2 mutant backgrounds.

Since our expectation was that the FAD3 proteins would be stabilized in a yeast system where relevant proteolysis pathways were defective, we conducted additional experiments in the yeast backgrounds which had been shown to stabilize BF3. Both steady-state amount and half-life of BF3 expressed in wild type, ΔDOA1, ΔUBR1 and ΔUFD2 yeast mutants were determined

when yeast cells were grown at both 30°C and 20°C (Figure 3-7). Panel A shows the steady-state amount of protein relative to BF3 expressed in wild type yeast grown at 30°C. Inspection and quantification of these bands versus DPMS (lower panel) demonstrate that BF3 protein expressed in ΔUBR1 and ΔUFD2 show similar changes in steady-state accumulation when grown at 30°C and 20°C as BF3 protein expressed in wild type yeast (i.e., more protein accumulates at 20°C compared to 30°C, suggesting that temperature-dependent regulation is still largely operational in these mutant yeast backgrounds). However, BF3 expressed in a ΔDOA1 mutant background did not show any significant difference in steady-stage amount when cells were grown at 30°C and 20°C, indicating that the loss of DOA1 activity somehow stabilized the protein at 30°C. Panel B confirms that there was no significant difference in protein half-lives of BF3 expressed in the ΔDOA1 deficient yeast at the two growth temperatures. Panel C shows a graphical representation of BF3 half-life determined from multiple experiments, comparing protein extracted from wild type and ΔDOA1 yeast cultured at both 30°C and 20°C. In contrast to the BY4742 wild type yeast where BF3 accumulation and half-life are significantly increased when the yeast is grown at 20°C, in the ΔDOA1 yeast mutant, there is no significant difference in BF3 steady-state accumulation or half-life between 30°C and 20°C growth temperatures.
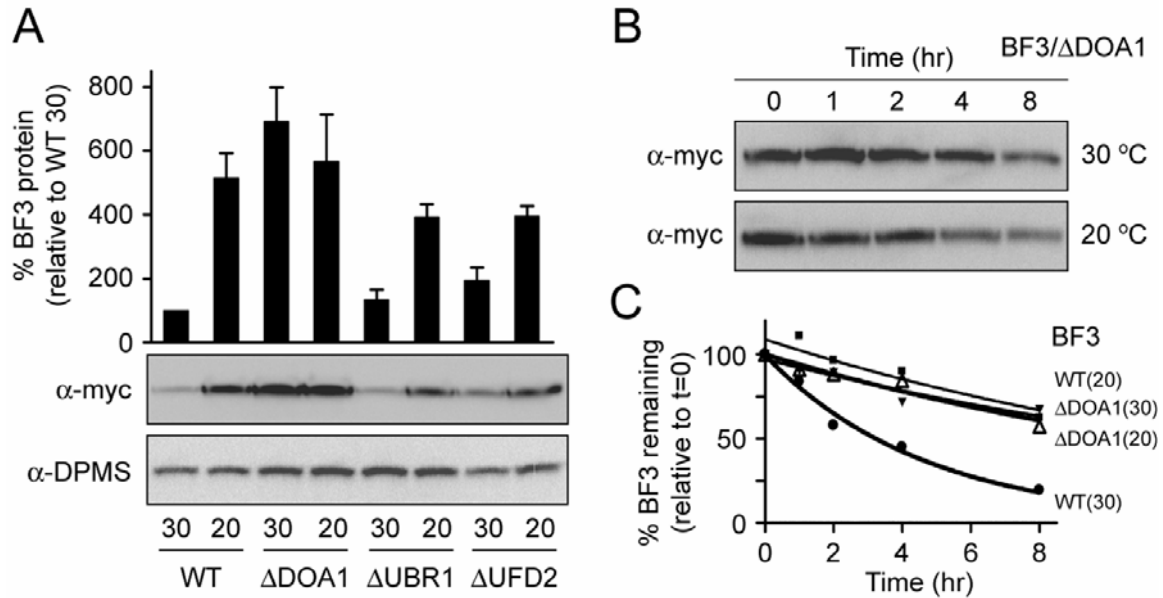
Figure 3-7. FAD3 expression in yeast mutants cultured at 30°C and 20°C. Comparison of BF3 protein extracted from either wild type yeast or ΔDOA1, ΔUBR1 and ΔUFD2 mutant yeast cells cultivated at 30°C and 20°C growth temperatures. (A) Western blot of steady-state accumulation probed with anti-myc antibody. Anti-DPMS blot provided loading control. Bar graph indicates quantified data with error bars indicating standard deviation. Data are expressed relative to BF3 WT grown at 30°C. (B) Western blot of BF3 half-life when expressed in BY4742 ΔDOA1 mutant background at 30°C and 20°C growth temperatures and probed with anti-myc antibody. (C) Line graph of BF3 half-life when expressed in BY4742 ΔDOA1 mutant background at 30°C and 20°C growth temperatures and probed with anti-myc antibody.

DOA1 functional complementation

We explored the possibility of complementing ΔDOA1 yeast deficiency with a plant DOA1 homolog. The *Arabidopsis thaliana* DOA1 gene (At3G18860) was amplified, T'tailed and ligated to pYES 2.1 vector with a galactose inducible promoter. Bacterial DH5α cells were transformed and grown up using LB-amp selection. Mini prep DNA was screened for insert and orientation using restriction enzyme digest and three potential candidates were identified. BY4742ΔDOA1 yeast was transformed with the plasmid DNA and plated on SD-URA. One clone showed growth in galatose media comparable to wild type indicating a possible successful functional complementation. These experiments need to be repeated and extended to determine if the *Arabidopsis* DOA1 homolog can indeed functionally complement the ΔDOA1 yeast strain.

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Discussion

Overview

FAD3s including BF3 and TF3 have been shown to vary differentially with respect to temperature stresses. In many of the FADs studied, a key component of this differential regulation has been shown to be restricted to a specific segment of the protein (usually in the N- and/or C-terminal regions). No individual amino acid residues in plant FADs have been explicitly defined as stand alone contributors to the differential regulation. This is true even in soybean FAD2-1 isoforms, where there is only a four amino acid residue difference between FAD2-1A and FAD2-1B (Tang et al., 2005). Ubiquitin-mediated proteolysis has also been suggested, and strongly supported, as a key component in the highly efficient yet rapid FAD protein turnover critical for maintaining proper lipid balances. My work attempted to explore each of these areas, experimenting with a number of N-terminal amino acids and taking a cursory look at ubiquitin-mediated degradation. My primary goal was to identify a single indispensable variable for the known differential stability between the BF3 and TF3 proteins.

Lysine residues and protein stability

It had previously been shown that swapping the N-terminal sequences (approximately first 65 amino acids) of TF3 and BF3 proteins significantly affects steady-state protein amounts (Bryan 2004). Additionally Bryan changed a conserved lysine in BF3 to an asparagine, like that found in the corresponding location in TF3. She found the BF3 K_N mutant had significantly more steady-state protein and a longer half-life than BF3. I explored the effect of mutating the corresponding asparagine in TF3 to lysine (TF3 N_K), with the expectation that the half-life would decrease. When I introduced a lysine back into this conserved region in TF3 (TF3 N_K), protein steady-state accumulation did decrease slightly, but there was significant experimental variation with a high standard deviation.

We hypothesized that other amino acid residues in close proximity to this conserved lysine residue, but which also differed between BF3 and TF3, might be contributing to the regulatory differences. To explore this theory, another mutant, TF3 LNN→PAK, was constructed. In this mutant, the leucine and asparagines residues (located 7 and 5 residues away from the conserved lysine) were changed to the corresponding residues in BF3 (proline and alanine, respectively). As was the case with the TF3 N→K mutant, TF3 LNN→PAK did not show a significant decrease in protein accumulation or half-life beyond what could be considered normal variation(results not shown), although once again a slight downward trend in steady-state amount was seemingly evident.

Ju and Xie's demonstration (2006) of a lysine that serves as a preferential ubiquitination site located proximal to an acidic region initiated a hypothesis that perhaps TF3 followed a similar course. TF3 has a lysine adjacent to a stretch of six acidic residues (glutamic acids) and there was also a lysine in the corresponding location in BF3 (although the stretch of acidic amino acids is shorter at two). Changing this lysine to asparagine in both proteins (BF3 K_N(2) and TF3 K_N(2)) resulted in a slight decrease in protein steady-state amount, suggesting that these lysines could also influence protein stability. These data support the general trend where lysine residues in the N-termini of the FAD3s confer instability to proteins.

Acidic residues and protein stability

Lysine mutations, while apparently affecting stability of the FAD proteins, did not reveal an all conclusive, definitive answer to the differences in BF3 and TF3 regulation, and it appeared that other N-terminal residues might play a larger contributory role. Since the six glutamic acid stretch found in TF3 is an obvious difference between the BF3 and TF3 proteins, it was the next

candidate for mutagenesis. Mutations were created that inserted four additional glutamic acid residues into BF3, and removed four glutamic acid residues from TF3.

Results from the glutamic acidic mutagenesis studies strongly suggest that the acidic-rich domain has a significant affect on protein stability. My experimental trials clearly showed that the expansion of the polyglutamic acid track in BF3 2_6E leads to a higher steady-state accumulation of this protein when compared to unaltered BF3, and conversely, the shortened polyglutamic acid track in TF3 6_2E leads to lower steady-state abundance of this protein relative to native TF3. Conclusive results were seen with the polyglutamic mutations, but an important question remains unanswered. Ultimately, that question is whether the key component in the success of the polyglutamic acid mutations was due to changes in length, charge, or a combination of both. Time constraints prohibited further investigation, but a possible next step might be to mutate the glutamic acids in TF3 to uncharged alanine residues, or to add four additional alanine residues to BF3. The first proposed mutation would change the net charge of TF3 but not its length, whereas the second would change the length of BF3 but not its charge. Together, both sets of mutants could potentially yield information to help answer the central question of length, charge, or a combination effect.

Further research may also provide a better understanding of how the polyglutamic region confers increased stability to the TF3 protein. Protective effects might be related to thermal stability and protein folding, electrostatic shielding, modulating degradation domains, post-translational modifications, or a number of other possibilities. In the area of thermal stability, temperature sensitivity of proteins is often related to thermal instability problems and increased degradation by the proteasome. A simple model for having a temperature-sensitive degradation signal in conjunction with enzyme activity is based on protein folding, where the protein is more

loosely folded at higher temperatures (resulting in reduced enzyme activity and more rapid protein degradation) and more tightly/properly folded at cooler temperatures (which would increase enzyme activity and stabilize protein half-life). In addition, there could be discrete degradation signals in the N-terminus that are exposed at higher temperatures due to improper protein folding, and masking of these degradation signals when the proteins are properly folded at lower temperatures. A third scenario involves protein partners that might bind to FADs to regulate their degradation, and the ability of the proteins to bind together is modulated by changes in ambient temperature. Since membrane proteins such as FADs are difficult to purify, *in vitro* protein folding /stability experiments are not really feasible, but I do think it would be interesting and potentially very revealing to compare the thermal stability of the BF3 and TF3 proteins at different temperatures. Although unsubstantiated, I tend to believe that the TF3 polyglutamic acid track with its strong negative charge and its potential interaction with surrounding residues (folding conformation), effectively shields the protein from more rapid degradation. This effect, known as electrostatic shielding or electrostatic repulsion, might occur because the highly negatively charged glutamic region pushes away ubiquitin ligases, which themselves likely have negatively charged regions since these proteins bind to and modify positively charged lysine residues on substrate proteins.

If TF3 is somehow initially protected from degradation, there is likely a degradation impetus. One factor commonly found in short-lived proteins and implicated in their rapid degradation, is the PEST site. PEST sites are hydrophilic stretches of amino acids that contain at least one proline (P), one glutamic (D) or aspartic (E) acid, and one serine (S) or threonine (T). A considerable body of evidence supports the idea that PEST sequences target proteins for degradation by the proteasome. Evidence also points to constitutive or automatic, and signal-

induced, degradation through PEST sequences. Many PEST signals appear to be induced or conditional signals with a number of ways to activate them, and it seems reasonable that PEST regions could be initially masked in the assembled enzyme, and then somehow later exposed initiating degradation. Several examples have been described in the literature where phosphorylation controls the metabolic stability of a protein. Phosphate addition to serines and threonines might prove to be a widespread mechanism for activating a latent PEST signal (Rechsteiner and Rogers, 1996). Notably, soybean FAD2s are apparently phosphorylated in developing soybean seeds (Tang et al., 2005).

The computer algorithm "PESTfind" indicates that TF3 has a potential PEST sequence that is not found in BF3. From our experimental results, where BF3 appears to be degraded much more quickly than TF3, one might expect the converse to be true, that BF3 would have a PEST site and TF3 would not. Interestingly, if you reduce the number of glutamic acids in TF3 from 6 to 4, no potential PEST is indicated. Conversely, adding 1 additional glutamic acid to BF3 results in it acquiring a potential PEST sequence. TF3 is itself a short-lived protein and its PEST sequence could be an important factor in regulating its degradation. BF3, on the other hand, may have a degradation regulation signal that has yet to be identified or characterized. But localization of protein degradation signals in similar regions of protein sequence (i.e., the N-termini) and similar changes in protein half-life in various mutant yeast cells suggests that the proteins are degraded by a similar pathway, and that differences in TF3 and BF3 stability are due to subtle variations in protein sequence that cause the proteins to interact in slightly different ways with these regulatory pathways.

Other peptide motifs besides PEST sequences have been identified that can target proteins for rapid destruction. These short sequence motifs are known as destruction boxes. A

search for potential destruction boxes and other predicted destruction motifs yielded three hits in BF3 and only one in TF3. It is my opinion that native conformation and thermal stability are key components to the protective effect TF3 seems to have, while BF3 seems open and vulnerable to expedient degradation. Little is know about the secondary and tertiary structure of these two proteins, although secondary structure predictors give slightly different results, especially with respect to structure in polyglutamic acid region. BF3 is predicted to contain a helix in that region while TF3 is predicted to contain a coil.

Another set of experiments that could provide additional information on FAD3 regulation would be to combine various mutations that affect FAD3 stability. For instance, mutation of lysines to asparagines was shown to increase stability of both TF3 and BF3, and introducing of additional glutamic acids into BF3 was also shown to increase stability. It would be interesting to combine the lysine and glutamic acid mutations in BF3 to see if stability is raised even further. I attempted to make several of these combined mutations, but after a number of trials, I was unable to create the desired mutants. Site-directed mutagenesis of the magnitude attempted in the polyglutamic region is not without inherent challenges. In this case I was attempting to delete 12 amino acids (4 glutamic acid codons) from TF3 and insert 12 into BF3. With site-directed mutagenesis, large deletion mutations are often more successful than large insertion mutations, mainly due to critically important primer annealing. This was my experience also, as deletion mutations in the TF3 and TF3 N_K and TF3 K_N(2) mutants proved more successful than the BF3 insertion mutations. I was only able to insert the additional four glutamic acids into the native BF3 sequence. Numerous attempts at inserting the mutation into the BF3 K_N(1) and BF3 K_N(2) mutants were unsuccessful, probably due to problems with the primers related to lower GC content and melting temperature issues. Additionally, an attempt to insert the K_N(1)

and K_N(2) mutations into the BF3 2_6E mutants also failed, most likely due to improper primer annealing.

To summarize my analysis of specific amino acids involved in the N-terminal cis-acting degradation signal(s), lysines are clearly important and, in general, the more lysine residues, the greater the instability of the protein. Acidic residues have an opposite effect, where an increase in acidic residues leads to greater stabilization of the proteins.

Ubiquitination and protein degradation

Proteasome Inhibitor studies

Our expectation was that if the proteasome was the primary mechanism by which FAD proteins were degraded, the accumulation of BF3 (in particular) would increase in the presence of a proteasome inhibitor such as MG132. This was not the case however, as protein amounts were identical in cultures with or without MG132. A number of variables could have contributed to the fact that BF3 expression did not change in the presence of the inhibitor. First, BF3 degradation may not be mediated by the proteasome. Second, the proteasome inhibitor might not have been properly mixed and only solvent was added to the cultures. Third, the proteasome inhibitor may not have been able to permeate the cell membrane of BY4742 yeast. Lee and Goldberg (1996) found that proteasome inhibitors were effective only in an ISE1 yeast mutant. The ISE1 strain is allelic to the ERG6 mutant, which is defective in the biosynthesis of the principal membrane sterol in yeast. They could not demonstrate any effects of proteasome inhibitors in wild type yeast, and they were also unsuccessful in demonstrating any effects in yeast spheroplasts prepared from wild type yeast cells.

I suspect that cell membrane permeability of BY4742 yeast may be the key factor in my negative proteasome inhibitor results. No reference could be located for an experiment using the

BY4742 yeast strain with the MG132 proteasome inhibitor. Tang used the yeast strain CTY182 in his MG132 proteasome inhibitor and soybean FAD-2-1 experiments. CTY182 genotype (mat a, ura3-52, his3Δ200, lys2-801, SEC14) is unremarkable and although there is no indication of increased permeability with this strain, perhaps it is a factor. Further experimental trials are necessary with both BY4742 and other known MG132-permeable yeast strains (such as ise1/erg6), to determine if my MG132 experiments failed because of BY4742 cell membrane permeability issues, or because BF3 and TF3 are not degraded by proteasome-mediated proteolysis. As a side note, I did not add solvent (DMSO) only controls to the -MG132 cultures. Although I expect this would have no obvious effects on my results, it should be done in future proteasome inhibitor trials. Another control and standardization benchmark that needs to be incorporated into future proteasome inhibitor trials is a positive control for MG132 activity. An especially good positive control would be an ER membrane protein previously shown to be stabilized by MG132, such as soybean FAD2-1. The use of a positive control would enable us to determine whether the proteasome inhibitor was actually working (something I was unsure of) and provide a reference point for FAD3 comparisons.

Immunoprecipitation

It was surmised that the difference between TF3 and BF3 proteins might be related to degradation processing, specifically ubiquitination and subsequent proteolysis by the proteasome. One potential hypothesis suggests that perhaps TF3 is not ubiquitinated as efficiently as BF3, and so TF3 degradation would occur more slowly than BF3. Another possibility is that TF3 is efficiently ubiquitinated, but not degraded as quickly as BF3 for other reasons. My numerous IP trials seemingly indicate that TF3 is indeed ubiquitinated, while data was inconclusive for BF3. The vast majority of my immunoprecipitation trials showed no

ubiquitinated BF3 protein. At this point I cannot determine whether (1) there was not enough BF3 protein to detect or (2) BF3 is not ubiquitinated. Perhaps refined immunoprecipitation procedures would allow for the detection of ubiquitinated BF3, or perhaps MG132 could be included in these experiments to block degradation, thereby allowing any ubiquitinated proteins to accumulate (which should improve their identification). As it stands, my (unsubstantiated) belief is that BF3 is indeed very efficiently ubiquitinated and rapidly degraded, leaving too little protein to immunoprecipitate and detect in my experimental trials. All I can say with confidence is that, using the immunoprecipitation and western blot protocols I used, no conclusive results were obtained. All my data supports is TF3 appears to be readily ubiquitinated, with no evidence to support the same for BF3. One conclusion that can be potentially drawn from these results is that since it appears that TF3 is effectively ubiquitinated, there is likely some other factor(s) delaying its degradation.

It is also possible that other post-translational modifications may be having a larger impact on plant FAD3 steady-state accumulation and half-life than ubiquitination (although they would also have to be modulated primarily by sequences in the N-termini). Tang demonstrated that in the soybean FAD2-1 proteins, Ser^{185} is a potential phosphorylation site since it is located within a motif that has been shown to be related to targets for phosphorylation by calcium-dependent protein kinases. Ser^{185} is also predicted to reside at the ER:cytosol interface in close proximity to the active site of the enzyme, making it entirely plausible that the introduction of a charged residue at this location would have an impact on enzyme activity.

Proteasome-mediated rapid turnover of proteins is often modulated by PEST sequence phosphorylation, normally at a serine residue and less frequently at a threonine residue. Phosphorylation of a PEST sequence located in a flexible "hinge" region of a short-lived protein

accelerates its degradation. It appears that the structure around the PEST regions has evolved marginal stability that is finely tunable by phosphorylation. If this is true, then conformational stability, rather than recognition of a phosphate modification, modulates the degradation of the PEST sequence by the proteasome machinery. Research supports that it is not the phosphate moiety that is recognized by the proteasome system, but rather a conformational switch that affects the local thermodynamic stability of the protein, which ultimately results in its degradation. Structural studies have confirmed the occurrence of phosphorylation induced conformational changes in peptide hinge regions (Garcia-Alai et al., 2006).

My research didn't include an analysis of post-translational modifications other than ubiquitination, but potential phosphorylation sites should likely be a consideration for future studies, with particular emphasis on conformational switch effects. Also, another potential difference in post-translational modification may be related to N-glycosylation. Post-translational modification prediction programs list a potential N-glycosylation site in TF3 which is not predicted in BF3, and there is some evidence for protein stabilization through glycosylation. Thus, there are a number of post-translational modifications that may be contributing to the differential stability of the BF3 and TF3 proteins.

FAD3 expression in yeast mutants

Since one of the primary regulators of BF3 and TF3 turnover may be ubiquitin-mediated proteolysis, studies with yeast mutants defective in proteolysis related genes could potentially reveal important clues to their differential regulation. The Cdc48/Ufd1/Npl4 protein complex has been discussed previously, with respect to its role in extracting proteins from the ER membrane and acting as a molecular chaperone in bringing targeted substrates to the proteasome. Yeast mutants deficient in Cdc48 and Ufd1 are inviable, since they function more globally

within the yeast cell and only a small portion of the Cdc48 pool is associated with the ER

(Schuberth and Buchberger, 2005). Cdc48 appears to be the central player in the molecular

chaperone process of ER-associated degradation, and is often associated with key cofactor

proteins including Ufd1 (ubiquitin fusion degradation protein 1), and Npl4 (nuclear protein

localization protein). NPL4 and another ubiquitination-related gene, CUE1 (coupling of

ubiquitin conjugation to ER degradation protein), were two proteolysis-related genes that Braun

et al. (2002) examined in the studies of yeast SCD protein. Their work demonstrated that SCD

was significantly stabilized in the NPL4 and CUE1 mutants, whereas in our studies, both BF3

and TF3 appear to be destabilized in these same mutants. We saw almost complete

destabilization in the NPL4 and CUE1 mutant backgrounds. Cue1 is an ER membrane protein

that recruits an ubiquitin conjugating enzyme in its role in the ubiquitin-mediated protein

degradation pathway. CUE1 has been shown to stabilize a number of ER degradation substrates

(Braun et al., 2002). In our case however, FAD3 actually became more destabilized when

expressed in the CUE1 deficient background, raising the possibility that cofactor binding

differences may exist with respect to the Cdc48 molecular chaperone and plant FAD3s. Since

our FAD3 results are in stark contrast with the yeast SCD studies, the possibility exists that plant

FADs do not use exactly the same degradation pathway that yeast SCD does. Experiments with

NPL4 and CUE1 need to be repeated to substantiate results. If these results are substantiated, it

would be interesting to include proteasome inhibitors with these yeast mutants to determine if the

FADs are more rapidly degraded by the proteasome in these mutant backgrounds.

Potentially what might be happening in the Npl4 deficient background, is that the absence

of Npl4 might be availing more opportunities for other proteins to bind to Cdc48. Although the

Cdc48 complex is still being characterized, it has already been shown to bind a variety of

proteins, a number of which might compete for binding, with the final result being the entire Cdc48 complex deciding which proteins are delivered for degradation. Cdc48 has been shown to be necessary for the removal of stable transmembrane domains (Rumpf and Jentsch, 2006), such as those known to exist in the FADs, another piece of evidence supporting FAD3 degradation through ERAD. Research also supports Cdc48 binding an ER receptor protein before binding a polyubiquitinated target substrate with the substrate being recruited to the Cdc48 chaperone via Ufd1 before ubiquitination (Ye et al., 2004).

We also looked at some of the proteolysis-related genes identified in a previous microarray analysis as being down regulated at 20°C when compared to 30°C, namely UBR1, UFD2 and DOA1. The idea is that down regulation of certain protease-related activities at 20°C might explain the increase in FAD protein half-life at cooler temperatures. BF3 appeared to be stabilized slightly in the UBR1 and UFD2 mutants while TF3 was approximately the same or potentially slightly destabilized in both. Ubr1 is an ubiquitin protein ligase required for the retrotranslocation or release of the polyubiquitinated substrate from the membrane bringing it into the cytosol for further transit to the proteasome (Ye et al., 2004). Ufd2, an ubiquitin chain assembly factor protein, places a ubiquitin molecule on the target protein and is known to competes with Ufd3 (Doa1) for Cdc48 binding.

The greatest changes in protein half-life of both BF3 and TF3 were observed in the DOA1 mutant background. Doa1 has been shown to be required for ubiquitin-mediated protein degradation. The Doa1 mutant was originally identified in screens for mutants that stabilize several normally short-lived proteins. Doa1 has been implicated in having a role in DNA damage response (Lis and Romesberg, 2006) and shown to be involved in the rapid recycling of ubiquitin from the proteasomal degradation pathway into transcriptional and damage response

pathways during stress. Reduced levels of free ubiquitin were also noted in Doa1 deficient

mutants, and it was shown that overexpression of ubiquitin can complement the mutant

phenotype. Lis and Romesberg (2006) concluded that DOA1 is active in stress response as a

regulatory component of the proteasomal pathway.

From my perspective, it is feasible that temperature sensitivity may be regulated by a

mechanism comparable to other inducible stresses. Rumpf and Jentsch (2006) believe that

DOA1 is a negative regulator of ubiquitin dependent proteolysis, as Doa1 has been functionally

linked to Otu1, a deubiquitinating enzyme that removes ubiquitin placed on the target protein by

Ufd2. Rumpf and Jentsch (2006) also showed that Doa1 directly competes with Ufd2 for Cdc48

binding, as they both use the same docking site on Cdc48. Doa1 can't bind simultaneously with

both Cdc48 and Otu1, so there is competitive binding for opposing activities, resulting in a

forced cooperation of both inhibitory mechanisms. Cdc48 can bind Otu1 and Doa1

simultaneously however, so it is plausible that a balance between substrate processing cofactors

may determine whether a substrate is routed to proteasome for degradation or deubiquitinated

and released for other purposes (Rumpf and Jentsch, 2006).

Dr. Dyer proposes a model where yeast SCD is delivered to the proteasome via the

typical Cdc48/Ufd1/Npl4 complex whereas plant FADs might be delivered by a Cdc48/Doa1

complex. If this was the case, conditions that increase the formation of the Cdc48/Doa1 complex

would mean faster degradation of Doa1-dependent proteins (potentially, such as plant FADs)

while removal of Doa1 promotes FAD3 stabilization. This theory would help explain the

apparent conflicting data derived from yeast SCD and plant FAD studies. If plant FADs were

delivered to the proteasome by a Cdc48/Doa1 complex, the expectation would be that plant

FADs steady-state amount will go up in the DOA1 mutant background, while yeast SCD amount

might go down, since deletion of Doa1 would allow Npl4 to bind Cdc48 more efficiently. In contrast, in an NPL4 deficient mutant background, the opposite would be true: yeast SCD would become more stable (as observed in the Braun 2002 paper) and plant FADs less stable (as we observed here). Plant FADs become less stable because deletion of NPL4 creates more binding opportunities for Doa1 to associate with Cdc48, which promotes more rapid degradation of the FAD3 proteins. Dr. Dyer is beginning a new set of experiments to test the Cdc48/Doa1 complex theory.

Kunze et al. (2007) determined that Doa1 had a catalytic function and that its absence resulted in a morphological problem, namely cell surface integrity defects. My experimental trials with the DOA1 deficient mutant presented its own set of challenges. Growth on synthetic dextrose media was slow but reliable, but growth was essentially absent in galactose media (with cells often clumping together in the bottom of liquid cultures). During our struggles with the DOA1 mutant, Mullaly et al. (2006) reported functional complementation of yeast DOA1 with a partial construct of its human homolog (PLAA). We attempted to do our own functional complementation, ultimately hoping to express both the FAD3 gene (BF3 or TF3) and the AtDOA1 gene from Arabidopsis in the DOA1 deficient yeast background. Initial attempts were made to amplify the AtDOA1 gene out of the pCMV SPORT 6 vector provided by the Arabidopsis repository and ligate it to a pBEVY-L vector, which contains a constitutive gene promoter. These attempts failed, likely during the restriction enzyme digest and/or ligation process, as uncut pCMV SPORT 6 vectors (still containing the AtDOA1 gene) were being carried through the process. Both the SPORT 6 and pBEVY vectors use an ampicillin selection process so this further exacerbated determining whether the correct plasmid had been constructed. To circumvent this problem, the AtDOA1 gene was amplified, T'tailed and ligated

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to pYES 2.1 vector with a galactose inducible promoter. Once we had the AtDoa1 gene in the

pYES2.1 vector, we believed that if functional complementation did occur, we would see better

growth in galactose cultures as the pYES galactose inducible promoter would activate expression

of the AtDOA1 gene rescuing the mutant phenotype. One BY4742Δdoa1 pYES AtDOA1 clone

showed growth comparable to wild type yeast in galactose media, suggesting that indeed

functional complementation did occur. However, further experimentation will be required to

verify and extend these initial results.

Trials with proteolysis-related yeast mutants have provided interesting results and much

food for thought. On the most basic level, since FAD3 protein accumulation changes in

backgrounds deficient in several proteolysis related genes, this would suggest that FAD3 levels

are indeed influenced by ubiquitin-mediated proteolysis, a key question we were exploring in its

regulation. Through my attempt to understand and explain the factors involved in ubiquitin-

mediated degradation, especially in regards to reading the relevant literature, I have discovered a

number of other proteolysis-related genes that could be investigated in future experiments.

Otu1 is a deubiquitinating enzyme that removes ubiquitin from substrates ubiquitinated

by Ufd2, a step in disassembling multiubiquitin chains shown to inhibit ubiquitin-dependent

proteolysis. Doa1 and Ufd2 compete for Cdc48 binding and Otu1 has been functionally linked

to Doa1 (Rumpf). In the absence of Doa1, there would no competition for Cdc48 binding

providing additional opportunity to deubiquitinate Ufd2 bound substrates. If this was the case,

the expectation would be less degradation with Doa1 mutants, which is precisely what we see.

Expressing BF3 and TF3 in an OTU1 deficient background should yield additional clues and

potentially confirm steps in the FAD3 degradation pathway.

Der1, a degradation in the ER protein, has homologs found in every eukaryote. DER1 was identified in a screen for components required for degradation of misfolded luminal proteins, and its expression has been shown to be upregulated during times of ER stress. If BF3 has a lower thermal stability and is subject to folding problems, perhaps it is also subject to Der1 action. In this case, the expectation would be that the amounts of BF3 increase in the DER1 deficient background while TF3 levels might remain unchanged.

Ubx2 is an ubiquitin regulatory integral membrane protein localized to the ER and proposed to coordinate the assembly of proteins involved in ERAD. UBX2 is required, but not sufficient, for Cdc48 recruitment to the ER and it has been shown to play a particularly important role under stress conditions. All ER substrates tested by Schuberth and Buchberger (2005) were significantly stabilized in an UBX2 deficient background. Expression of FAD3s in this mutant would show if they also are stabilized, like the ER substrates tested, or if they go against this trend.

Final thoughts

My research primarily involved analyzing protein steady-state accumulation. Although previous work has shown a direct relationship between steady-state protein amounts and half-life in the BF3 and TF3 proteins (Bryan 2004), additional half-life studies will need to be done to further validate my results, especially with respect to the BF3 2_6E and TF3 6_2E mutants. Fatty acid analysis trials could also be done to investigate the relationship between FAD3 protein abundance and the amount of linolenic acid, the enzyme product. Fatty acid content should mirror protein accumulation amounts. Additionally, although cells yeast often provide an ideal model system for rapidly characterizing plant genes, it will be important to determine the degree to which yeast-based observations are repeatable within the native plant cell environment.

Evaluating mutant versus wild type genes in a plant environment is essential in substantiating and extending the conclusions I have reached.

Although great care was taken to standardize my experimental trials, a large amount of variability was seen within, and between, repetitions. Some factors which may have influenced my experimental variability include, differences in yeast viability, protein extraction efficiency, protein loading accuracy, gel electrophoresis, protein transfer, antibody hybridization and chemi-luminescent development. Variability, seemingly innate to experimental trials, tends to result in high standard deviations and makes it difficult for me to draw many strong definitive conclusions.

I can say however that, through my mutagenesis experiments, some general trends did emerge with respect to the effects of lysine and acidic amino acid residues on protein stability. My experimental results help support that lysine residues confer a certain amount of instability to proteins and that acidic residues, such as the polyglutamic run in TF3, offer an additional measure of stability. I can also substantiate that I was able to reduce the differences in protein accumulation between BF3 and TF3. Ultimately though I was unable to increase the amount of BF3 to the TF3 level, or decrease the amount of TF3 to the BF3 level. In his work with soybean FAD2s, Tang stated he believed his results were indicative of a complex model, where interaction among multiple domains is ultimately responsible for determining enzyme stability. Although time constraints prohibited me from fully exploring the many differences between the N-terminals of BF3 and TF3, the results I did obtain seem to support this complex model, where numerous factors contribute to the differential stability of these two enzymes

That said, I am especially satisfied, and relatively confident, with the results from the polyglutamic mutation trials with BF3 (BF3 2_6E) and TF3 (TF3 6_2E). Trials with these two

mutants were relatively consistent throughout numerous repetitions. When expressed in yeast grown at 30°C, increasing the glutamic acids in BF3 increased its accumulation, and decreasing the glutamic acids in TF3 decreased its accumulation. Consequently, I conclude that the polyglutamic region found in TF3 is a significant factor in the protein's increased steady-state accumulation and half-life, and that the lack of a polyglutamic region in BF3 results in a reduction of steady-state accumulation and half-life. What remains to be determined is whether the impact of the stretch of glutamic acids is due exclusively to the content of negative charge, changes in the length of the N-terminus, or a combination of both. This is an especially important consideration since, even with the glutamic acid mutations I completed, TF3 6_2E is still longer and more negative than BF3 and BF3 2_6E is still shorter and more neutral than TF3. Once the key variable (length, charge or a combination of the two) has been revealed, another important consideration is how this polyglutamic region is affecting the protein. A few possible effects might relate to protein folding, thermal stability, steric hindrance, active site conformation changes, post-translational modifications including but not limited to ubiquitination, phosphorylation or glycosylation, or other protein to protein reactions within the ubiquitination-proteasomal pathway. Much more experimentation needs to be done to fully elucidate the BF3 and TF3 regulatory mechanisms, but I have shown the TF3 polyglutamic region to be an important variable in this process.

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