Characterization of proteins influencing the nutritional qualityof maize (Zea mays L.) endosperm

Item type	text; Dissertation-Reproduction (electronic)
Authors	Lopez-Valenzuela, Jose A.
Publisher	The University of Arizona.
Rights	
Downloaded	
Link to item	

CHARACTERIZATION OF PROTEINS INFLUENCING THE NUTRITIONAL QUALITY OF MAIZE (*Zea mays* L.) ENDOSPERM

by

Jose Angel Lopez Valenzuela

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PLANT SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2 0 0 3

UMI Number: 3107017

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Jose Angel Lopez Valenzuela

entitled CHARACTERIZATION OF PROTEINS INFLUENCING THE NUTRITIONAL QUALITY OF MAIZE (Zea mays L.) ENDOSPERM

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Brian A. Larkins — Date 8/12/03

Robert T. Leonard — Date 8/12/03

Ramin Yadegari — Date 8/12/03

Richard A. Jorgensen — Date 8/12/03

Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Brian A. Larkins — Date 8/12/03

STATEMENT BY AUTHOR

ACKNOWLEDMENTS

I would like to express my gratitude to Consejo Nacional de Ciencia y Tecnologia (CONACyT, Mexico) and the Universidad Autonoma de Sinaloa -Programa de Mejoramiento del Profesorado (UAS-PROMEP, Mexico), for providing the fellowship that made my training possible. I also thank the support from the Marshall Foundation and the Department of Plant Sciences at the University of Arizona.

I am very grateful to my advisor, Dr. Brian Larkins, for his guidance, support and patience that made this a great learning experience. Thanks to my dissertation committee members, Robert Leonard, Ramin Yadegari, Vicki Chandler, Richard Jorgensen, Karen Shumaker and Gary Thompson, for their suggestions and constructive criticisms.

I would like to acknowledge my lab colleages for their friendship and help. Special thanks go to Dr. Peter Hughes, Dr. Bryan Gibbon, Dr. David Holding, Dr. Hong Nguyen and Mr. Roberto Lizarraga Guerra. Thanks to Dr. Theo Dreher (Dept. of Agricultural Chemistry, Oregon State University) for his collaboration. I also thank Dr. David Galbraith and his group for the use of some equipment and for technical advice.

Very special thanks to my parents, parents in law, brother and systers for their unlimited support.

My most sincere appreciation goes to my wife, Jeanett, and my sons, Jose Angel and Antonio, for their love and companionship, as well as their patience and emotional support.

Dedicated to

Jeanett, Jose Angel and Antonio

TABLE OF CONTENTS

TABLE OF CONTENTS – Continued

TABLE OF CONTENTS - Continued

LIST OF FIGURES

LIST OF FIGURES - Continued

LIST OF TABLES

ABSTRACT

Elongation factor 1A is one of the lysine-rich proteins increased in *o2* mutants, and its concentration is highly predictive of the protein-bound lysine content of the endosperm. Understanding the biological basis of this relationship could help to explain the mechanisms of lysine accumulation in the endosperm, providing new insights for developing maize genotypes with better nutritional quality.

Three different eEF1A isoforms were purified from developing endosperm and investigated in their accumulation, structural and functional activities. The accumulation of the isoforms appears to be developmentally regulated and independent of the *o2* mutation. The purified proteins differed in their ability to bind F-actin *in vitro*, suggesting thy are functionally distinct. The isoform that binds actin most effectively was the most predominant in high eEF1A genotypes, which may be related to enhanced cytoskeleton formation, and therefore increased synthesis of cytoskeleton-associated proteins in these genotypes. Tandem mass spectrometry revealed each isoform is composed of the four same gene products, which are modified post-translationally by methylation and phosphorylation. The chemical differences that account for their different actin binding activities could not be determined.

Recombinant inbred lines varying in eEF1A content were developed from a cross between a high (Oh51A*o2*) and a low (Oh545*o2*) eEF1A inbred. The parental inbreds and RILs with the highest and lowest eEF1A content were used to investigate patterns of gene expression and protein synthesis. Transcript profiling with an endosperm EST microarray

identified about 110 genes coordinately regulated with eEF1A. These genes encode proteins involved in several biological structures and processes, including the cytoskeleton, the endoplasmic reticulum and the protein synthesis apparatus. The content of α-zein and several cytoskeletal proteins was measured in high and low eEF1A inbred lines, and the levels of these proteins were found to correlate with that of eEF1A. Thus, higher levels of eEF1A may be related with a more extensive cytoskeletal network surrounding the rough ER and increased translation of mRNAs encoding cytoskeleton-associated proteins, all of which contribute significantly to the lysine content of the endosperm.

CHAPTER 1

CHARACTERIZATION OF PROTEINS INFLUENCING THE NUTRITIONAL QUALITY OF MAIZE (*Zea mays* L.) ENDOSPERM

INTRODUCTION

Cereals play an important role in human nutrition by contributing about 50% of the calories and proteins in our diet (FAOSTAT, 2002). Among cereal crops, maize has the highest production (609 million tons in 2002); it contributes about 30% of total cereal production (FAOSTAT, 2002). Unfortunately, like many other cereals, maize does not provide a nutritionally balanced source of protein, because the most abundant it contains, prolamins or zeins, are devoid of several essential amino acids, lysine being the most limiting. Maize and other cereals generally contain between 1.5% and 2.5% lysine, which is much lower than the 5% recommended by the Food and Agriculture Organization (Young et al., 1998) for human nutrition.

The lysine content of maize endosperm can be significantly increased by the *opaque2* (*o2*) mutation (Mertz et al., 1964), which reduces α-zein content and increases the accumulation of several other proteins that contain lysine (Damerval and Devienne, 1993; Habben et al., 1993). Elongation factor 1A (eEF1A) is one of the proteins generally increased in *o2* mutants, and its concentration is highly predictive of the lysine content of the endosperm (Habben et al., 1995). As described later in this chapter, several studies from this laboratory have demonstrated a relationship between eEF1A and other lysine-containing proteins in the endosperm. However, these studies have neither explained the biological basis for this relationship, nor led to the identification of other proteins whose concentration is aligned with eEF1A.

The main goal of my dissertation research was to understand the biological basis of the relationship between eEF1A and other major proteins that contribute to the lysine content of maize endosperm. Understanding this relationship could allow us to develop an effective approach for improving the protein quality of maize and other cereal grains. To approach this goal, I purified and characterized isoforms of eEF1A to investigate their function in maize endosperm. I also developed recombinant inbred lines (RILs) from the F2 progeny of a cross between Oh51A*o2* (high eEF1A) and Oh545*o2* (low eEF1A), and used them for analysis of genes encoding proteins that are coordinately increased with eEF1A, in order to explain the pleiotropic effect by which other lysine-rich proteins are increased in *o2* mutants.

As background for this study, the nature of maize storage proteins, high lysine mutants in maize, the nature of eEF1A and its relationship with endosperm lysine content, are reviewed. Finally, the questions and major technical approaches used in this dissertation are described.

MAIZE STORAGE PROTEINS

General properties and structure

Osborne (1924) developed a classification for seed proteins based on their solubility in a series of solvents: albumins, water soluble proteins; globulins, salt soluble proteins; prolamins, alcohol soluble proteins; and glutelins, the remaining acid- or alkaline-soluble proteins. Although this classification is still widely used, it is also common to classify seed proteins into three groups based on their function: storage proteins, structural and metabolic proteins, and protective proteins. The storage proteins of maize endosperm, prolamins or zeins, account for about 70% of the endosperm protein and 50-60% of the total kernel protein in wild type genotypes. Since zeins are nearly devoid of lysine and tryptophan, they dilute the concentration of these essential amino acids in the total endosperm/kernel protein, significantly affecting its nutritional quality. Albumins and globulins contain high levels of lysine and threonine, but the abundance of these proteins is usually low.

Landry and Moureaux (1970) modified the classical Osborne fractionation procedure by adding the reducing agent β-mercaptoethanol and/or sodium dodecyl sulfate (SDS) to some of the solvents. Based on this procedure, maize endosperm proteins were divided into five classes: the 0.5 M NaCl-soluble albumins and globulins; the 70% ethanol-soluble zeins; the 70% ethanol- and 0.6% β-mercaptoethanol-soluble zein-like proteins; the glutelin-like proteins extracted with a combination of borate buffer pH 10, 70% ethanol and 0.6% β-mercaptoethanol; and the glutelins extracted with the same buffer plus 0.5% SDS. Wallace et al. (1990) developed a simplified procedure to separate maize endosperm proteins into storage proteins (zeins) and all other proteins (non-zeins). Total endosperm protein was extracted with 0.0125 M sodium borate (pH 10) containing 1% (w/v) SDS and 2% (v/v) β-mercaptoethanol. Non-zeins were precipitated from the mixture with 70% ethanol, while zeins remained in solution.

Although zeins share the property of solubility in 70% ethanol, they are structurally distinct. Classification of these proteins was confusing because of their differential solubility in aqueous solvents and the ability of several proteins to form disulfide bonds (Wilson, 1985). A widely accepted nomenclature system was developed that classified zein proteins on the basis of their solubility and structural similarities (Esen, 1987). The proteins were identified as α-, β-, γ-, and δ-zeins. A typical SDS-PAGE separation of zeins from wild type and *o2* endosperm is shown in **Figure 1.1**. The most abundant zein proteins in wild type kernels are α-zeins (~70%), which resolve into two main bands of molecular mass 19-kD and 22-kD by SDS-PAGE **(Fig. 1.1)**, although they have true molecular masses of 23-24 kD and 26.5-27 kD, respectively (Shewry and Halford, 2002). Both of these α-zein subclasses contain 9 to 10 repeats of about 20 amino acids (Argos et al., 1982). Separation of α-zeins by isoelectric focusing electrophoresis showed the two bands are composed of a mixture of related polypeptides (Righetti et al., 1977). α-zeins have moderate levels of proline and high levels of glutamine, leucine and alanine (Marks et al., 1985).

γ-zeins are the second most abundant subclass of zein proteins (~20%) and correspond to the 16-, 27-, and 50-kD bands following SDS-PAGE separation **(Fig. 1.1)**. These

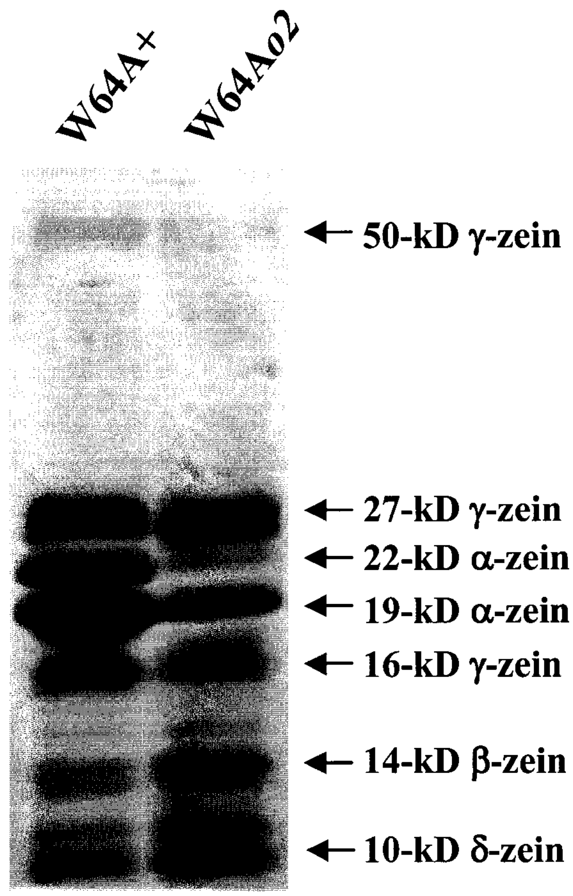


Figure 1.1. SDS-PAGE of the zein proteins in wild type and *opaque2* maize endosperm. Zein fractions from 750 mg of endosperm flour of W64A wild type and *opaque-2* were separated by 12.5% SDS-PAGE. The molecular mass of each band is indicated on the right.

proteins differ in their N-terminal sequence (Prat et al., 1985; Prat et al., 1987; Woo et al., 2001). The 27-kD γ-zein protein contains eight tandem haxepeptide repeats (PPPVHL), while the 16-kD γ-zein protein contains three degenerate versions of this proline-rich repeat. The N-terminal sequence of the 50-kD γ-zein protein contains several polyglutamine repeats and it is remarkably rich in histidine (Woo et al., 2001). Besides being very rich in glutamine and proline, γ-zeins contain high levels of cysteine, and require the presence of reducing agents to be soluble (Prat et al., 1985). β- and δ-zein together account for about 10% of the total zein and have a molecular mass of 14- and 10-kD, respectively **(Fig. 1.1)**. β-zein contains no repetitive peptides and is related to the γ-zeins (Woo et al., 2001), but compared to the γ-zeins, β-zein has greater amounts of the sulfur-containing amino acids, cysteine and methionine, and less glutamine, leucine and proline (Pedersen et al., 1986). δ-zein contains no repetitive peptides and is very rich in methionine (22%) (Kirihara et al., 1988).

Synthesis, deposition and spatial distribution of zeins within the endosperm

Maize storage proteins are synthesized by membrane bound polyribosomes and associate within the lumen of the rough endoplasmic reticulum (RER) to form discrete protein bodies 1 to 2 μm in diameter (Khoo and Wolf, 1970; Larkins and Hurkman, 1978). **Figure 1.2** shows the development of protein bodies (Lending and Larkins, 1989). Protein bodies appear to form sequentially from by the association of different types of zeins. Immunolocalization studies showed that the smallest protein bodies consist mainly

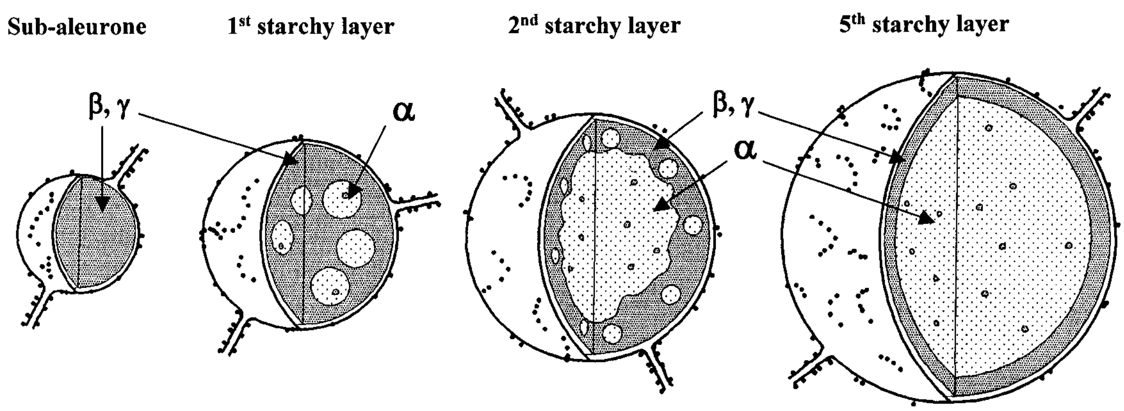


Figure 1.2. Developmental pattern of protein body formation in maize endosperm (Adapted from Lending and Larkins, 1989). The most immature protein bodies are on the left, and development is from left to right. Greek letter designations indicate the location of the corresponding zein classes as determined by immunolocalization.

of β- and γ-zeins, and as they enlarge, α- zeins fill the center partitioning β- and γ-zeins to the periphery (Lending et al., 1988; Lending and Larkins, 1989). Similar to α-zeins, δ-zeins are located in the center of the protein body (Esen and Stetler, 1992). Consistent with this pattern of zein protein accumulation, *in situ* hybridization studies showed that γ-zein mRNA is expressed throughout the endosperm before α- and δ-zein transcripts appear (Woo et al., 2001). This study also described the expression of three novel storage proteins, including a 50-kD γ-zein, an 18-kD α-globulin and a legumin-like protein. As for other γ-zeins, the 50-kD γ- zein was located mainly at the periphery of the protein body, while the 18-kD α-globulin was found primarily in vacuole-like structures not reported previously in maize endosperm (Woo et al., 2001).

Even though the mechanisms of protein body formation are not well understood, studies describing differences in the temporal and spatial accumulation of zeins imply a functional role for γ-zein in the initiation process, and suggest that specific interactions are important for their association to form protein bodies. Experiments with transgenic plants and the yeast two-hybrid system support the hypothesis that specific interactions between zeins are important for their assembly into protein bodies. Transgenic tobacco plants were used to express α- and γ-zein in endosperm (Coleman et al., 1996) and β- and δ-zein in other tissues (Bagga et al., 1997). When synthesized individually, γ- and β-zein were accumulated in the ER, while α- and δ-zein were not. The latter required the coexpression of γ- and β-zein for stability and assembly into protein bodies.

Previous studies expressing γ-zein in Arabidopsis indicated the PPPVHL repeats at the N-terminus are required for ER retention, and in addition to this repeat, the C-terminus cysteine-rich domain is required for protein body formation (Geli et al., 1994). More recently, Kim et al (2002) used the yeast two-hybrid system to examine interactions between zein proteins and their role in protein body assembly. The results showed strong interactions between α- and δ-zeins, as well as 16-kD γ-zein and 15-kD β-zein, suggesting an important role for the latter two proteins in the binding and assembly of α-zeins within the protein body. A truncated 22-kD α-zein (N terminus, first and last two repeats, plus 17 aminoacids of the C terminus) interacted strongly with the 22-kD α-, 15-kD β-, 16-kD γ-, and 10-kD δ-zein proteins. This truncated α-zein could be useful to produce proteins that assemble into proteins bodies but have more nutritional value.

MAIZE HIGH LYSINE MUTANTS WITH ALTERED STORAGE PROTEIN ACCUMULATION

Mutations that affect zein synthesis can lead to protein bodies with altered morphology, size or number, and they usually result in kernels with a soft texture. At least 18 mutations have been described that cause a soft, starchy endosperm phenotype (Thompson and Larkins, 1994). The starchy endosperm mutants *opaque2 (o2)* and *fluory2 (fl2)*, originally described by Emerson et al. (1935), contain lower levels of zeins and higher levels of lysine and tryptophan compared to their wild type counterparts (Mertz et al., 1964; Nelson et al., 1965). The lower α-zein content in these mutants results in small, unexpanded protein bodies (Geetha et al., 1991), but they are also deformed in *fl2* (Coleman et al., 1995). Similar to *fl2,* the opaque mutants *Mucuronate* (*Mc*) and

Defective endosperm B30 (*DeB30*) contain reduced levels of α-zeins and increased levels of nonzeins, as well as deformed protein bodies (Coleman and Larkins, 1999). *opaque7 (o7)* decreases the accumulation of 19-kD α-zeins (Di Fonzo et al., 1979), while *opaque15* (*o15*) lowers the synthesis of γ-zein and leads to a smaller number of protein bodies (Dannenhoffer et al., 1995).

Only in the case of *o2* and *fl2* is the basis of the genetic defect known. *O2* encodes a transcription factor that regulates the expression of genes encoding the 22-kD α- zeins (Kodrzycki et al., 1989; Schmidt et al., 1990), as well as several other lysine-containing proteins (Damerval and Devienne, 1993; Habben et al., 1993). *fl2* corresponds to a point mutation in the signal peptide of a 22-kD α-zein protein (Coleman et al., 1995; Coleman et al., 1997), which appears to anchor the protein to the ER membrane resulting in the accumulation of α-zeins at the surface of the protein body (Gillikin et al., 1997). However, the basis for the pleiotropic effects of these mutations that leads to a significant increase in nonzein proteins is not well understood.

Comparing the mechanisms responsible for the opaque kernel phenotype in other mutants has been difficult, because they are often in different genetic backgrounds (Balconi et al., 1998). Hunter et al. (2002) backcrossed the *o1*, *o5*, *o9*, *o11*, *DeB30*, and *Mc* mutants into W64A to develop nearly isogenic lines (NILs). These NILs, plus W64A*o2*, and W64A*fl2*, were compared for protein synthesis, amino acid composition, and global patterns of gene expression. The amount of total protein in the mutants varied from 10% in *o2*, to about 12% for the others. The largest reduction in zein synthesis occurred in *o2*, *DeB30*, and *fl2* mutants, which contained between 35 and 55% of the zein in wild type. As reported previously (Nelson et al., 1965), *o1* was indistinguishable from wild type in the amount of zein and nonzein protein, suggesting that the opaque phenotype cannot be explained by a reduction in the amount of zeins, nor total protein. Except for *o1*, the lysine content in the mutants was higher than in wild type; it was about 1.4-times higher for *o5*, *o9* and *Mc* and more than double in *o2*. In general, the increase in lysine content coincided with a decrease in the amino acids Gln/Glu, Leu, and Pro, which are normally abundant in zein proteins. Transcript profiling of 18-days after pollination (DAP) endosperms from each of the mutants was conducted using Affimetrix oligonucleotide arrays. Genes associated with physiological stress and the unfolded protein response, such as genes encoding molecular chaperones, were consistently affected in all the opaque mutants. Based on this analysis, the mutants were divided in four phenotypic groups: W64A+ and *o1*; *o2*; *o5/o9/o11*; and *Mc* and *fl2* (Hunter et al., 2002). Although endosperm transcript profiling provided information about common features of gene expression between the mutants, the results also suggested different mechanisms are responsible for the opaque kernel phenotype. As in other microarray experiments, these results represent a framework for additional genetic and biochemical experiments.

ELONGATION FACTOR 1 ALPHA (eEF1A)

Relationship with the lysine content of maize endosperm

To identify lysine-rich proteins that are increased in *o2* mutants, a complex polyclonal antiserum was developed against the nonzein endosperm proteins and used to screen an endosperm cDNA expression library (Habben et al., 1993). The cDNAs found in this screen were used as probes in RNA blots of mutant and wild type endosperm. One of the mRNAs found significantly increased in W64A*o2* compared to W64A+ was eEF1A. Subsequent analysis of this protein using ELISA assays revealed a 2- to 3-fold increase in *o2* genotypes relative to their wild type counterparts (Habben et al., 1995). A survey of several maize genotypes showed a 4-fold variation in lysine content and a highly significant relationship (r=0.9) between lysine and eEF1A concentration (Habben et al., 1995; Moro et al., 1996). This relationship was also found in other cereals such as barley and sorghum. On the other hand, the correlation between lysine and sucrose synthase, which accounts for about 3% of the nonzein protein, was much lower (r=0.57), as was the correlation for ADP glucose pyrophosphorylase (r=0.21) and the protein synthesis factor EF-2 (r=0.07). These results suggested the relationship between eEF1A and lysine is neither a general feature of nonzein proteins nor protein synthesis factors.

The basis for the correlation between eEF1A and endosperm lysine content is not obvious. eEF1A contains about 10% lysine and constitutes 1 to 3% of the total soluble protein in eukaryotic cells (Merrick, 1992). However, it accounts for only 1% of the protein and 2% of the lysine associated with proteins in maize W64A*o2* endosperm (Sun et al., 1997). Therefore, eEF1A itself cannot account for the increase in lysine content, suggesting a stoichiometric relationship between eEF1A and other lysine containing proteins in the endosperm.

Because eEF1A interacts with components of the cytoskeleton in other eukaryotes (Durso and Cyr, 1994), Clore et al (1996) used immunofluorescence and confocal microscopy to visualize and determine the spatial relationship *in situ* of eEF1A, actin, tubulin, and protein bodies in intact endosperm cells. The results showed that protein bodies are juxtaposed to a reticulate array of microtubules and are surrounded by eEF1A and actin. Treatment with cytochalasin D, an actin disrupter, resulted in redistribution of eEF1A within the cells, suggesting that eEF1A and actin exist in a complex. In fact, eEF1A purified from developing maize endosperm was shown to interact with yeast actin *in vitro* (Sun et al., 1997). The association of eEF1A with the cytoskeleton may help to explain its relationship with the lysine content.

Genetic analysis of the progeny from a cross between a high (Oh51A*o2*) and a low (Oh545*o2*) eEF1A maize inbred, showed a QTL linked with a cluster of 22-kD α-zein genes (Wang et al., 2001). High levels of α-zeins were found to cosegregate with high eEF1A content, suggesting that the difference in eEF1A between the two inbred lines may be due to an increase of the surface area of protein bodies in the endosperm, creating a more extensive network of cytoskeletal proteins. Thus, the relationship between eEF1A and lysine content may arise from eEF1A providing an index of the amount of cytoskeletal proteins, which are presumably lysine rich.

These studies demonstrated a relationship between eEF1A and other endosperm lysine-containing proteins. However, the nature of the other lysine-rich proteins with which eEF1A is allied is unknown. As discussed in the following section, eEF1A appears to be a multifunctional protein and the basis for its increased synthesis in *o2* mutants may result from one or more of its activities. The research described in this dissertation was conducted to address these questions.

Functional activities and post-translational modifications

Elongation factor 1 (eEF1) is a protein synthesis factor composed of four subunits: A, Bα, Bβ and Bγ (Browning, 1996). Subunit eEF1A, also known as eEF1α, binds aminoacyl-tRNAs in a GTP-dependent manner and positions them in the acceptor (A) site of the ribosome. After GTP hydrolysis, eEF1A-GDP is released from the ribosome, allowing peptide bond formation with the peptidyl-tRNA in the P site.

Beyond its role in protein synthesis, several other activities have been reported for eEF1A (reviewed in chapter 2), ranging from its interaction with components of the cytoskeleton (Durso and Cyr, 1994a), where it may play a role in regulating cytoskeletal structure, to its involvement in ubiquitin-dependent degradation of N-acetylated proteins (Gonen et al., 1994) and quality control of newly synthesized proteins (Hotokezaka et al., 2002).

At least four types of post-translational modifications have been reported for eEF1A: lysine residues are methylated (Hiatt et al., 1982), serine residues are phosphorylated (Yang et al., 1993), glutamic or aspartic residues are modified by the attachment of phoshoglycerylethanolamine (Whiteheart et al., 1989; Ransom et al., 1998), and the C-terminus is modified by methylesterification (Zobel-Thropp et al., 2000). In contrast to the amino acid sequence, the post-translational modifications of eEF1A are not highly conserved between different species (Cavallius et al., 1993). The diversity of post-translational modifications of the protein raises the possibility that some of eEF1A's activities are associated with different modifications. However, the functional significance of these modifications is poorly understood (discussed in chapter 2).

Gene family

eEF1A is typically encoded by multiple genes. There are two genes in yeast (Nagata et al., 1984), three genes in *Mucor* (Linz et al., 1986), and four genes in *Artemia* (van Hemert et al., 1983). Humans contain more than 18 eEF1A genes (Lund et al., 1996) and there are four genes in Arabidopsis (Axelos et al., 1989) and rice (Kidou and Ejiri, 1998). There are 10 to15 genes in maize (Carneiro et al., 1999), four to eight genes in tomato (Pokalsky et al., 1989), and about 20 genes in sugarcane (Vijaykumar et al., 2002). The amino acid sequences encoded by these genes are highly conserved, even between different species (Browning, 1996).

Some studies have provided evidence for tissue- or development-specific expression of eEF1A genes. For example, three eEF1A genes were characterized in *Xenopus laevis*: 42Sp50, EF-1αO and EF-1αS (Dje et al., 1990). 42Sp50 is expressed exclusively in oocytes, while EF-1αO is active from fertilization until early onset of neurolation. Several eEF1A-like sequences exist in the mammalian genome, but only eEF1A-1 and eEF1A-2 appear to be actively transcribed eEF1A genes (Lund et al., 1996). eEF1A-2 is only expressed in the terminally differentiated cells of skeletal muscle, heart, brain, and aorta, while eEF1A-2 is expressed ubiquitously (Lund et al., 1996; Kahns et al., 1998).

There is not clear evidence for tissue- or development-specific expression of eEF1A genes in plants. The expression of some eEF1A genes in soybean and sugarcane appears to be specific for expanded leaves and stimulated by light (Aguilar et al., 1991; Vijaykumar et al., 2002). In maize, most of the eEF1A genes appear to be expressed and the relative level of their transcripts varies in different tissues (Carneiro et al., 1999). At least five eEF1A genes are expressed in the endosperm.

OBJECTIVES AND RESEARCH APPROACHES

Previous work from our laboratory suggested there are multiple isoforms of eEF1A proteins in maize endosperm (Sun et al., 1997), which may reflect different physiological functions of the protein. Therefore, one objective of this dissertation was to characterize isoforms of eEF1A to understand their function in maize endosperm. Three eEF1A isoforms were purified and characterized by (1) investigating their accumulation during endosperm development; (2) comparing their ability to bind F-actin *in vitro*; and (3) investigating their amino acid sequence and post-translational modifications using tandem mass spectrometry. Tandem mass spectrometry combined with electrospray ionization provides a method to determine amino acid sequences and post-translational modifications with sensitivity at the fentomole concentration (Wilm et al., 1996; Dongre et al., 1997). **Figure 1.3** shows a typical experiment using this approach.

Investigating the nature of the other lysine-rich proteins with which eEF1A is allied was the second major objective of this dissertation. Recombinant inbred lines (RILs) were developed from the F2 progeny of the cross of Oh51A*o2* (high eEF1A content) by Oh545*o2* (low eEF1A content) and used for analysis of genes encoding proteins that are coordinately increased with eEF1A. Proteins whose levels are coordinately increased with eEF1A were identified by (1) ELISA measurements of cytoskeleton-related proteins in high and low eEF1A genotypes, and (2) transcript profiling of high and low eEF1A genotypes using cDNA microarrays. In this case, I assumed that although protein abundance is not strictly controlled by the amount of mRNA, physiological changes in the cell are often correlated with changes in transcript levels, providing clues to gene function.

The two types of microarray methods commonly used for transcript profiling are based on oligonucleotide arrays (Fodor et al., 1991), where the array elements are synthetic oligonucleotides (~20 bp), and cDNA microarrays (Schena et al., 1995), where the array elements are longer DNA fragments (~400-2,000 bp), usually PCR products or isolated cloned cDNAs. In both cases the basic principle is the same: hybridization of a set of transcripts (target) to an ordered array of immobilized DNA molecules of known sequence (probe) produces a specific pattern that can be analyzed and compared to a given standard. **Figure 1.4** shows the basic principle of production and use of a cDNA microarray.

Figure 1.3. Tandem mass spectrometry. The protein to be analyzed is digested with a protease, usually trypsin, to obtain a peptide mixture. Peptides are then separated by liquid-based chromatography tools and eluted into an electrospray ion source to produce protonated peptides. After ionization, the mass-to-charge ratio (*m/z*) of the peptides is measured in the first mass analyzer. This mass spectrum (MS) provides a peptide fingerprint of the protein. Individual peptide ions are selected for fragmentation by energetic collision with gas, followed by analysis with a second mass analyzer to obtain a tandem or MS/MS spectrum, which in addition to the mass of the peptide also provides sequence information (Aebersold and Mann, 2003). The identity of the peptide/protein is usually obtained by comparison of the experimental MS/MS spectrum with MS/MS spectra predicted from protein sequence databases using different algorithms such as SEQUEST (Yates, 1998).

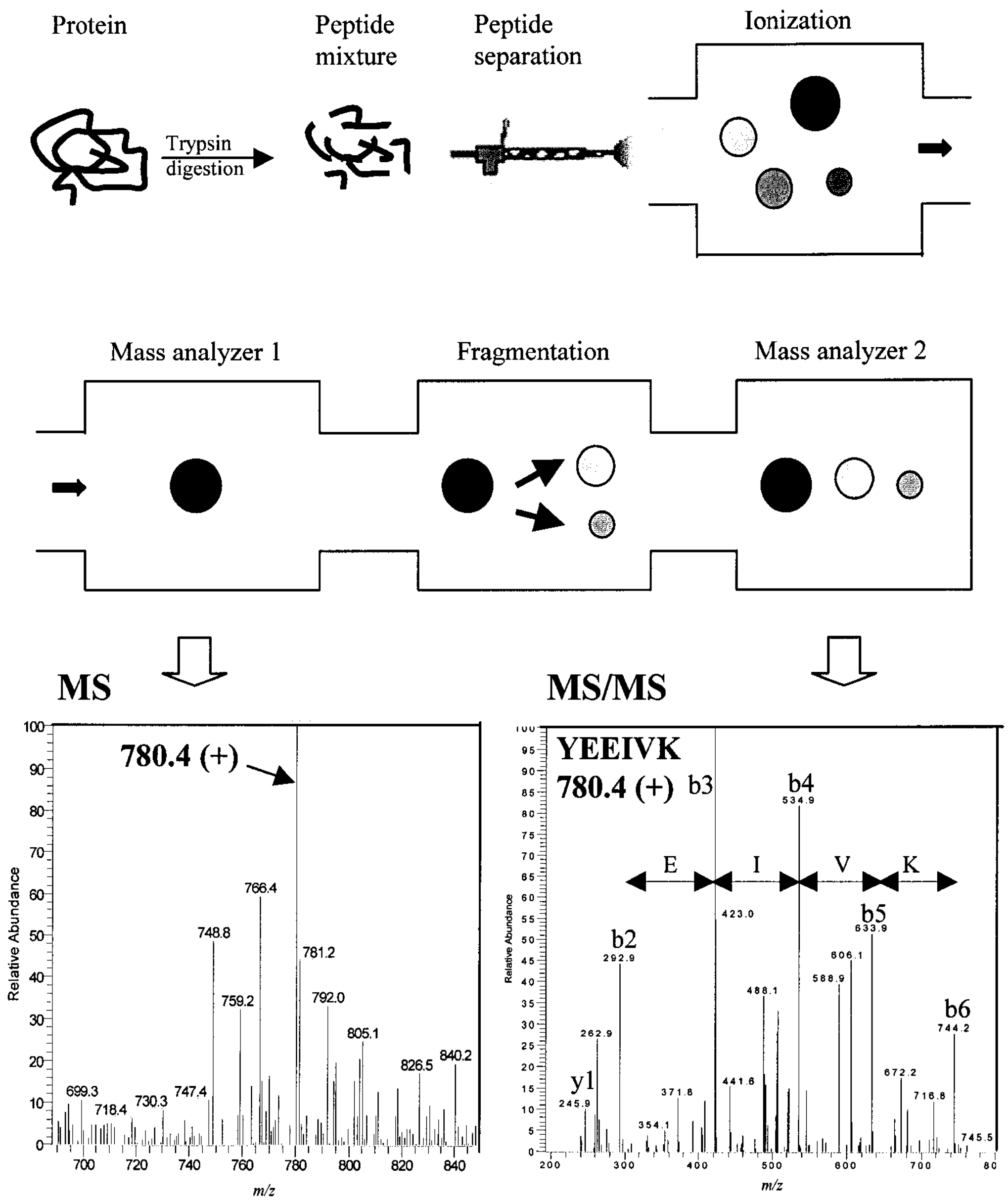
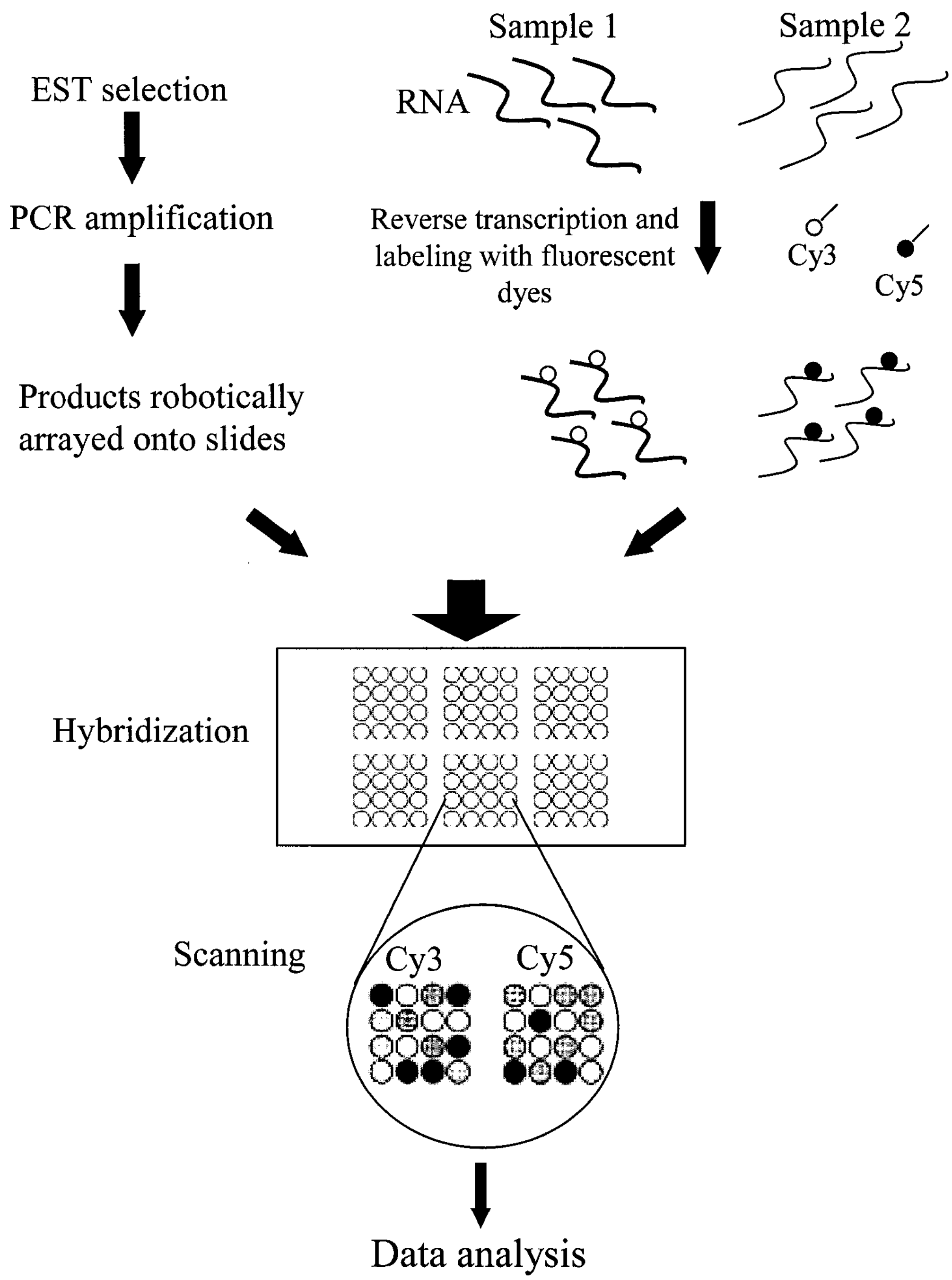

Protein
Trypsin digestion
Peptide mixture
Peptide separation
Ionization
Mass analyzer 1
Fragmentation
Mass analyzer 2
MS
MS/MS
780.4 (+)
Relative Abundance
699.3
718.4
730.3
747.4
748.8
759.2
766.4
781.2
792.0
805.1
826.5
840.2
m/z
YEEIVK
780.4 (+)
b3
b4
534.9
E
I
V
K
423.0
b5
633.9
b2
292.9
606.1
588.9
488.1
b6
744.2
262.9
y1
245.9
371.8
441.6
672.2
716.8
354.1
745.5
m/z

Figure 1.4 cDNA microarrays. The first step in cDNA microarray production is the selection of probes. These are normally produced by PCR amplification of cDNA and EST libraries. DNAs are robotically printed onto chemically coated glass microscope slides to produce the microarray. To prepare the targets, RNA is extracted from the biological samples to be compared and labeled by reverse transcription using nucleotides fluorescently-labeled with Cy3 or Cy5. These dyes are normally paired due to their distinct absorption/emission wavelengths. Equal amounts of both labeled cDNA samples are mixed and then hybridized to the array. Fluorescence from the array is detected for each dye by laser scanning, and the output is typically stored as two separate image files. Image analysis software is used to calculate pixel intensities for each element for both dyes. The resulting data files can be used for subsequent analysis with other software packages, such as Excel (Deyholos and Galbraith, 2001).


Sample 1
Sample 2
EST selection
RNA
PCR amplification
Reverse transcription and labeling with fluorescent dyes
Cy3
Cy5
Products robotically arrayed onto slides
Hybridization
Scanning
Cy3
Cy5
Data analysis

CHAPTER 2

eEF1A ISOFORMS CHANGE IN ABUNDANCE AND ACTIN-BINDING ACTIVITY DURING MAIZE ENDOSPERM DEVELOPMENT

INTRODUCTION

Translation elongation factor 1A (eEF1A) is a protein synthesis factor that binds aminoacyl-tRNAs to the acceptor (A) site of ribosomes during peptide chain elongation (Browning, 1996). Several other activities have been described for this protein, including interactions with the valyl-tRNA synthetase complex (Motorin et al., 1988), actin (Yang et al., 1990), tubulin (Durso and Cyr, 1994b), calmodulin (Kaur and Ruben, 1994), and calcium/calmodulin-dependent protein kinase (Wang and Poovaiah, 1999). eEF1A has also been associated with activation of phosphatidylinositol 4-kinase (Yang et al., 1993), ubiquitin-dependent degradation of N-acetylated proteins (Gonen et al., 1994), and quality control of newly synthesized proteins (Hotokezaka et al., 2002). As a cytoskeleton-associated protein, eEF1A bundles actin in a pH-dependent manner (Edmonds et al., 1995), and it associates with microtubules *in vitro* and *in vivo* (Moore and Cyr, 2000). The basis for the association of eEF1A with elements of the cytoskeleton is unclear, but given the increasing evidence for the role of the cytoskeleton as a scaffold for protein synthesis (Hesketh, 1994), it may be that crosslinkage of the cytoskeleton by eEF1A is essential for transport, anchorage, and/or translation of mRNAs associated with microfilaments and microtubules (Condeelis, 1995). Liu et al. (2002) have shown that the eEF1A-F-actin complex may function as a scaffold for β-actin mRNA anchoring in chicken embryo fibroblasts.

eEF1A contains 10% lysine, and it is subject to several types of posttranslational modifications, including methylation of lysine residues (Hiatt et al., 1982), addition of glycerylphosphorylethanolamine to glutamic or aspartic acid residues (Whiteheart et al., 1989; Ransom et al., 1998), phosphorylation (Venema et al., 1991), and methylesterification at the C terminus (Zobel-Thropp et al., 2000). In higher eukaryotes, eEF1A is typically encoded by a multigene family: there are 18 genes in humans (Lund et al., 1996), four genes in Arabidopsis (Axelos et al., 1989) and 10 to15 genes in maize (Carneiro et al., 1999). The amino acid sequences encoded by these genes are highly conserved, even between different species (Browning, 1996). Several studies have provided evidence for tissue-specific expression of members of the eEF1A gene family (Dje et al., 1990; Aguilar et al., 1991; Lund et al., 1996; Kahns et al., 1998), but the importance of this has not been widely investigated. Thus, the different biological activities of eEF1A could result from isoforms of the protein generated by posttranslational modifications and/or expression of different eEF1A genes.

eEF1A is an abundant protein in maize endosperm, where its concentration is highly correlated (r^2=0.9) with the lysine content (Habben et al., 1995). Moro et al. (1996) documented a five-fold variation in the concentration of eEF1A in the endosperm of several maize genotypes and found that eEF1A levels were consistently increased on a dry weight basis in *opaque2* (*o2*) mutants, which normally contain a higher than average lysine content. In W64A*o2*, eEF1A accounts for about 1% of the total endosperm protein and 2.3% of the lysine content (Sun et al., 1997). Thus, there appears to be a stoichiometric relationship between eEF1A and the other major lysine-containing proteins in the endosperm.

Immunocytochemistry studies showed eEF1A colocalizes with a network of F-actin surrounding the rough ER (RER) at sites where protein bodies are forming in maize endosperm cells (Clore et al., 1996). This localization was disrupted with cytochalasin D, indicating that eEF1A interacts with F-actin. eEF1A purified from developing endosperm was shown to bundle actin *in vitro* (Sun et al., 1997). Wang et al. (2001) found a QTL associated with eEF1A content genetically linked to a cluster of 22-kDa α-zein genes and proposed a positive correlation between eEF1A content and the surface area of protein bodies, hence the network of cytoskeletal proteins in the endosperm. Thus, the relationship between eEF1A concentration and lysine content may arise from eEF1A providing an index of the amount of cytoskeletal proteins.

Previous work from our lab suggested there are different isoforms of eEF1A in maize endosperm (Sun et al., 1997), which may reflect different physiological functions of the protein. In the present study, we purified three eEF1A isoforms from developing endosperm and compared their F-actin and aminoacyl-tRNA binding activities *in vitro*. We also investigated their amino acid sequences and post-translational modifications by tandem mass spectrometry. Our results showed the purified eEF1A isoforms differ in their ability to bind F-actin, but share similar aminoacyl-tRNA binding activities. They consist of the same four gene products modified post-translationally by methylation and phosphorylation.

MATERIALS AND METHODS

Plant materials

The maize (*Zea mays* L.) inbreds W64A+ and W64A*o2* were grown at the University of Arizona West Agricultural Center (Tucson, AZ). The maize inbreds Oh545*o2* (low eEF1A) and Oh51A*o2* (high eEF1A), as well as the recombinant inbred lines (RIL) developed from their cross, 31-5 and 34-3, were grown in the greenhouse at the University of Arizona Campus Agricultural Center. The 31-5 (low eEF1A) and 34-3 (high eEF1A) RILs were developed by single seed descent and were chosen because of their extreme eEF1A content. Developing kernels from W64A+ and *o2* were harvested at 11-, 16-, and 20- days after pollination (DAP). Developing kernels from Oh545*o2*, Oh51A*o2*, and the RILs were harvested at 10-, 15-, and 20-DAP. Harvested kernels were frozen in liquid nitrogen and stored at –80°C.

Purification of eEF1A isoforms

Seventy grams of maize kernels were homogenized in 140 mL of buffer A (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM DTT, 10% [v/v] glycerol, 1 mM EDTA, 1 mM PMSF) using a Polytron (Kinematica Gmbh, Switzerland). The homogenate was centrifuged at 10,000*g*, filtered though two layers of Miracloth (Calbiochem, La Jolla, CA), and adjusted to 40% and then 80% ammonium sulfate. The protein precipitated between 40% and 80% ammonium sulfate was collected by centrifugation at 20,000*g* for 30 min with a Sorvall RC-5B centrifuge, resuspended in 10 mL of buffer A and desalted by gel filtration (G25, Pharmacia Biotech). The protein was loaded onto a Q Sepharose (Pharmacia Biotech) column connected to a SP Sepharose (Pharmacia Biotech) column, which were both pre-equilibrated with buffer A. The SP column was eluted with a 40-min linear gradient (1.5 mL min^{-1}) of 0.05 to 0.5M KCl in buffer A, and the fractions containing eEF1A were monitored by immunoblot analysis using a rabbit polyclonal antibody raised against recombinant maize eEF1A (Habben et al., 1995). Pooled fractions containing eEF1A were dialyzed against buffer B (20 mM HEPES [pH 7.5], 2 M ammonium sulfate, 1 mM DTT, 1 mM EDTA) and loaded onto a Phenyl (GMB 1000 PHE, Biospher) column pre-equilibrated with the same buffer. The column was eluted with a 40-min linear gradient (1 mL min^{-1}) of 2 M to 0 M ammonium sulfate. Fractions containing eEF1A were dialyzed against buffer C (20 mM HEPES [pH 7.5], 10% [v/v] glycerol, 1 mM DTT, 1 mM EDTA) and loaded onto a cation exchange (GMB 1000 SB, Biospher) column pre-equilibrated with the same buffer. The column was eluted with a 40-min linear gradient (1 mL min^{-1}) of 0 to 0.5 M NaCl in buffer C. The amount of protein eluting from the column was measured by absorbance at 280 nm.

For analysis of the eEF1A isoforms from different genotypes and at different developmental stages, 15 g of frozen, developing maize kernels were homogenized with 30 mL of buffer C and the homogenate was adjusted to 40% and then 80% ammonium sulfate. The protein precipitated between 40% and 80% ammonium sulfate was collected by centrifugation, resuspended in 5 mL of buffer B, and loaded directly onto the phenyl column. The remainder of the procedure was as described above. Purification of eEF1A was conducted at least two times for each genotype/developmental stage combination. To estimate the relative abundance of each isoform, the area of the corresponding peak, as measured by absorbance at 280 nm, was divided by the total of all eEF1A peaks in the chromatogram.

Actin binding assay

The ability of each eEF1A isoform to associate with filamentous actin (F-actin) (rabbit skeletal muscle, Cytoskeleton Inc., Denver, CO) was tested in a co-sedimentation assay according to Demma et al. (1990) with some modifications. Actin and each of the eEF1A isoforms were combined in a final equimolar concentration of 2.5 μM and then incubated for 1h at room temperature in 200 μL of actin polymerization buffer (20 mM HEPES [pH 7.5], 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA). As controls, actin and each of the isoforms were incubated alone in polymerization buffer. The samples were then centrifuged at 200,000*g* for 30 min with the TLA 100.3 rotor of a Beckman TL-100 ultracentrifuge. The supernatant (S) was recovered and adjusted to 1X SDS-PAGE buffer (Laemmli, 1970). The pellet (P) was dissolved in 1X SDS-PAGE buffer to a final volume equal to the supernatant. In both cases, a 30 μL aliquot was separated by 12.5% (w/v) SDS-PAGE and stained with Comassie Blue R. The relative amount of protein in the pellets and supernatants was estimated by densitometry. The proportion of eEF1A binding to actin was calculated as the eEF1A in the pellet minus eEF1A in the pellet of the control, divided by the total (pellet + supernatant).

Analysis of ternary complex formation by eEF1A

Purified eEF1A isoforms were compared for their ability to form ternary complexes with aminoacylated tRNA (aa-tRNA•eEF1A•GTP). These experiments were conducted by Dr. Theo W. Dreher, Oregon State University, Corvallis, OR. $tRNA^{Gly}$ purified from bovine liver was a gift to T.W.D. from Dr. Boris Negrutskii, Institute of Molecular Biology and Genetics, Kiev, Ukraine. $tRNA^{Val}$ was purified from wheat germ by hybridization affinity selection as described (Dreher et al., 1999); eEF1A was also purified from wheat germ.

$tRNA^{Gly}$ and $tRNA^{Val}$ were aminoacylated with ^{3}H-labeled glycine and valine (specific activities of 41.1 and 40.9 Ci/mmole, respectively; New England Nuclear, Boston, MA) using aminoacyl-tRNA synthetase activities present in a partially purified extract made from wheat germ (Dreher et al., 1992). The formation of ternary complex (aminoacylated tRNA•eEF1A•GTP) was assayed by a ribonuclease protection assay as described (Dreher et al., 1999). The molar concentrations of eEF1A were based on total protein concentrations determined by Coomassie Blue R staining assays (Pierce, Rockford, IL).

Mass spectrometry analysis

The purified eEF1A isoforms were digested with the proteases Trypsin (Roche), Glu-C (V8, Sigma) and Asp-N (Sigma) according to the manufacturer. For trypsin digestion, 50 μg of eEF1A protein was digested with 1 ug of protease in 25 mM NH_4HCO_3 (pH 7.8) for 20 h at 37°C in a 100 μL reaction. For Glu-C digestion, 50 μg of eEF1A protein was digested with 1 μg of protease in 25 mM of NH_4HCO_3 (pH 7.8) for 20 h at room temperature in a 100 μL reaction. For Asp-N digestion, 50 μg of eEF1A protein was digested with 1 μg of protease in 50 mM sodium phosphate buffer (pH 8.0) for 20 h at 37°C in a 100 μL reaction. In all cases, the reactions were stopped by freezing and then used for tandem mass spectrometry (LC-MS-MS) analysis. The instrument used was a Finnigan LCQ HPLC/MS combined with an electrospray ionization source (ESI) available at the University of Arizona Chemistry Department MS facilities. A 20 μL aliquot of the peptide mixture was first separated by reversed phase HPLC using a C18 column and a 0.1% TFA/acetonitrile gradient. Peptides eluting from the column were ionized and their masses determined by an ion trapping mass analyzer. Individual ions were selected, subjected to fragmentation and the ion products characterized in a second mass analyzer. The SEQUEST program (Yates, 1998) was used to identify the protein(s). The experimental fragmentation patterns produced for each peptide were compared to those predicted from eEF1A protein sequences using the same protease. Post-translational modifications were investigated by introducing a mass shift in a specific amino acid, such as 14 for methylation in lysine residues, to generate the predicted fragmentation patterns. In addition to SEQUEST identification, the nature of selected peptides was confirmed manually by analysis of the fragmentation pattern.

RESULTS

Purification of eEF1A isoforms

eEF1A was purified from 16- to 20-DAP frozen endosperm of several maize inbred lines. After comparing different approaches for extraction and separation, the following procedure was found to yield highly purified preparations of eEF1A. Proteins in endosperm extract were initially fractionated by ammonium sulfate (40-80%) precipitation, followed by liquid chromatography using anion-exchange on Q Sepharose, cation-exchange on SP Sepharose, hydrophobic interaction on Phenyl PHE, and cation-exchange on SB, as described in Materials and Methods. Since eEF1A is a very basic protein (pI ca 9.2), the Q Sepharose column was used only to clarify and reduce the complexity of the extract before binding to the SP Sepharose column. The SP Sepharose column was used to enrich the protein extract for eEF1A and prevent saturation of the phenyl column with protein when using more than 20 g of frozen endosperm tissue.

Three major eEF1A isoforms (eEF1A22, eEF1A24 and eEF1A26) were separated by SB cation-exchange chromatography and identified based on their elution profile. **Figure 2.1** shows SDS-PAGE analysis of two micrograms of proteins recovered after each step of purification; proteins were visualized by staining with Coomassie Blue R **(Fig 2.1A)**, and by probing with eEF1A polyclonal antibodies (Habben et al., 1995) after transfer to

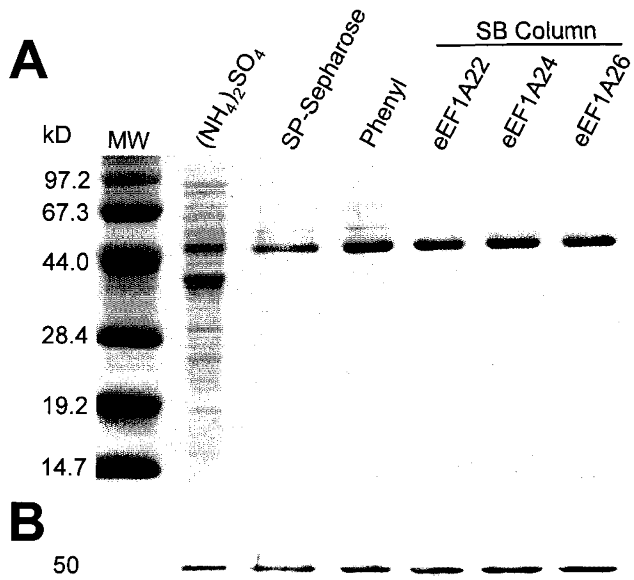


Figure 2.1. SDS-PAGE and immunoblot analysis of maize endosperm eEF1A obtained during steps of purification. eEF1A from crude endosperm extract was purified by ammonium sulfate precipitation (40-80%), cation-exchange chromatography on SP Sepharose, hydrophobic interaction on Phenyl PHE, and cation-exchange on SB. The major isoforms (eEF1A22, eEF1A24 and eEF1A26) separated by SB cation exchange chromatography were identified according to their approximate retention time. Two micrograms of protein from each step of purification were separated by 12.5% SDS-PAGE and stained with Coomassie Blue R (A) or transferred to nitrocellulose membrane and probed with polyclonal antibodies against maize eEF1A (B). The purified proteins were the only bands detected in the immunoblot. MW estimates were based on migration of prestained protein markers.

nitrocellulose membrane **(Fig. 2.1B)**. Based on this analysis, the different eEF1A isoforms were more than 95% pure. Typically, 600 μg of eEF1A22, 700 μg of eEF1A24 and 650 μg of eEF1A26 were purified from 70 g of 16 DAP W64A*o2* maize kernels. After purification, the proteins were used for comparison of their functional and chemical properties.

The relative abundance of eEF1A isoforms changes during endosperm development

Because differences in the accumulation of the eEF1A isoforms were observed in W64A+ and *o2*, we compared their relative levels at early and middle stages of endosperm development. **Figure 2.2** shows a comparison of the eEF1A isoforms obtained from W64A*o2* and wild type endosperm at 11-, 16- and 20-DAP. In W64Ao2 **(Fig. 2.2A)** at 11-DAP, eEF1A26 contributed about 70% of the protein, eEF1A24 contributed 30% and eEF1A22 was barely detectable. At 16-DAP, the proportions of eEF1A26, eEF1A24 and eEF1A22 were about 30%, 40%, and 30%, respectively. By 20-DAP, eEF1A26 contributed about 10% of the protein, eEF1A24 contributed 30%, and eEF1A22 contributed 60%. Thus, the isoform with the longest retention time (eEF1A26) was most abundant at 11-DAP, and by 20- DAP the isoform with the shortest retention time (eEF1A22) was the most abundant. Similar qualitative changes in the accumulation of the eEF1A isoforms, although not exactly in the same proportion, were observed during development of W64A+ endosperm **(Fig. 2.2B)**, suggesting their accumulation is developmentally regulated and independent of the *o2* mutation.

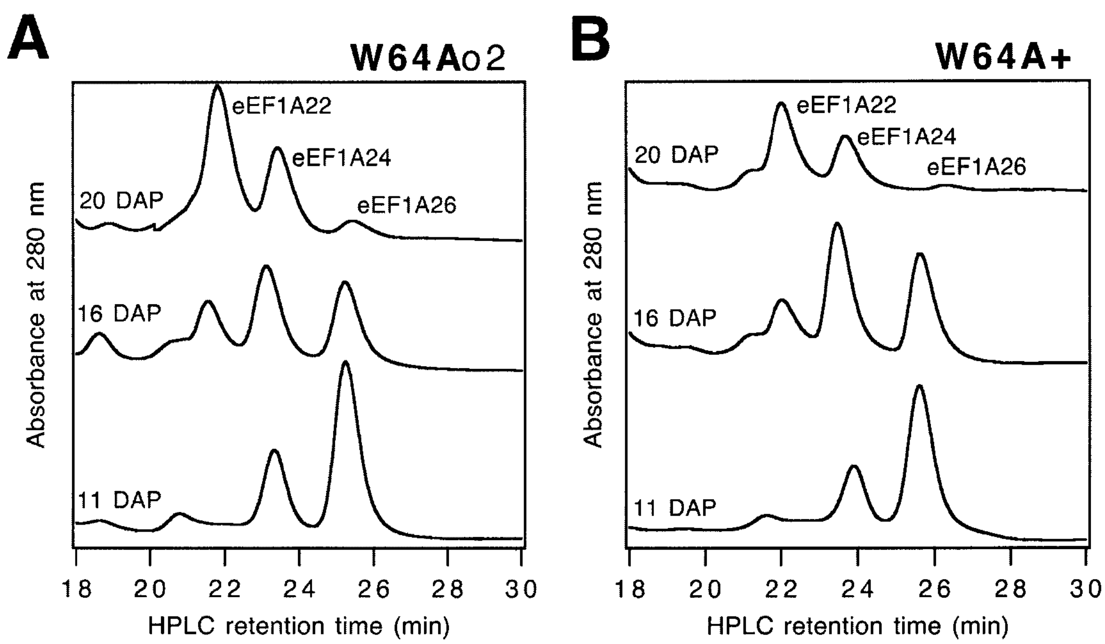


Figure 2.2. Accumulation of eEF1A isoforms during development of W64A*o2* and W64A+ endosperm. eEF1A was purified from W64A*o2* (A) and W64A+ (B) endosperm at 11-, 16- and 20-DAP. Three eEF1A isoforms (eEF1A22, eEF1A24 and eEF1A26), measured by absorbance at 280 nm and identified by immunoblot analysis, were separated by SB cation exchange chromatography and labeled according to their retention time.

Previous studies showed a broad range of eEF1A concentration among various maize inbreds (Moro et al., 1996). The amount of eEF1A in mature endosperm of Oh51A*o2* is twice that in Oh545*o2* (Wang et al., 2001), and the levels in the reciprocal F1s are intermediate between the parental inbreds. Recombinant inbred lines (RIL) derived from the F2 progeny of this cross show continuous variation in eEF1A concentration that ranges between the parents. To investigate the abundance of the three isoforms in these genotypes, eEF1A was purified from 10-, 15-, and 20-DAP endosperms **(Fig. 2.3)** as previously described. Accumulation of the isoforms during development of Oh545*o2* endosperm was similar to W64A*o2* **(Fig 2.3A).** At 10-DAP, eEF1A26 contributed about 80% of the protein and eEF1A24 contributed 20%; there was no eEF1A22 detectable. At 15-DAP, the proportions of eEF1A26, eEF1A24 and eEF1A22 were about 50%, 10%, and 40%, respectively. At 20-DAP, eEF1A26 contributed about 30% of the protein, eEF1A24 contributed 10%, and eEF1A22 contributed 60%. Thus, eEF1A26 was the most abundant isoform at 10-DAP, while eEF1A22 was the most abundant at 20-DAP. However, only eEF1A26 (with possibly very low levels of eEF1A24) was observed during development of Oh51A*o2* endosperm **(Fig. 2.3B)**. Accumulation of eEF1A isoforms in RILs 31-5 (low eEF1A content) and 34-3 (high eEF1A content) was analyzed only at 20-DAP. In RIL 31-5 endosperm, the proportions of eEF1A26, eEF1A24 and eEF1A22 were about 30%, 20%, and 50%, respectively **(Fig. 2.3C)**; in RIL 34-3, eEF1A26 contributed about 85% of the protein, while eEF1A24 and eEF1A22 contributed only about 10% and 5% of the protein, respectively **(Fig. 2.3D)**. The eEF1A

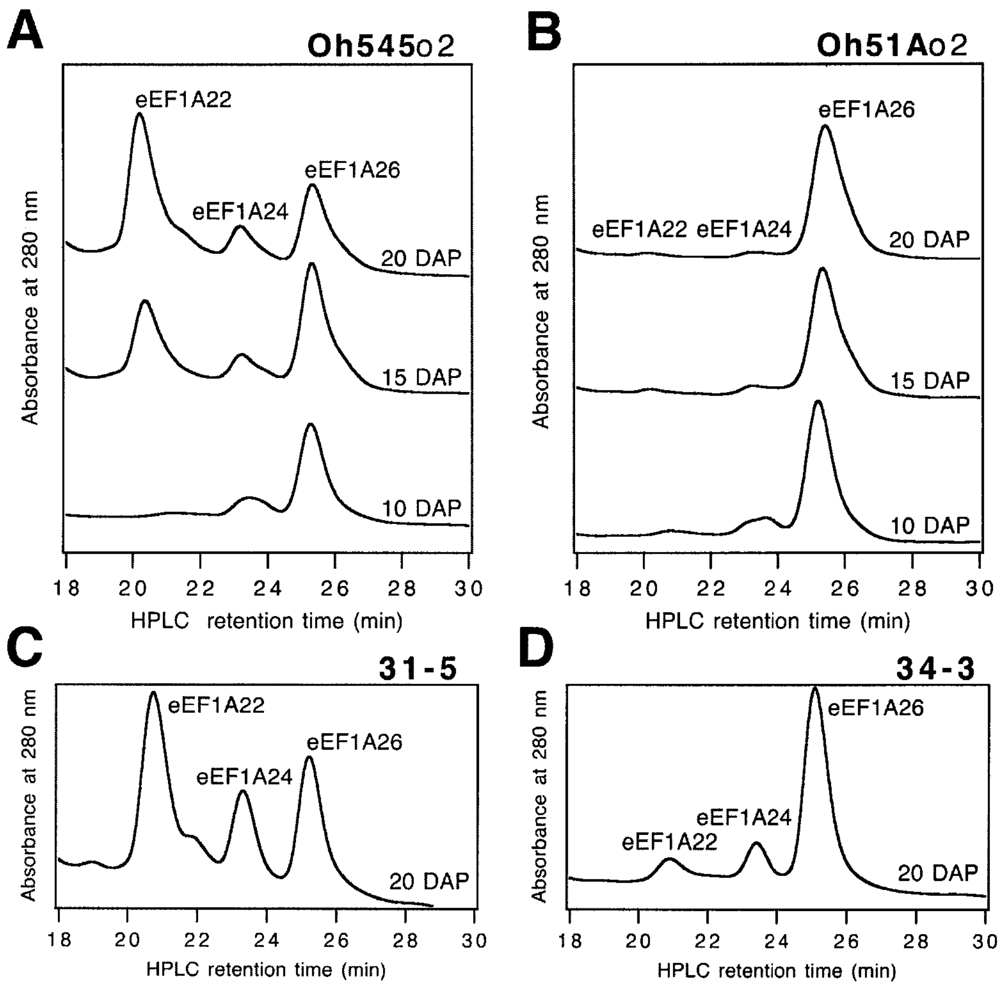


Figure 2.3. Comparison of protein isoforms in low (Oh545*o2*) and high (Oh51A*o2*) eEF1A maize inbreds. eEF1A was purified from endosperm of Oh545*o2*(A), a low eEF1A inbred and Oh51A*o2*(B), a high eEF1A inbred, at 10-, 15- and 20-DAP. eEF1A was purified from 20-DAP endosperm of the recombinant inbred lines 31-5 (C, low eEF1A content) and 34-3 (D, high eEF1A content) derived from a cross between Oh545*o2* and Oh51A*o2*.

profiles of the two RILs resembled those of their phenotypically similar parents at 20-DAP.

eEF1A isoforms differ in their ability to bind F-actin *in vitro*

eEF1A appears to be a multifunctional protein, since several biochemical activities have been reported for this protein in addition to its classic role in the elongation cycle of protein synthesis (Durso and Cyr, 1994a; Gonen et al., 1994; Hotokezaka et al., 2002). The existence of a variety of post-translational modifications of the protein raises the possibility that some of eEF1A's activities are associated with different modifications.

To investigate the functional activities of the isoforms from developing endosperm, we analyzed their ability to interact with rabbit muscle F-actin *in vitro,* using an assay based on that described by Demma et al. (1990). Equimolar concentrations of actin and each of the eEF1A isoforms were incubated in actin polymerization buffer for one h at room temperature. After centrifugation at 200,000*g*, the eEF1A associated with F-actin (pellet) was compared to that remaining soluble (supernatant) by SDS-PAGE **(Fig. 2.4)**. Monomeric actin (G-actin), used as a control, was found mainly in the supernatant (**Fig. 2.4**, lane 2), while filamentous actin (F-actin) was recovered in the pellet (**Fig. 2.4**, lane 3). When eEF1A22 was incubated with actin in polymerization buffer, the amount of eEF1A22 in the pellet (lane 5) did not increase when compared to eEF1A22 alone (lane 7). The amount of eEF1A24 in the pellet with F-actin (lane 9) was 34±5% more than that of eEF1A24 alone (lane 11), while the amount of eEF1A26 in the pellet with F-actin (lane 13) increased 64±5% when compared to eEF1A26 alone (lane 15). These results

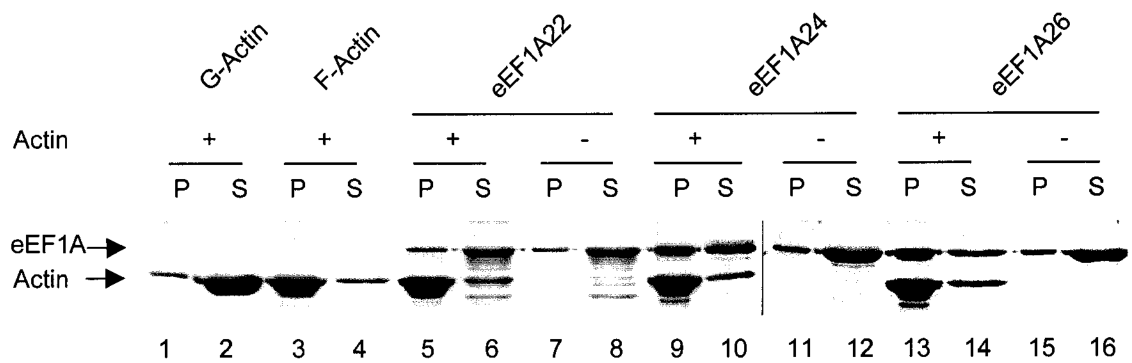


Figure 2.4. F-actin binding activities of eEF1A isoforms. Equimolar amounts of rabbit muscle actin and each of the eEF1A isoforms were incubated in actin polymerization buffer for 1 h at room temperature. After centrifugation at 200,000g, the pellet (P) and supernatant (S) were analyzed by 12.5% SDS-PAGE and stained with Commassie Blue R. As controls, actin and each of the eEF1A isoforms were incubated alone. The eEF1A associated with polymerized actin (P) was compared to that not bound to polymerized actin (S).

suggested the isoforms differ in their ability to associate with F-actin. eEF1A22, the isoform most predominant at later stages of endosperm development (20-DAP), had no apparent F-actin binding activity, while about 64% of eEF1A26, the isoform most predominant at the early stages of endosperm development (11-DAP), binds F-actin. Similar results were obtained when the analysis was conducted using actin purified from maize pollen (data not shown).

eEF1A isoforms share similar participation in ternary complex formation with aminoacylated tRNA (aa-tRNA)

To investigate whether the purified eEF1A isoforms differ in their interaction with aminoacyl tRNA during protein synthesis, the formation of aa-tRNA•eEF1A•GTP ternary complexes was studied and compared against a reference wheat germ eEF1A preparation. The fraction of protein capable of participating in ternary complex formation was assayed in eEF1A•GTP binding experiments conducted in the presence of excess ^{3}H-Gly-tRNA^{Gly}, while binding affinities were measured by determining dissociation constants (K_d) from binding experiments in which ^{3}H-aminoacyl-tRNA was present at 2 to 5 nM and eEF1A•GTP concentrations varied from 0 to 300 nM (Dreher et al., 1999). All three maize endosperm eEF1A fractions were active for ternary complex formation. There were no large differences between the three preparations (8 to 12% active; **Table 2.1**), although rather small fractions of the maize eEF1As were active in ternary complex formation. For unknown reasons, eEF1A preparations commonly are only partially

Table 2.1. Formation of aa-tRNA•eEF1A•GTP ternary complex by various preparations of eEF1A

Source	Active Molecules (%)	Kd Val-tRNA^{Val} (nM)	Kd Gly-tRNA^{Gly} (nM)
Wheat Germ eEF1A	26	3.74 ± 0.46	1.85 ± 0.31
Maize eEF1A22	7.8	3.04 ± 0.32	1.31 ± 0.25
Maize eEF1A24	9.4	2.85 ± 0.40	1.10 ± 0.16
Maize eEF1A26	12.2	3.36 ± 0.38	2.01 ± 0.26

active in these assays; while this wheat germ eEF1A preparation was 26% active, a previous one was only 14% active.

The aa-tRNA binding affinities of the active eEF1A molecules present in each of the maize endosperm preparations were similar and were equivalent to the binding affinity of wheat germ eEF1A (**Fig. 2.5, Table 1**). Dissociation constants of about 1.5 nM and 3 nM were measured for binding to bovine Gly-tRNA^{Gly} and wheat germ Val-tRNA^{Val}, respectively, indicating tight interactions.

Mass spectrometry analysis

To investigate the nature of the eEF1A isoforms as a possible explanation for their chromatographic and functional differences, we used liquid chromatography-coupled tandem mass spectrometry (LC/MS/MS) to determine their amino acid sequences and post-translational modifications. Each of the isoforms was digested with trypsin, Glu-C, and Asp-N proteases, as described in Materials and Methods. The resultant peptides mixtures were separated by liquid chromatography and characterized by two consecutive mass analyzers. Multiple analyses were conducted for each isoform/protease combination.

The peptide patterns obtained for the three isoforms were very similar. Because eEF1A contains about 11% lysine, trypsin generated a complex mixture of peptides, most of which were between 500 and 3,000 daltons. Consequently, trypsin was the most informative protease. Glu-C was the second most informative protease, and its coverage overlapped extensively with trypsin, although it generated some additional peptide

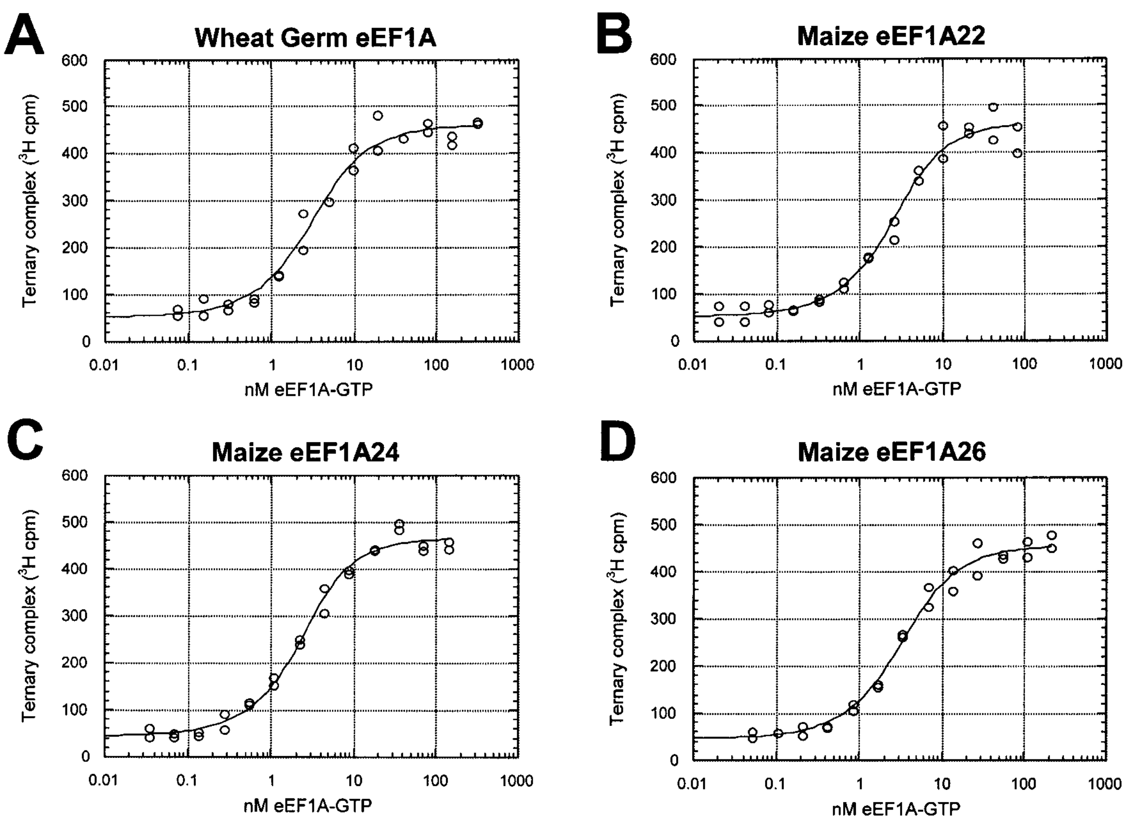
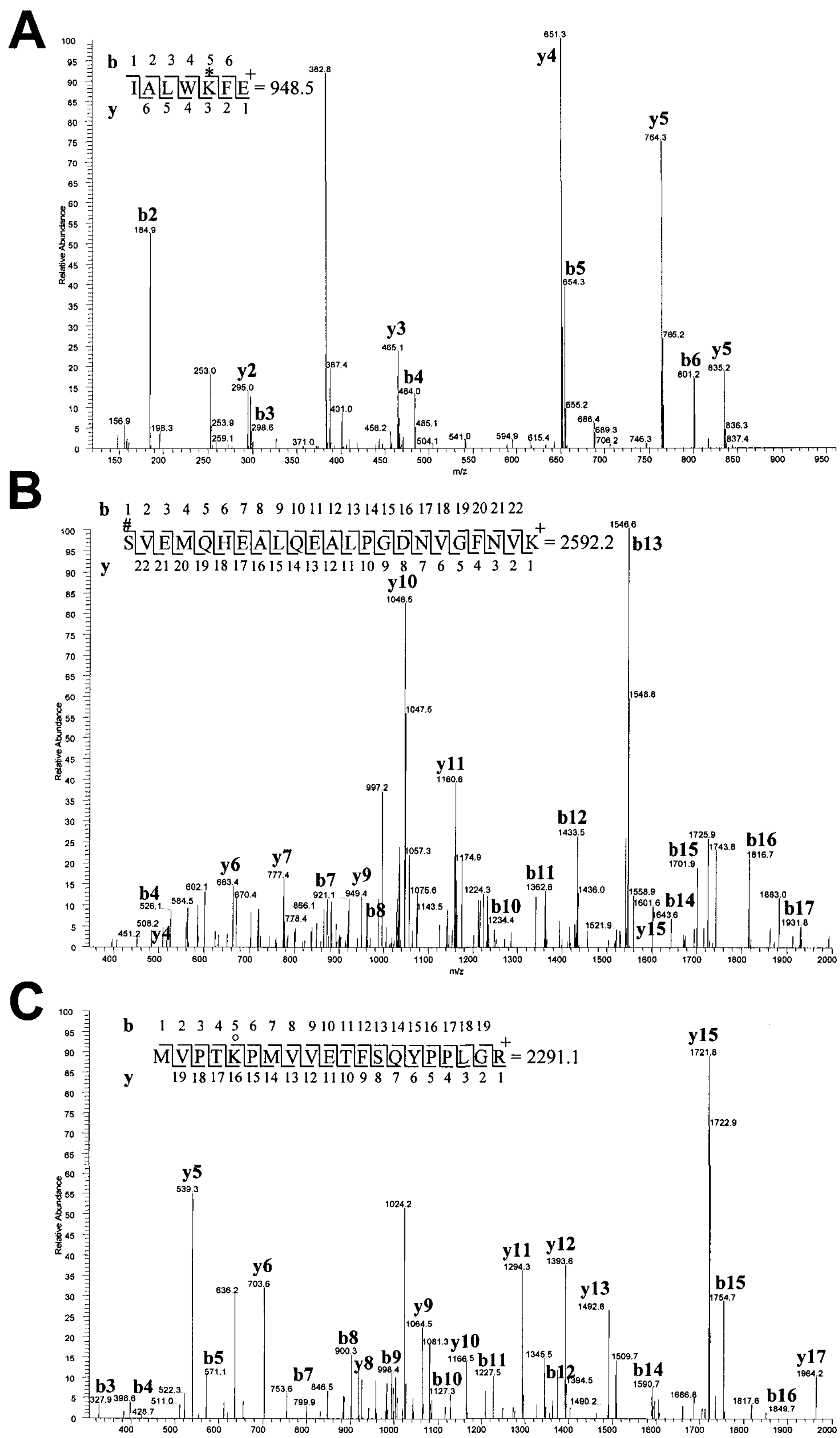


Figure 2.5. Interaction of wheat germ Val-tRNA^{Val} with eEF1A from maize endosperm and wheat germ. Ternary complex formation was estimated by liquid scintillation counting of ^{3}H-Val-tRNA^{Val} bound to paper filters after ribonuclease protection assay (Dreher *et al.*, 1999). The curve fits (KaleidaGraph) were used to generate the K_d values reported in Table 1.

sequences. AspN produced a smaller number of peptides, most of which corresponded to peptide sequences covered by the other two proteases. Each of the peptides was identified by comparison of the fragmentation pattern obtained in the second mass analyzer with that predicted from eEF1A protein sequences using the SEQUEST program (Yates, 1998).

Analysis of the data with SEQUEST, as well as manually, revealed the presence of peptide sequences corresponding to the same four gene products in each eEF1A isoform. This complexity made it difficult to identify chemical differences unique to the purified proteins. Analysis of the data revealed the purified proteins were modified by methylation and phosphorylation, although the same pattern of modifications was found in each of them. **Figure 2.6** shows selected LC/MS/MS spectra of peptides found in the eEF1A26 isoform; however, similar results were obtained for eEF1A22 and eEF1A24. **Figure 2.6A** shows the spectrum of a Glu-C derived peptide of apparent $(M+H)^+$ m/z = 948.5. This value corresponds to the addition of 42 daltons (tri-methylation) to the unmodified peptide "IALWKFE" (calculated $[M+H]^+$ m/z = 906.5), which corresponds to amino acid residues 75-81 (e. g. Genbank accession AAB64207.1). The product ions are consistent with the predicted amino acid sequence of eEF1A, provided that the 42 daltons increment is located on the lysine residue at position 79. The mass difference of 170 daltons between ions y3 and y2 and between ions b5 and b4 corresponds to the mass of lysine plus 42 daltons. **Figure 2.6B** shows the spectrum of a tryptic peptide with apparent $(M+H)^+$ m/z = 2592.2. This value corresponds to the addition of 80 daltons to the unmodified peptide "SVEMQHEALQEALPGDNVGFNVK" (calculated $[M+H]^+$ m/z =

Figure 2.6. LC/MS/MS spectra of peptides identified in the eEF1A26 isoform. eEF1A26 was purified by HPLC and digested with trypsin, Glu-C and Asp-N proteases, as described in Materials and Methods, and the digestion products were analyzed by tandem mass spectrometry (LC/MS/MS). Identification of the peptides and post-translational modifications were conducted by comparison of the fragmentation patterns of individual peptides with those predicted from protein databases using SEQUEST. (A) LC/MS/MS spectrum of a Glu-C derived peptide trimethylated at a lysine residue. (B) LC/MS/MS spectrum of a tryptic peptide phosphorylated at a serine residue. (C) LC/MS/MS spectrum of a tryptic peptide monomethylated at a lysine residue.


A
b 1 2 3 4 5 6
IALWKFE = 948.5
y 6 5 4 3 2 1
Relative Abundance
m/z
y4
y5
b2
b5
y3
y2
b3
b4
b6
B
b 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
SVEMQHEALQEALPGDNVGFNVK = 2592.2
y 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
b13
y10
y11
b12
b15
b16
b4
y6
y7
b7
y9
b8
b11
b10
b14
b17
y4
y15
C
b 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
MVPTKPMVVETFSQYPPLGR = 2291.1
y 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
y15
y5
y6
y11
y12
y13
b15
y9
y10
b8
y8
b9
b5
b7
b10
b11
b12
b14
b3
b4
b16
y17

2512.2), which is predicted by accession AAF42978.1 and corresponds to amino acid residues 279-301. Although the product ion corresponding to the phosphorylated serine residue was not observed, this is perhaps because the peptide is rather long; there is a consistent shift of 80 daltons in the mass of the b ion series. For example, the predicted $(M+H)^+$ *m/z* value for the unmodified ion, b13, is 1466.6, while that observed in Figure 6B is 1546.6. **Figure 2.6C** shows the spectrum of a tryptic peptide of apparent $(M+H)^+$ *m/z* = 2291.1. This value corresponds to the addition of 14 daltons (mono-methylation) to the unmodified peptide "MVPTKPMVVETFSQYPPLGR" (calculated $[M+H]^+$ *m/z* = 2277.1), which corresponds to amino acid residues 392-411 (e. g. accession AAB64207.1). The product ions are consistent with the predicted amino acid sequence of eEF1A, provided that the 14 daltons increment is located on the lysine residue at position 396. The mass difference of 142 daltons between ions b5 and b4 corresponds to the mass of lysine plus 14 daltons. On the other hand, ion y16 was not observed and therefore cannot be compared with y15. However, the *m/z* of ion y17 corresponds to an addition of 14 daltons to the calculated *m/z* of ion y17 in the unmodified peptide. Analyses with eEF1A22 and eEF1A24 produced results similar to these. While it was possible to identify specific peptides and modifications, none were found to be unique for a given eEF1A isoform.

Figure 2.7 shows the amino acid sequences of the eEF1A proteins identified in each of the isoforms and their post-translational modifications. Highlighted sequences correspond to peptides differing between the gene products, which in combination with the post-translational modifications allowed us to distinguish between the protein

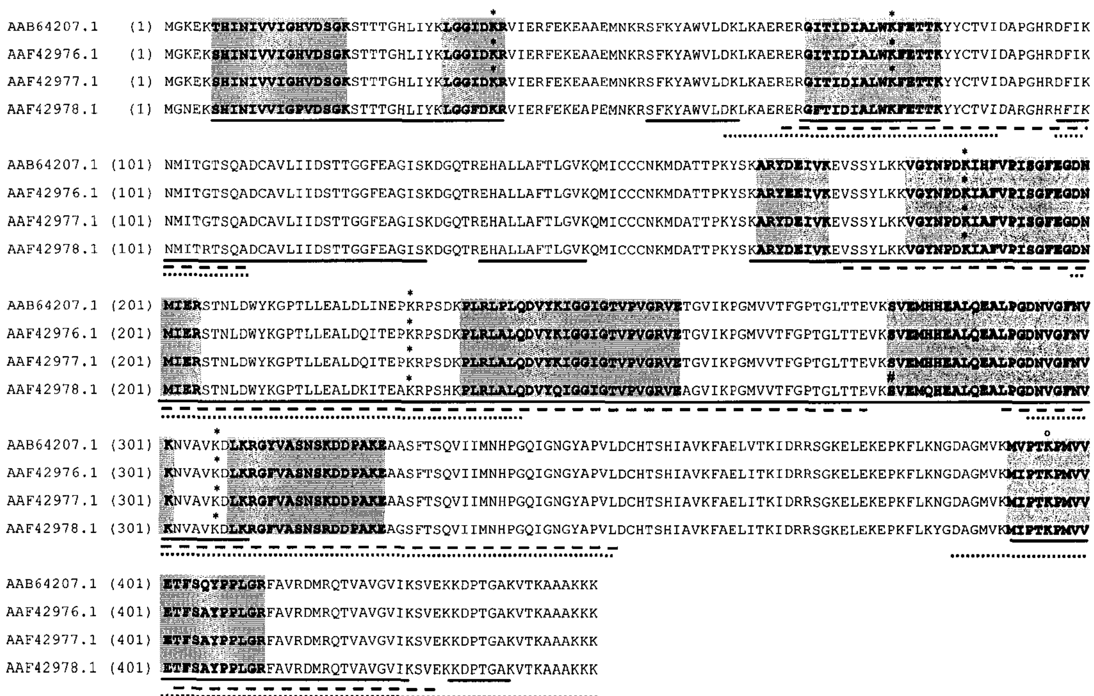


Figure 2.7. Tandem mass spectrometry analysis of eEF1A isoforms. eEF1A isoforms purified by HPLC were digested with trypsin, Glu-C and Asp-N proteases, as described in Materials and Methods. The digestion products were analyzed by tandem mass spectrometry (LC/MS/MS). Four eEF1A gene products were identified, all present in each of the three isoforms. Solid lines indicate the amino acid sequences obtained by trypsin digestion, while dashed and dotted lines indicate the sequences obtained by Glu-C and AspN digestion, respectively. Peptides distinguishing the four proteins are highlighted; the post-translational modifications identified are designated as follows: (°) monomethylation, (*) trimethylation and (#) phosphorylation.

sequences. For example, the amino acid sequence of the phosphorylated peptide spanning residues 279-301 is unique to gene product AAF42978.1. Similarly, the amino acid sequence of the mono-methylated peptide spanning amino acids 392-411 is specific for AAB64207.1, as well as the peptide spanning amino acids 6-20. AAF42976.1 contains a glutamic acid residue at position 168 while the other gene products contain aspartic acid at this position, and both amino acid sequences were identified in the analysis. This is the only amino acid difference between AAF42976.1 and AAF42977.1; therefore AAF42977.1 was also included. The amino acid sequence coverage obtained with trypsin (solid line) was about 60%, followed by Glu-C (dashed line) with 50%, and AspN (dotted line) with about 40%. The combined amino acid sequence coverage obtained with the three proteases was about 80%.

The eEF1A isoforms were highly modified **(Fig. 2.7)**. Lysine residues at positions 36, 79, 187, 227, and 306 were tri-methylated, while lysine at position 396 was mono-methylated. Since tri-methylated lysines were found in peptides of identical sequence for at least three of the four proteins, they could not be assigned to a specific gene product. Tri-methylated lysines at positions 36 and 79 were not found in AAF42978.1. Mono-methylated lysine appeared to be specific for AAB64207.1. Serine at position 279 was phosphorylated specifically in AAF42978.1. We did not identify glycerylphosphoryl-ethanolamine modifications in glutamic acid at position 285, the site of modification reported in carrot eEF1A (Ransom et al., 1998). From the amino acid sequence coverage obtained in our LC/MS/MS analysis (~80%), it was not possible toidentify isoform-specific sequences or post-translational modifications that could be correlated with or explain the functional differences of the eEF1A isoforms.

DISCUSSION

The ability to separate maize endosperm eEF1A into three chromatographic forms **(Figs. 2, 3)** suggests the existence of chemical diversity in amino acid sequence, post-translational modification, or both. At least five eEF1A genes producing proteins with minor sequence differences are expressed in maize endosperm (Carneiro et al., 1999), and diversity is also documented at the post-translational level. The functional significance of these modifications is poorly understood. Phosphorylation of eEF1A has been associated with increased translational activity (Venema et al., 1991), activation of PI-4K (Yang and Boss, 1994) and decreased F-actin binding *in vitro* (Izawa et al., 2000). In the fungus *Mucor racemosus*, methylation of eEF1A was associated with an increase in protein synthetic activity after sporulation (Fonzi et al., 1985). This methylation did not affect the affinity of eEF1A for GTP, aminoacyl-tRNA and ribosomes, although it may affect the ability of eEF1A to form complexes with other EF1 subunits (Sherman and Sypherd, 1989). In yeast, mutation of methylation sites in eEF1A did not affect its ability to promote protein synthesis *in vitro* (Cavallius et al., 1997). Two isoforms from rabbit, eEF1A-1 and eEF1A-2, showed similar patterns of glycerylphosphoryl-ethanolamine, but two of the lysine residues dimethylated in eEF1A-1 were tri-methylated in eEF1A-2 (Kahns et al., 1998; Kristensen et al., 1998). These forms were indistinguishable with regard to *in vitro* protein synthesis activity, although the GDP dissociation rate constant was higher for eEF1A-1 than eEF1A-2. Nevertheless, the functional significance of these isoforms of eEF1A is unclear.

The changes we observed in the relative abundance of the three different eEF1A isoforms W64A*o2* and W64A+ during endosperm development **(Fig. 2.2)** suggest their accumulation is developmentally regulated. Although the total amount of eEF1A is higher in W64Ao2, this does not appear to be a consequence of the accumulation of a specific isoform. Together with the qualitative differences observed between high and low eEF1A genotypes **(Fig. 2.3)**, these results also suggest the isoforms have different functions, which is supported by their different actin binding activities **(Fig. 2.4)**.

In order to explain why the purified eEF1A isoforms differ in their affinity for F-actin, we investigated their chemical properties by mass spectrometry. LC/MS/MS analysis identified the same four gene products in each of the purified isoforms **(Fig. 2.7)**, suggesting they have different patterns of post-translational modifications. We found the isoforms are modified by methylation and phosphorylation **(Fig. 2.7)**. Only tri-methylation of lysines at positions 36, 79 and 306 have been reported in other organisms, although only that at position 79 appears to be conserved (Cavallius et al., 1993). Mono-methylated lysine at position 396 and phosphorylation of serine at position 279 have not been reported before. In contrast to the amino acid sequence of eEF1A, it is well documented that post-translational modifications of the protein are not highly conserved.

The presence of multiple gene products in each of the isoforms made the peptide analysis complex, and made it difficult to identify isoform-specific modifications that could distinguish between them. In addition to conducting the protease digestions multiple times, these experiments were also done in the presence of 2 to 4 M urea. However, these treatments did not allow us to recover any new peptides that increased the protein sequence coverage, which was about 80%. Peptide fingerprinting by MALDI-TOF also failed to distinguish between the isoforms. It is unlikely this was due to cross contamination of the isoform peaks during purification, because products similar to those obtained from eEF1A26 of W64A*o2* were recovered when eEF1A26 was purified from Oh51A*o2*. eEF1A26 was the only isoform observed during development of Oh51A*o2* endosperm **(Fig. 2.3)**.

Phosphorylation of eEF1A has been suggested to interfere with its actin binding activity *in vitro* (Izawa et al., 2000). eEF1A26, the isoform that binds actin most efficiently, could be phosphorylated *in vitro* by a calcium dependent protein kinase (CDPK) four times more efficiently than eEF1A24 and eEF122, but this phosphorylation did not appear to affect the *in vitro* actin binding activity of the protein (data not shown). Phosphorylation has also been associated with the ability of eEF1A to be a functional activator of phosphatidylinositol-4 kinase (PI-4K)(Yang et al., 1993; Yang and Boss, 1994). In collaboration with Dr. Wendy Boss at North Carolina State, we analyzed the ability of the isoforms to activate PI-4K and found no significant differences between them, even though they differ in their phosphorylation levels when treated with a CDPK (data not shown).

As a further characterization of the eEF1A isoforms, they were compared with wheat germ eEF1A for the formation of aa-tRNA•eEF1A•GTP ternary complexes. The isoforms had similar aa-tRNA binding affinities **(Fig. 2.5, Table 1)**, suggesting the nature of their chemical differences had no significant effect on their interaction with aminoacyl tRNA during protein synthesis. Although we did not measure protein synthesis activity, these results are in agreement with other studies reporting similar *in vitro* protein synthesis activities for eEF1A proteins from yeast and rabbit, even though they are only about 80% identical and have different patterns of methylation and glycerylphosphorylethanolamine modifications (Merrick et al., 1990; Cavallius et al., 1993).

At present is not clear why there are multiple eEF1A isoforms in maize endosperm. In addition to the qualitative and quantitative differences in their accumulation during endosperm development, the only apparent biological difference we found is their F-actin binding activity. eEF1A from rat metastatic cells had a 10 to 20% reduction in the binding affinity to F-actin *in vitro* when compared to eEF1A from nonmetastatic cells (Edmonds et al., 1996). Both eEF1A proteins had similar protein synthesis activities and the different eEF1A activities with F-actin were proposed to be important for cytoskeletal organization and differential translation of mRNAs associated with the cytoskeleton. A similar situation may occur in maize endosperm. The isoform that binds actin most efficiently was predominantly accumulated in endosperm of high eEF1A genotypes **(Fig. 2.3)**. This may be related to enhanced cytoskeleton formation, and therefore increased synthesis of cytoskeleton- associated proteins, which are presumably lysine rich (Habben et al., 1995).

CHAPTER 3

GENE EXPRESSION PROFILING OF HIGH AND LOW EEF1A GENOTYPES REVEALS COORDINATED CHANGES IN TRANSCRIPTS ENCODING CYTOSKELETAL PROTEINS

INTRODUCTION

The discovery that the *opaque2* (*o2*) mutation almost doubles the lysine content of maize endosperm (Mertz et al., 1964) led to extensive efforts to develop genotypes with better nutritional quality for human and animal consumption. Unfortunately, the inferior agronomic traits associated with this mutation, reduced yield and protein content, soft kernels, and pathogen susceptibility, made it difficult to develop *o2* in breeding programs (Glover and Mertz, 1987). To overcome these problems, maize breeders at CIMMYT, Mexico (Villegas et al., 1992), and the University of Natal, South Africa (Gevers and Lake, 1992), developed modified *o2* mutants called Quality Protein Maize (QPM). These genotypes combine the nutritional quality of *o2* with a hard, vitreous endosperm and normal yield. However, additional improvement of the protein quality is required for QPMs to meet the minimal lysine content (5 mg /100 mg of protein) recommended for human diets (Young et al., 1998).

Several studies have documented a broad range of variability in lysine content among normal, *o2* and modified *o2* maize genotypes (Moro et al., 1996; Zarkadas et al., 2000), showing the effect of the mutation is highly dependent on the genetic background. These studies suggested the lysine content of the kernel can be improved by appropriate selection. However, identification of more nutritional maize genotypes has been limited by knowledge of the genetic and biochemical basis for lysine accumulation in the endosperm. *O2* encodes a basic domain/leucine zipper (bZIP) transcription factor that regulates the expression of a number of genes, including the highly abundant α-zeins (Kodrzycki et al., 1989; Schmidt et al., 1990). Defects in O2 lead to a significant reduction in α-zein synthesis and a pleiotropic increase in the synthesis of several lysine-rich, non-zein proteins (Damerval and Devienne, 1993; Habben et al., 1993). Both of these effects contribute to the increased lysine content of the mutant (Moro et al., 1996), but the molecular mechanisms responsible for the larger amount of lysine-rich proteins are unknown.

Habben et al. (1993) investigated the nature of the increased lysine-containing proteins in *o2* and found that elongation factor 1A (eEF1A), a protein synthesis factor, is highly increased compared to wild type. Analysis of a large number of normal and *o2* inbreds with extensive variability in lysine and eEF1A content showed this protein is highly correlated (r=0.9) with the protein-bound lysine of the endosperm (Habben et al., 1995; Moro et al., 1996). However, the biological basis of this relationship is unclear. eEF1A contains 11% lysine, but it only accounts for 2.3% of the lysine in W64A*o2* endosperm (Sun et al., 1997). This suggests a stochiometric relationship exists between eEF1A and the other major proteins contributing to the lysine content of the grain.

One possible explanation for the relationship between eEF1A and other lysine-rich proteins in maize endosperm is that eEF1A is a component of the cytoskeleton (Durso and Cyr, 1994). Biochemical characterization of eEF1A from maize endosperm revealed this protein binds F-actin (Sun et al., 1997; Lopez-Valenzuela et al., 2003), and immunolocalization showed eEF1A is associated with an F-actin network that surrounds the rough ER (RER) at sites where protein bodies are forming (Clore et al., 1996). Wang et al. (2001) found a QTL linked with eEF1A content that mapped with a cluster of 22-kD α-zein genes. Based on this association, they proposed a positive correlation between eEF1A content and the surface area of protein bodies, hence the network of cytoskeletal proteins surrounding the RER in the endosperm. Thus, the level of eEF1A may reflect the amount of proteins that are components of the cytoskeleton in maize endosperm.

This study was initiated to identify proteins that vary in abundance in parallel with eEF1A. The inbred lines Oh51A*o2* (high eEF1A) and Oh545*o2* (low eEF1A), as well as high and low eEF1A recombinant inbred lines (RILs) derived from their cross, were used for mRNA transcript profiling with cDNA microarrays and for ELISA measurement of endosperm proteins. The results of this study revealed a number of genes that are coordinately regulated with eEF1A content, several of which encode proteins associated with the cytoskeleton and the ER, as well as components of the protein synthesis machinery.

MATERIALS AND METHODS

Plant materials

The maize inbred lines Oh51A*o2* (high eEF1A content) and Oh545*o2* (low eEF1A content) were obtained from Crow's Hybrid Corn Company, Milford, IL. F1 and F2 progeny were created from a cross between these two inbreds (Wang et al., 2001), and the recombinant inbred lines (RIL) were created by single seed descent from the F2 progeny. Parental inbreds and RILs were grown at the University of Arizona West Agricultural Center in Tucson, AZ, and at the University of Florida, Gainesville, FL. Developing kernels were harvested at 18-DAP, frozen in liquid nitrogen and stored at –80°C until use. Prior to RNA extraction, the embryo and pericarp were removed by hand dissection and the endosperm placed in liquid nitrogen.

RNA purification and labeling

Total RNA was extracted from 18-DAP developing endosperms. Frozen endosperms were ground with a mortar and pestle and the powder was homogenized in one volume of NTES buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% [w/v] SDS) and one volume of phenol/chloroform saturated with Tris pH 8.0. This extraction buffer allowed the precipitation and removal of starch, which is highly abundant in 18-DAP endosperm. Following centrifugation, nucleic acids were precipitated from the aqueous phase with three volumes of ethanol and resuspended in DEPC water. RNA was precipitated with three volumes of 4M LiCl at –20°C for 4 h, resuspended in DEPC water and purified using TRIzol (Invitrogen, Carlsbad, CA). Poly$(A)^+$ RNA was purified from total RNA using a Qiagen oligotex mRNA kit (Qiagen, Valencia, CA). RNA quantity was estimated by uv absorption at 260 nm, and its quality was assessed by visual inspection following agarose gel electrophoresis. For synthesis of fluorescently-labeled cDNA targets, 2 μg of poly $(A)^+$ RNA was first hybridized to 1 μg oligo-(dT) primer at 65°C for 10 min. The reaction was completed by addition of 1X reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 500 μM dNTPs (dATP, dCTP, dGTP), 100 μM dTTP, 50 μM Cy3- or Cy5-dUTP, 10 mM DTT, and 400 units Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), followed by incubation at 42°C for 90 min.

Microarray hybridization and analysis

Glass slides of the maize endosperm 605.03 microarray were obtained from the Maize Gene Discovery Project (Fernandes et al., 2002). A detailed description of the array design can be found at http://zmdb.iastate.edu/zmdb/microarray. Hybridization was conducted following the protocols described by Fernandes et al (2002) and the details available at http://zmdb.iastate.edu/zmdb/microarray/protocols.html. The slides were analyzed with a ScanArray 3000 scanner (GSI Lumonics, Watertown, MA) for both channel 1 (Cy3) and 2 (Cy5) at 10-μm resolution. Photomultiplier settings were adjusted manually to reduce the percentage of spots on the array with saturated signal values and to obtain a Cy3:Cy5 signal ratio close to one for the control genes. The image files obtained were analyzed using Imagene software (Biodiscovery, Inc., Marina del Rey, CA). Quality control measurements produced by the Imagene software were used to ensure that only spots of good quality were used in the analysis. In addition, spots that hybridized unevenly were flagged manually and excluded from the analysis. Further analysis of the data was conducted using Microsoft Excel (Microsoft, Redmond, WA). Net signal intensity was computed from the median signal minus the local background, minus the signal background (mean of the local background for every spot plus two standard deviations). Very low or negative net signal values were set to a minimum positive equal to the signal background. To normalize the signal intensities, the intensities of the 27-kD γ-zein (glutelin-2) gene probes in one channel (genotype 1) were compared with the corresponding intensities in the second channel (genotype 2) on the same slide. The slope of the trend line was used for the normalization of all the elements on the slide.

For each high *versus* low eEF1A genotype comparison, we used two slides, one with direct and one with reverse labeling. Ratios from reverse labeling were inverted to compare with the ratios from direct labeling. Therefore, for all slides, ratios >1 and <1 indicated increased and decreased mRNA levels, respectively, in the high eEF1A *versus* low eEF1A genotypes. An average and standard deviation of the ratios from eight replicates were determined and used as final ratio of mRNA levels.

RNA blot hybridization

RNA (10 μg of total RNA per sample) was separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde (Lehrach et al., 1977) and blotted onto nylon membranes (MSI, Westboro, MA). DNA probes were obtained by labeling with α-^{32}P-dCTP using the random prime method, according to the manufacturer (Invitrogen, Carlsbad, CA). EST-specific cDNAs were obtained from the corresponding 605 EST clones by PCR amplification as described by Fernandes et al (2002) and their identity confirmed by partial sequencing. Primer sequences used for amplification of the 605 EST clones were as follows: forward 5'-CTGCAGTAATACGACTCACTATAG- 3' and reverse 5'- CTATTCGATGATGAAGATACC- 3'. The cDNAs corresponding to zeins, glyceraldehyde 3-phosphate dehydrogenase (*GAPC2*), and granule-bound starch synthase (*Waxy1*) were obtained by PCR using gene-specific primers. Amplified DNA products were analyzed by agarose gel electrophoresis and purified using the GeneClean III kit from Amersham (Piscataway, NJ). The RNA gel blots were hybridized using the indicated ^{32}P-labeled probes. As a loading control, the blots were hybridized with a ^{32}P-labeled cDNA corresponding to the 27-kD γ-zein. Hybridizations were performed at 65°C in a solution containing 1.5X SSPE, pH 7.7, 6% PEG 8000, 1% SDS, 1% non-fat dried milk (Carnation), 0.1% (v/v) DEPC, and 30 μg mL^{-1} freshly denatured salmon sperm DNA (Sabelli and Shewry, 1993). After hybridization, the blots were washed once with 1X SSC and 0.1% SDS at room temperature, then twice with 1X SSC and 0.1% SDS for 15 min at 65°C, and then a final wash with 0.1X SSC and 0.1% SDS for 15 min at 65°C. Quantification of hybridization signals was obtained using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Blots were stripped between hybridizations with boiled 0.5% SDS, followed by a 20 min incubation at 65°C.

Zein and non-zein protein extraction, quantification and SDS-PAGE analysis

Protein fractions were obtained from mature endosperms or lyophilized, developing endosperms as described by Wallace et al. (1990). Fifty milligrams of flour was extracted overnight with 1 mL of borate buffer (12.5 mM $Na_2B_4O_7$, 1% [w/v] SDS, 2% 2-mercaptoethanol, pH 10) at 37°C, and the soluble proteins were partitioned into zeins (supernatant) and non-zeins (pellet) by the addition of absolute ethanol to a final concentration of 70% (v/v). Zein proteins (equivalent to an extract from 15 mg of flour) were lyophilized and resuspended in 100 μL of deionized water. Non-zein proteins obtained from the same amount of flour were resuspended in 100 μL of 8 M urea.

The concentration of zein proteins was determined by the Bicinchoninic acid (BCA) method (Brown et al., 1989), using the BCA protein assay reagents from Pierce (Rockford, IL). The concentration of non-zein proteins was determined according to Bradford (1976), using the Bradford protein assay reagent from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin (BSA) was used as a standard for both zein and non-zein quantification. The results reported (mg of protein per 100 mg of flour) correspond to the average of two independent experiments in which three measurements were taken. Five microliters of the original zein extract were used for analysis with 12.5% (w/v) SDS-PAGE and the gel was stained with Coomassie Blue R.

ELISA measurements

Endosperm protein extraction and zein/non-zein fractionation were performed as described by Wallace et al. (1990). ELISA of eEF1A was similar to that described by

Habben et al. (1995), Moro et al. (1996) and Wang et al. (2001). Total endosperm protein extract was diluted 1,000-fold in carbonate coating buffer (CCB; 0.1 M $Na_2CO_3/NaHCO_3$, pH 9.6), and 150 μL of the sample was loaded in the well of an ELISA plate (Immulon2, Dynatech Laboratories, Inc., Chantilly, VA). A multi-channel pippette was used to mix 50 μL of the sample with 100 μL of CCB to make four three-fold dilutions into adjacent wells containing CCB. The protein was allowed to bind to the plate overnight at 4°C. Subsequently, the wells were washed twice using PBS plus Tween-20 (PBST, 0.05 M phosphate, 145 mM NaCl, 0.05% [v/v] Tween-20, pH 7.4) and 100 μL of rabbit eEF1A antiserum (Habben et al., 1995) diluted 1:1000 in PBST was added and incubated for 3 h. After incubation, the primary antibody was removed, the wells were washed twice with PBST, and the secondary antibody, goat anti-rabbit IgG alkaline conjugate (Sigma Chemical Co., St. Louis) diluted 1:1000 in PBST, was added and allowed to bind for 2 h. After removal of the secondary antibody, the wells were washed twice with PBST and 200 μL of the alkaline phosphatase substrate (Sigma) diluted in diethanolamine buffer (50mM diethanolamine, pH 9.8) was added. The color reaction was allowed to develop for 30-45 min, and the absorbance was read at 420 nm with a micro plate reader (MRX, Dynatech).

An ELISA to measure actin, profilin and α-tubulin was performed as for eEF1A, but with some modifications. Non-zein pellets from 15 mg of flour were resuspended in 100 μL of 8 M urea. The non-zein protein solution was diluted 2,500-, 4,000- and 10-fold in CCB for actin, profilin and α-tubulin, respectively. Two hundred microliters of the samples were loaded in the well of the ELISA plate and 100 μL were mixed with 100 μL of CCB to make four two-fold dilutions into adjacent wells. The rest of the procedure was similar to that described for eEF1A. The primary antibodies were from rabbit antiserum produced against actin and profilin from maize pollen (Gibbon et al., 1998; Gibbon et al., 1999) and recombinant α-tubulin of human origin (Santa Cruz Biotechnology, Inc.); they were diluted 1:500, 1:500 and 1:50, respectively, in PBST.

The ELISA for α-zein was similar to the procedure described by Moro et al. (1996), with some modifications. Following the initial extraction of endosperm flour, the protein extract was diluted 20,000-fold and used to make four two-fold dilutions into adjacent wells of the ELISA plate. The dilution and antigen binding steps were done using a 40% (v/v) ethanol, 10% (v/v) acetic acid solution (Wallace et al., 1990) and the primary antibody (rabbit anti-α-zein) dilution was 1:2000 in PBST. The range of protein concentration for all the ELISA assays was such that the relationship between absorbance and relative antigen concentration was linear, and a regression analysis was performed. The slope of the regression is proportional to the antigen concentration, and it was used to measure the relative protein content. The results were normalized to the values of the Oh545*o2* inbred and represent the average of two independent experiments in which three measurements were taken.

RESULTS

Development of recombinant inbred lines with high and low eEF1A content

To monitor patterns of gene expression and protein synthesis in high and low eEF1A genotypes, we developed a set of 75 recombinant inbred lines (RIL) by single seed descent from a cross between Oh51A*o2*, a high eEF1A inbred, and Oh545o2, a low eEF1A inbred. After six generations of self-pollination, each of the inbred lines appeared to be homogeneous based on phenotypic uniformity in the field. The level of eEF1A in these lines at the F6 generation was measured by ELISA and found to exist in a two-fold concentration range, similar to that observed in the F3 (Wang et al., 2001). The variation in eEF1A content of these genetically fixed and related lines allowed us to study gene expression patterns and their relationship to endosperm lysine content in *o2* mutants.

The parental inbreds, Oh545*o2* and Oh51A*o2*, and a subset of 10 RILs representing the range of eEF1A variation were first compared for total zein and non-zein protein content in mature endosperm. Protein extracts from equal amounts of endosperm flour of each genotype were partitioned into zein and non-zein fractions (Wallace et al., 1990). **Figure 3.1A** illustrates a Comassie blue-stained gel showing the nature and relative abundance of various zein proteins in the parental inbreds and the RILs. The accumulation of subfamilies of zein proteins is quite different between Oh545*o2* and Oh51A*o2*: the amount of 19- and 22-kD α-zeins is much lower in Oh545*o2* than Oh51A*o2* (**Fig. 3.1A**), and this appears to account for most of the 50% decrease in total

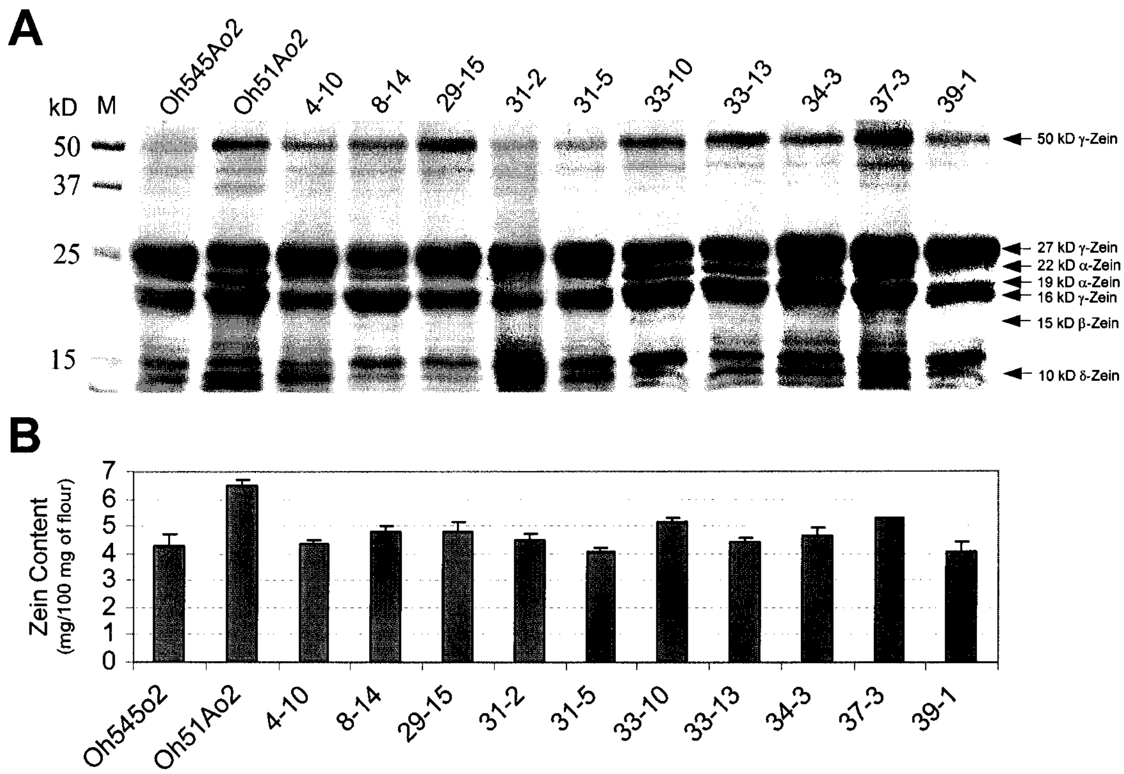


Figure 3.1. SDS-PAGE and quantification of zeins in mature endosperm of Oh545*o2*, Oh51A*o2*, and 10 RILs with high and low eEF1A contents. (A) Total zein extracts were prepared from 750 μg of endosperm flour of the indicated genotypes, separated by 12.5% (w/v) SDS-PAGE, and stained with Coomassie blue R. Pre-stained molecular mass standards are shown on the left. (B) Total zein content was estimated using BCA reagent and BSA as standard. The values are the average of two independent extractions in which three measurements were taken.

zein content in this inbred compared to Oh51A*o2* (**Fig. 3.1B**). The amount of 50-kD γ-zein is less in Oh545*o2* than Oh51A*o2*, while the amount of 27-kD γ-zein appears to be slightly greater in this inbred compared to Oh51A*o2*. The relative abundance of 16-kD γ-zein could not be estimated, because this protein migrated too close to the 19-kD α-zeins in the gel. The abundance of 15-kD β-zein was too low to be compared among these inbreds. The 10-kD δ-zein appears to be less abundant in Oh545*o2* than Oh51A*o2*.

A diverse pattern of zein accumulation was observed in the RILs developed from the Oh51A*o2* by Oh545*o2* cross (**Fig. 3.1A**), but some of these lines closely resembled the parental inbreds. For example, the zein profiles of RILs 4-10, 31-5, and 39-1 are very similar to that of Oh545*o2* (**Fig. 3.1A**), and the amount of total zein in these lines is also similar to Oh545*o2* (**Fig. 3.1B**). On the other hand, the zein profiles of RILs 34-3 and 37-3 are qualitatively similar to that of Oh51A*o2* (**Fig 3.1A**), but the amount of total zein in these lines is 20 to 25% less than in Oh51A*o2* (**Fig. 3.1B**). This is mainly because of a reduction in α-zeins, although there appeared to be a slight increase in the synthesis of the 27-kD γ-zein (**Fig. 3.1A**).

We measured the non-zein content in endosperms of these inbreds using the Bradford assay, and the eEF1A content by ELISA (**Fig. 3.2**). There is about two-fold more non-zein protein in Oh51A*o2* (5.7 mg/100 mg of flour) than Oh545*o2* (2.8 mg/100 mg of flour) (**Fig. 3.2A**). The non-zein content of the RILs ranged from about 2.5 mg per 100 mg of flour in 31-5 to about 4.5 mg per 100 mg of flour in 34-3 (**Fig. 3.2A**). The pattern of eEF1A accumulation in the parental inbreds and the RILs (**Fig. 3.2B**) was similar to

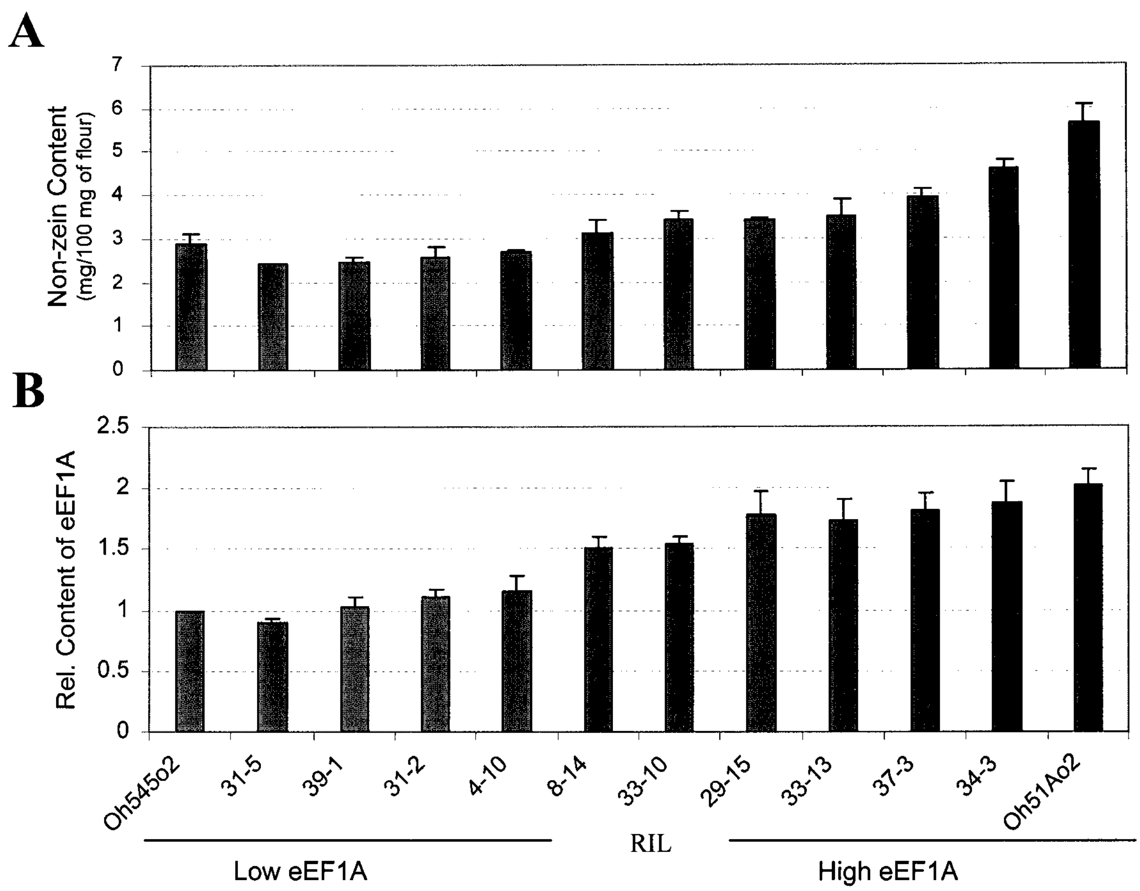


Figure 3.2. Non-zein quantification and ELISA measurement of eEF1A content in mature endosperm of Oh51A*o2*, Oh545*o2* and 10 RILs with high and low eEF1A content (see Fig. 3.1). Protein extracts were prepared from endosperm flour as described in Materials and Methods. (A) Non-zein content was estimated using Bradford reagent and BSA as standard. The order of samples is the same as that identified in B. (B) ELISA measurement of eEF1A was conducted as described in Materials and Methods, and the results were normalized to the values of the Oh545*o2* inbred. In both (A) and (B), the results are the average of two independent extractions in which three measurements were taken.

that observed for non-zein content (**Fig. 3.2A**). The difference in eEF1A content between Oh545*o2* and Oh51A*o2* is about two-fold, and the range of eEF1A contents in the RILs was also about two-fold. As with non-zein content, the lines with the lowest and highest eEF1A content were RILs 31-5 and 34-3, respectively (**Fig. 3.2B**). It is worth noting that these two lines also have zein profiles similar to those of the corresponding low and high eEF1A parents (**Fig 3.1A**).

Microarray analysis

The pattern of gene expression in selected high and low eEF1A inbreds was monitored using an endosperm cDNA microarray from the Maize Gene Discovery Project (Fernandes et al., 2002). Poly(A)$^+$ RNA was extracted from 18 days-after-pollination (DAP) endosperms of the high eEF1A inbred lines, Oh51A*o2* and RIL 34-3, and the low eEF1A inbreds, Oh5454*o2* and RIL 31-5. This stage of development (18-DAP) was chosen because the majority of endosperm cells are differentiated and have high levels of gene expression, as reflected by large amounts of starch and storage protein synthesis (Demason, 1997). For each genotype, RNA samples were prepared from two different ears to account for biological variability. Following poly(A)$^+$ RNA isolation, cDNA was synthesized and labeled with Cy3- and Cy5-dUTP. For comparison of each of the high and low eEF1A inbreds, we used two microarray slides with reciprocal labeling, for a total of four slides. DNA microarray hybridization data were normalized as described in Materials and Methods and the ratios of the fluorescence intensities of the two probes were determined. The average ratio from the replicates was taken and used for comparative analysis. We only considered probes where the ratio was consistent for at least three of the four replicate slides. All the replicate slides were analyzed in parallel and only the probes with an average intensity ratio >2.0-fold or <0.5-fold were considered.

We initially identified about 450 probes that showed marked differences in gene expression levels, of which about 200 corresponded to zein sequences, mainly 19-kD α–zeins. The 605 array is not normalized, and consequently these probes are highly redundant. A significant amount of redundancy was also found for a number of other genes. This array contains about 2,800 tentative unique genes (TUGs) among 5,534 ESTs (Fernandes et al., 2002). However, the probe redundancy allowed us to accurately document some measurements, because in many cases different ESTs of the same gene showed similar changes in expression levels. In some cases, multiple ESTs corresponding to the same gene showed slightly different expression ratios, which may have resulted from differences in hybridization efficiency due to variable target/probe length and overall base-pair composition (Xu et al., 2001). After correction for redundancy, about 110 genes encoding proteins of known function were found to be differentially expressed, the majority of which are up-regulated in high eEF1A genotypes.

Table 3.1 shows a summary of the genes that change expression in parallel with eEF1A transcript abundance. The functional classification of these genes is based on their identification by BLAST, which corresponded to the most similar sequences in Genbank. Proteins encoded by these genes include several structural and metabolic proteins, such as enzymes involved in amino acid, carbohydrate, and cell wall metabolism, as well as

Table 3.1. **Summary of microarray results**

		Oh51A*o2*/Oh545*o2*		34-3/31-5	
EST ID	**Blast Name**	**Average**	**SD**	**Average**	**SD**
Amino acid metabolism					
AI673901	Acetohydroxyacid synthase	**16.17**	6.00	**6.20**	2.68
AI664892	Ketol-acid reductoisomerase	**15.91**	4.29	**3.59**	1.84
AI664918	3-methylcrotonyl-CoA carboxylase	**8.87**	3.89	**3.63**	1.54
AI668434	Branched chain alpha-keto acid dehydrogenase	**8.80**	4.42	**6.72**	3.52
AI665546	Putative tryptophan synthase alpha	**7.02**	3.52	**2.75**	1.33
AI665281	Putative sarcosine oxidase	**5.83**	2.48	**3.39**	1.30
AI714578	Indole-3-glycerol phosphate synthase	**4.85**	2.02	**3.61**	1.39
AI833818	Aspartate aminotransferase	**4.75**	2.45	**3.44**	1.68
AI665202	Leucine aminopeptidase	**4.17**	2.56	**3.44**	1.91
Carbohydrate metabolism					
AI833417	Pyruvate orthophosphate dikinase*	**7.53**	2.01	**3.99**	1.24
AI745791	Pyrophosphate fructose 6-phosphate 1-phosphotransferase*	**6.48**	1.85	**3.53**	0.38
AI714607	Sorbitol dehydrogenase*	**6.33**	2.80	**3.11**	0.40
AI739986	Putative fructose-1,6-bisphosphate aldolase*	**6.11**	3.18	**3.97**	0.20
AI711638	6-phosphogluconate dehydrogenase*	**5.61**	2.58	**3.26**	0.37
AI668401	Putative malate dehydrogenase	**5.06**	1.65	**2.90**	1.11
AI711820	Brittle-1 protein*	**4.58**	1.45	**3.48**	1.04
AI677069	Sucrose synthase*	**3.78**	0.38	**3.51**	0.93
AI737050	Starch branching enzyme II	**3.33**	1.66	**2.61**	1.65
AI665404	Starch branching enzyme I	**2.88**	1.36	**2.21**	0.86
AI833421	Glyceraldehyde-3-phosphate dehydrogenase (*GAPC2*)	**2.55**	0.79	**2.51**	0.98
AI740122	Starch debranching enzyme (*Zpu1*)	**2.11**	0.86	**2.42**	0.91
AI745808	Granule-bound starch synthase 1 (*Waxy1*)	**2.01**	0.72	**2.03**	0.38
Cell wall synthesis/turnover					
AI712112	Beta-D-xylosidase	**11.62**	4.09	**4.32**	1.88
AI665255	Putative wall-associated kinase	**8.65**	2.57	**3.70**	1.48
AI668305	Extensin-like protein	**8.28**	3.32	**4.10**	2.23
AI759118	Glycine-rich protein*	**5.15**	2.11	**3.77**	1.09
AI739985	Golgi associated protein se-wap41*	**4.51**	1.76	**3.46**	0.14
AI665065	Putative xyloglucan endo-1,4-beta-D-glucanase	**4.35**	1.07	**2.61**	0.70
AI833575	Putative beta-1,3-glucanase	**4.06**	0.85	**3.42**	1.71
AI737116	Alpha-expansin	**2.72**	0.54	**2.76**	0.97
Cytoskeleton					
AI740030	Actin (*Maz65*) like	**12.26**	3.47	**4.61**	1.36
AI740025	Myosin heavy chain like protein	**6.84**	2.78	**5.22**	2.62
AI670309	Dynein light chain like protein	**6.01**	2.00	**4.24**	1.39
AI665295	Actin (*Maz87*)	**2.73**	0.97	**2.02**	0.63
AI746097	Alpha1-tubulin*	**2.19**	0.34	**2.04**	0.06
AI820423	Actin depolymerizing factor (*ZmADF3*)	**2.03**	0.66	**2.90**	1.34
AI677353	Actin 1 (*MAc1*)	**0.19**	0.05	**0.17**	0.13
Molecular chaperonin					
AI677488	Protein disulfide isomerase*	**7.66**	2.80	**3.94**	1.41
AI665165	Small heat shock protein*	**7.59**	1.09	**3.77**	1.46
AI665058	Calmodulin-binding heat shock like protein	**6.99**	2.74	**4.19**	1.28
AI745903	Cyclophilin*	**4.90**	2.56	**3.54**	0.39
AI664970	Putative prolyl 4-hydroxylase	**4.74**	1.53	**2.56**	1.19

Table 3.1. (Continued)

EST ID	Blast Name	Oh51A*o2*/Oh545*o2* Average	Oh51A*o2*/Oh545*o2* SD	34-3/31-5 Average	34-3/31-5 SD
AI795286	*Hsp70*	**3.90**	1.98	**3.32**	1.42
AI665294	*BiPe2**	**2.73**	0.90	**3.54**	0.28
Membrane transport					
AI665354	Voltage-dependent anion channel protein 1a	**14.02**	3.45	**5.10**	1.88
AI665187	Plasma membrane H+ ATPase	**5.11**	1.39	**5.08**	2.42
AI668193	H+-pyrophosphatase	**2.82**	1.18	**3.61**	1.85
Metabolism/oxidoreductase					
AI665244	Cytosolic ascorbate peroxidase	**17.54**	2.66	**4.85**	1.69
AI739829	Glutathione S Transferase*	**8.69**	1.86	**5.25**	2.98
AI677477	Mitochondrial Rieske Fe-S protein*	**5.81**	2.11	**3.28**	0.74
AI670285	Cytochrome P450*	**4.91**	1.42	**4.86**	0.09
AI711662	Cytosolic aldehyde dehydrogenase RF2C	**4.26**	0.83	**3.00**	1.05
AI759131	4-hydroxyphenylpyruvate dioxygenase	**2.39**	0.29	**4.29**	2.11
Lipid metabolism					
AI668552	Sterol 4-alpha-methyl-oxidase	**13.94**	4.49	**3.95**	1.73
AI668423	Lipid transfer protein*	**6.23**	2.22	**3.93**	0.64
AI795367	Putative acyl-CoA synthetase	**5.89**	2.73	**7.15**	2.09
AI674009	Delta-12 desaturase	**5.55**	2.21	**3.22**	1.85
AI833884	Beta-keto acyl reductase gene	**4.55**	1.83	**2.88**	1.00
Protein turnover					
AI714537	ATP-dependent Clp protease regulatory subunit	**9.12**	5.68	**4.23**	1.69
AI665136	E2 ubiquitin-conjugating enzyme	**8.43**	2.14	**3.14**	1.16
AI795496	Proteinase inhibitor*	**8.25**	0.52	**4.57**	0.33
AI833831	Polyubiquitin*	**6.54**	1.39	**3.49**	0.59
AI664917	Ubiquitin/ribosomal protein S27a*	**4.72**	0.83	**2.48**	0.05
AI657385	20S proteasome alpha subunit F	**4.38**	1.83	**4.09**	1.16
AI833826	Aspartic protease	**3.70**	1.15	**4.02**	2.28
AI665632	Hageman factor inhibitor	**0.66**	0.10	**0.63**	0.12
Seed protein					
AI665146	19 kD alpha zein*	**22.75**	10.11	**4.50**	1.13
AI745984	Endosperm specific protein*	**8.06**	2.93	**3.74**	0.88
AI665066	Putative protein body membrane protein MP27	**6.51**	1.82	**3.14**	0.92
AI745801	22 kD alpha zein*	**5.96**	1.84	**4.69**	1.18
AI746046	LEA-like protein	**5.95**	2.08	**3.80**	1.10
AI668466	Legumin-like protein	**2.48**	1.11	**2.52**	1.14
AI739978	10 kD zein*	**0.22**	0.10	**0.15**	0.04
Secondary metabolism					
AI665286	Glucosyltransferase-like protein*	**5.35**	2.04	**2.77**	0.44
Signal transduction					
AI676990	Putative inorganic pyrophosphatase	**14.90**	3.60	**5.48**	1.71
AI664994	Putative calcium binding protein	**12.90**	5.62	**4.74**	2.33
AI674011	Phosphoprotein phosphatase	**11.01**	5.25	**4.99**	2.50
AI670625	Putative phytosulfokine peptide precursor (*PSK1*)*	**8.91**	1.30	**3.38**	1.23
AI711812	Protein phosphatase 2C*	**7.35**	1.56	**4.34**	0.98
AI664997	Adenosine kinase 2	**6.11**	2.25	**4.07**	1.80
AI711834	GTP-binding protein*	**5.33**	2.49	**3.66**	1.34
AI739858	Guanine nucleotide binding protein	**4.15**	1.48	**4.49**	2.27

Table 3.1. (Continued)

EST ID	Blast Name	Oh51A*o2*/Oh545*o2* Average	SD	34-3/31-5 Average	SD
AI745933	Serine/threonine protein kinase	**3.41**	0.24	**2.96**	0.29
AI711751	LRR receptor-like protein	**3.41**	1.47	**4.02**	1.70
AI712212	ADP-ribosylation factor	**2.88**	0.81	**2.93**	1.35
AI739988	Serine/threonine phosphatase PP1	**0.58**	0.21	**0.26**	0.11
Stress/defense response					
AI740176	Stress-related protein	**5.10**	2.28	**2.73**	1.16
AI670652	Putative metallothionein*	**4.00**	0.93	**4.30**	1.63
Transcription/translation					
AI833456	ETTIN-like protein	**15.39**	2.76	**4.91**	1.58
AI664855	Trancription factor-like protein*	**14.33**	1.76	**3.73**	0.11
AI665388	Chloroplast elongation factor EF-Tu*	**11.62**	2.48	**4.69**	0.38
AI711673	60S ribosomal protein L27	**11.18**	3.30	**4.74**	2.66
AI665325	40S ribosomal protein S2	**9.85**	2.96	**2.90**	0.50
AI711629	Putative uridylyl transferase	**9.10**	3.32	**2.96**	1.05
AI664888	Histone H3 gene (H3C4)*	**7.76**	0.57	**2.73**	0.32
AI737090	60S ribosomal protein L7	**7.03**	2.03	**3.11**	1.16
AI833442	Putative zinc finger transcription factor	**7.01**	1.62	**5.41**	2.37
AI711653	60S ribosomal protein L19*	**6.69**	2.40	**3.16**	0.01
AI664978	Initiation factor 3g	**6.16**	1.07	**2.59**	0.96
AI665326	RNA binding protein*	**6.04**	1.03	**3.36**	1.11
AI711841	60S ribosomal protein L3	**4.92**	1.31	**2.57**	0.86
AI677610	40S ribosomal protein S11	**4.90**	1.87	**3.22**	0.67
AI657447	Dead box RNA helicase	**4.87**	1.90	**2.24**	0.66
AI833834	Histone H2A	**4.83**	2.20	**4.64**	2.44
AI714569	40S ribosomal protein S4*	**4.80**	0.45	**3.30**	1.66
AI665170	40S ribosomal protein S24	**4.80**	1.21	**3.23**	0.90
AI745832	Histone H2B*	**4.58**	1.73	**3.54**	0.49
AI737088	Initiation factor eIF-2*	**4.42**	0.32	**2.74**	0.20
AI795427	60S ribosomal protein L2*	**4.38**	0.97	**3.17**	0.75
AI665352	60S acidic ribosomal protein P*	**4.29**	0.98	**4.26**	1.68
AI740119	40S ribosomal protein S14	**3.92**	1.94	**4.35**	2.59
AI833851	60S ribosomal protein L1	**3.42**	1.34	**2.28**	0.80
AI712164	Elongation factor 1 beta*	**3.38**	0.45	**2.68**	0.62
AI745952	Elongation factor 1 alpha*	**2.39**	0.26	**2.25**	0.23

* The expression ratio of these genes correspond to the average of two or more ESTs.

storage proteins, proteins involved in stress responses, and components of the cytoskeleton, signal transduction pathways and the transcription/translation machinery.

Among genes encoding seed storage proteins, 19- and 22-kD α-zeins showed the largest difference in expression between high and low eEF1A genotypes. The transcript levels of these genes were about 23- and 6-fold higher, respectively, in Oh51A*o2* than Oh545*o2* (**Table 3.1**). The 19- and 22-kD α-zeins are encoded by multigene families, and our results most likely are representative of the gene subfamilies, due to cross-hybridization among the members (Marks et al., 1985). The transcript level of the 10-kD δ-zein correlated inversely with that of eEF1A; the amount of 10-kD δ-zein mRNA was about five-times lower in high versus low eEF1A genotypes.

The mRNA levels of genes encoding enzymes involved in the synthesis of branched amino acids, such as acetohydroxyacid synthase, ketol-acid reductoisomerase and branched chain alpha-keto acid dehydrogenase, were several-fold greater in high compared to low eEF1A genotypes. Similarly, several genes involved in carbohydrate metabolism, including several starch synthesis enzymes, were up-regulated in the high eEF1A genotypes. Genes encoding some of the proteins in these two groups were reported to be direct or indirect targets of *O2* regulation (Damerval and Le Guilloux, 1998).

Genes encoding cytoskeletal proteins, such as actin (*Maz87*) and α-tubulin were also up-regulated in high eEF1A genotypes (**Table 3.1**). Conversely, one actin probe (*MAc1*) was down-regulated in high eEF1A genotypes. The nucleotide sequence identity between *MAc1* and *Maz87* is about 60%, so it is likely their transcripts did not cross-hybridize.

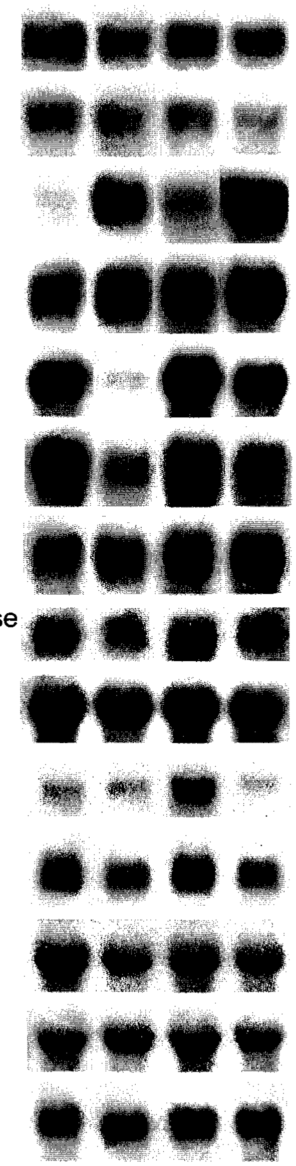
Other actin genes represented in the array and not included in **Table 3.1** are *Maz83* (Genbank accession AI677580), *Maz89* (AI745940) and *Maz95* (AI677101). There were no significant differences in the expression levels of *Maz83* in high and low eEF1A genotypes, while the hybridization signals of *Maz89* and *Maz95* were similar to background.

One group of genes shown in **Table 3.1** that paralleled expression of eEF1A has been implicated in stress responses and includes ascorbate peroxidase, a proteinase inhibitor, a glycine-rich (cell wall) protein and ER-resident molecular chaperones, such as binding protein (Bip), and protein disulfide isomerase (PDI). Another group of genes upregulated in high eEF1A genotypes included several sequences encoding phosphatases, ribosomal proteins, histones and protein synthesis factors.

RNA blot analysis

To validate the results of the microarray hybridization, the transcript levels of 13 genes were investigated by RNA gel blot analysis using total RNA prepared from the same samples used for the microarray experiments (**Fig. 3.3**). The intensities of the radioactive signals were normalized to the 27-kD γ-zein RNA, because its level was found to be similar among these genotypes at 18-DAP. For most of these genes, the expression level determined by RNA gel blot analysis correlated with that detected with microarrays. However, with the exception of the zein genes, the differences in mRNA abundance were generally estimated to be smaller using RNA blot analysis (**Fig. 3.3**).

Figure 3.3. RNA gel blot analysis for validation of microarray hybridization data. Lanes contained 10 μg of total RNA extracted from 18-DAP endosperms of Oh51A*o2*, Oh545*o2*, and their RILs: 34-3 (high eEF1A) and 31-5 (low eEF1A). Blots were hybridized with ^{32}P-labeled probes corresponding to the genes indicated. GeneBank accession numbers of EST-specific probes are indicated in parentheses. The values given are the ratio of signals between Oh51A*o2* and Oh545*o2*, as well as RILs 34-3 and 31-5, normalized relative to the 27-kD γ-zein RNA signal. For each gene, the expression ratio of high *versus* low eEF1A genotypes is compared to that obtained by microarray hybridization (**Table 3.1**).



	Oh51A*o2*/Oh545*o2*		34-3/31-5	
	RNA blot	Microarray	RNA blot	Microarray
Waxy1 (X03935)	1.5	2.0	1.4	2.0
GAPC2 (L13432)	1.6	2.5	1.7	2.5
10-kD δ-zein (AF371266)	0.14	0.22	0.15	0.15
16-kD γ-zein (AF371262)	1.0	1.0	1.0	1.0
19-kD α-zein (M12146)	25.7	22.7	3.3	4.5
22-kD α-zein (J01246)	6.1	5.9	3.1	4.7
27-kD γ-zein (AF371261)	0.78	1.0	0.86	1.0
Asc. Peroxidase (AI665244)	1.9	17.5	1.7	4.8
α-tubulin (AI746097)	1.25	2.2	1.15	2.0
Bip (AI665294)	1.5	2.7	25.3	3.5
Profilin (AI668265)	1.5	ND	1.6	ND
eEF1A (AI745952)	1.6	2.4	1.5	2.2
Actin (*Maz87*) (AI665295)	1.15	2.7	1.25	2.0
27-kD γ-zein (AF371261)	0.94	1.0	0.96	1.0

According to microarray data, the expression ratios of eEF1A, granule-bound starch synthase (*Waxy1*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPC2*) were between 2.0- and 2.5-fold greater in the high *versus* the low eEF1A genotypes, while in gel blot analysis the expression ratios were between 1.4- and 1.7-fold greater in these genotypes. The expression levels determined by microarray hybridization for actin (*Maz87*) and α-tubulin were about two-fold greater in high compared to low eEF1A genotypes; however, the differences in gene expression detected by gel blot analysis were approximately 1.2-fold larger. In the case of ascorbate peroxidase, the expression ratio obtained by microarray hybridization with Oh51A*o2* *versus* Oh545*o2* was 17.5, while the expression ratio obtained by gel blot analysis was 1.9. Conversely, according to microarray data the expression ratio of Bip in RIL 34-3 *versus* 31-5 was 3.5, while by gel blot analysis the ratio was 25.3. In spite of the discrepancy in the RNA expression ratios for ascorbate peroxidase, the pattern of expression in high and low eEF1A genotypes was consistent. However, it is more difficult to explain the unexpectedly high level of Bip expression in RIL 34-3. This result was confirmed in multiple RNA blots.

The expression of profilin was also determined by RNA gel blot analysis, and it was found to be about 1.5-fold higher in Oh51A*o2* and RIL 34-3 compared to Oh545*o2* and RIL 31-5 (**Fig. 3.3**). Profilin transcript levels were not considered to be significantly different between high and low eEF1A genotypes in the microarray experiments, because the signal was too close to background.

Patterns of protein synthesis

To further assess the gene expression results obtained by RNA hybridization, we measured protein accumulation in 18-DAP and mature endosperms of the genotypes used for transcript profiling. In addition, we analyzed four RILs that differ in eEF1A content. Protein extracts from equal amounts of lyophilized endosperm of each genotype were partitioned into zein and non-zein fractions (Wallace et al., 1990) and analyzed using SDS-PAGE. **Figure 3.4A** illustrates a Comassie blue-stained gel showing the relative abundance of zein proteins in 18-DAP endosperm of the parental inbreds and six RILs. The level of 27-kD γ-zein appeared to be similar between Oh545*o2* and Oh51A*o2*, and between RILs 31-5 and 34-3, although the amount of this protein is greater in the inbred lines. These observations were in agreement with the RNA levels obtained by microarray hybridization and RNA blot analysis, and further validated the use of the 27-kD γ-zein gene to normalize the microarray hybridization data. The accumulation of 19- and 22-kD α-zein proteins was greater in Oh51A*o2* than Oh545*o2* (**Fig. 3.4A**) and correlated with the RNA hybridization results (**Fig. 3.3**). Based on Coomassie blue staining, the differences in α-zein content in RILs 31-5 and 34-3 were not as obvious as for the parents, consistent with the smaller differences in RNA transcript levels measured for these lines compared to Oh545*o2* and Oh51A*o2* (**Fig. 3.3**). Except for RIL 33-13, the accumulation of zein proteins in RILs 39-1, 37-3 and 4-10 at 18-DAP appeared to be very similar. **Figure 3.4B** shows there were only small differences in total zein content at this stage of development for the six genotypes. The amount of total zein ranged from about

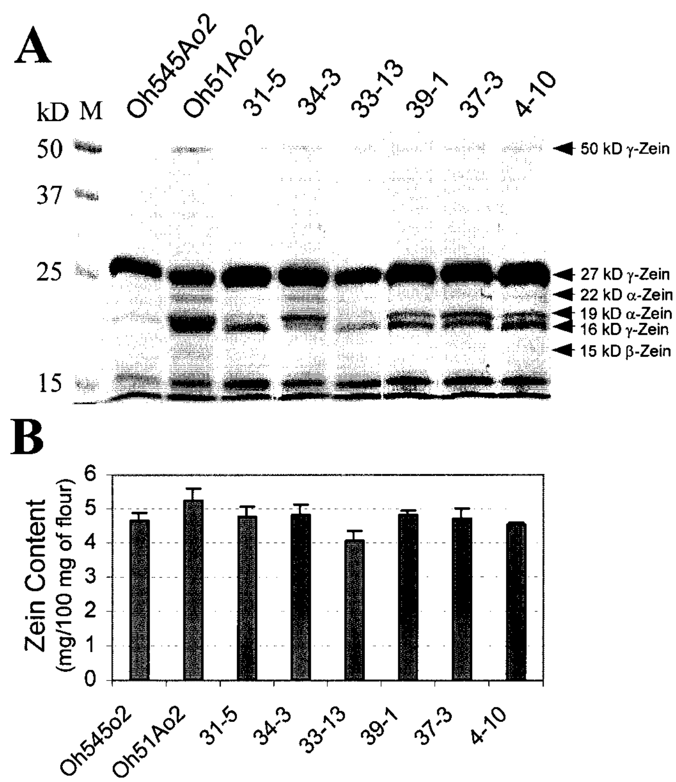


Figure 3.4. SDS-PAGE and quantification of total zein in developing endosperm of Oh545*o2*, Oh51A*o2* and six RILs with high and low eEF1A content. (A) For each lane, total zein extracts were prepared from 750 μg of lyophilized 18-DAP endosperms of the indicated genotypes, separated by 12.5% (w/v) SDS-PAGE, and stained with Coomassie blue R. Pre-stained molecular mass standards are shown on the left. (B) Total zein content was estimated using BCA reagent with BSA as standard. The results are the average of two independent extractions in which three measurements were taken.

4.1 mg per 100 mg of flour in RIL 33-13 to about 5.2 mg per 100 mg of flour in Oh51A*o2*.

The non-zein content in 18-DAP endosperms of Oh545*o2*, Oh51A*o2* and the six RILs was estimated by Bradford assay and the relative levels of eEF1A, actin, profilin and α-tubulin by ELISA. **Figure 3.5A** shows the non-zein content at this stage of development was similar in all genotypes, and ranged from about five mg per 100 mg of flour in RIL 31-5 to about six mg per 100 mg of flour in Oh51A*o2*. **Figure 3.5B** shows the ELISA quantification of eEF1A, actin, profilin, and α-tubulin in these genotypes. The parental inbreds and the RILs were organized according to their relative eEF1A content. In general, the relative level of eEF1A, actin, and profilin in the RILs was lower than in the high and low eEF1A parents. There was about 1.3-fold more eEF1A in Oh51A*o2* than in Oh545*o2*. The difference in eEF1A content between the highest and lowest RILs, 34-3 and 31-5, was also about 1.3-fold. The levels of actin, profilin and α-tubulin protein paralleled those of eEF1A (**Fig. 3.5B**). The amount of these proteins in Oh51A*o2* compared to Oh545*o2* was about 1.2-, 1.3-, and 1.8-fold greater, respectively. Among the RILs, the greatest difference in the amount of these proteins was in RIL 34-3 (high eEF1A) and RIL 31-5 (low eEF1A), where the levels of actin, profilin, and α-tubulin were about 1.3-, 1.5- and 1.4-fold higher in RIL 34-3 than in RIL 31-5, respectively.

When the levels of eEF1A, actin, profilin, and α-zein were measured in mature endosperms of Oh545*o2*, Oh51A*o2*, and 10 of their RILs by ELISA (**Fig. 3.6**), we found the relative contents of actin and profilin closely paralleled those of eEF1A, as observed in developing endosperm (**Fig. 3.5B**), but the range of variation was larger. The levels of

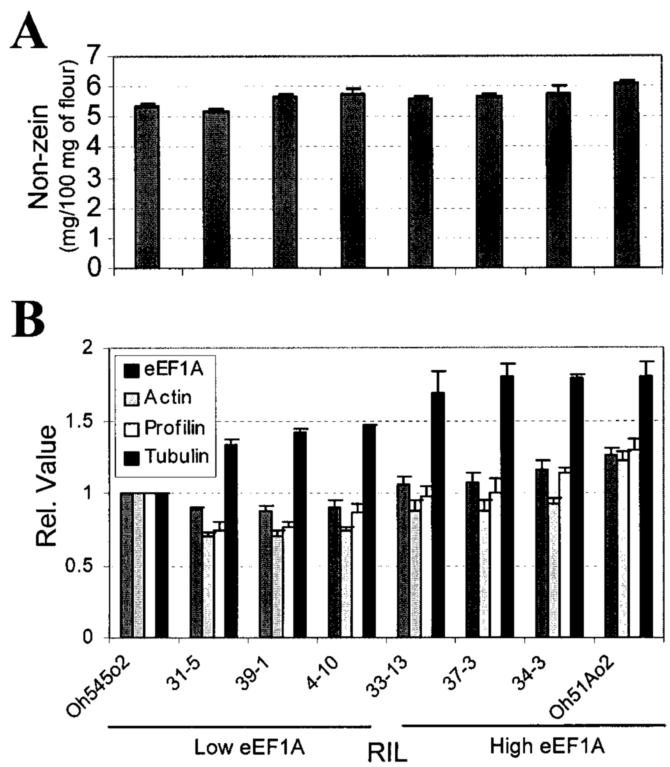


Figure 3.5. Non-zein quantification and ELISA measurement of eEF1A, actin, profilin and α-tubulin content in developing endosperm of Oh51A*o2*, Oh545*o2* and six RILs with high and low eEF1A content. Protein extracts were prepared from lyophilized 18-DAP endosperms. (A) Non-zein content was estimated using the Bradford reagent with BSA as standard. (B) ELISA measurements of the indicated proteins were conducted as described in Materials and Methods and the results were normalized to the values of the Oh545*o2* inbred. In both (A) and (B), the results are the average of two independent experiments in which three measurements were taken.

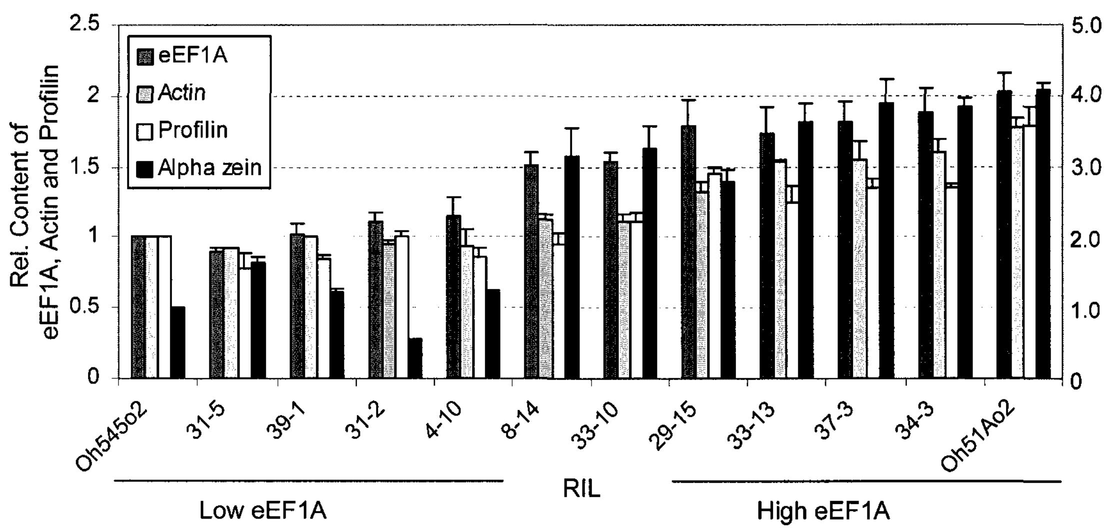


Figure 3.6. ELISA measurement of eEF1A, actin, profilin and α-zein content in mature endosperm of Oh51A*o2*, Oh545*o2* and 10 RILs with high and low eEF1A content. Protein extracts were prepared from endosperm flour. ELISA measurements of the indicated proteins were conducted as described in Materials and Methods and the results were normalized to the values of the Oh545*o2* inbred. The results are the average of two independent experiments in which three measurements were taken.

actin and profilin proteins were about 1.8-fold higher in Oh51A*o2* than Oh545*o2*, and the differences in their protein levels in RIL 34-3 compared to RIL 31-5 were about 1.9 and 1.7-fold greater, respectively. The relative amounts of eEF1A shown in **Figure 6** correspond to those in **Figure 2**, but they are included here for reference. The levels of α-tubulin could not be determined in mature endosperms, perhaps because of protein instability. The relative content of α-zein was determined using antibodies that do not distinguish between the 19- and 22-kD subclasses. There was about four-times more α-zein in Oh51A*o2* than Oh545*o2*, and about two-times more α-zein in RIL 34-3 than RIL 31-5 (**Fig. 3.6**). Except for RIL 31-2, which had only about 50% of the α-zein content of Oh545*o2*, the levels of α-zein proteins correlated with those of eEF1A.

DISCUSSION

The quality of data from comparative RNA/protein expression profiling is directly related to the genetic material being analyzed and its phenotypic evaluation. For this study, we selected the maize inbreds Oh51A*o2* and Oh545*o2*, because they have among the highest degree of variability reported for eEF1A content (Moro et al., 1996), and the RILs created from a cross between them showed the same range of variation for eEF1A content as the parental genotypes (~2-fold). This difference allowed us to compare multiple, genetically fixed and related inbred lines for patterns of gene expression and protein synthesis relative to eEF1A protein content. Unfortunately, the cost of the microarray experiments prevented us from analyzing more genotypes than the parents and one high and one low eEF1A RIL. However, we measured the accumulation of selected proteins by ELISA in multiple high and low eEF1A RILs, both at 18-DAP and maturity.

Using an endosperm EST microarray, we were able to conduct a broad survey of genes that change in expression parallel with eEF1A in high and low eEF1A genotypes. Our goal was to identify genes with an expression level that was higher in Oh51A*o2* compared to Oh545*o2*, and higher in RIL 34-3 compared to RIL 31-5. We tested the microarray results for several genes by RNA gel blot analysis and generally found a good correspondence between the expression levels estimated by the two techniques. In a few cases the differences in RNA levels detected by gel blot analysis and microarray hybridization did not correspond. This may have been a consequence of inherent differences in the stringency of the two hybridization procedures or the specific alleles present, or absent, on the microarray. These results emphasize the importance of validating microarray data with additional methods. However, the general consistency between the results obtained by the RNA gel blot analysis and microarray hybridization largely confirmed the utility of using the EST microarray to identify genes differentially expressed in high and low eEF1A genotypes.

The microarray hybridization identified about 110 genes coordinately increased with eEF1A (**Table 3.1**). These genes encode proteins involved in a variety of cellular processes, and several of them have been previously reported to be differentially expressed at the transcript and protein level in wild type and *o2* mutant inbreds, although not necessarily in parallel with eEF1A. For example, the levels of α-zeins, pyruvate orthophosphate dikinase and acetohydroxyacid synthase were decreased in *o2* mutants compared to wild type (Damerval and Le Guilloux, 1998; Hunter et al., 2002), while that of eEF1A was increased (Habben et al., 1993; Sun et al., 1997). For some enzymes involved in carbohydrate metabolism, such as glyceraldehyde-3-phosphate dehydrogenase and sorbitol dehydrogenase, earlier studies showed these proteins are increased in *o2* mutants (Damerval and Le Guilloux, 1998).

One explanation for the relationship between eEF1A and endosperm lysine content is that eEF1A is part of an elaborate cytoskeletal network that surrounds the rough ER, particularly at sites where protein bodies are forming (Clore et al., 1996). Other lysine-rich proteins that exist in association with this cytoskeletal network may be increased as the consequence of a greater protein body surface area (Sun et al., 1997). Oh51A*o2* has more α-zein and also appears to have a larger number of small protein bodies (larger RER surface area) than Oh545*o2* (Wang et al., 2001), which could reflect in a more extensive cytoskeleton. Consistent with this hypothesis, our microarray results indicated the expression of α-zeins and a number of genes encoding cytoskeletal and ER proteins are coordinately increased in high eEF1A genotypes (**Table 3.1**). In addition, the levels of α-zeins and some cytoskeletal proteins corresponded well with those of eEF1A in the parental inbreds and some high and low eEF1A RILs (**Figs. 3.5 and 3.6**). This could explain a significant portion of the increased eEF1A content in Oh51A*o2* and its related high eEF1A RILs. Microscopy and biochemical studies will be required to examine the postulated ultrastructural differences in protein body size and number and ER development in RILs with differing levels of eEF1A.

The RNA expression data also showed that several genes encoding carbohydrate-metabolizing enzymes were up-regulated in high eEF1A genotypes. Interestingly, some of these enzymes, such as sucrose synthase (Carneiro, 1998; Winter et al., 1998), glyceraldehyde-3-phosphate dehydrogenase, and fructose-1,6-bisphosphate aldolase were recently reported to be associated with the cytoskeleton (Azama et al., 2003). The lysine content of these proteins varies from about 5 to 8.5% and their masses, together with those of cytoskeletal proteins (actin, tubulin and eEF1A), were reported to contribute significantly (~67%) to the total endosperm lysine content of W64A*o2* (Azama et al., 2003). Several endosperm proteins found to associate with actin in yeast two-hybrid interactions (Carneiro, 1998) included a serine-threonine protein kinase, 60S acidic ribosomal protein, E2 ubiquitin-conjugating enzyme, and a putative beta-1,3-glucanase, and genes encoding these proteins were also up-regulated in high eEF1A genotypes (**Table 3.1**).

In a previous study, we characterized isoforms of eEF1A from developing maize endosperm and found that they differ in their F-actin binding activities (Lopez-Valenzuela et al., 2003). The isoform that binds actin most efficiently was the predominant form accumulated in endosperm of the high eEF1A inbreds, Oh51*o2* and RIL 34-3, which may reflect enhanced cytoskeleton formation and therefore increased synthesis of cytoskeleton-associated proteins in these genotypes. Consistent with this hypothesis, our results show the mRNA levels of several genes encoding ribosomal proteins and protein synthesis factors are significantly higher in Oh51A*o2* and RIL 34-3, compared to Oh545*o2* and RIL 31-5 (**Table 3.1**).

Differences in transcript levels between genotypes are often consistent with variation in protein levels, although this need not be the case. Furthermore, differences in RNA levels in developing seeds need not necessarily be related to protein content in the mature seed. However, it is the latter that ultimately determines the lysine content of the grain. This study was predicated on the hypothesis that by measuring differences in transcript levels at mid-development (18-DAP), we could identify proteins linked with eEF1A concentration in mature maize endosperm. To a large extent, this hypothesis proved to be correct. At 18-DAP, the differences in eEF1A protein between Oh51A*o2* and Oh545*o2* and between RIL 34-3 and RIL 31-5 (~1.3-fold, **Fig. 3.5B**) were smaller than those measured at the RNA transcript level by microarrays (~2-fold, **Table 3.1**) and were more in agreement with the 1.5-fold difference observed by RNA gel blot analysis **(Fig. 3.3)**. According to microarray hybridization **(Table 3.1)**, actin RNA (Maz87) transcripts were more than two-fold greater in Oh51A*o2* and RIL 34-3 (high eEF1A) compared to Oh545*o2* and RIL 31-5 (low eEF1A), while only about 1.2-fold differences in actin transcript levels were measured by RNA blot analysis (**Fig. 3.3**). The protein measurements indicated there was about 20 to 30% more actin in Oh51A*o2* and RIL 34-3 (high eEF1A) compared to Oh545*o2* and RIL 31-5 (low eEF1A) at 18-DAP **(Fig. 3.5B)**. The differences obtained for α-tubulin protein content (1.8- fold greater in Oh51A*o2* than Oh545*o2* and 1.4-fold greater in RIL 34-3 than RIL 31-5) corresponded better with the microarray results (~2-fold, **Table 3.1**), compared with 1.2-fold difference obtained by RNA blot analysis (**Fig. 3.3**). Differences in profilin RNA levels were not apparent by microarray hybridization, but differences in the amount of this protein in Oh51A*o2* versus Oh545*o2* and RIL 34-3 versus RIL 31-5, 1.3- and 1.5-fold, respectively, were in good agreement with the transcript levels determined by gel blot analysis (~1.5-fold, **Fig. 3.3**). In mature endosperm, the differences in eEF1A and actin protein content between Oh51A*o2* and Oh545*o2* and between RIL 34-3 and RIL 31-5 (~2-fold, **Fig. 3.6**) were similar to those measured at the RNA transcript level by microarrays (**Table 3.1**), while the differences in α-zein protein content (2- to 4-fold) were smaller than those measured at the transcript level in 18-DAP endosperm (**Table 3.1**, **Fig. 3.3**). Thus, in general there was a good relationship between RNA transcript levels at 18-DAP and protein levels in developing and mature endosperm.

In conclusion, the results of this study support the hypothesis that eEF1A concentration in maize endosperm is related to the concentration of cytoskeleton-related protein components, and this may explain the high correlation between eEF1A and the concentration of protein-bound lysine in endosperm (Habben et al., 1995). Cytoskeleton-associated proteins are not as abundant as zeins, but combined, their masses appear to make a substantial contribution to the lysine content of the grain (Azama et al., 2003). By selecting for maize genotypes, particularly Quality Protein Maize lines (Moro et al., 1996; Zarkadas et al., 2000) with high levels of eEF1A, it should be possible to create maize kernels that meet the lysine requirement for humans and other monogastric animals.

CHAPTER 4

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Several decades of research have provided limited progress in understanding the mechanisms of lysine accumulation in maize endosperm. Most of our knowledge has come from the identification and characterization of "high lysine" mutants with altered synthesis of storage proteins. The best characterized of these mutants is *o2*, which increases the lysine content as a consequence of reduced levels of zein storage proteins and the pleoitropic increase of non-zein proteins. However, one important question that remains unanswered is how the regulatory activity of *o2* is translated into an increase of lysine-containing proteins. Studies addressing this question could reveal an effective approach for improving the protein quality of maize and other cereal grains.

One strategy to understand the increased lysine content of *o2* mutants has been to identify lysine-rich proteins that are synthesized at higher levels in *o2* backgrounds compared to their wild type counterparts. Translation elongation factor-1A (eEF1A) is one of these proteins, and its concentration is highly correlated ($r = 0.9$) with the lysine content of maize endosperm. However, the biological basis of this relationship is unclear. eEF1A contains 11% lysine, but it accounts for only about 2% of the total endosperm lysine, suggesting a stoichiometric relationship between eEF1A and other lysine-rich proteins. The research described herein has provided some insights about the basis of this relationship.

eEF1A is a multifunctional protein and the basis for the enhanced levels of this protein in *o2* maize endosperm could result from one or more of its apparent activities. To begin investigating this conundrum, I purified three isoforms of the protein from developing endosperm. One of the questions addressed was whether these isoforms change during endosperm development and if there are specific isoforms affected by the *o2* mutation. The results of this study suggested the accumulation of eEF1A isoforms is developmentally regulated and independent of the *o2* mutation.

Although eEF1A has been implicated in a variety of cellular processes and there is evidence for tissue-specific gene expression, no one has demonstrated a distinct physiological activity for any particular isoform. The purified eEF1A isoforms differed in their ability to bind F-actin *in vitro*, suggesting they are functionally distinct. To my knowledge, this is the first time eEF1A isoforms have been shown to have different actin binding activities.

In order to explain why the purified eEF1A isoforms differ in their affinity for F-actin, their chemical properties were investigated by tandem mass spectrometry (LC/MS/MS). LC/MS/MS revealed each isoform is composed of the four same gene products, which are modified post-translationally by methylation and phosphorylation. However, it was not possible to identify isoform-specific sequences or post-translational modifications that could be correlated with or explain the functional differences of the eEF1A isoforms. Since the amino acid sequence covered by the LC/MS/MS analysis was about 80% of the protein, it appears that chemical differences between the purified proteins occur in sequences corresponding to the 20% not covered in this analysis. Improvements in mass spectrometry-based analysis of proteins and peptides could eventually allow the identification of these structural differences.

When the accumulation of the purified isoforms was investigated in maize inbreds that vary in eEF1A levels, it was found the isoform that binds actin most efficiently predominates in high eEF1A genotypes. This may be related to enhanced cytoskeleton formation, and therefore increased synthesis of cytoskeleton-associated proteins, which are presumably lysine-rich. Analysis of patterns of gene/protein expression between high and low eEF1A genotypes could help identify other lysine-containing proteins with which eEF1A is allied, and therefore explain the physiological and biochemical basis for the high eEF1A/lysine phenotype.

Because eEF1A isoforms have different F-actin binding activities *in vitro*, it is possible they are differentially localized in endosperm cells. One common way to investigate the intracellular localization of proteins is by using translational fusions to a florescent protein, such as green fluorescent protein (GFP). However, mass spectrometry analyses showed each of the isoforms consists of the four same gene products, and most likely each of them are the result of post-translational modifications. Therefore, it may not be informative to use eEF1A-GFP translational fusions to dissect their function/subcellular localization, because they are likely to be distributed in each of the isoforms via post-translational modification. As an alternative approach to determine whether the eEF1A isoforms localize differently in endosperm cells, I initiated some experiments where the purified proteins were fluorescently labeled *in vitro*, introduced into endosperm cells by microinjection and their localization visualized by confocal microscopy. The activity of the labeled proteins was confirmed by *in vitro* actin binding assays as described in Chapter 2. eEF1A26, the isoform that binds actin most effectively, was found to form hollow spheres similar to those observed by immunocytochemistry (Clore et al., 1996), suggesting this isoform co-localizes with actin around protein bodies. eEF1A22 appeared to localize similarly, but with a weaker interaction. Additional experiments are required to evaluate the significance of these results. Other proteins, such as bovine serum albumin, could be labeled and injected as controls to ensure they are non-specifically localized in the cell. The microinjected cells could then be fixed and localization of the fluorescently-labeled eEF1A isoform compared with that of actin, γ-zein, ribosomal proteins and ER by immunocytochemistry.

One hypothesis to explain the stoichiometric relationship between eEF1A and endosperm lysine content is that eEF1A is associated with a number of other lysine-containing proteins that form the cytoskeletal network around the endoplasmic reticulum. The basis of this relationship was investigated by analyzing patterns of gene expression and protein synthesis between a high (Oh51A*o2*) and a low (Oh545*o2*) eEF1A maize inbred, as well as in high and low eEF1A recombinant inbred lines (RIL) obtained from a cross of these two inbreds (Chapter 3). Transcript profiling with an endosperm EST microarray identified about 110 genes coordinately regulated with eEF1A: these genes encode proteins involved in several biological structures and processes, including the cytoskeleton, the ER and the protein synthesis apparatus. The content of α-zein and several cytoskeletal proteins was measured in high and low eEF1A inbred lines, and the levels of these proteins were found to correlate with that of eEF1A. These results support the hypothesis that eEF1A concentration in maize endosperm is related to the concentration of cytoskeleton protein components. This may explain the high correlation between eEF1A and the concentration of protein-bound lysine in endosperm (Habben et al., 1995; Azama et al., 2003), since cytoskeleton-associated proteins appear to contribute significantly to the lysine content of the endosperm (Azama et al., 2003).

Another way to identify proteins that are coordinately regulated with eEF1A content is via proteomic analyses. Endosperm proteins can be separated by 2D SDS-PAGE and individual spots increased in abundance with eEF1A content excised and digested with trypsin. Afterward, the tryptic peptides can be analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), followed by comparison of the mass list against databases of protein sequences using internet-based programs such as Profound, Mascot or Protein Prospector. I used this approach to identify some proteins differentially accumulated in Oh51A*o2* and Oh545*o2* and also changed accordingly in RILs 34-3 and RIL 31-5. One of the protein spots corresponded to a small (17.4 kD) heat shock protein, while the identification of several others was not possible. The main limitation of this approach was the small amount of protein sequence information currently available in databases. A postdoctoral associate in the laboratory, Dr. Bryan Gibbon, is developing algorithms suitable for searching maize unigene compilations derived from EST sequences, which are available from TIGR and the Maize Gene Discovery Project. Alternatively, proteins that cannot be identified by peptide mass fingerprinting could be analyzed by LC/MS/MS operating in nanospray mode, followed by database searches with the SEQUEST program (Yates, 1998). The cost per sample using this instrument can be significantly greater than MALDI-TOF, but it has the advantage of providing information about the sequence of individual peptides and the potential to identify proteins not represented in the databases. Proteomics could be also used to characterize proteins associated with the cytoskeleton recovered from the protein body fraction of high and low eEF1A genotypes.

To investigate the pattern of gene expression associated with a high lysine content, the cDNA clones identified in Chapter 3 can be used to examine transcript levels in other genotypes that differ in eEF1A levels, including several high and low eEF1A RILs and other inbreds found to differ significantly in eEF1A content. In addition, proteins produced from these clones, as well as those identified by proteomics, could be used to create antibodies. These antibodies can be used to determine the amount of each protein in genotypes that differ in eEF1A levels, determine their relationship to lysine content, and examine their subcellular distribution by immunocytochemistry with confocal microscopy.

Based on the hypothesis that the increased content of eEF1A/lysine in *o2* genotypes is associated with a larger number of protein bodies and the consequent increase in the surface of the RER, it would be expected that there are differences in the ultrastructure of genotypes with variable amounts of eEF1A. Developing kernels (~20-DAP) from high and low eEF1A RILs can be used to assess ultrastructural differences by microscopy and immunocytochemistry techniques. One half of each kernel can be used to determine the levels of α-zein, eEF1A, and cytoskeletal proteins by ELISA, as described in Chapter 3, and determine the amount of ER using a specific marker such as NADH-cytochrome C reductase. The other half of the kernel can be used to examine cellular ultrastructure and estimate the number and surface area of protein bodies using electron microscopy techniques.

The results of this study support the hypothesis that selection for high eEF1A content can be used to develop maize genotypes with better nutritional quality. A higher lysine modified *o2* inbred (QPM) could be created by recurrent backcrossing, using a well-adapted QPM as a recurrent parent and a high eEF1A QPM as the male donor. After backcrossing and self-pollination, F2 kernels can be analyzed for eEF1A content by ELISA. Kernels with the highest eEF1A content can be planted and the plants backcrossed to the recurrent parent. This cycle would have to be repeated about six times for the inbred being developed and the recurrent parent to be nearly isogenic (~98% homozygous). These nearly isogenic QPM lines (NILs) could be also very valuable to investigate the mechanisms of lysine accumulation in maize endosperm. Although the present study has provided some insights about the basis of the correlation between eEF1A concentration and lysine content, this relationship could be further investigated by comparing QPM NILs contrasting in eEF1A content, instead of genotypes with distinct genetic backgrounds.

Although two QTLs linked with eEF1A content have been identified previously (Wang et al., 2001), they account for only about 25% of the variability in eEF1A content. A large population segregating for eEF1A content could be created from a cross between the two QPM NILs and used to identify other loci linked with eEF1A content. These markers could also facilitate the development of higher protein quality maize.

REFERENCES

Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. Nature 422: 198-207

Aguilar F, Montandon PE, Stutz E (1991) Two genes encoding the soybean translation elongation factor eEF-1 alpha are transcribed in seedling leaves. Plant Mol Biol 17: 351-360

Argos P, Pedersen K, Marks MD, Larkins BA (1982) A structural model for maize zein proteins. J Biol Chem 257: 9984-9990

Axelos M, Bardet C, Liboz T, Le Van Thai A, Curie C, Lescure B (1989) The gene family encoding the *Arabidopsis thaliana* translation elongation factor EF-1 alpha: molecular cloning, characterization and expression. Mol Gen Genet 219: 106-112

Azama K, Abe S, Sugimoto H, Davies E (2003) Lysine-containing proteins in maize endosperm: a major contribution from cytoskeleton-associated carbohydrate-metabolizing enzymes. Planta In press

Bagga S, Adams HP, Rodriguez FD, Kemp JD, Sengupta-Gopalan C (1997) Coexpression of the maize delta-zein and beta-zein genes results in stable accumulation of delta-zein in endoplasmic reticulum-derived protein bodies formed by beta-zein. Plant Cell 9: 1683-1696

Balconi C, Berardo N, Reali A, Motto M (1998) Variation in protein fractions and nitrogen metabolism of developing normal and opaque endosperm mutants of maize. Maydica 43: 195-203

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Brown RE, Jarvis KL, Hyland KJ (1989) Protein measurement using bicinchoninic acid: elimination of interfering substances. Anal Biochem 180: 136-139

Browning KS (1996) The plant translational apparatus. Plant Mol Biol 32: 107-144

Carneiro N (1998) Patterns of gene expression in maize endosperm: characterization of the eEF1A gene family. PhD thesis. University of Arizona, Tucson

Carneiro NP, Hughes PA, Larkins BA (1999) The eEFIA gene family is differentially expressed in maize endosperm. Plant Mol Biol 41: 801-813

Cavallius J, Popkie AP, Merrick WC (1997) Site-directed mutants of post-translationally modified sites of yeast eEF1A using a shuttle vector containing a chromogenic switch. Biochim Biophys Acta-Gene Struct Expression 1350: 345-358

Cavallius J, Zoll W, Chakraburtty K, Merrick WC (1993) Characterization of yeast EF-1-Alpha - nonconservation of posttranslational modifications. Biochim Biophys Acta 1163: 75-80

Clore AM, Dannenhoffer JM, Larkins BA (1996) EF-1 alpha is associated with a cytoskeletal network surrounding protein bodies in maize endosperm cells. Plant Cell 8: 2003-2014

Coleman CE, Clore AM, Ranch JP, Higgins R, Lopes MA, Larkins BA (1997) Expression of a mutant alpha-zein creates the *floury2* phenotype in transgenic maize. Proc Natl Acad Sci USA 94: 7094-7097

Coleman CE, Herman EM, Takasaki K, Larkins BA (1996) The maize gamma-zein sequesters alpha-zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm. Plant Cell 8: 2335-2345

Coleman CE, Larkins BA (1999) The prolamins of maize. *In* PR Shewry, R Casey, eds, Seed proteins. Kluwer Academic Publishers, Dordrecht, pp 109-139

Coleman CE, Lopes MA, Gillikin JW, Boston RS, Larkins BA (1995) A defective signal peptide in the maize high-lysine mutant *floury2*. Proc Natl Acad Sci USA 92: 6828-6831

Condeelis J (1995) Elongation factor 1 alpha, translation and the cytoskeleton. Trends Biochem Sci 20: 169-170

Damerval C, Devienne D (1993) Quantification of dominance for proteins pleiotropically affected by *opaque-2* in maize. Heredity 70: 38-51

Damerval C, Le Guilloux M (1998) Characterization of novel proteins affected by the *o2* mutation and expressed during maize endosperm development. Mol Gen Genet 257: 354-361

Dannenhoffer JM, Bostwick DE, Or E, Larkins BA (1995) *opaque-15*, a maize mutation with properties of a defective *opaque-2* modifier. Proc Natl Acad Sci USA 92: 1931-1935

Demason DA (1997) Endosperm structure and development. *In* BA Larkins, IK Vasil, eds, Cellular and molecular biology of plant seed development. Kluwer Academic Publishers, Dordrecht, pp 73-115

Demma M, Warren V, Hock R, Dharmawardhane S, Condeelis J (1990) Isolation of an abundant 50,000-dalton actin filament bundling protein from *Dictyostelium amoebae.* J Biol Chem 265: 2286-2291

Deyholos MK, Galbraith DW (2001) High-density microarrays for gene expression analysis. Cytometry 43: 229-238

Di Fonzo N, Gentinetta E, Salamini F, Soave C (1979) Action of the *opaque-7* mutation on the acccumulation of storage products in maize endosperm. Plant Sci Lett 14: 345-354

Dje MK, Mazabraud A, Viel A, le Maire M, Denis H, Crawford E, Brown DD (1990) Three genes under different developmental control encode elongation factor 1-alpha in *Xenopus laevis*. Nucleic Acids Res 18: 3489-3493

Dongre AR, Eng JK, Yates JR (1997) Emerging tandem-mass-spectrometry techniques for the rapid identification of proteins. Trends Biotechnol 15: 418-425

Dreher TW, Tsai CH, Florentz C, Giege R (1992) Specific valylation of turnip yellow mosaic virus RNA by wheat germ valyl-tRNA synthetase determined by three anticodon loop nucleotides. Biochemistry 31: 9183-9189

Dreher TW, Uhlenbeck OC, Browning KS (1999) Quantitative assessment of EF-1alpha. GTP binding to aminoacyl-tRNAs, aminoacyl-viral RNA, and tRNA shows close correspondence to the RNA binding properties of EF-Tu. J Biol Chem 274: 666-672

Durso NA, Cyr RJ (1994) Beyond translation - elongation factor-1 alpha and the cytoskeleton. Protoplasma 180: 99-105

Durso NA, Cyr RJ (1994) A calmodulin-sensitive interaction between microtubules and a higher-plant homolog of elongation factor-1 alpha. Plant Cell 6: 893-905

Edmonds BT, Murray J, Condeelis J (1995) pH regulation of the F-actin binding properties of Dictyostelium elongation factor 1 alpha. J Biol Chem 270: 15222-15230

Edmonds BT, Wyckoff J, Yeung YG, Wang Y, Stanley ER, Jones J, Segall J, Condeelis J (1996) Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma. J Cell Sci 109: 2705-2714

Emerson RA, Beadle GW, Frazer AG (1935) A summary of linkage studies in maize. Cornell University Agricultural Experimental Station Report 180.

Esen A (1987) A proposed nomenclature for the alcohol-soluble proteins (zeins) of maize (*Zea mays* L.). J Cereal Sci 5: 117-128

Esen A, Stetler DA (1992) Immunocytochemical localization of delta-zein in the protein bodies of maize endosperm cells. Am J Bot 79: 243-248

FAOSTAT (2002) FAO Statistical Databases. http://apps.fao.org/default.htm.

Fernandes J, Brendel V, Gai X, Lal S, Chandler VL, Elumalai RP, Galbraith DW, Pierson EA, Walbot V (2002) Comparison of RNA expression profiles based on maize expressed sequence tag frequency analysis and micro-array hybridization. Plant Physiol 128: 896-910

Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D (1991) Light-directed, spatially addressable parallel chemical synthesis. Science 251: 767-773

Fonzi WA, Katayama C, Leathers T, Sypherd PS (1985) Regulation of protein synthesis factor EF-1 alpha in *Mucor racemosus*. Mol Cell Biol 5: 1100-1103

Geetha KB, Lending CR, Lopes MA, Wallace JC, Larkins BA (1991) *opaque-2* modifiers increase gamma-zein synthesis and alter its spatial distribution in maize endosperm. Plant Cell 3: 1207-1219

Geli MI, Torrent M, Ludevid D (1994) Two structural domains mediate two sequential events in gamma-zein targeting: protein endoplasmic reticulum retention and protein body formation. Plant Cell 6: 1911-1922

Gevers HO, Lake JK (1992) Development of modified *opaque-2* maize in South Africa. *In* ET Mertz, ed, Quality Protein Maize. The American Society of Cereal Chemists, St. Paul, pp 49-78

Gibbon BC, Kovar DR, Staiger CJ (1999) Latrunculin B has different effects on pollen germination and tube growth. Plant Cell 11: 2349-2363

Gibbon BC, Zonia LE, Kovar DR, Hussey PJ, Staiger CJ (1998) Pollen profilin function depends on interaction with proline-rich motifs. Plant Cell 10: 981-993

Gillikin JW, Zhang F, Coleman CE, Bass HW, Larkins BA, Boston RS (1997) A defective signal peptide tethers the *floury-2* zein to the endoplasmic reticulum membrane. Plant Physiol 114: 345-352

Glover DV, Mertz ET (1987) Corn. *In* RA Olson, KT Frey, eds, Nutritional quality of cereal grains: genetic and agronomic improvement. ASA-CSSA-SSSA, Madison, pp 183-336

Gonen H, Smith CE, Siegel NR, Kahana C, Merrick WC, Chakraburtty K, Schwartz AL, Ciechanover A (1994) Protein synthesis elongation factor EF-1 alpha is essential for ubiquitin-dependent degradation of certain N alpha-acetylated proteins and may be substituted for by the bacterial elongation factor EF-Tu. Proc Natl Acad Sci USA 91: 7648-7652

Habben JE, Kirleis AW, Larkins BA (1993) The origin of lysine-containing proteins in *opaque-2* maize endosperm. Plant Mol Biol 23: 825-838

Habben JE, Moro GL, Hunter BG, Hamaker BR, Larkins BA (1995) Elongation factor 1-alpha concentration is highly correlated with the lysine content of maize endosperm. Proc Natl Acad Sci USA 92: 8640-8644

Hesketh J (1994) Translation and the cytoskeleton: a mechanism for targeted protein synthesis. Mol Biol Rep 19: 233-243

Hiatt WR, Garcia R, Merrick WC, Sypherd PS (1982) Methylation of elongation factor 1 alpha from the fungus Mucor. Proc Natl Acad Sci USA 79: 3433-3437

Hotokezaka Y, Tobben U, Hotokezaka H, van Leyen K, Beatrix B, Smith DH, Nakamura T, Wiedmann M (2002) Interaction of the eukaryotic elongation factor 1A with newly synthesized polypeptides. J Biol Chem 277: 18545-18551

Hunter BG, Beatty MK, Singletary GW, Hamaker BR, Dilkes BP, Larkins BA, Jung R (2002) Maize opaque endosperm mutations create extensive changes in patterns of gene expression. Plant Cell 14: 2591-2612

Izawa T, Fukata Y, Kimura T, Iwamatsu A, Dohi K, Kaibuchi K (2000) Elongation factor-1 alpha is a novel substrate of Rho- associated kinase. Biochem Biophys Res Commun 278: 72-78

Kahns S, Lund A, Kristensen P, Knudsen CR, Clark BF, Cavallius J, Merrick WC (1998) The elongation factor 1 A-2 isoform from rabbit: cloning of the cDNA and characterization of the protein. Nucleic Acids Res 26: 1884-1890

Kaur KJ, Ruben L (1994) Protein translation elongation factor-1 alpha from *Trypanosoma brucei* binds calmodulin. J Biol Chem 269: 23045-23050

Khoo U, Wolf MJ (1970) Origin and development of protein granules in maize endosperm. Am J Bot 57: 1042-1050

Kidou S, Ejiri S (1998) Isolation, characterization and mRNA expression of four cDNAs encoding translation elongation factor 1A from rice (*Oryza sativa* L.). Plant Mol Biol 36: 137-148

Kim CS, Woo Ym YM, Clore AM, Burnett RJ, Carneiro NP, Larkins BA (2002) Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membranes, influence protein body formation in maize endosperm. Plant Cell 14: 655-672

Kirihara JA, Petri JB, Messing J (1988) Isolation and sequence of a gene encoding a methionine-rich 10-kDa zein protein from maize. Gene 71: 359-370

Kodrzycki R, Boston RS, Larkins BA (1989) The *opaque-2* mutation of maize differentially reduces zein gene transcription. Plant Cell 1: 105-114

Kristensen P, Lund A, Clark BF, Cavallius J, Merrick WC (1998) Purification and characterization of a tissue specific elongation factor 1 alpha (EF-1 alpha 2) from rabbit muscle. Biochem Biophys Res Commun 245: 810-814

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Landry J, Moureaux T (1970) Heterogeneite des glutelines du grain de mais: extraction selective et composition en acides amines des trois fractions isolees. Bull Soc Chim Biol 52: 1021-1037

Larkins BA, Hurkman WJ (1978) Synthesis and deposition of zein in protein bodies of maize endosperm. Plant Physiol 62: 256-263

Lehrach H, Diamond D, Wozney JM, Boedtker H (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry 16: 4743-4751

Lending CR, Kriz AL, Larkins BA, Bracker CE (1988) Structure of maize protein bodies and immunocytochemical localization of zeins. Protoplasma 143: 51-62

Lending CR, Larkins BA (1989) Changes in the zein composition of protein bodies during maize endosperm development. Plant Cell 1: 1011-1023

Linz JE, Katayama C, Sypherd PS (1986) Three genes for the elongation factor EF-1 alpha in *Mucor racemosus*. Mol Cell Biol 6: 593-600

Liu G, Grant WM, Persky D, Latham VM, Singer RH, Condeelis J (2002) Interactions of elongation factor 1 alpha with F-actin and beta-actin mRNA: Implications for anchoring mRNA in cell protrusions. Mol Biol Cell 13: 579-592

Lopez-Valenzuela JA, Gibbon BC, Hughes PA, Dreher TW, Larkins BA (2003) eEF1A isoforms change in abundance and actin-binding activity during maize endosperm development. Plant Physiol In press

Lund A, Knudsen SM, Vissing H, Clark B, Tommerup N (1996) Assignment of human elongation factor 1 alpha genes: eEF1A maps to chromosome 6q14 and eEF1A2 to 20q13.3. Genomics 36: 359-361

Marks MD, Lindell JS, Larkins BA (1985) Nucleotide sequence analysis of zein mRNAs from maize endosperm. J Biol Chem 260: 16451-16459

Merrick WC (1992) Mechanism and regulation of eukaryotic protein synthesis. Microbiol Rev 56: 291-315

Merrick WC, Dever TE, Kinzy TG, Conroy SC, Cavallius J, Owens CL (1990) Characterization of protein synthesis factors from rabbit reticulocytes. Biochim Biophys Acta 1050: 235-240

Mertz ET, Nelson OE, Bates LS (1964) Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145: 279-280

Moore RC, Cyr RJ (2000) Association between elongation factor-1 alpha and microtubules *in vivo* is domain dependent and conditional. Cell Motil Cytoskeleton 45: 279-292

Moro GL, Habben JE, Hamaker BR, Larkins BA (1996) Characterization of the variability in lysine content for normal and *opaque2* maize endosperm. Crop Sci 36: 1651-1659

Motorin YA, Wolfson AD, Orlovsky AF, Gladilin KL (1988) Mammalian valyl-tRNA synthetase forms a complex with the first elongation factor. FEBS Lett 238: 262-264

Nagata S, Nagashima K, Tsunetsugu-Yokota Y, Fujimura K, Miyazaki M, Kaziro Y (1984) Polypeptide chain elongation factor 1 alpha (EF-1 alpha) from yeast: nucleotide sequence of one of the two genes for EF-1 alpha from *Saccharomyces cerevisiae*. Embo J 3: 1825-1830

Nelson OE, Mertz ET, Bates LS (1965) Second mutant gene affecting amino acid pattern of maize endosperm proteins. Science 150: 1469-1470

Osborne TB (1924) The vegetable proteins. Longmans, Green, London

Pedersen K, Argos P, Naravana SVL, Larkins BA (1986) Sequence analysis and characterization of a maize gene encoding a high-sulfur zein protein of Mr 15,000. J Biol Chem 261: 6279-6284

Pokalsky AR, Hiatt WR, Ridge N, Rasmussen R, Houck CM, Shewmaker CK (1989) Structure and expression of elongation factor 1 alpha in tomato. Nucleic Acids Res 17: 4661-4673

Prat S, Cortadas J, Puigdomenech P, Palau J (1985) Nucleic acid (cDNA) and amino acid sequences of the maize endosperm protein glutelin-2. Nucleic Acids Res 13: 1493-1504

Prat S, Perezgrau L, Puigdomenech P (1987) Multiple variability in the sequence of a family of maize endosperm proteins. Gene 52: 41-49

Ransom WD, Lao PC, Gage DA, Boss WF (1998) Phosphoglycerylethanolamine posttranslational modification of plant eukaryotic elongation factor 1 alpha. Plant Physiol 117: 949-960

Righetti PG, Gianazza E, Viotta A, Soave C (1977) Heterogeneity of storage proteins in maize. Planta 136: 115-123

Sabelli PA, Shewry PR (1993) Nucleic acid blotting and hybridisation. Methods Plant Biochem 40: 79-100

Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270: 467-470

Schmidt RJ, Burr FA, Aukerman MJ, Burr B (1990) Maize regulatory gene *Opaque-2* encodes a protein with a "leucine-zipper" motif that binds to zein DNA. Proc Natl Acad Sci USA 87: 46-50

Sherman M, Sypherd PS (1989) Role of lysine methylation in the activities of elongation factor 1 alpha. Arch Biochem Biophys 275: 371-378

Shewry PR, Halford NG (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. J Exp Bot 53: 947-958

Sun Y, Carneiro N, Clore AM, Moro GL, Habben JE, Larkins BA (1997) Characterization of maize elongation factor 1A and its relationship to protein quality in the endosperm. Plant Physiol 115: 1101-1107

Thompson G, Larkins BA (1994) Characterization of zein genes and their regulation in maize endosperm. *In* M Freeling, V Walbot, eds, The Maize Handbook. Springer-Verlag, Berlin, pp 639-646

van Hemert FJ, Lenstra JA, Moller W (1983) Genes for elongation factor EF-1 alpha in the brine shrimp Artemia. FEBS Lett 157: 295-299

Venema RC, Peters HI, Traugh JA (1991) Phosphorylation of valyl-tRNA synthetase and elongation factor 1 in response to phorbol esters is associated with stimulation of both activities. J Biol Chem 266: 11993-11998

Vijaykumar D, Ramachander TVN, Mahishi LH, Kaul R, Pyati P, Paul B, Rawal SK (2002) Molecular cloning, characterization and tissue specific expression of an elongation factor 1A gene in *Saccharum officinarum* L. Plant Sci 162: 315-321

Villegas E, Vasal SK, Bjarnarson M (1992) Quality protein maize -what is it and how was it developed. *In* ET Mertz, ed, Quality Protein Maize. The American Society of Cereal Chemists, St. Paul, pp 27-48

Wallace JC, Lopes MA, Paiva E, Larkins BA (1990) New methods for extraction and quantitation of zeins reveal a high content of gamma-zein in modified *opaque-2* maize. Plant Physiol 92: 191-196

Wang W, Poovaiah BW (1999) Interaction of plant chimeric calcium/calmodulin-dependent protein kinase with a homolog of eukaryotic elongation factor-1alpha. J Biol Chem 274: 12001-12008

Wang X, Woo YM, Kim CS, Larkins BA (2001) Quantitative trait locus mapping of loci influencing elongation factor 1alpha content in maize endosperm. Plant Physiol 125: 1271-1282

Whiteheart SW, Shenbagamurthi P, Chen L, Cotter RJ, Hart GW (1989) Murine elongation factor 1 alpha (EF-1 alpha) is posttranslationally modified by novel amide-linked ethanolamine-phosphoglycerol moieties. Addition of ethanolamine-phosphoglycerol to specific glutamic acid residues on EF-1 alpha. J Biol Chem 264: 14334-14341

Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T, Mann M (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 379: 466-469

Wilson CM (1985) Nomenclature of zein polypeptides based on isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Cereal Chem 62: 361-365

Winter H, Huber JL, Huber SC (1998) Identification of sucrose synthase as an actin-binding protein. FEBS Lett 430: 205-208

Woo YM, Hu DW, Larkins BA, Jung R (2001) Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. Plant Cell 13: 2297-2317

Xu W, Bak S, Decker A, Paquette SM, Feyereisen R, Galbraith DW (2001) Microarray-based analysis of gene expression in very large gene families: the cytochrome P450 gene superfamily of *Arabidopsis thaliana*. Gene 272: 61-74

Yang F, Demma M, Warren V, Dharmawardhane S, Condeelis J (1990) Identification of an actin-binding protein from Dictyostelium as elongation factor-1A. Nature 347: 494-496

Yang W, Boss WF (1994) Regulation of phosphatidylinositol 4-kinase by the protein activator PIK-A49 - activation requires phosphorylation of PIK-A49. J Biol Chem 269: 3852-3857

Yang WN, Burkhart W, Cavallius J, Merrick WC, Boss WF (1993) Purification and characterization of a phosphatidylinositol 4- kinase activator in carrot cells. J Biol Chem 268: 392-398

Yates JR (1998) Mass spectrometry and the age of the proteome. J Mass Spectrom 33: 1-19

Young VR, Scrimshaw NS, Pellet PL (1998) Significance of dietary protein source in human nutrition: animal and/or plant proteins? *In* JC Waterlow, DG Armstrong, L Fowden, R Riley, eds, Feeding a world population of more than eight billion people. Oxford University Press in association with Rank Prize Funds, New York, pp 205-221

Zarkadas CG, Hamilton RI, Yu ZR, Choi VK, Khanizadeh S, Rose NG, Pattison PL (2000) Assessment of the protein quality of 15 new northern adapted cultivars of quality protein maize using amino acid analysis. J Agric Food Chem 48: 5351-5361

Zobel-Thropp P, Yang MC, Machado L, Clarke S (2000) A novel post-translational modification of yeast elongation factor 1A. Methylesterification at the C terminus. J Biol Chem 275: 37150-37158