THE CONSTRUCTION AND TESTING OF MAIZE TRANSCRIPTIONAL FUSIONS IN YEAST(SACCHAROMYCES CEREVISIAE)

by

Selester Bennett

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APPROVED:

A. Esen, Chairman

K. Pedersen

C. Cramer

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Committee Chairman: Asim Esen

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(ABSTRACT)

The specific goal of this study was to construct and test transcriptional fusions of zein promoters and a yeast reporter gene that will serve as part of a two plasmid system that will allow for the identification of maize transcriptional regulators of zein genes. Zein genes are expressed coordinately and temporally during endosperm development and are controlled at the transcriptional level (Pedersen et al., 1980; Kodryzcki et al., 1989). The accumulation of zein proteins in the endosperm presents an ideal model system to study plant gene regulation. These proteins are synthesized only in the endosperm tissue, and their concentration in the endosperm determine the nutritional quality of the seed.

Because of the coordinate and temporal regulation of zein gene transcription, there is a strong likelihood that there exists positive regulatory elements of zein gene expression during early endosperm development. We know that the control of storage protein gene expression is mediated by regulatory elements in the endosperm of maize seeds. It has been shown that the recessive mutation opaque-2 (o2) specifically reduces the 22,000 zein polypeptide. Schmidt et al. (1990) and Aukerman et al. (1991) show that the wild-type O2 encodes a protein containing a basic leucine zipper domain

(bZIP) and binds specifically to the promoter region of 22kD zein genes, the target of the O2 regulatory locus. This binding is all that is necessary for transcriptional activation (Aukerman et al., 1991). This makes it possible to identify maize transcriptional activators by their function in the heterologous yeast cell.

In this study, a heterologous system *(Saccharomyces cerevisiae)* was used to investigate the potential for the selection of maize regulatory genes by their function in yeast. This study utilized an *in vivo* assay to demonstrate that zein gene promoters were capable of initiating transcription of the CUP1 gene in yeast and thereby conferring a basal level of copper tolerance to a host cell that was completely deleted in its genome for the CUP1 gene. The CUP1 gene enables yeast to tolerate copper in the enviroment. Copper tolerance was demonstrated to be confered from functional zein promoters by plating yeast cells on selective plates with increasing concentrations of copper. Additionally, results from analysis of Southern blot DNA hybridization experiments show that conferred copper tolerance is due to the function of an active zein gene promoter-CUP1 transcriptional fusion.

Our results clearly show that the minimal requirement of the native zein promoters being expressed at a basal level by the yeast transcription system is satisfied. This basal level of expression is necessary for the isolation of maize transcription factors specific for zein gene promoters. The functional zein promoter-CUP1 fusions provide the foundation for a two plasmid system that will allow for the selection of maize regulatory genes.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The overall goal of my thesis work has been to construct and test transcriptional fusions of zein promoters and a yeast reporter gene as part of an overall yeast system that will allow for the functional identification of transcription factors involved in the regulation of maize storage protein (zein) genes. Many heterologous genes have been isolated by function or shown to function in this simple eukaryotic yeast cell. A minimal requirement for the isolation of maize transcription factors specific for zein gene promoters is that the native zein promoters be expressed at a basal level by the yeast transcription system. The yeast cell provides a complete set of transcription factors in addition to the RNA polymerase II.

We believe that the accumulation of maize storage proteins presents an ideal model system to study plant gene regulation. Maize is the fourth largest cereal crop in the world (Food and Agricultural Organization, United Nations, 1992); thus, maize plays a major role in human and monogastric animal nutrition. However, the nutritional quality of maize is considered poor largely due to an imbalance with respect to the amino acid content of its seed protein. The cause of this nutritional imbalance is ascribed to the fact that maize storage proteins (zeins) contain no lysine and very little tryptophan, and high levels of zein accumulation (e.g. 50-60%) lowers the overall balance of these essential amino acids in maize seed. Zeins, the major storage proteins in maize seed, comprise a group of alcohol-soluble polypeptides that are synthesized by rough endoplasmic reticulum (RER) bound polyribosomes. Zein proteins are produced in the endosperm of the developing seed between 10 to 50 days after pollination, and account for 50 to 60 percent of the total endosperm protein. Zeins are separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis into six size classes that are further categorized into four structurally distinct groups: Alpha zeins, generally the most abundant proteins (Mr 19,000 and 22,000), Beta zein (Mr 15,000); Gamma zeins, (Mr 27,000 and 16,000); and Delta zein, (Mr 10,000). The different relative molecular weight groups have been examined by two-dimensional gel analysis and revealed up to 28 peptides, the majority of which belong to the 19,000 and 22,000 weight class (Righetti et al., 1977). The alpha zein (19,000 and 22,000) polypeptide components are encoded by multigene families which is the basis for charge heterogeneity revealed by two-dimensional gel electrophoresis.

The zein genes are arranged in several linkage groups on chromosomes 4, 7, and 10 (Soave et al., 1981). During the early stages of endosperm development, zein mRNAs accumulate to high concentrations. Expression of zein genes is developmentally and coordinately regulated (Boston et al., 1986) and is controlled at the transcriptional level (Pedersen et al., 1980; Kodryzcki et al., 1989). It is the level to which zeins accumulate in the maize endosperm that determines its nutritional quality. Zein gene expression is controlled by several regulatory loci (Pedersen et al., 1980; Kodryzcki et al., 1989). These regulatory loci affect zein transcription and thereby the accumulation of zein proteins during early endosperm development (Motto et al., 1988). These regulatory loci have been characterized by the identification of recessive mutants which exert regulation on the timing and rate of zein accumulation. These recessive mutants are grouped by their mode of action: those that delay or reduce the accumulation of all the zein polypeptides or those that affect some specific zein components. The recessive mutants opaque-2 (o2), opaque-7 (o7), and floury-2 (fl2) all reduce the accumulation of zein protein. These mutations have been mapped to chromosomes 7, 10, and 4, respectively. Through genetic linkage analysis several of the genes for the 22kD zeins have been mapped to chromosome 4 and 10, and the genes for the 19kD zeins have been mapped to chromosome 7 (Soave et al., 1981). The effects of each recessive mutation are as follows: o2 primarily reduces the 22kD zeins, o7 reduces the 19kD zeins, and fl2 reduces both the 19kD and 22kD zeins (DiFonzo et al., 1980). The o2 locus is found on chromosome 7 while the regulated functional 22kD zein genes are found on chromosome 4 and 10 which suggest that the o2 gene is a transacting regulator of zein expression; both o7 and fl2 also regulate their respective zein genes by way of transacting elements. The recessive alleles, o2 and o7, act additively in reducing zein accumulation, 22kD is reduced by o2, and 19kD is reduced by o7 (DiFonzo et al., 1980); whereas, o2 and o7 show epistatic effects when paired with floury-2 (fl2) (Soave et al., 1978).

Several mutations (e.g. opaque-2) have been identified which exert a regulatory effect on the synthesis of zein proteins during endosperm development (Aukerman et al., 1991), and as a result zein protein accumulation is reduced in these mutants, thereby changing the overall balance of the essential amino acids, lysine and tryptophan in total seed protein. This results in seeds with improved nutritional quality. However, these mutations cause a phenotypic change from hard glassy kernels to seeds with a soft powdery endosperm, resulting in a seed with inferior harvesting, storage, and milling properties. Through genetic selection efforts "modified" opaque-2 mutants have been developed to have a hard, vitreous seed phenotype. The modified opaque-2 mutants that have been identified all apparently activate 27,000 gamma-zein gene transcription in all genetic backgrounds (Geetha et al., 1991). The "modifier" genes have the same effect on gamma zein synthesis in wild-type backgrounds although there is no phenotypic change associated with "modifier" genes in the wild-type background. The identification of these "modifier" genes in opaque-2 backgrounds strongly suggests that the regulation of zein gene transcription is governed by an complex regulatory circuitry and that there must be other factors that contributed to the coordinate and temporal control of zein gene expression.

It has been shown by Pedersen et al. (1980) that the effect of the o2 mutation is at the transcriptional level and that there is a selectable reduction of the 22kD zein transcripts over all other zein transcripts. From work done on the opaque-2 (o2) system, it is know that the recessive nature of the mutation and other recessive mutations like it (e.g. o7 and fl2) exert their effects by way of transacting elements and that the wild-type loci encodes functional regulatory elements that activates transcription by selectively interacting with individual zein gene promoters. The o2 locus has been tagged by Ds, Bg, Spm, and Ac transposable elements (Motto et al., 1988, Schmidt et al., 1987) which led to the successful cloning of the wild-type allele of the o2 locus.

It has been demonstrated by Schmidt et al. (1990) and Aukerman et al. (1991) that the wild-type O2 encodes a protein containing a basic leucine zipper (bZIP) domain typical of eukaryotic transcription factors (Landschulz et al., 1988). The basic leucine zipper (bZIP) domain consists of heptameric repeats of leucine that function as a dimerization interface, while the basic region is responsible for direct contact with its DNA target. It has been shown by Aukerman et al. (1991) that a ethylmethane sulfonate (EMS) induced opaque-2 mutant (o2-676) protein does not display specific DNA binding to 22,000 zein gene promoters; the o2 mutant phenotype was due to a single amino acid change (arginine to lysine) within the basic domain of the bZIP domain. The substitution of lysine for arginine abolishes the binding to 22,000 zein gene sequences and binding is not restored until arginine is reinstated by oligo-directed in vitro mutagenesis, indicating that direct DNA binding is necessary for function of the O2 protein and this specific binding is all that is necessary for transcriptional activation.

The known maize transcription factors contain domains typical of eukaryotic transcription factors. The Knotted-1 regulatory gene contains a homeo-domain (Volbrecht et al., 1991) and O2 is a basic leucine-zipper protein (Hartings et al., 1989; Schmidt et al., 1990). Because of the coordinate and temporal regulation of zein gene transcription, there is a strong likelihood that there exists both positive and negative regulation of zein gene expression during early endosperm development. To date no plant repressors have been identified, but there is an example of negative cis-acting DNA element responsible for the correct temporal regulation of the major storage protein gene from Phaseolus (Bustos et al., 1991). We believe that the control of expression of storage protein genes is mediated by regulatory elements in the endosperm of maize seeds. It has been shown that the recessive mutations opaque-7 (o7) and opaque-2 (o2) specifically reduces 19,000 and 22,000 polypeptides, respectively. In addition to these regulatory loci affecting alpha zeins, the "modifier" genes demonstrate that many regulatory genes are involved in zein gene expression even without phenotypic manifestations.

Currently, all the known molecular regulation of zein genes is of a positive nature. For example, the protein encoded by the wild-type allele of the O2 gene binds specifically to the 22kD promoters and has a positive effect on the transcriptional levels of the 22kD zein genes. Evidence presented by Schmidt et al. (1992) has shown that O2 can activate lacZ gene transcription in yeast in the absence of other maize proteins when the lacZ gene promoter contains a multimer of the O2 target site. Furthermore, the O2 gene product (bZIP) has been shown to complement the function of yeast GCN4 mutants (Mauri et al., 1993) demonstrating that a maize transcription factor can substitute for yeast GCN4 function; therefore, the known maize transcription factors should function in yeast cells.

We will utilize yeast molecular biology and genetics to screen for corn regulatory elements as a result of their function in yeast in order to isolate cDNAs for wild-type regulatory genes affecting the transcription rate of zein genes. The advantage of using the yeast system is that it is possible to screen large numbers of cells, so that basically all mRNAs present in the maize endosperm can be screened for transcriptional regulatory function in this simple eukaryotic cell. The yeast system has been used to identify many eukaryotic genes by their function (e.g. estrogen receptors, Metzger et al. 1988; transcription factors, Berger et al. 1990; glucocorticoid receptors, Schena and Yamamoto, 1988; and cell cycle genes, Lee and Nurse, 1987). Plant transcriptional activators share binding motifs typical of a number of other eukaryotic transcriptional factors and are likely to recognize their target sequence in this simple eukaryotic cell.

We believe that there exist a complex network of regulation in zein gene expression, and that this system is best studied by analyzing each component individually. We will be able to take advantage of the sensitivity of a reporter gene in yeast to allow us to identify genes that may play a part in zein gene expression.

As a means of understanding the molecular events that control the expression of maize storage protein (zein) genes, we have constructed transcriptional cassettes with zein gene promoters fused to a yeast reporter genes that will allow us to select for transcriptional regulators of maize zein genes.

The reporter gene will enable yeast cells to grow under defined conditions, only when activated. We have used the copperthionein gene from the CUP1 locus of yeast *Saccharomyces cerevisiae* (Karin et al., 1984; Butt et al., 1984) as our reporter gene.

The yeast CUP1 gene is found on chromosome VIII and confers tolerance to exogenous copper ions in the environment and in our system provides a tool for positive selection for positive, activating factors. The yeast CUP1 gene is usually present in multiple copies in the yeast genome and its native promoter is induced upon growth on a copper substrate. We have chosen a yeast strain that is completely deleted for the CUP1 gene in its genome, resulting in cells that are extremely sensitive to copper. Any cells with the CUP1 deletion that are transformed with a plasmid containing the zein-CUP1 fusions will reflect copper tolerance that is conferred by the transcriptional activation of the zein promoter. We have zein promoter-CUP1 fusion in which zein promoters have replaced the native CUP1 promoter resulting in constructs that confer a basal level of CUP1 gene activity. Therefore based on the strength of transcriptional activation we will be able to identify factors that influence the activity of the zein promoter as a result of increase CUP1 gene activity.

Figure 1 shows a model of the ultimate strategy of our system. We have fashioned a functional assay for the identification of maize transcriptional regulators of zein genes in yeast by positive selection. It consists of both a single copy target plasmid which contains a transcriptional fusion between individual zein gene promoters and a yeast reporter, and a high copy expression plasmid for the expression of maize cDNA libraries under the genetic control of an inducible yeast promoter. The difference in copy number between the transcriptional fusions and the cDNA libraries ensure maximal interaction between the promoter and the expressed maize transcription factors. Expression of maize transcriptional factors that interact specifically with zein promoters would lead to altered expression patterns of the yeast reporter gene. The advantage of our system over conventional methods is that there is no requirement for phenotypic change in order to score for positive results.

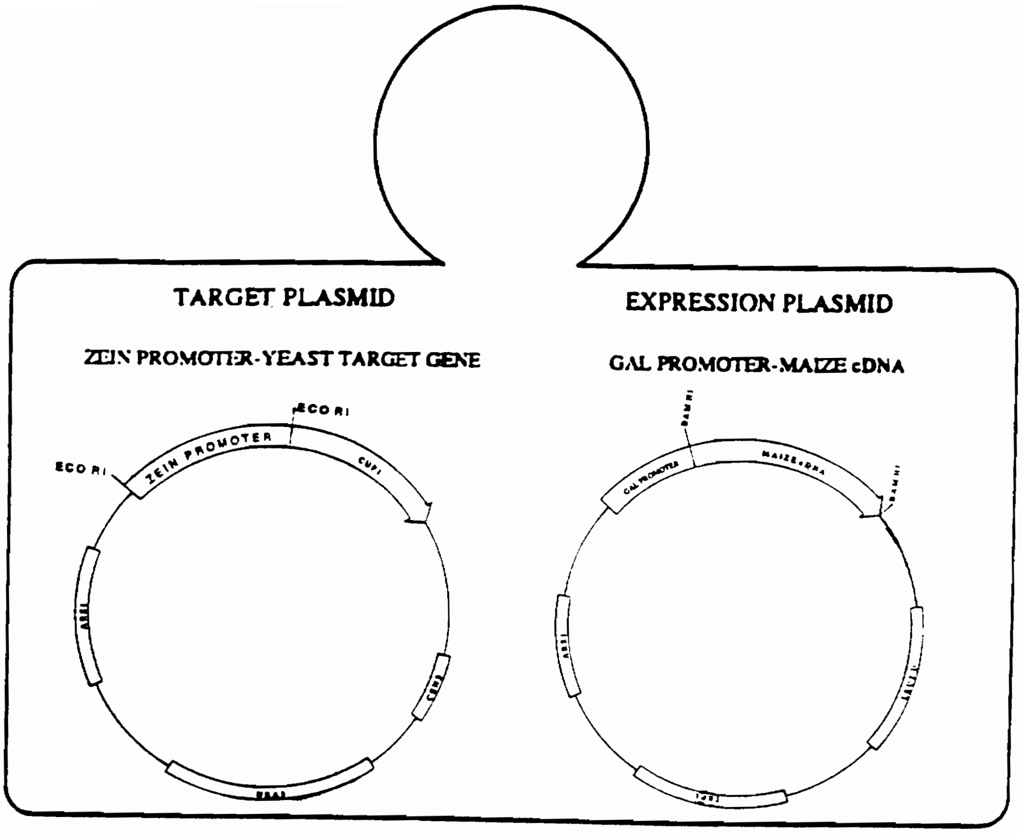

Figure 1. Model of two plasmid system

We have constructed transcriptional fusions between individual zein promoters and a yeast reporter or target gene, and a high copy expression plasmid for the expression of maize cDNA libraries under the control of a yeast inducible promoter. Expression of maize transcriptional factors from cDNAs in *trans* that interact specifically with zein promoters would lead to altered expression patterns of the reporter gene, which in turn will allow for the identification of cDNAs encoding transcription factors.

It has been demonstrated that zein gene promoters function and are recognized correctly in yeast cells and initiate transcripts at the same position as in maize (Pedersen, personal communication). In the case of one 19,000 zein gene (Pedersen et al., 1982), the 5' cis-elements necessary for maximal expression (Roussell et al., 1988) and tissue specificity (Schernthaner et al., 1988) have been determined. We feel that the correct recognition and initiation from zein promoters in yeast will enable us to examine positive modulation of zein gene promoters that have been transcriptionally fused to a yeast reporter gene. This makes it possible to identify maize transcriptional activators of zein promoters by their function in the heterologous yeast cell.

LITERATURE REVIEW

1. Mutations Affecting Zein Synthesis and Accumulation

Zein genes have been located by genetic linkage analysis to chromosomes 4,6,7, and10 (Soave et al., 1981). The alpha zeins have been located to the short arm of chromosome 4 and 7 (Thompson and Larkins 1989) and the long arm of chromosome 10. In addition to the alpha zeins, the gamma zeins have been located to chromosome 7 by RFLP mapping (Murray et al., 1988), and the delta zeins to the short arm of chromosome 7 (Thompson and Larkins, 1989). Beta zeins have been located to chromosome 6 (Murray et al., 1988).

Studies based on sequence analysis of zein cDNA and genomic clones (Marks and Larkins, 1982; Burr et al., 1982) show that zein genes contain no intervening sequences (Pedersen et al., 1982; Messing et al., 1983). Inheritance studies of crosses between maize inbreds have identified twenty zein structural genes (Soave et al., 1981; Soave and Salamini, 1984), many of which belong to a complex multigene family; for example, the 19 and 22kD zein genes. All other zein genes: beta , gamma, and delta are encoded by one or two genes. It has been demonstrated based on the degree of hybridization of zein cDNA clones as probes that there is an estimated 50-75 genomic gene copies and only approximately 50% of the genes are expressed and have been identified by isoelectric focusing (Pedersen et al., 1980; Park et al., 1980; Hagen and Rubenstein, 1981; Marks et al., 1985). Marks et al. (1985) have shown that the only sequence homology demonstrated between the zein genes is that shared between the alpha zeins, 19 and 22kD polypeptide genes found to have a homology of 60 to 65%, which suggest that these genes may have a common ancestral gene (Spena et al., 1982; Wilson and Larkins, 1984; Heidecker et al., 1991).

Further comparisons of zein genes reveals that their upstream regions share common features typical of eukaryotic promoter regions. The zein genes contain a TATA box (TATTA) and CAAT consensus sequence located within -180 from the 5' transcription start site (Pedersen et al., 1982; Boronat et al., 1986; Pedersen et al., 1986). The AGGA box represents the only consensus sequence in the 5' region of zein genes (Messing et al., 1983). Brown et al. (1986) have shown that among zein genes there is a highly conserved 15-bp CACATGTGTAAAGGT located between -310 and -340, with the 15kD zein gene being the exception having its conserved sequence at -274 (Drong and Slightom; 1993). This conserved sequence known as a prolamin box shares homology with the sucrose synthase gene of maize (Werr et al., 1985), and prolamin genes from other cereal crops such as H. vulgare (Barley),and T. aestivum (Wheat) (Kreis et al., 1985; Forde et al., 1985), and the SV40 enhancer core sequence GTGGAAG. Maier et al., (1987) have demonstrated that this highly conserved 15-bp sequence binds nuclear proteins that may serve to regulate gene expression. Further inspection of upstream sequences of zein genes has resulted in the determination of the nucleotides -337 and -125 that is required for maximal transcription of the 19kD class of genes (Roussell et al., 1988).

The 3' flanking DNA of zein genes contains multiple polyadenylation sequences which are common for zein genes (Marks et al., 1985). The consensus polyadenylation sequences found in zein genes are similar in sequence and position to those reported in other eukaryotic genes (Pedersen et al., 1982; 1986; Messing et al., 1983) while some zein cDNA clones have polyadenylation signals that are identical to those of mammalian genes (AATAAA); for example, nt 657-672 (AATAAA) of the 15kD zein gene, in addition several variants, (AATAAT), (TATAAA), (ATAAAA), (AATAAG), (AATGAA) have been reported (Drong and Slightom; 1983; Marks et al., 1985), and 3' flanking region mapping with S1 nuclease has shown that all of these polyadenylation signal are used. A nine base pair element (CATGTGTGG) that is important for efficient mRNA 3' end formation in plants (Mogen et al., 1990) had been identified in the sequence of a 15kD zein gene starting at nt 624 (Drong and Slightom, 1993).

The 5' flanking sequences of zein genes are divided into two regions, one that controls tissue-specific temporal and spatial regulation, and one that is responsible for the rate of transcription, thus these regions play a role in the activation of transcription at the correct time during seed development.

2. Mutations in Maize That Alter Zein Gene Expression

The production of seed storage proteins at specific developmental stages in plants is a highly regulated and coordinated process. Maize storage protein genes are specifically expressed in a specific tissue (endosperm), and these genes are controlled by complex regulatory mechanisms that control the initiation and rate of zein synthesis and accumulation. Several mutations that affect zein gene expression and timing have been studied and characterized (Manzocchi et al., 1980). These mutatations have been shown to affect endosperm integrity and reduce total zein content in maize seeds (Motto et al., 1989). These mutants are grouped by their mode of action: those that delay or reduce the accumulation of all the zein polypeptides or those that target some specific zein components. The recessive mutants opaque-2 (o2), opaque-6 (o6), opaque-7 (o7), and floury-2 (fl2) all reduce the accumulation of zein protein. These mutations have been mapped to chromosomes 7, 10, and 4 respectively. The effects of each recessive mutations is as follows: o2 primarily reduces the 22kD zeins, o7 reduces the 19kD zeins, and fl2 reduces both the 19kD and 22kD zein proteins (DiFonzo et al., 1980). The o2 locus is found on chromosome 7 while the regulated functional 22kD zeins genes are found on chromosome 4 and 10 which suggests that the O2 gene is a transacting regulator of zein expression (both o7 and fl2 also regulate their respective zein genes by way of transacting elements). The recessive alleles o2 and o7 act additively in reducing zein accumulation: 22kD is reduced by o2 and 19kD is reduced by o7 (DiFonzo et al., 1980); whereas, o2 and o7 showed epistatic effect when paired with floury-2 (fl2).

The dominant mutants defective endosperm -B30 (De-B30), and Mucronate (Mc) (Salamini et al., 1979; 1983) delay the synthesis of zein by at least 10 days, particularly the 22kD zein class.

The maize seed storage protein mutation that receives the most research attention is opaque-2 because of its effect on nutritional value of maize seed. This mutation has been shown to exert a regulatory effect on the production of zein proteins during endosperm development, and as a result zein protein accumulation is reduced, approximately 47%, in these mutants, thereby changing the overall balance of the essential amino acids, lysine and tryptophan, in total seed protein.

However, these mutations cause a phenotypic change from hard glassy kernels to seeds with a soft powdery endosperm, resulting in a seed with inferior harvesting, storage, and milling properties.

It has been shown through the work of Dalby and Tsai, (1974) and Jones et al., (1977) that in opaque-2 endosperm, the synthesis of zein is reduced, and terminates at 30 days after pollination (DAP), some 15 days earlier than wild-type maize. Pedersen et al. (1980) have demonstrated that the effect of the O2 mutation is at the transcriptional level and that there is a selective reduction (100 fold) of the 22kD zeins over all other zein transcripts. From work done on the opaque-2 (o2) system, we know that the recessive nature of the mutation and other recessive mutations like it, e.g. o7 and fl2 exert their effects by way of transacting elements and that the wild-type loci encodes functional regulatory elements that activates transcription by selectively interacting with individual zein genes.

The o2 locus is found on chromosome 7 while the regulated functional 22kD zein genes are found on chromosome 4 and 10 which indicate that the O2 gene is a transacting regulator of zein expression (Schimdt et al., 1987; Motto et al., 1989). Some early investigators had reasoned that the O2 protein might regulate gene expression through a soluble cytoplasmic factor, b-32, which presumed to mediate transcriptional activation (Soave et al., 1981; Motto et al., 1988). Information obtained from cDNA and genomic DNA indicates that the O2 protein contains a "leucine zipper" dimerization/DNA-binding (bZIP) motif commonly found in mammalian proto-oncogene binding proteins (Fos, Jun, Myc) and yeast transcriptional activator (GCN4) (Hartings et al., 1989; Landschulz et al., 1988).

Opaque-7 mutants reduce the 19kD zein classes primarily (DiFonzo et al., 1979). The genes for the 19kD zeins have been mapped to chromosome 7 (Soave et al., 1981) while the 07 locus is located on the long arm of chromosome 10 (Motto et al., 1989) which suggest that the O7 gene, like the O2 gene, is a transacting regulator of zein expression.

Mutations that reduce the synthesis of all zein proteins to the same extent have been studied. The mutation opaque-6 (o6) is reported to be non-specific with respect to any one zein class and result in a decrease in zein accumulation when compared to wild-type maize. Additionally, opaque-6 (o6) has an additive effect when it is paired with opaque-2 (o2) or opaque-7 (o7).

Floury-2 (fl2) is another example of a mutation that reduces the synthesis of all classes of zein genes, most noticeably the 19kD and 22kD zein proteins (DiFonzo et al., 1980). Soave and Salamini (1984) have shown a relationship between the fl2 mutation and overproduction of a soluble protein b-70 (70kD) located in the zein protein bodies of fl2. This association between the overproduction of b-70 and the abnormal protein body formation is thought to be a cellular response to the activity associated with the factor encoded by the O7 locus (Boston et al., 1991).

The dominant mutations defective endosperm-B30 (De-B30) and Mucronate (Mc) (Salamini et al, 1979, 1983) are further examples of developmental mutants that control zein synthesis and accumulation. Defective endosperm-B30 (De-B30) allele has been mapped to chromosome 7, closely linked to O2, and reduces the expression of the 22kD class of zeins. Mucronate (Mc) reduces all zein protein levels to the same extent. Additionally, Boston et al. (1991) have reported that an overexpression of b-70 is also associated with zein regulatory mutants: Defective endosperm -B30 (De-B30) and Mucronate (Mc).

Other mutations that negatively affect zein accumulation are the starch mutants. These mutants are categorized into two groups: starch-deficient mutants and mutants with modified starch. Shrunken-1 (sh1), shrunken-2 (sh2), shrunken-4 (sh4), brittle-1 (bt1), brittle-2 (bt2), and sugary-1 (su1) are examples of mutants which are exemplified by a reduction in their starch biosynthesis (starch-deficient). Those mutants that affect the normal amylose to amylopectin ratio are dull (du), waxy (wx), sugary-2 (su2), and amylose-extender (ae).

Synergistic interactions between starch mutants, shrunken-4 (sh4), brittle-2 (br2), and opaque-2 (o2) result in significant reduction in zein accumulation, especially the alpha zein, suggesting a complex cross-regulation of protein and starch synthesis in the endosperm.

To date all mutations effecting zein synthesis and, thereby, accumulation show a particular effect on the alpha zein, the only example of a mutation effecting only non-alpha zeins in the "opaque-2 modifier". This mutation has been identified in certain opaque-2 genetic backgrounds. "Modified" opaque-2 mutants have been developed by selection for hard, vitreous seed phenotype. The identification of "modifiers" in opaque-2 backgrounds indicates further the presence of additional factors required for the control of zein gene expression. "Modified" opaque-2 mutants (Quality Protein Maize-QPM) have been identified and are proposed to increase 27,000 gamma zein gene transcription independent of the opaque-2 mutation (Geetha et al., 1991; Lopes and Larkins, 1991), and as a result returns the seed to normal glassy phenotype. The "modifier" genes have the same effect on gamma zein synthesis in wild-type backgrounds although there is no phenotypic changes associated with"modifier" genes in the wild-type background. Quality Protein Maize may carry a positive cis-acting or trans-acting factor that is responsible for increasing gene transcription affecting 27kD gamma zein protein synthesis causing an extensive cross-linking of this protein in "modified" opaque-2 endosperm.

3. Plant Transcriptional Elements Share Structural Motifs Typical of Eukaryotic Transcriptional Factors

The known maize transcriptional factors contain domains typical of eukaryotic transcription factors. The maize Knotted-1, Kn1, regulatory gene contains a homeodomain, a 61-amino acid helix-turn-helix motif which is responsible for DNA binding. The Knotted-1 (Kn1) homeodomain shares 35% homology with the human Prl protein of pre-B cell leukemia (Nourse et al., 1990) and the MATP protein of Schizosaccharomyces pombe (Kelly et al., 1988). Opaque-2 (O2) mutation in maize contains a bZIP or leucine zipper motif (Harting et al., 1989; Schmidt et al., 1990); the Opaque -2 (O2) is a member of the basic domain/leucine zipper (bZIP) family of transcriptional activators. The bZIP class of transcriptional activators bind to DNA through a bipartite structural motif, consisting of a dimerization motif termed the "leucine zipper" and a "basic region" involved in DNA contact. Examples of the bZIP family of transcriptional activators are the GCN4 gene of yeast involved in amino acid biosynthesis pathway, the rat liver protein C/EBP, the wheat histone gene transcription factor HBP1, the oncogenes Fos and Jun, and the human transcriptional activator CREB (Vinson et al., 1989). The maize transcriptional activators C1, Zm1, Zm38 and the barley (Hordeum vulgare) Hv1, Hv33 share similarities with the myb proto-oncogene family (Paz-Ares et al., 1987; Goff et al., 1990,1991). Schmidt et al (1993) have shown that there are mechanisms ZAG1 and ZAG2 that are responsible for floral development in maize that share homology with floral homeotic genes of Arabidopsis, APETALA1 (AP1), APETALA3 (AP3), AGAMOUS (AG) (Yanofsky et al., 1990; Jack et al., 1992; Mendel et al., 1992) and Antirrhinum, DEFIENCS (Mueller and Nordheim, 1991; Schwarz-Sommer et al., 1992) suggesting that the mechanisms controlling floral development in dicotyledonous species are conserved in sequence and function to those for floral development in monocotyledonous species (maize). Additionally, the plant floral homeotic genes share sequence similarity to eukaryotic transcription factors MCM1 in yeast (Passmore et al., 1988) and serum response factors (SRF) in humans (Norman et al., 1988). The products of these eukaryotic developmental genes have been shown to be sequence-specific DNA binding proteins containing a highly conserved stretch of more than 50 amino acids called the "MADS domains" which have been shown to be DNA binding motifs (Norman et al., 1988; Mueller and Nordheim, 1991; Schwarz-Sommer et al., 1992).

Finally, the maize regulatory genes R and B encode proteins homologous to the helix-loop-helix dimerization region of the Myc oncogene family of proteins (Ludwig et al., 1989; Chandler et al., 1989). The regulatory gene B is required for pigmentation in the plant body of maize (Gerats et al., 1984), and R is required for anthocyanin pigmentation in the aleurone, coleoptile, and anthers (Styles et al., 1973).

4. Yeast as a Heterologous System for the Isolation of Maize Regulatory Genes.

It has been demonstrated by Ma et al. (1988) that a yeast transcriptional activator (GAL4) can stimulate plant gene expression in tobacco-leaf protoplasts when GAL4 binding sites are introduced upstream of the 35S promoter from Cauliflower Mosaic Virus. Furthermore, the maize transcriptional activator O2 has been shown to complement the function of yeast GCN4 mutants (Mauri et al., 1993) demonstrating that a maize transcription factor can function as a yeast transcription factor. Evidence presented by Schmidt et al. (1992) has shown that O2 can activate lacZ gene transcription in yeast in the absence of other maize proteins when the lacZ gene promoter contains a multimer of the O2 target sites, further illustrating that the specific binding of O2 is all that is necessary for transcriptional activation (Aukerman et al., 1991). We believe that given an appropriate DNA binding site, plant activators should function in yeast providing an excellent system for studying plant regulatory genes.

We propose a functional assay to identify transcription factors that are responsible for the regulation of zein gene transcription as a part of a two plasmid system. We have constructed transcriptional fusion cassettes with zein gene promoters fused to a yeast reporter gene (CUP1). The CUP1 gene confers copper tolerance to exogenous copper ions in the environment. In our selection system maize cDNA libraries will be introduced into cells containing the transcriptional fusion cassettes and the expression of these cDNAs in *trans* will allow for the identification of cDNAs encoding proteins that specifically interact with zein promoters (Figure1). The direct isolation of the regulatory genes will allow us to determine how these genes contribute to the control of zein gene expression. As part of our overall strategy to identify regulatory factors we have constructed transcriptional fusion cassettes between zein promoters and a yeast CUP1 gene on shuttle plasmids in yeast and tested for the basal level of expression from zein promoters. This basal level of expression is scored by the yeast ability to tolerate increasing levels of copper in the medium.

MATERIALS AND METHODS

A. Strains and Media

1. Strains

Escherichia coli HB101 (E.coli) [F^- hsdS20 (r^-_{β} m^-_{β}) recA13 ara-14 proA2 lacY1 galK2 rpsL20 (Sm^r) xyl-5 mtl-1 supE44λ^-] was used for transformation and amplification of plasmid DNA. The auxotrophic *Saccharomyces cerevisiae* CUP1 deletion strain ABDE1 [MATa ade2 his7-2 leu2-3 112 arg4-8 cup1::ura3 thr1-4 trp1-289 ura3-52] was used as the host for plasmid transformations and as the test strain in the measurement of copper sensitivity to determine the basal level of expression from zein promoter-CUP1 fusions.

2. Media for Bacteria

For growth of E.coli liquid LB-Broth containing 1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 0.5% NaCl per liter of medium was used. For LB-Broth agar plates, 2% Bacto-agar was added and 40 μg/ml final concentration of ampicillin per liter was added for medium requiring an antibiotic.

3. Media for Non-Selective Growth of Yeast

For the non-selective growth of yeast [ABDE1] a complete liquid YPD medium containing 2% glucose (dextrose), 2% peptone, and 1% yeast extract per liter of medium was used. YPD is complex medium that was used for the routine growth of yeast. All cells were thoroughly washed with cold sterile water before all electro-transformation experiments to remove any carry-over of excess nutrients from YPD.

4. Media for the Selection of Cells Containing a Shuttle Plasmid

For the selection of yeast cells containing a shuttle plasmid Synthetic selective medium containing 0.67% yeast nitrogen base without amino acids, 2% galactose, L-arginine (20μg/ml), L-leucine (30μg/ml), L-histidine (20μg/ml), L-tryptophan (20μg/ml), L-threonine (200μg/ml), and adenine sulfate (20μg/ml) was used to select for the growth of the auxotroph ABDE1 [Materials and Methods] bearing a URA3 containing shutlle plasmid. Synthetic selective medium was used to test the growth requirements of auxotrophs. It is a medium in which commonly encountered auxotrophies are supplemented except for the one component of interest, all of our shuttle vector constructs were based on the auxotrophic marker gene URA3. Therefore, all Synthetic selective medium used in this study lacked uracil. For Synthetic selective agar plates 2% Bacto-agar was added per liter of medium.

5. Media to Score for Zein Promoter-CUP1 Gene Activity

To score for zein promoter activity in yeast cells the CUP1 gene was used as the reporter gene in all of our zein promoter-CUP1 gene transcriptional fusions The CUP1 gene confers tolerance to copper ions in a fashion reflecting copy number and promoter activity. The copy number is fixed to one copy per cell in the present study by the incorporation of the centromere from chromosome 4 on the shuttle plasmids. To score for zein promoter activity plates containing the appropriate levels of copper sulfate (20-200μM) was added to a Synthetic selection medium for the selection of cells containing an active zein promoter-CUP1 gene.

Copper-containing liquid Synthetic selection medium was made by adding the appropriate dilution of a 100mM stock solution of copper sulfate to the liquid Synthetic selective medium.

B. Electro-Transformation of Bacteria and Yeast

1. Electro-Transformation of Bacteria

The electro-transformation method was used for transforming Escherichia coli (E. coli). An early-to-mid-log phase culture of E. coli strain HB101 grown at $37^{o}C$ to an optical density (660 nm) of 0.5-0.7 in LB-Broth and was used in all bacterial transformation experiments. The procedures for preparation of cells and electro-transformation are described in BIO-RAD publication number 165-2101 (BIO-RAD). Following electro-transformation E. coli are allowed to recover in LB- Broth for 1 hour at $37^{o}C$ before plating.

2. Electro-Transformation of Yeast

The electro-transformation method was used for transforming *Saccharomyces cerevisiae* (yeast). The procedures for preparation of cells and electro-transformation were described by Becker and Guarente (1991). In short the procedure is as follows: a mid-to-late-log phase culture of *Saccharomyces cerevisiae* strain ABDE1 was grown at $30^{o}C$ to an optical density (660nm) of 1.3-1.5 in liquid YPD medium. The cells were then collected by centrifugation and washed in ice-cold sterile water. The cells were centrifuged again, resuspended in 20ml of ice-cold 1M sorbitol followed by a third centrifugation. Finally the cells are resuspended in 0.5ml 1M sorbitol divided into 200μl aliquots and stored at $-70^{o}C$ until needed. These electro-competent cells were used in all yeast transformation experiments.

Following all electro-transformations yeast were allowed to recover in Synthetic selective medium for 1 hour before plating. All electro-transformation samples for both yeast and bacteria were pulsed at the following parameters: 1.5kV, 25μF, and 200 ohms on a Gene Pulser System (BIO-RAD).

C. Restriction Digestion Conditions and Enzymes

All digestions involving EcoR1 (Boehringer Mannheim) [90mM Tris-HCL, pH 7.5, 10mM $MgCl_2$, 50mM NaCl] were incubated at $37^{o}C$ for 8 hours.

All digestion involving Taq1 (Boehringer Mannheim) [10mM Tris-HCL, pH 8.5, 10mM $MgCl_2$, 100mM NaCl, 10mM 2-mercaptoethanol] were incubated at $65^{o}C$ for 5 hours.

All digestions involving BamH1 (Boehringer Mannheim) [5mM NaCl, 1mM Tris-HCL, pH 7.5, 1mM $MgCl_2$, 0.1mM Dithiothreitol (DTT)] were incubated at $37^{o}C$ for 8 hours.

All digestions involving Sal1/Eag1 (New England Biolabs, Inc.) were carried out in reaction buffer 3 supplied by New England Biolabs, Inc. (100mM NaCl, 50mM Tris-HCL, 10mM $MgCl_{2,}$ 1mM pH 7.9) and incubated at $37^{o}C$ for 8 hours.

D. "Fill-in", Kinase, Ligase, and Phosphatase Reactions

The "fill-in" reaction by Klenow enzyme contained 1μg of digested 220bp fragment (15kDzein promoter DNA), 2.0 mM of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 3 μl (3 units) Klenow fragment of DNA polymerase I (Boehringer Mannheim) and 1X Klenow buffer (50mM Tris-HCL, pH 7.6, 10mM $MgSO_4$, 1mM DTT). The reaction was allowed to proceed at $37^{o}C$ for 30 minutes, the reaction mixture was then loaded onto an 0.8% low melting point agarose gel (FMC BioProducts) to remove the deoxyribonucleotide triphosphates. The blunt-ended DNA fragment was purified from the agarose by using the Geneclean Kit (Bio101).

The kinase reaction mixture contained 0.5-1μg of DNA, 1X Kinase buffer (70mM Tris-HCL, pH 7.6, 10mM $MgCl_2$, 5mM DTT, 0.2 mM ATP, 2μl [10 units] T4

polynucleotide kinase (Boehringer Mannheim). The reaction was incubated for 30 minutes at 37^{o}C. Following the kinase reaction the DNA was purified by one cycle of phenol/chloroform extraction taking care to remove the aqueous phase to a fresh microcentrifuge tube.

All ligase reactions contained 1X ligase buffer (30mM tris-HCL, pH 7.8, 10mM $MgCl_2$, 10mM DTT, 2mM ATP), 3 units T4 DNA ligase (1 unit/µl) (Boehringer Mannheim), and were incubated at 16^{o}C for 16 hours. Following the ligation reaction the ligase in the ligase was heat inactivated by heating the sample at 75^{o}C for 15 minutes.

All DNA samples were dephosphorylated using 1.0 units/µg DNA Calf intestinal phosphatase (Boehringer Mannheim) [50mM Tris-HCL, pH 9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine] and were incubated at 37^{o}C for 1 hour. Calf intestinal phosphatase was removed from the DNA samples by two cycles of phenol extraction. The DNA is then precipitated by adding 2.5 volumes of 95% ethanol and 0.1 volume of 5M ammonium acetate. Precipitations were performed two times to remove any trace of phenol from the DNA.

E. <u>PCR Reaction</u>

The isolation of a 19kD zein-encoding gene promoter was accomplished by polymerase chain reaction (PCR) from the pZG 99.178 plasmid by using a upstream primer C91 5'-CCG<u>GAATTC</u>AACTAGCAATCTCTTC-3' and a downstream primer G95 5' CCG<u>GAATTC</u>TCAATAATATGTGCG-3' with EcoR1 restriction sites incorprated into the primer sequence to amplify a 589bp 19kD zein-encoding gene promoter fragment. The PCR reaction mixture contained 0.5 µg of plasmid pZG 99.178, 200 µM deoxynucleotide triphosphates, 1X Vent DNA polymerase buffer

[New England Biolabs, Inc. (10mM KCl, 20mM Tris-HCL, pH 8.8, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100], 1.5 mM $MgCl_2$, 10mM of each primer, 0.5µl (2.5 unites) Vent DNA polymerase. PCR was performed for 30 cycles with the following profile: denaturation at 94°C, 1 minute; annealing at 45°C, 1 minute; and extension at 72°C, 1 minute, with a final 8 minute extension step at 72°C. The PCR reaction was carried out in the MJ Research PTC-100 Thermal cycler (MJ Research, Inc.).

Following the PCR reaction the mineral oil was removed from the sample. The reaction mixture was then supplemented with EcoR1 restriction buffer, 10 units of EcoR1 restriction enzyme (Boehringer Mannheim), and incubated at 37°C for 8 hours to generate DNA fragments with cohesive EcoR1 ends. An aliquot of the reaction was then electrophoresed in a 6% polyacrylamide gel with pBR322 digested with HaeIII as a size marker in 1x TBE buffer at 150 volts for 8 hours to confirm a PCR product of 589bp. The remaining of the PCR reaction was electrophoresed in a 0.8% low melting point agarose gel. PCR fragments were then purified from the agarose on a Magic PCR column (Promega) per the manufacturer's specifications and resuspended in sterile water and stored at -20°C. All primers were synthesized in house on the Applied Biosystems 381A DNA synthesizer (Applied Biosystems).

F. Gel Electrophoresis and DNA Visualization

All polyacrylamide and agarose gels were electrophoresed in 1X Tris-Borate EDTA buffer [89mM Tris-HCL, ph 8.0, 8.9mM Boric acid, 2.5mM EDTA (TBE) along with lambda phage DNA digested with Hind III as a size marker for agarose gels and pBR 322 digested with HaeIII to generate DNA size markers for polyacrylamide gels. A UV trans-illuminator was used to visualize all digestion products on ethidium bromide (0.5μg/ml) stained gels.

G. <u>DNA Elution from Polyacrylamide Gel</u>

Polyacrylamide gel pieces containing DNA was excised by using a sharp razor blade. The gel pieces were then transferred to a 1.5ml microcentrifuge tube (GIBCO BRL/Life Technologies) and crushed into fine pieces with a pasteur pipette which had been heat sealed on its tapered end. The DNA was then eluted from the crushed gel pieces by adding 1 volume of Gel Elution Buffer [0.1% sodium dodecyl sulfate (SDS), 0.5M ammonium acetate, 1mM EDTA, pH 8.0] and eluting overnight at 37^{o}C in a rotating incubator. Following the incubation the sample was centrifuged at 14,000 RPM for 10 minutes at room temperature. The supernatant was transferred to a fresh 1.5ml polyallomer microcentrifuge tube (Beckman Instruments), taking care to avoid transferring fragments of acrylamide. An additional 0.5 volume of elution buffer was added to the crushed gel pieces, vortexed briefly, and recentrifuged at 14,000 RPM for 10 minutes at room temperature. The two supernatants were combined, and the DNA was precipitated by adding 2.5 volumes of 95% ethanol and incubating at -20^{o}C for eight hours.

Following the -20^{o}C incubation the DNA was centrifuged in a TL100 table top ultracentrifuge (Beckman Instruments) at 65,000 RPM for 45 minutes at 4^{o}C. The DNA was air-dried for 1 hour at room temperature and resuspended in a 200μl of sterile water. The DNA was precipitated twice more to remove any residual SDS by adding 2.5 volumes of 95% ethanol and 0.1 volume of 5M ammonium acetate with each precipitation. Following the additional precipitations the DNA was resuspended in sterile water and stored at -20^{o}C.

H. Colony Screening and Plasmid Confirmation

A number of aliquots of each ligation reaction were removed and mixed with 40µl of electro-competent HB101. The DNA and cell mixtures were incubated on ice for 1 minute and then loaded into chilled electroporation cuvettes with a 0.2cm electrode gap (BIO-RAD). The chilled cuvettes containing the sample are then loaded into the Gene Pulser System (BIO-RAD) and pulsed for 4.5 milliseconds at 1.50kV. Following the electroporation 1ml of LB-Broth was added to the cell mixtures and transferred to a 1.5ml microcentrifuge tube and incubated at 37^{o}C for 1 hour. Once the incubations were complete 100µl aliquots of the cell mixtures were spread onto LB-Broth plates with ampicillin (40µg/ml final concentration of ampicillin) and incubated at 37^{o}C overnight. After the incubation cells were selected at random from a pool of 2000 to 2500 recombinants for each construct and screened. The recombinants were screened by first isolating plasmid DNA by a standard protocol for isolating small amounts of plasmid DNA (Ausubel et al., 1988). Isolated DNAs were then subjected to a series of restriction endonuclease mapping experiments in either 0.8% agarose or 6% polyacrylamide gels to verify the presence fragments and their proper orientations. All postive clones were amplified and their plasmid DNAs were isolated and purified by a standard large scale cesium chloride procedure (Maniatis et al., 1982).

I. Direct Plating Assay to Determine Basal Level of CUP1 Gene Activity

Transformed Yeast cultures were grown in liquid Synthetic selection medium at 30^{o}C to stationary phase so that the cell numbers were constant for all cultures. Cells were initially grown in liquid Synthetic selection medium to select for presence of the URA3 gene on the plasmid. Equal sample volumes of the stationary phase cultures were then streaked onto Synthetic selection medium agar plates containing increasing concentrations of copper sulfate. After 5 days of incubation at $30^{o}C$ the plates were examined for the growth capability of cells containing the individual plasmid constructs, in this assay survival is based on the growth capability of individual cells in a population.

J. Liquid Replica Plating Assay to Accurately Measure Percentage Cell Survival

In order to accurately measure uniformity in the transfer of cells from individual colonies to medium containing copper, liquid replica plating was used instead of the traditional velvet imprint replica plating method. Non-transformed and transformed yeast cultures were grown in either liquid non-selective YPD or liquid Synthetic selection medium, respectively. All cultures were grown at $30^{o}C$ to stationary phase so that the cell numbers were constant for all cultures. Equal samples volumes of the stationary phase cultures in YPD and Synthetic selection medium were then transferred into 1 ml aliquots of Synthetic selection medium containing increasing concentrations of copper sulfate and incubated for 8 hours at $30^{o}C$. Following the incubation 150 μl aliquots were plated on Synthetic selective medium agar plates containing increasing concentrations of copper sulfate. After 5 days of incubation at $30^{o}C$, the percentage of copper tolerant cells was scored. All samples were done in triplicate and the average values are presented. ABDE1 cells grown in YPD medium served as the negative control in our assay. The resistance level of cells containing an active zein promoter-CUP1 gene fusion corresponds to the highest copper concentration at which there was no growth.

K. <u>Gel Electrophoresis and Hybridization Analysis of DNA</u>

DNA samples were isolated from yeast and amplified in E. coli or directly isolated from E. coli by a standard protocol for isolating small amounts of plasmid DNA (Ausubel et al., 1988). Analysis of DNA was carried out in 1X TBE 0.8% agarose gels along with a lambda DNA/HindIII size marker run at 85 volts for 2 hours. All DNA samples were digested with BamHI restriction enzyme (Boehringer Mannheim). Southern transfer of DNA from agarose gels to nitrocellulose filters (Schleicher and Schuell) was performed as described by Manaitis et al. (1982). Random priming of the probe DNA was performed as described by Boehringer Mannheim publication number 1006 878. Probe DNA was added to the hybridization buffer at $2X10^5$ cpm/ml and incubated at 65^oC for 16 hours. [$\alpha^{35}S$] dATP (10µci/µl) was the isotope used in all probe labeling (New England Nuclear/DuPont Research Products). Autoradiography was performed for 48 hours at room temperature with Kodax XAR-5 film (Eastman Kodak).

RESULTS

The overall goal of this study was to construct and test transcriptional fusion cassettes between zein promoters and yeast reporter genes on shuttle vectors in yeast. The ability of the zein promoter-CUP1 gene fusions to confer copper ion tolerance to the yeast CUP1 deletion strain ABDE1 was determined by transforming these cells with various zein promoter-CUP1 transcriptional fusion plasmids and examining basal expression levels.

A. Construction of Zein Gene Promoter-CUP1 Transcriptional Fusions.

1. Zein Gene Promoter Isolation

Plasmids pZG 99.178 and pZG 86.183 were supplied by Dr. Karl Pedersen (Pedersen et al., 1982; 1986). These plasmids contain maize genomic clones that were isolated from maize genomic library constructed with DNA isolated from Black Mexican Sweet Corn.

Plasmid pZG 86.183 which contains a 5.6kb EcoR1 15kD zein-encoding gene fragment was subjected to a double digest with EcoR1 followed by a Taq1 digest. Following the digestion reaction, the reaction products were analyzed on a 0.8% agarose gel electrophoresed at 85 volts for 2 hours. Following the verification of a complete restriction digest of the plasmid DNA, a 220 bp EcoR1/Taq1 fragment was separated by electrophoresis in a 6% polyacrylamide gel run at 100 volts overnight. The 220 bp EcoR1/Taq1 fragment represents the 15kD zein gene promoter as determined from DNA sequence data (GCG data base, Wisconsin, Accession Number M13507), and restriction endonuclease mapping.

In order to generate DNA fragment with compatible EcoR1 ends, to enhance the cloning of the promoter fragment, the 220 bp EcoR1/Taq1 fragment was first subjected to a brief "fill-in" reaction by Klenow enzyme to blunt end the DNA fragment followed by a blunt-end ligation reaction to add EcoR1 linkers to the DNA fragment. Following the "fill-in" reaction EcoR1 linkers were phosphorylated by a kinase reaction in preparation for the linker-addition to the 220 bp blunt-ended fragment. When the kinase reaction was complete the phosphorylated EcoR1 linkers (CCGGAATTCCGG; New England Biolabs) were added by a routine ligation procedure (Maniatis et al., 1982). In a fresh microcentrifuge tube the 220 bp blunt-ended 15kD zein promoter fragment was added to a 50-fold molar excess EcoR1 linkers, and incubated at $16^{o}C$ for 16 hours. Following the ligation reaction the ligase in the reaction was heat inactivated by heating the sample at 75 degrees celsius for 15 minutes. The ligation mixture was then supplemented with EcoR1 restriction buffer and EcoR1 restriction enzyme and incubated at $37^{o}C$ to produce EcoR1 sticky ends. The reaction was loaded onto a 0.8% low melting point agarose gel (FMC BioProducts) to remove unligated linkers and subsequently a 232bp DNA fragment was purified from the agarose by using the Geneclean kit (Bio101).

The isolation of a 19kD zein-encoding gene promoter was accomplished by polymerase chain reaction (PCR) from the pZG 99.178 (which contains a 7.7kb EcoR1 19kD zein gene fragment) plasmid by using a upstream primer C91 5'-CCG<u>GAATTC</u>AACTAGCAATCTCTTC-3' and a downstream primer G95 5' CCG<u>GAATTC</u>TCAATAATATGTGCG-3', with EcoR1 restriction sites incorporated into the primer sequence, to amplify a 589bp 19kD zein-encoding gene promoter fragment (Six base pair EcoR1 site underlined).

2. Constuction of the CUP1 Promoterless Vector

The 4.381kb pRS306 plasmid vector (Sikorski and Hieter, 1989) was linearized by digestion with BamH1. The DNA was then dephosphorylated using Calf intestinal phosphatase (Boehringer Mannheim). An aliquot of the digested dephosphorylated DNA was electrophoresed in a 0.8% agarose gel at 85 volts for 4 hours to verify complete digestion of the pRS306 vector.

The copperthionein gene fragment CUP1 was derived from the pACUP plasmid (Thiele and Hamer, 1986) by a BamH1 digestion. Following the restriction digest the DNA was loaded into a 0.8% low melting point agarose gel and electrophoresed at 85 volts for 4 hours. After the electrophoresis a 3.4kb copperthionein gene fragment was purified from the agarose using the Geneclean kit (Bio101). The copperthionein gene from the CUP1 locus of yeast enables cells to tolerate high levels of exogenous copper ions in its environment (Fogel and Welch, 1983).

The pRS306 plasmid vector was modified by ligating a 3.4kb BamH1 copperthionein gene fragment into the BamH1 site in the multiple cloning region of this plasmid. A group 100 colonies were selected at random from a pool of 2000 recombinants and were screened. Isolated DNAs were then subjected to a series of restriction endonuclease mapping experiments in 0.8% agarose gels to verify the presence of the 3.4 CUP1 fragment and its proper orientation.

The hybrid, pRSCUP1 (7.781kb), contains the pRS306 plasmid and a 3.4kb CUP1 fragment. The 3.4kb CUP1 fragment consist of a 2.9kb BamH1/HindIII CUP1 gene fragment and a 500bp HindIII/BamH1 alcohol dehydrogenase (ADH) terminator fragment. (Figure 2).

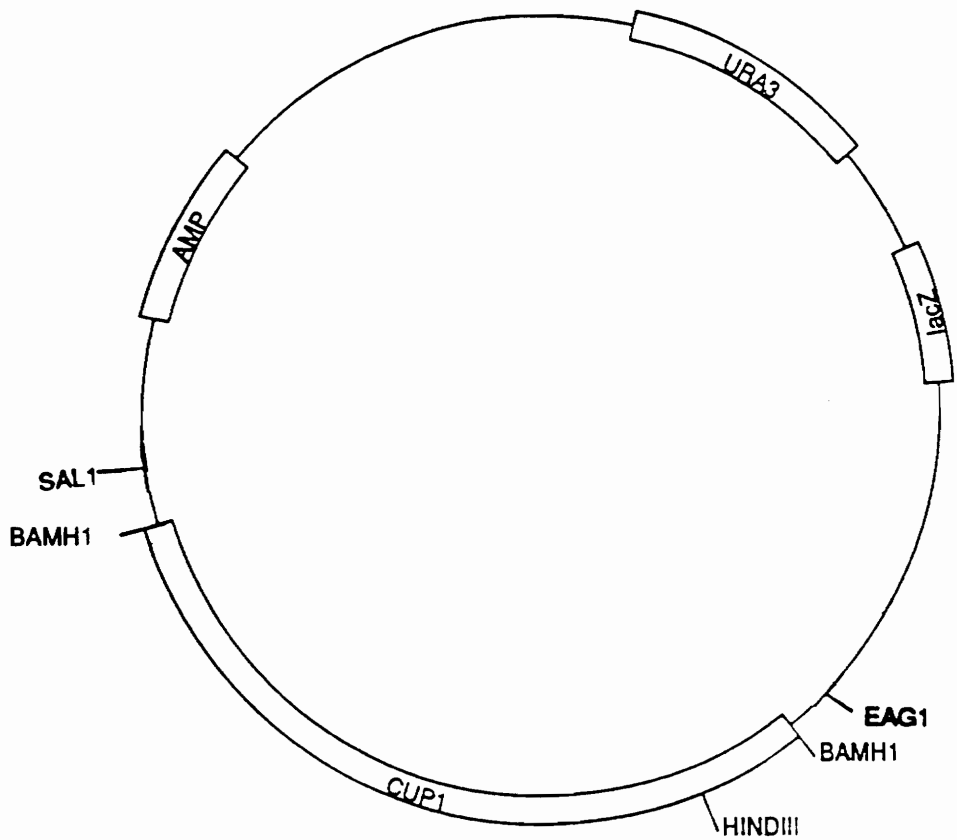

Figure 2. pRSCUP1 Promoterless Vector.

The hybrid, pRSCUP1 (7.781kb), contains the pRS306 plasmid and a 3.4kb CUP1 fragment. The 3.4kb CUP1 fragment consist of a 2.9kb BamH1/HindIII CUP1 gene fragment and a 500bp HindIII/BamH1 alcohol dehydrogenase (ADH) terminator fragment.

3. Construction of Transcriptional Fusion Cassettes Between Zein Promoters and a Yeast Reporter Gene (CUP1)

Plasmids pRS15zein-CUP1 and pRS19zein-CUP1(Figures 3and 4) were constructed by converting the CUP1 gene fragment of plasmid pRSCUP1 into a zein promoter-CUP1 gene fusion cassette by the addition of zein promoters. The pRSCUP1 plasmid vector was digested with EcoR1. After the restriction reaction the sample was dephosphorylated by adding Calf intestinal phosphatase (Boehringer Mannheim). An aliquot of the digested DNA was analyzed on a 0.8% agarose gel and electrophoresed at 85 volts for 4 hours to verify complete digestion of vector DNA.

Both the purified 15kD and 19kD zein promoters were ligated into the EcoR1 site of pRSCUP1 vector as 232bp and 589bp EcoR1 fragments respectively. From a pool $2X10^4$ recombinants 150 colonies were selected at random and screened for each individual plasmid construct. In total, 400 cells were screened for both the 15kD and 19kD zein promoter CUP1 fusion clones. The need to isolate such a large number of cells was due to the difficulty of identifying clones with the proper orientation. Isolated DNA were subjected to a series of restriction endonuclease mapping experiments in a 6% polyacrylamide gel to verify the presence of a 232bp 15kD zein promoter and a 589 bp 19kD zein promoter and their proper orientation with respect to the CUP1 gene clone into pRSCUP1. The zein promoters were linked as transcriptional fusions to the CUP1 coding region. The fusion points are between +1 and the ATG initiation codon for the zein promoters and the -1 for the CUP1 gene (Figures 3 and 4).

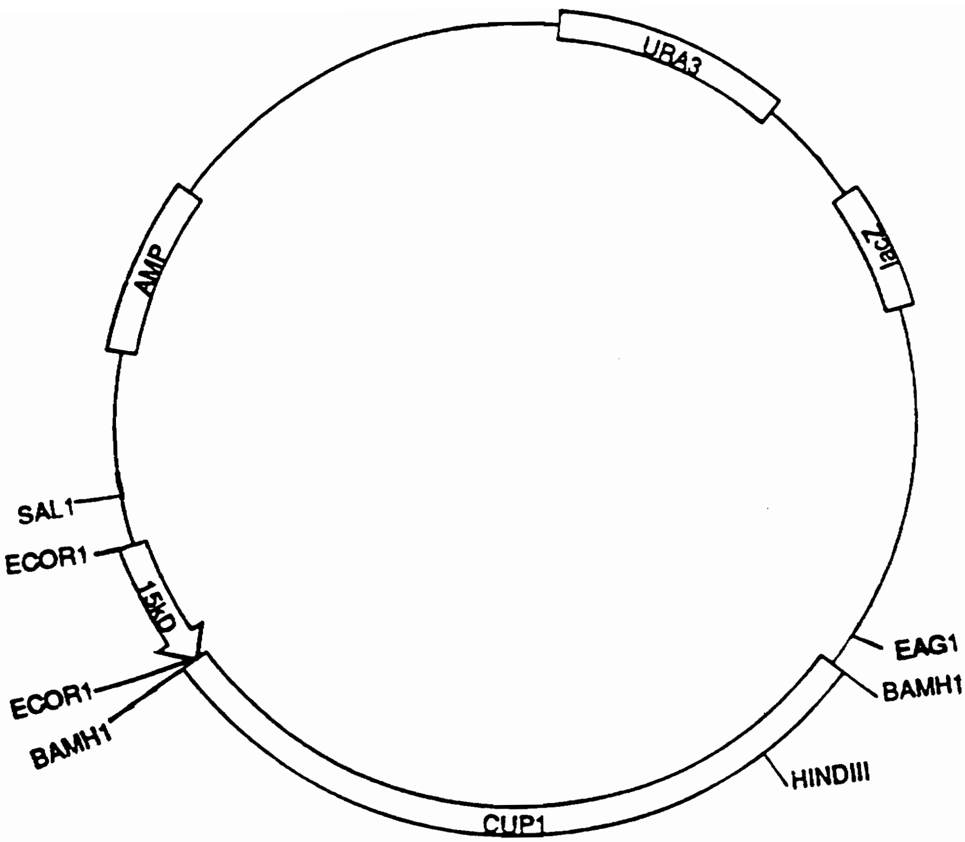

Figure 3. pRS15zein-CUP1 transcriptional fusion.

The 15kD zein promoter was linked as a transcriptional fusion to the CUP1 coding region and ADH terminator. The fusion points are between +1 and the ATG initiation codon for the zein promoter and the -1 for the CUP1 gene.

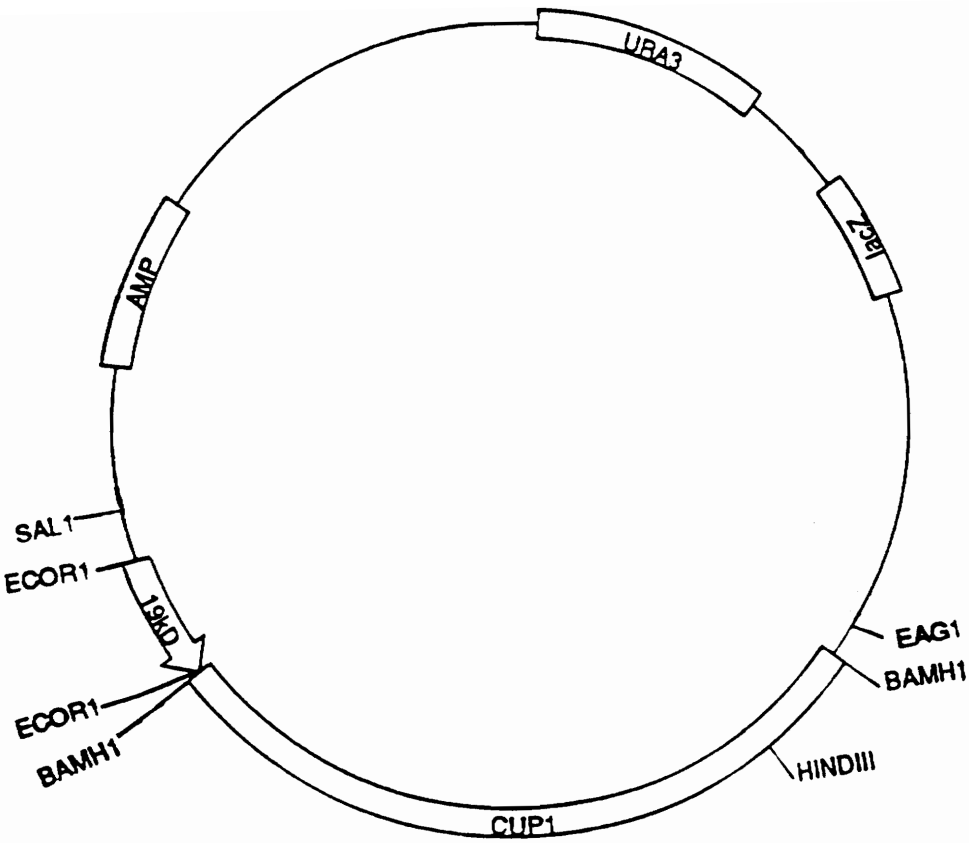

Figure 4. pRS19zein-CUP1 transcriptional fusion.

The 19kD zein promoter was linked as a transcriptional fusion to the CUP1 coding region and ADH terminator. The fusion points are between +1 and the ATG initiation codon for the zein promoter and the -1 for the CUP1 gene.

4. <u>Construction of Shuttle Vectors for Testing the Zein Promoter-CUP1 Gene Fusions</u>

The plasmid pYC^2 is an *Escherichia coli*-yeast shuttle vector that serves as the basis for our test constructs in yeast. The pYC^2 plasmid contains a conditional centromere, CEN3 that is placed under the genetic control of the inducible GAL1-10 promoter which impairs centromere function (Figure 5).

It has been demonstrated that when a centromere is placed near an actively transcribed promoter, centromere function is lost (Hill and Bloom, 1987). Yeast shuttle vectors require a centromere sequence for mitotic stability and for single copy number control. Plasmid pYC^2 must be propagated on galactose containing medium on order to function as a monocentric plasmid that is maintained intact throughout cell divisions. Under galactose conditions CEN4 functions as the sole centromere on the plasmid since it is not affected by the selection of the carbon source. Other features of the shuttle vector include a yeast replication origin, ARS1, and a selectable yeast marker, URA3, an ampicillin resistance gene marker for selection in E. coli, AMP, and the pBR322 origin of replication (Figure 5). We choose the pYC^2 vector because it was the only shuttle plasmid we had in stock that reqiured no modifications and contained the selectable markers we needed in order to run our test and we were able to clone directly into it. This plasmid also allows us to select for markers under very defined conditions.

The construction of pYC^{2}15 zein-CUP1, pYC^{2}19 zein-CUP1, and pYC^{2}CUP1 shuttle vectors was accomplished by ligating a 3632bp Sal1/Eag1 15 zein CUP1 fragment, a 3989bp Sal1/Eag1 19 zein-CUP1 fragment, and a 3400bp Sal1/Eag1 CUP1 fragment into the Sal1/Eag1 site of pYC^2 vectors.

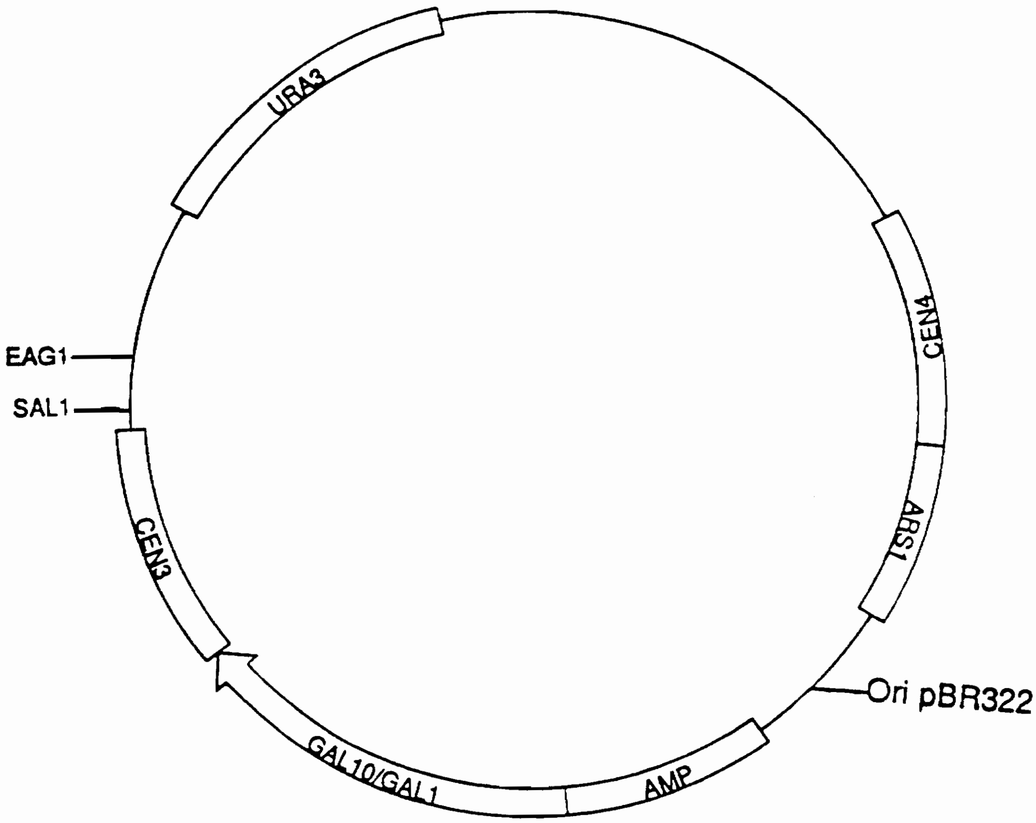

Figure 5. Shuttle vector pYC^2

pYC^2 is a plasmid that contains a conditional centromere, CEN3 that is placed under the genetic control of the inducible GAL1-10 promoter which impairs centromere function. pYC^2 must be propagated on galactose containing medium in order to function as a monocentric plasmid that is maintained intact throughout cell divisions. Under galactose conditions CEN4 functions as the only centromere on the plasmid since it is not affected by the selection of the of the carbon source. Other features of the shuttle vector include a yeast replication origin, ARS1, an selectable yeast marker, URA3, an ampicillin resistance gene marker for selection in *E.coli*, AMP, and the pBR322 origin of replication (Hill and Bloom, 1987).

The pYC^2 vector was digested with a combination of Sal1/Eag1 (New England Biolabs, Inc.) After the digestion was complete the sample was dephosphorylated and analyzed in a 0.8% agarose gel run at 85 volts for 4 hours.

In order to isolate the 15 zein-CUP1, 19 zein-CUP1, and CUP1 fragments, their respective pRS306 based construct were also subjected to a Sal1/Eag1 (New England Biolabs) double digest. Following the restriction endonuclease digestion the reactions were loaded into a 0.8% low-melting point gel (FMC) and electrophoresed at 85 volts for 5 hours. After the electrophoresis a 3632bp Sal1/Eag1 15 zein-CUP1 fragment, a 3989bp Sal1/Eag1 19 zein-CUP1 fragment, and 3400bp Sal1/Eag1 CUP1 fragment were isolated and purified from the agarose by using the Gene Clean kit (Bio101).

The dephosphorylated pYC^2 and the individual zein-CUP1 and CUP1 fragments were mixed in a clean microcentrifuge tube and ligated. Following the ligation reaction a 2µl aliquot of the reaction was removed and mixed with 40µl of elector-competent HB101, the cells were transformed and grown on LB-Broth plates with ampicillin. A group of 150 cells was selected at random from a pool of 2000 to 2500 recombinants for each construct and screened. Isolated DNAs were then subjected to a series of restriction endonuclease mapping experiments in a 6% polyacrylamide gel electrophoresed at 100 volts for 16 hours to verify the presence of the zein promoter, and CUP1 fragments and their proper orientations. The pYC^2 CUP1 promoterless, pYC^2 15 zein promoter-CUP1, and pYC^2 19 zein promoter-CUP1 positive clones (Figure 6, 7, and 8) were amplified and their plasmid DNAs were isolated and purified by a standard large scale cesium chloride procedure (Maniatis et al. 1982).

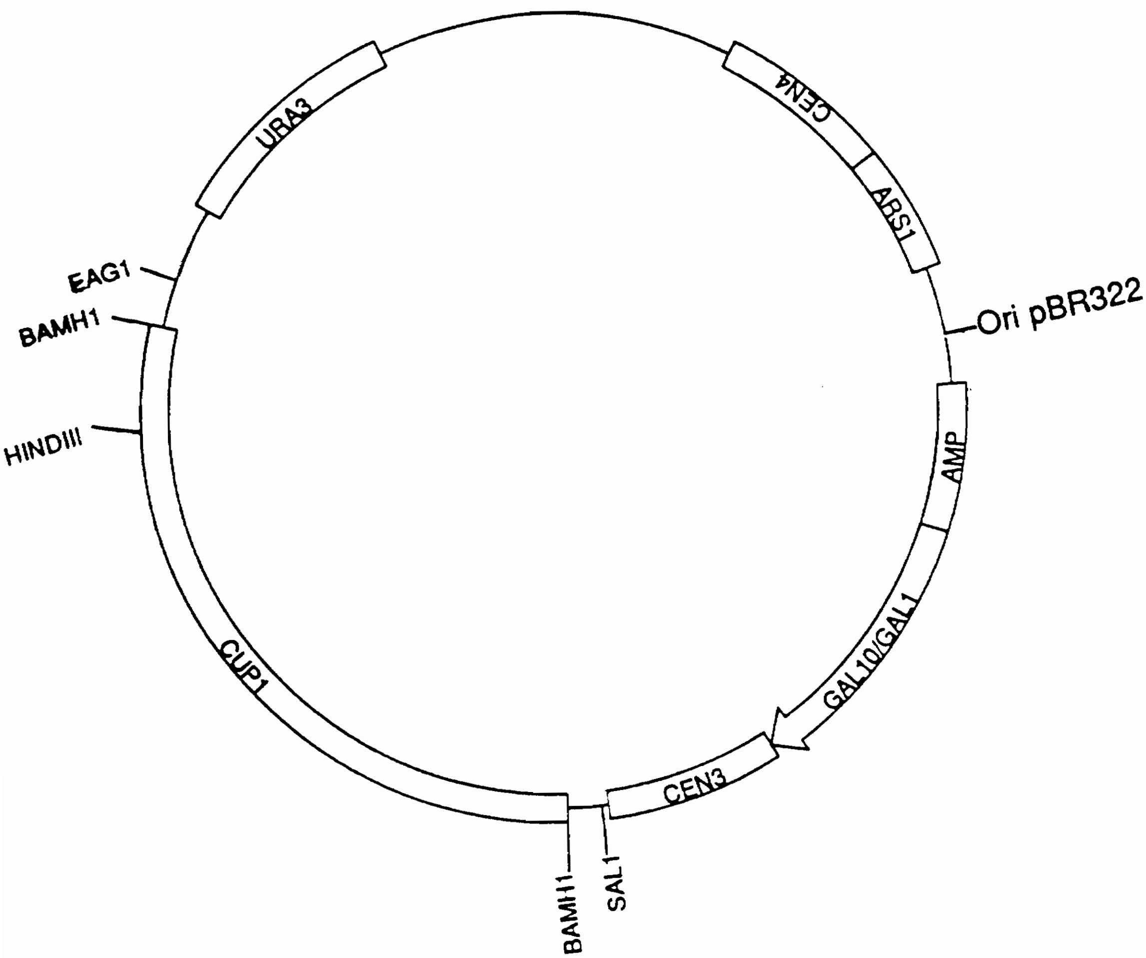

Figure 6. pYC^{2}CUP1

Plasmid YC^{2}CUP1 serves as a control construct for our copper sensitivity assay. The CUP1 gene was cloned into the Sal1/Eag1 site of pYC^2.

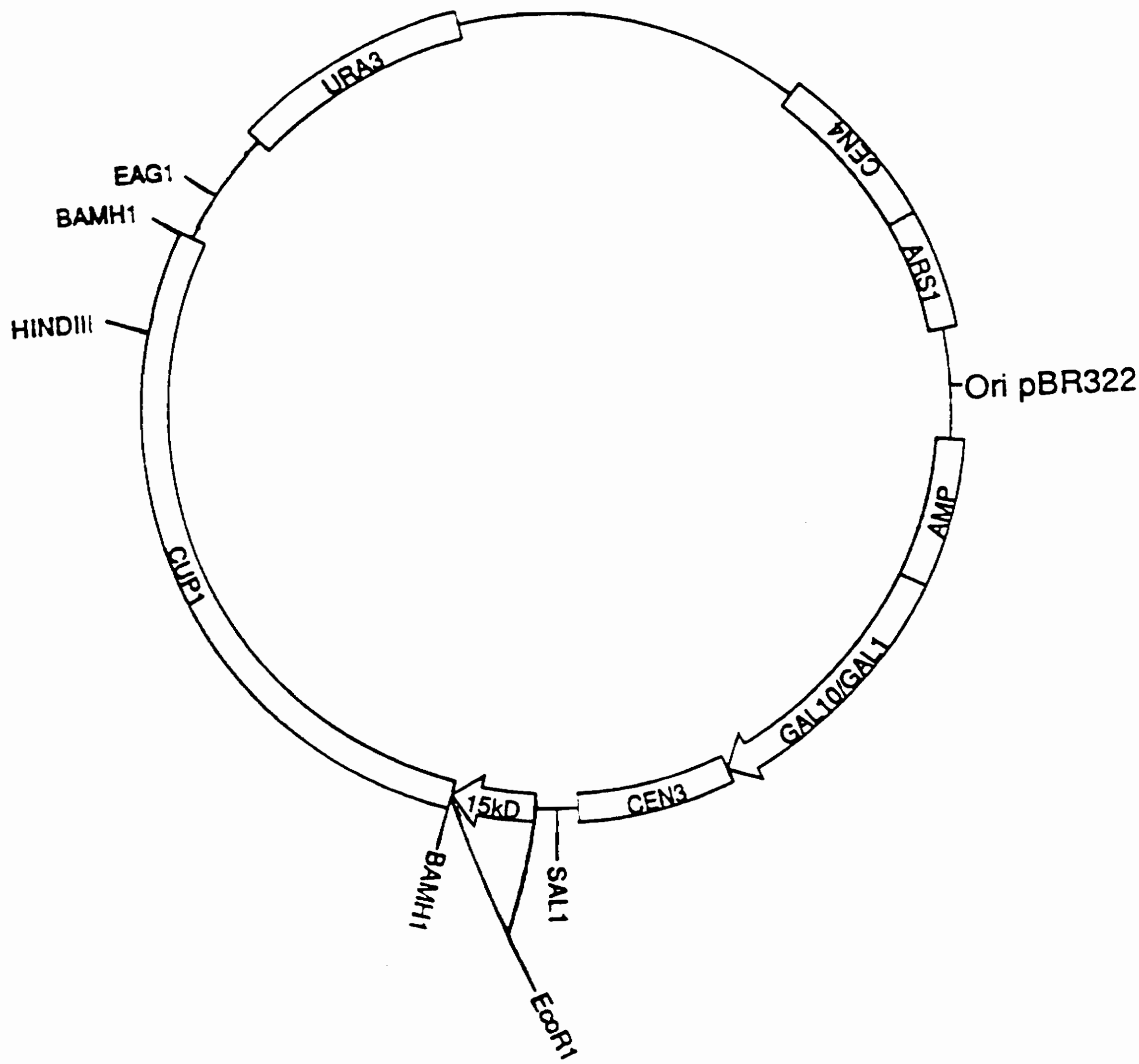

Figure 7. pYC^{2}15zein-CUP1

The 15kD zein promoter was linked as a transcriptional fusion to the CUP1 coding region and ADH terminator. The fusion points are between +1 and the ATG initiation codon for the zein promoter and the -1 for the CUP1 gene. Other features of the shuttle vector include a yeast replication origin, ARS1, an selectable yeast marker, URA3, an ampicillin resistance gene marker for selection in *E.coli*, AMP, and the pBR322 origin of replication.

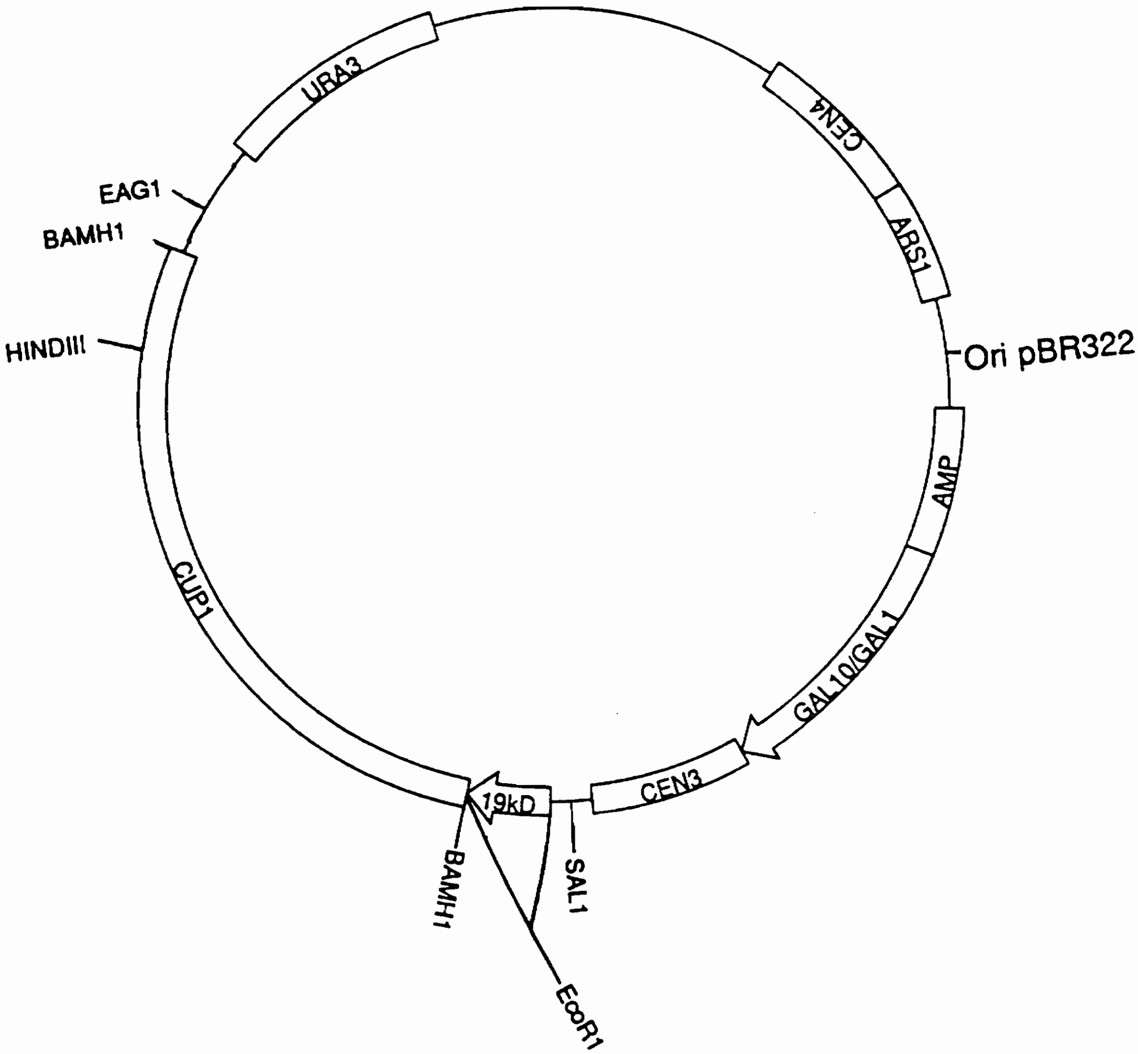

Figure 8. pYC^{2}19zein-CUP1

The 19kD zein promoter was linked as a transcriptional fusion to the CUP1 coding region and ADH terminator. The fusion points are between +1 and the ATG initiation codon for the zein promoter and the -1 for the CUP1 gene. Other features of the shuttle vector include a yeast replication origin, ARS1, an selectable yeast marker, URA3, an ampicillin resistance gene marker for selection in *E. coli*, AMP, and the pBR322 origin of replication.

B. A Direct Plating Assay Demonstrates That Copper Tolerance is Conferred by Functional Zein Promoter-CUP1 Transcriptional Fusions

To demonstrate that zein promoters are capable of regulating the expression of a yeast reporter gene, we constructed transcriptional fusion cassettes between zein promoters and a yeast CUP1 gene on a shuttle vector in yeast. The reporter gene enables yeast cells to grow under defined conditions, only when activated. We have used the copperthionein gene from the CUP1 locus of yeast as our reporter gene (Karin et al., 1984; Butt et al., 1984). This gene enables yeast to tolerate high levels of copper ions in its environment (Fogel and Welch, 1982).

In determining the basal level of expression conferred by zein promoter-CUP1 fusions ABDE1 cells were transformed with pYC^2, pYC^2 15 zein-CUP1, or pYC^2 19 zein-CUP1 shuttle vector constructs (Figures 5, 7, and 8) [see Materials and Methods] and streaked directly on Synthetic selection medium agar plates with increasing concentrations of copper sulfate to determine the basal level of copper sensitivity conferred by zein promoter-CUP1 transcriptional fusions. The rationale for this approach is that the CUP1 deletion strain, ABDE1, is extremely sensitive to copper ions as a result of the complete CUP1 gene deletion in its genome. Therefore, only cells that contain an active CUP1 gene will be able to tolerate increased copper in the medium.

It is clear that cells without a functional CUP1 gene are extremely sensitive to copper ions. In contrast, cells containing functional zein promoter-CUP1 gene fusions show plasmid mediated copper tolerance as a result of the basal gene activity conferred by an active zein promoter. Yeast cells containing a functional zein promoter-CUP1 fusions tolerate copper ions to a concentration of 100μM (Figure 9).

C. Liquid Replica Plating Assay to Determine Percent Cell Survival

Percent cell survival was measured by liquid replica plating onto Synthetic selection plates containing increasing concentrations of copper and determining plating efficiency (see Materials and Methods). This method allows us to obtain a accurate measure of the percentage of cell survival in the presence of copper. The data generated from this method is presented in Table 1 and Figure 10, respectively. Four ABDE1 cell cultures containing either non-transformed or transformed cells bearing pYC^{2}CUP1, pYC^{2} 15 zein-CUP1, and pYC^{2} 19 zein-CUP1 shuttle vectors were used in this experiment. Figure 10 and Table1 illustrate that zein promoters are capable of conferring about 48% survival to cells with deletions of the CUP1 gene on medium containing 30uM copper. In this assay the percent survival is based on clonal growth capability. The difference in the number of cells from one concentration of copper to the next is a reflection of both the total precentage of viable cells at that particular concentration of copper and the differences of the efficiency of transcription from the CUP1 gene from the individual zein promoters.

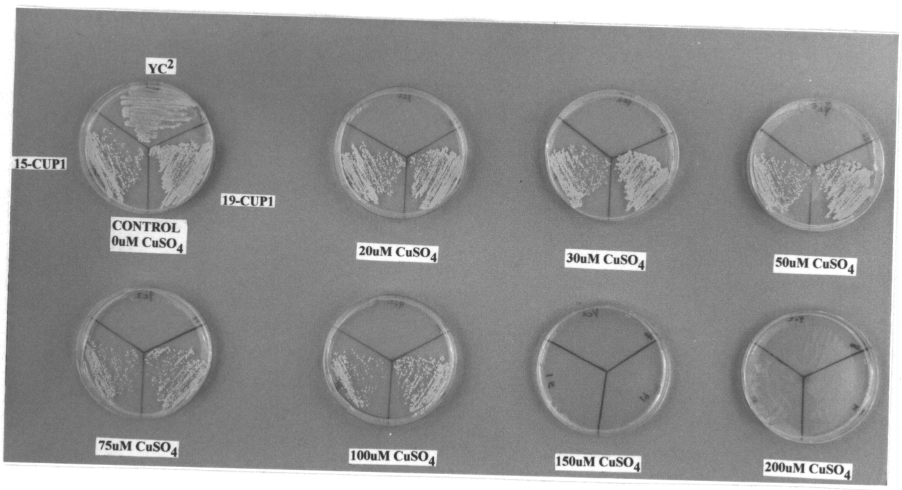

Figure 9. Direct Plating Assay

Functional zein promoters confer a basal level of CUP1 zein activity in yeast

Eight Synthetic selection plates, divided into thirds, were streaked with pYC^2, pYC^2 15 zein-CUP1, and pYC^2 19 zein-CUP1 transformed ABDE1 cells. Plates were incubated for 5 days at 30^oC. Following incubation the plates were examined to determine the basal level of copper tolerance conferred by functional zein promoter-CUP1 transcriptional fusions. Both pYC^2 15 zein-CUP1 and pYC^2 19 zein-CUP1 allow transformed cells to tolerate copper ions to a concentration of 100μM. Cells containing the pYC^2 shuttle plasmid show absolutely no growth at 20mM copper sulfate.

Table 1. Plating assay to determine the basal level of copper tolerance confered by zein promoters [α]

Promoter	PERCENT SURVIVAL							
	0uM	20uM	30uM	50uM	75uM	100uM	150uM	200uM
none(nontransformed)	0	0	0	0	0	0	0	0
15kD zein gene	100	69	48	27	12	3	0	0
19kD zein gene	100	63	42	21	15	1	0	0
Promoterless CUP1	100	0	0	0	0	0	0	

[α] All copper tolerant cells were transformed with plasmids that contain a of the CUP1 gene. The CUP1 deletion strain ABDE1 [MAT a ade2 hi arg4-8 cup1:: URA3 thr1-4 trp1-289 ura3-52] was used for all yeas cells were plated on galactose containing Synthetic selective med centrations of copper sulfate.

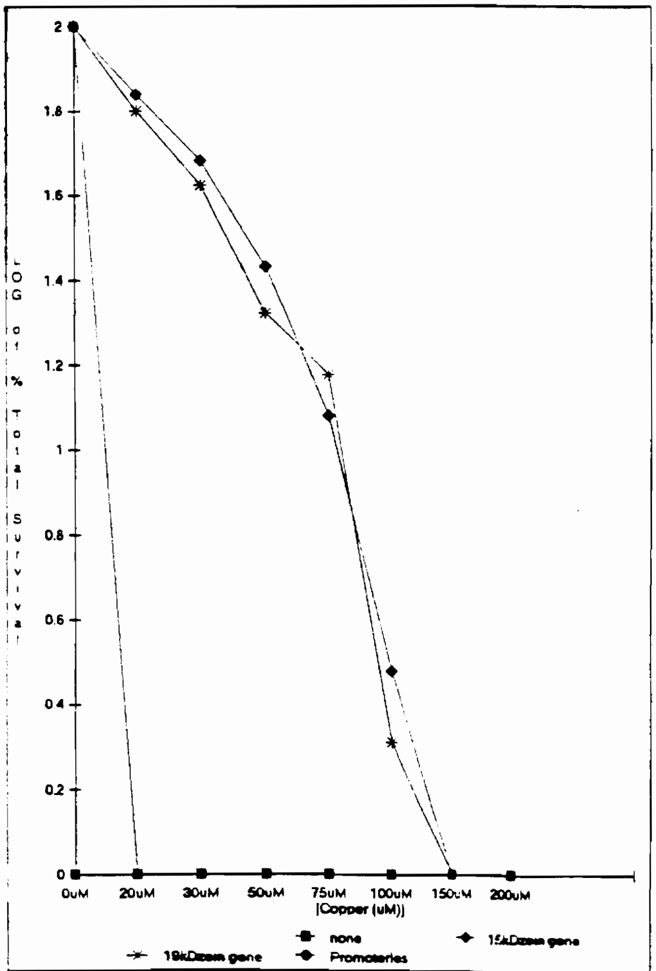

Figure 10. LOG of Percent Total Survival.

The LOG of percent total survival of cells containing a single copy of the zein promoter-CUP1 gene fusion on a shuttle plasmid is plotted. Non-transformed ABDE1 (closed square); cells bearing the 15kD zein promoter-CUP1 fusion(closed diamond); cells bearing the 19kD zein promoter-CUP1fusion(*star); and cells bearing the promoterless CUP1 construct(closed circle). Zein promoters are capable of conferring about 48% survival to ABDE1 cells on medium containin 30uM copper.

D. Confirmation of the Presence of the CUP1 gene

Analysis of the presence of the CUP1 gene in transformed ABDE1 cells was done through Southern Blot Hybridization. DNA was isolated from pYC^2, pYC^2 15 zein-CUP1, and pYC^2 19 zein-CUP1 transformed ABDE1 cells according to the procedure described by Kaiser and Auer (1993). DNA isolated from yeast transformants was then electroporated into *E. coli* strain HB101 in order to remove contaminating yeast chromosomal DNA that would hybridized to the centromere portions of the pYC^2 vector that is found in all of our yeast shuttle vectors. Plasmid DNA that was isolated from *E. coli* was subsequently digested with BamH1. Additionally, pBR322 was linearized with BamH1 under the same condition (see Materials and Methods). Following the restriction digest the DNA samples were loaded into three 0.8% agarose gels. The agarose gels were then subjected to Southern transfer of DNA to Schleicher and Schuell nitrocellulose filters (Maniatis et al., 1982). Following the Southern transfer two ^{35}S-labelled DNA probes were prepared, a 3.4kb BamHI CUP1 fragment was isolated from plasmid pACUP (Thiele and Hamer, 1986) [Materials and Method] and the linearized pBR322 was used to make the second probe. The fragments were labelled according to the Random Primer procedure (Boehringer Mannheim publication number 1006 878). We used linearized pBR322 as a probe because plasmid pYC^2, the basis for all of our yeast shuttle vectors, contains a large portions of pBR322. The use of pBR322 allows us to avoid possible cross-hybridization with any centromere containing yeast DNA that we would encounter if we were to use the pYC^2 plasmid as a probe. We chose Southern hydribrization to demonstrate the presence of CUP1 gene because of problems associated with isolating plasmid DNA from yeast for restriction endonuclease analysis. Some of the more common problems include co-isolated internal 2-micron circle DNA, yeast chromosomal DNA, and recombination which make restriction pattern analysis difficult.

The nitrocellulose filters containing the digested DNAs were prepared and hybridized according to the procedure described by Maniatis et al. (1982). The filter represented in Figure 11A was hybridized with the pBR322 probe and as a result lanes 2-5 yield positive hybridization signals, as expected. Lane 2 shows a 8.9 kb fragment that was generated by a BamH1 digest of pYC^2. In contrast to lane 2 of Figure 11A lanes 4, and 5 show a rearrangement in the pYC^2 based 15 zein-CUP1 and 19 zein-CUP1 constructs as well as the pYC^2 plasmid isolated from yeast (lane 3; Figure 11A). In Figures 11A, 11B, and 11C lane 2 represents pYC^2 shuttle vector DNA that has been amplified and isolated from *E. coli* without ever having being transformed into yeast. DNAs in lanes 3, 4, and 5 contain DNA that was isolated from yeast, followed by transformation into *E. coli* and plasmid DNA isolation from E. coli. The BamHI digest of these DNAs shows fragments of approximately 3.4kb in size when probed with pBR322. We had expected a 3.4kb CUP1 BamHI fragment and a 8.9kb BamHI fragment representing the pYC^2 assuming no rearrangement in yeast. We contribute the discrepancy to the relative size difference of the BamHI fragments to a rearrangement event in yeast. However, as indicated by the cell culture experiments, the function of the zein promoter-CUP1 transcriptional fusions are not affected; as evidenced by the fact that only cells containing functional zein promoter-CUP1 transcriptional fusions tolerate copper ions in the growth medium (Figure 9).

Lanes 4 and 5 of both Figures 11B and 11C show that only DNA isolated from yeast cells that were transformed with the 15 zein-CUP1 and 19 zein-CUP1 transcriptional fusions yield positive hybridization signals to the CUP1 probe. The positive hybridization signals shown on lanes 4 and 5 are approximately 3.4kb in size

which corresponds to the correct size of the CUP1 probe. In our system the CUP1 gene is maintained at one copy per cell on all shuttle plasmid constructs in yeast. Copper tolerance is a reflection of the basal activity of the zein promoter.

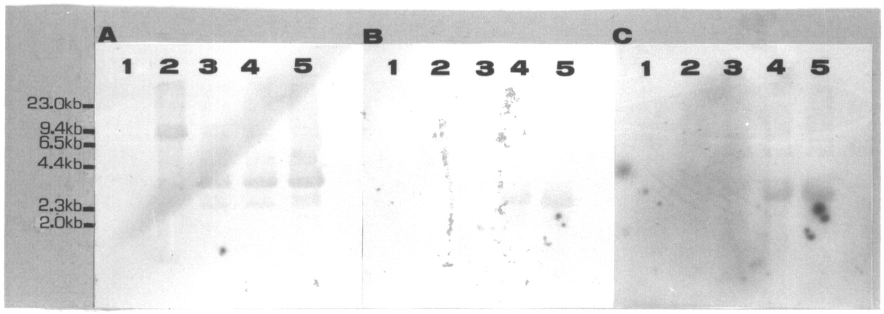

Figure 11. Hybridization Analysis of Shuttle Vector DNA

Figure 11A illustrates that all transformed cells harbor the pYC^2 construct and that there is a rearrangement in pYC^2 isolated from yeast. There is a shift from an 8.9kb BamHI pYC^2 fragment isolated from E. coli to a 3.4kb BamHI pYC^2 fragment isolated from yeast. Figures 11B and 11C show that only the cells that contain the pYC^2 15 zein-CUP1 and pYC^2 19 zein-CUP1 yield a positive hybridization signal to the CUP1 probe. In Figure 11A, 11B, and 11C lane 1 contains a lambda/HindIII size marker, lane 2 contains pYC^2 DNA isolated from E. coli, lane 3 contains pYC^2 DNA isolated from yeast, lane 4 comtains pYC^2 15 zein-CUP1 DNA isolated from yeast, and lane 5 contains pYC^2 19 zein-CUP1 DNA isolated fom yeast.

DISCUSSION

We have demonstrated that zein promoters can be substituted for the endogenous CUP1 promoter from yeast resulting in zein promoter-CUP1 transcriptional fusions that confer a basal level of CUP1 gene activity.

The success of our experiments was dependent on the use of a sensitivity assay system. The reporter gene[CUP1] (Thiele and Hamer, 1986) was placed under the control of zein gene promoters. Attempts to demonstrate conferred copper tolerance to exogenous copper ions in the CUP1 deletion strain ABDE1 were entirely successful. In our study the ability of the zein promoter-CUP1 transcriptional fusions to confer copper ion tolerance resulted from two factors: (1) the total absence of endogenous CUP1 gene activity in *Sacchromyces cervisiae* strain ABDE1; and (2) the ability of zein gene promoters to be correctly recognized and to initiate transcription of the CUP1 gene in the heterologous yeast system as part of centromere (CEN) containing plasmids.

This system will allow us the opportunity through the introduction and expression of maize cDNA libraries to identify factors that modulate the activity of zein promoters that are transcriptionally fused to the CUP1 gene.

We believe that there exists a complex network of regulation in zein gene expression and that this system is best studied by analyzing each component individually. We will be able to take advantage of the sensitivity of our reporter gene in yeast to allow us to identify genes that may play a part in zein gene expression, while only mildly effecting transcription levels. We have a yeast strain (ABDE1) in which the CUP1 gene sequence is completely deleted from its genome, resulting in cells that are extremely sensitive to copper ions. We have shown that transformation of plasmids containing functional zein promoter-CUP1 fusions into ABDE1 cells will confer tolerance to copper and will reflect the level of transcriptional activation (Resnick et al., 1990) of the target zein promoter. The zein promoter-CUP1 transcriptional fusion constructs consist of the selectable marker *URA3* and a centromere from a yeast chromosome, which is needed to maintain the target plasmid at a single copy number per cell in yeast (Figures 7 and 8). Cells containing the zein promoter-CUP1 transcriptional fusion constructs will be co-transformed with high copy number expression plasmids consisting of a maize cDNA transcriptional fusion to a inducible yeast promoter as well as the selectable markers *trp1* and *leu2-d* (Figure1). The difference in copy number between the zein promoter-CUP1 fusions and the expression plasmids ensures maximal interaction between the zein promoter-CUP1 fusion and the expressed maize transcription factors.

The ability of cells to tolerate copper conferred by zein promoter-CUP1 gene fusions will be measured by plating directly on plates with increasing concentrations of copper sulfate in the medium and determining plating efficiency. We have shown that zein promoter-CUP1 fusions confer ~50% survival to CUP1 deletion ABDE1 cells on selective medium containing 30μM copper sulfate (Table 1), whereas wild-type copper resistant strains, which contain 10 or more tandemly repeated copies of CUP1, can grow in the presence of up to 2mM copper sulfate (Fogel et al., 1983). Through selection on increased levels of copper the copy number of the CUP1 locus in wild-type yeast can be further increased from the usual 10 or more copies which results in increased copper tolerance. It has been shown by Resnick et al., (1990) that yeast strains that are completely deleted for the CUP1 gene in its genome which contain increased copies of a plasmid borne CUP1 gene require greater than 300 times the

copper to abolish cell growth relative to the CUP1 deletion strain containing no plasmid. We expect a similar affect in our selection system as a result of increased expression from zein promoters in the presence of regulatory factors. Cells containing a single copy of the zein promoter-CUP1 fusion construct should show increased tolerance to copper because of higher levels of activation from the zein promoter in much the same way as an increase the CUP1 gene copy number does in wild-type yeast as reported by Resnick et al., (1990). Therefore cells showing increased tolerence will be examined for the presence of regulatory factors.

The advantage of our system over conventional methods such as transposon tagging is that there is no requirement for phenotypic change in order to score positive results and it is possible to screen large numbers of cells so that basically all mRNAs present in the maize endosperm can be screened for transcriptional regulatory function. This approach is not limited to the isolation of zein regulatory factors. It can be used for the isolation of any developmental regulatory factor that has the ability to act independently in a manner similar to that of O2 and Kn1. For example O2 binds to the 22 kD zein promoters without accessory proteins and Kn1 has been shown to dimerize on its own. Our system should be able to isolate regulatory factors that promote transcripition without the need of accessory proteins. Therefore the production of transcription factors from our expression plasmid is dependent on full-length cDNAs.

We have discovered during the development of this system that one of the critical parameters for a reasonably stable yeast *in vivo* screen is that there is an absolute requirement for a recipient yeast strain that has a low reversion rate and therefore, stable selectable markers. In our previous attempts at developing this system we have encountered a reversion frequency of about 5X 10^{-5} to the *trp1*$^{+}$ phenotype, which

presented a major problem since we relied on *trp1* as our selectable marker for our initial zein promoter-CUP1 fusions plasmids. We have come to realize that the key to our system is the strength of our selectable markers and the requirement of an auxotrophic recipient strain of yeast that has multiple deletions in its genome for our desired markers in order to circumvent the problem encountered by using a recipient strain with a single point mutation in the gene(s) that corresponds to our choosen markers. Ideally a reversion rate of 10^{-8} is desired for the selectable marker. We had tried to use the yeast strain J17 (MATa his2 ade1 trp1 met14 ura3); however, its reversion frequency was greater than our transformation efficiency therefore we were unable to distinguish between those cells that were transformed and those that were revertants. We also tried to use strain DG 275(MATa trp1-1 his3-200 ura3-52 leu2-3,112). Although DG 275 contained the desired multiple deletion for our choosen selectable markers, it lacked the genomic deletion for the CUP1 gene. After screening other possible yeast strains we chose to use the ABDE1 CUP1 deletion strain as the host in our screening system. ABDE1 (MATa ade2 his7-2 leu2-3,112 arg4-8 cup1::URA3 thr1-4 trp1-289 ura3-52) was found to contain genomic deletions for all the marker deletions we chose for selection of both our target and expression plasmid vectors.

We feel that our assay system will allow us to identify the elements of a complex regulatory network of transcription factors that are likely to exist for the regulation of zein gene expression with the need for phenotypic change in order to score for positive identification; however, our system is limited to the identification of single genes which encodes transcription factors that interact with zein promoters; therefore, we are unable to select for transcription factors that require accessory proteins to

system has the power to expand our understanding of how zein genes are regulated as well as plant gene regulation in general.

LITERATURE CITED

Aukerman, M. J., Schmidt, R. J. Burr, B., and Burr, F. A. (1991). An arginine to lysine substitution in the bZIP domain of an opaque-2 mutant in maize abolishes specific DNA binding. Genes Dev. 5: 310-320.

Ausubel, F. M., Brent, R., Kingston, R., Moore, D., Seidman, J. J., Smith, J., and Struhl, K. (1988). Current Protocols in Molecular Biology (New York: John Wiley & Sons).

Becker, D. M., and Guarente, L. (1991) High-efficiency transformation of yeast by electroporation. In: Guide to Yeast Genetics and Molecular Biology. Guthrie, C., Fink, G. R., (eds.), Academic Press, Inc. Methods in Enzymology 194: 182-186.

Berger, S. L., Cress, D. W., Cress, A., Triezenberg, S. J., Guarente, L. (1990). Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. Cell 61: 1199-1208.

Boronat, A., Martinez, M. C., Reina, M., Puigdomenech, P., and Palau, J. (1986). Isolation and sequencing of a 28kD glutelin-2 gene from maize. Common elements in the 5' flanking regions among zein and glutelin genes. Plant Sci. 47: 95-102.

Boston, R. S., Fontes, E. B. P., Shank, B. B. and Russell, L.W. (1991). Increased expression of the maize immunoglobulin binding protein homologue b-70 in three zein regulatory mutants. Plant Cell 3: 497-505.

Boston, R. S., Kodrzycki, R. and Larkins, B. A., (1986) Transcriptional and post-transcriptional regulation of maize zein genes. In: Molecular biology of seed storage proteins and lectins. Ninth Symposium in Plant Physiology. Shannon, L. M. and Chrispeels, M. J. (eds.), University of California, Riverside, CA., pp. 117-126.

Brown, J. W. S., Wandelt, C., Feix, C., Neuhaus, G., and Schweiger, H. G. (1986). The upstream regions of zein genes-sequence analysis and expression in the unicellular alga *Acetabularia*. Eur. J. Cell Biol. 42: 161-170.

Burr, B., Burr, F. A., ST. John, T. P., Thomas, M., and Davis, R. D. (1882) Zein storage protein gene family of maize. J. Mol. 154: 33-49.

Bustos, M. M., Begum, D., Kalkan, F. A., Battraw, M. J., and Hall, T. C. (1991). Postive and negative cis-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter. EMBO J. 6: 1469-1479.

Butt, T. R., Sternberg, E., Herd, J., and Crooke, S. T. (1984). Cloning and expression of a yeast copper metallothionein gene. Gene 27: 23-33.

Chandler, V. L., Radicella, J. P., Robbins, T. P., Chen, J., and Turks, D. (1989). Two regulatory genes of the maize anthocyanin pathway are homologous: Isolation of B utilizing R genomic sequences. Plant Cell 1: 1175-1183.

Dalby, A., and Tsai, C. Y. (1974). Zein accumulation in phenotypically modified lines of opaque-2 maize. Cereal Chem. 51:821-825.

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

Di Fonzo, N., Fornasari, E., Salamini, F., Reggiani, R. and Soave, C. (1980). Interaction of maize mutants floury-2 and opaque-2 in the synthesis of endosperm proteins. J. Hered. 71: 397-402.

Drong, R. F., and Slightom, J. L. (1993). Analyses of genes that encode the 15kD zein protein of maize: identification of potential gene regulatory elements. Gene 123: 245-248.

Fogel, S., and Welch, J. W. (1982). Tandem gene amplication mediates copper resistance in yeast. Proc. Natl. Acad. Sci. USA 79: 5342- 5346.

Fogel, S., Welch, J. W., Cathala G., Karin, M. (1983). Gene amplification in yeast: CUP1 copy number regulates copper resistance. Curr. Genet. 7: 347-355

Forde, B. G., Heyworth, A., Pywell, J., and Kreis, M. (1985). Nucleotide sequence of a B1 hordein gene and the identification of possible upstream regulatory elements in endosperm storage protein genes from barley, wheat and maize. Nucleic Acids Res. 13: 7327-7339.

Geetha, K. B., Lending, C. R., Lopes, M. A., Wallace, J. C., and Larkins, B. A. (1991). opaque-2 modifiers increase γ-zein synthesis and alter its spatial distribution in maize endosperm. Plant Cell 3: 1207-1219.

Gerats, A. G. M., Bussard, J., Coe, E, H., and Larson, R. (1984). Influence of B and P1 onUDPG: Flavonoid-3-O-glucosyltransferase in Zea mays. Biochem. Genet. 22: 1161-1169.

Goff, S. A., Klein, T. M., Roth, B. A., Fromm, M. E., Cone, K. C., Radicella, J. P., and Chandler, V.L. (1990). Transactivation of anthocyanin biosynthetic genes following transfer of B regulatory genes into maize tissues. EMBO J. 9: 2517-2522.

Goff, S. A., Cone, K. C., and Fromm, M. E. (1991). Functional analysis of the maize Cl transcriptional activator: Comparison of wild-type and dominant inhibitor proteins. Genes & Dev. 5: 298-309.

Hagen, G., and Rubenstein, I. (1981). Complex organization of zein genes in maize. Gene 13: 239-249.

Hartings, H., Maddaloni, M., Lazzaroni, N., Di Fonzo, N., Motto, M., Salamini, F., and Thompson, R. (1989). The O2 gene which regulates zein deposition in maize endosperm encodes a protein with structural homologies to transcriptional activators. EMBO J. 8: 2795-2801.

Heidecker, G., Chaudhuri, S., and Messing, J. (1991). Highly clustered zein gene sequences reveal evolutionary history of the multigene family genomics. 10: 716-732.

Hill, A., and Bloom, K. S. (1987). Genetic manipulation of centromere function. Mol. Cell Biol. 7: 2397-2405.

Jack, T., Brockman, L.L., and Meyerowitz, E. M. (1992). The homeotic gene APETALA3 of Arabridopsis thaliana encodes a MADS-box and is expressed in petals and stamens. Cell 68: 683-688.

Jones, R. A., Larkins, B. A., and Tsai, C. Y. (1977). Storage protein synthesis in maize II. Reduced synthesis of a major zein component by the opaque-2 mutant of maize. Plant Physiol. 59: 525-529

Kaiser, P., and Auer, B. (1993). Rapid shuttle plasmid preparation from yeast cells by transfer to *E. coli.* BioTechniques 14: 552

Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welch, J., Fogel, S. (1984). Primary structure and transcription of an amplified genetic locus: the CUP1 locus of yeast. Proc. Natl. Acad. Sci. USA 81: 337-341.

Kelly, M., Burke, J., Smith, M., Klar, A., and Beach, D., (1988). Four mating-type genes control sexual differentiation in the fission yeast. EMBO J. 7:1537-1547.

Kodrzycki, R., Boston, R. S., and Larkins, B. A. (1989). The opaque-2 mutation of maize differentially reduces zein gene transcription. Plant Cell 1: 105-114.

Kreis, M., Forde, B.G., Rahman, S., and Miflin, B. J. (1985). Molecular evolution of the seed storage proteins of barley, rye, and wheat. J. Mol. Biol. 183:499-502.

Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988). The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. Science 240: 1759-1764.

Lee, M. G., and Nurse, P. (1988). Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. Nature 327: 31-35.

Lopes, M. A., and Larkins B. A. (1991). Gamma-zein content is related to endosperm modification in Quality Protein Maize. Crop Sci. 31:1655-1662.

Ludwig, S. R., Habera, L. F., Dellaporta, S. L., and Wessler,S. R. (1989). Lc, a member of the maize R gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region. Proc. Natl, Acad. Sci. 86: 7092-7096.

Ma, J., Przibilla, E., Hu, J., Bogorad, L., and Ptashne, M. (1988). Yeast activators stimulate plant gene expression. Nature 334: 631-633.

Maier, U. G., Brown, J. W. S., Toloczyki, C., and Feix, G. (1987). Binding of a nuclear factor to a consensus sequence in the 5' flanking region of zein genes from maize. EMBO J. 6: 17-22.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Mannzocchi, L. A., Daminati, M. G., and Gentinetta, E. (1980). Viable defective endosperm mutants in maize . EMBO J. 6:17-22.

Marks, M. D. and Larkins, B. A. (1982). Analysis of sequence microheterogeneity among zein messenger RNAs. J. Biol. Chem. 260: 9976-9983.

Marks, M. D., Lindell, J. S., and Larkins, B. A. (1985). Nucleotide sequence analysis of zein mRNAs From maize endosperm. J. Biol. Chem. 260: 16451-16459.

Mauri, I., Martegani, E., Maddaloni, M., Salamini, F., Thompson, R., and Motto, M. (1993). Functional expression of the transcriptional activator opaque-2 of Zea mays in transformed yeast. Maize Genetics Coop. Newsletter 67: 14-15.

Mendel, M. A., Gustafson-Brown, C., Savidge, B., and Yanofsky, M. F. (1992) Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. Nature 360: 273-277.

Messing, J., Geraghty, D., Heidecker, G., Hu, N. T., Kridl, J., and Rubenstein, I. (1983). Genetic engineering of plant genes. In: Genetic Engineering of Plants. Hollaender, A., Kosuge, T., and Meredith, C. A. (eds.), Plenum Press, N. Y., pp. 211-228.

Metzger, D., White, J., and Chambon, P. (1988). The human oestrogen receptor functions in yeast. Nature 334: 31-36.

Mogen, B. D., MacDonald, M. H., Graybosch, R., and Hunt, A. G. (1990). Upstream sequences other than AAUAAA are required for efficient messenger RNA 3'-end formation in plants. Plant Cell 2:1261-1272.

Motto, M., Maddaloni, M., Ponziani, G., Brembilla, M., Marotta,R., Di Fonzo, N., Soave, C., and Thompson, R. D. (1988). Molecular cloning of the o2-m5 allele of Zea mays using transposon marking. Mol. Gen. Genet. 212: 488-494.

Motto, M., Di Fonzo, N., Hartings, H., Maddaloni, M., Salamini, F., Soave, C., and Thompson, R. D. (1989). Regulatory genes affecting maize storage protein synthesis. *Oxford Surveys of Plant Molecular and Cell Biology* (Eds: B. J. Miflin and H. F. Miflin). Oxford University Press,New York.

Mueller, C. G. F., and Nordheim, A. (1991). A protein domain conserved between yeast MCM1 and human SRF directs ternary complex formation. EMBO J. 10: 4219-4229.

Murray, M., Cramer, J., Ma, Y., West, D., Romero-Severson, J., Pitas, J., DeMars, S., Vilbrandt, L., Krishman, J. (1988). Agrigenetic maize RFLP linkage map. Maize Genetics Coop. Newsletter 62: 89-90.

Norman, C., Runswick, M., and Pollock, R. (1988). Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response elememt. Cell 55: 989-1003.

Nourse, J., Mellentin, J. D., Galili, N., Wilkinson, J., Stanbridge, E., Smith, S. D., and Cleary, M. L., (1990). Chromosomal translocation t(1;19) result in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell 60: 535-545.

Park, W. D., Lewis, E. D., and Rubenstein, I. (1980). Heterogeneity of zein mRNA and protein in maize. Plant Physiol. 65: 98-106.

Passmore, S., Maine, G. T., Elble, R., Christ, C., and Tye, B. K. (1988). A Saccharomyces cerevisiae protein involved in plasmid maintenance is necessary for mating of MATa cells. J. Mol. Biol. 204: 593-606.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P.A., and Saedler, H. (1987) The regulatory c1 locus of Zea mays encodes a protein wiyh homology to myb proto-oncogene products and with structural similarities to transcriptional activators. EMBO J. 5: 829-833.

Pedersen, K., Bloom, K. S., Anderson, J. N., Glover, D. V., and Larkins, B. A. (1980). Analysis of the complexity and frequency of zein genes in the maize genome. Biochemistry 19: 1644-1650.

Pedersen, K., Devereux, J., Wilson, D. R., Sheldon, E., and Larkins, B. A. (1982). Cloning and sequence analysis reveal structural variation among related zein genes in maize. Cell 29: 1015-1026.

Pedersen, K., Argos, P., Nayarana, S. V. L., and Larkins, B. A. (1986). Sequence analysis and characterization of a maize zein gene encoding a high sulfur zein protein of Mr 15,000. J. Biol. Chem. 261: 6279-6284.

Resnick, M. A., Westmoreland, J., and Bloom, K., S. (1990). Heterogeneity and maintenance of centromere plasmid copy number in *Saccharomyces cerevisiae*. Chromosoma 99: 281-288.

Righetti, P. G., Gianazza, E., Viotti, A., and Soave, C. (1977). Heterogeneity of storage proteins in maize. Planta 136: 115-123.

Roussell, P. G., Boston, R. S., Goldsbrough, P. B. and Larkins, B. A. ((1988). Deletion of DNA sequences flanking an Mr 19,000 zein gene reduces its transcriptional activity in heterologous plant tissues. Mol. Gen. Genet. 211: 202-209.

Salamini, F., Di Fonzo, N., Gentinetta, E., and Soave, C. , (1979). A dominant mutation interfering with protein accumulation in maize seed. In: Seed protein Improvement in Cereals and Grain Legumes. Ed. IAEA, Vienna. Vol. 1: 97-108.

Salamini, F., Di Fonzo, N., Fornasari, E., Gentinetta, E., Reggiani, R., and Soave, C. (1983). Mucronate, Mc, a dominant gene of maize which interacts with Opaque-2 to suppress zein synthesis. Theor. Appl. Genet. 65: 123-128.

Schena, M., and Yamamoto, K. R. (1988). Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. Science 241:965-968.

Schernthaner, J. P., Matzke, M. A., and Matzke, A. J. M. (1988). Endosperm specific activity of a zein gene promoter in transgenic tobacco plants. EMBO J. 7: 1249-1255.

Schmidt, R. J., Burr, F. A., and Burr, B. (1987). Transposon tagging and molecular analysis of the maize regulatory locus opaque-2. Science 238: 960-963.

Schmidt, R. J., Burr, F. A., Aukerman, M. J., and 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.

Schmidt, R. J., Ketudat, M., Aukerman, M. J., and Hoschek, G. (1992). Opaque-2 is a transcriptional activator that recognizes a specific target site in 22kD zein genes. Plant Cell 4: 689-700.

Schmidt, R. J., Veit, B. Alejandra, M., Mena, M., Hake, S., and Yanofsky, M. F. (1993). Identification and molecular characterization of ZAG1, the maize homolog of the arabidopsis floral homeotic gene AGAMOUS. Plant Cell 5: 729-737.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lonnig, W-E., Saedler, H., and Sommer, H. (1992). Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: Evidence for DNA binding and autoregulation of its persistent expression throughout flower development. EMBO J. 11: 251-263.

Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae.* Genetics *122:19-27.*

Soave, C., Dossena, S., Lorenzoni, C., Di Fonzo, N., and Salamini, F. (1978). Expressivity of the *floury-2* allele at the level of zein molecular components. Maydica 23: 145-152.

Soave, C., Reggiani, R., Di Fonzo, N., and Salamini, F. (1981). Clustering of genes for 20k zein subunits in the short arm of maize chromosome 7. Genetics 97: 363-377.

Soave. C., and Salamini, F. (1984). Organization and regulation of zein genes in maize endosperm. Phil. Trans. T. Soc. London 304: 341-347

Spena, A., Viotti, A., and Pirrotta, V. (1982). A homologous repetitive zein gene. EMBO J. 1: 1589-1594.

Styles, E. D., Ceska, O., and Seah, K.T. (1973). Developmental difference in action of R and B alleles in maize. Can. J. Genet. Cytol. 15: 59-72.

Thiele, D. J., and Hamer, D. H. (1986). Tandemly duplicated upstream control sequences mediate copper induced transcription of the *Saccharomyces cerevisiae* copper metallothionein gene. Mol. Cell. Biol. 6: 1158-1163.

Thompson, G. A., and Larkins, B. A. (1989). Structural elements regulating zein gene expression . BioEssays 10: 108-113.

Vinson, C. R., Sigler, P. B., and McKnight, S. L. (1989). Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science 246: 911-916.

Volbrecht, E., Veit, B., Sinha, N., Hake, S. (1991). The developmental gene Knotted-1 is a member of a maize homeobox gene family. Nature 350: 241-243.

Werr, W., Frommer, W. B., Mass, C., and Starlinger, P. (1985). Structure of the sucrose synthase gene on chromosome 9 of Zea mays L. EMBO J. 4: 1373-1380.

Wilson, D. R., and Larkins, B. A. (1984). Zein gene organization in maize and related grasses. J. Mol. Evol. 20: 330-340.

Yanofsky, M. F., Ma, H., Bowman, J. L., Drews, G., Feildman, K., and Meyerowitz, E. M. (1990). The protein encoded by the Arabidopsis homeotic gene AGAMOUS resembles transcription factors. Nature 346: 53-39.

APPENDIX 1

LIST OF ABBREVIATIONS

15kD zein gene: zein gene encoding a protein of relative molecular weight of 15,000 on a polyacrylamide gel

19kD zein gene: zein gene encoding a protein of relative molecular weight of 19,000 on a polyacrylamide gel

22kD zein gene: zein gene encoding a protein of relative molecular weight of 22,000 on a polyacrylamide gel

kD: Kilodalton

bp: base pair

kb: Kilobase

VITA

Selester Averette Bennett was born in Danville, Virginia January 10, 1964. He received a Bachelors of Science degree in biology form Virginia Polytechnic Institute and State University in the spring of 1987 and his Masters of Science degree from Virginia Polytechnic Institute and State University in the fall of 1993.

Selester Bennett