1997

Characterization of the expression of two maize root-preferential genes at the protein level

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Characterization of the expression of two maize root-preferential genes at the protein level

by

Lathika Pinto Moragoda

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Botany (Physiology and Molecular Biology)
Major Professor: James T. Colbert

Iowa State University
Ames, Iowa
1997

Graduate College
Iowa State University

This is to certify that the Doctoral Dissertation of

Lathika Pinto Moragoda

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

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For the Major Program

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For the Graduate College

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GENERAL INTRODUCTION

Root structure and development

The primary function of roots is in anchorage and in absorption of nutrients and water. In addition, roots also serve as storage organs, interact with microbes in the soil and synthesize hormones.

Monocotyledons, such as maize, possess a fibrous root system that is composed largely of adventitious roots. A feature of grasses, including maize, is the presence of a rhizosheath which provides an amplified root surface in intimate contact with a considerable volume of soil, and interaction with characteristic bacteria found in the rhizosheath (McCully, 1995).

The growth of roots is a continuous process that is maintained throughout the life cycle of a plant. Several regions of development can be identified during growth of roots. The apex of the root is protected by a root cap. The apical meristem and the nearby portion of the root in which cell division occurs is called the region of cell division. Behind the region of cell division is the region of elongation which is usually only few mm in length (Raven et al., 1992). The elongation of cells in this region results in most of the increase in length of the root. The region of elongation is followed by the region of maturation, in which most of the cells of the primary tissues mature. Root hairs are also found in this region (Raven et al., 1992). These regions are not sharply delimited, rather a gradual transition from one region to the other is

observed. In maize roots, a fourth region called the distal elongation zone (DEZ) has been identified based on the special physiological properties these cells exhibit (Ishikawa and Evans, 1995). Although, the root structure has traditionally been considered in terms of different developmental regions, recent structural analyses have considered root as being composed of several developmental units, which consist of cylinders, sectors, packets (merophytes), and cell files (Rost and Bryant, 1996). For example, the vascular cylinder, which is considered as one of the cylinders of the root, is further subdivided into xylem and phloem sectors based on the differences between these two types of cells.

The internal structure of the root is relatively simple. Three tissue systems of the root in primary stage of growth can be readily distinguished. The epidermis (dermal tissue system), the cortex (ground tissue system), and the vascular cylinder (vascular tissue system) can be clearly distinguished from one another (Raven et al., 1992). In maize, the outer cell layer of the cortex differentiates into a compactly arranged exodermis in contrast to the loosely arranged typical cortical cells. The secondary cell walls of the exodermis contain suberin and lignin, unlike the typical cortical cells which produce cellulosic cell walls. The innermost layer of cortex, the endodermis, is compactly arranged and lacks intercellular air spaces. This layer is characterized by the presence of Casparian strips in its anticlinal walls which are impregnated with suberin initially and with lignin subsequently (Raven et al., 1992). The plasma membranes of the endodermal cells are firmly attached to these Casparian strips. Casparian strips are impermeable to

water and ions, thus all substances entering or leaving the vascular cylinder are forced through the symplastic pathway. The recent discovery of Casparian strips in the exodermis of maize, and the lack of permeability of the exodermis to apoplastic tracers indicate that the initial filtration of the soil solution occurs in this layer rather than in the endodermis (Perumalla and Peterson, 1986). However, the Casparian bands of the exodermis mature further from the root tip than its counterpart in the endodermis (Perumalla and Peterson, 1986). Hence, at least in the region where Casparian bands of the exodermis are absent, the endodermis appears to function in filtration of the soil solution.

Organ-preferential gene expression

From a morphological perspective, plants are relatively simple organisms. The mature sporophyte has just three vegetative organ systems (leaf, stem, root) and four reproductive organ systems (petal, sepal, pistil, stamen). Each organ system expresses a unique and complex set of structural genes in addition to the overlapping set of genes shared by all organ systems. Organ-specific mRNAs represent 25-40% of the structural gene set expressed in each organ (Okamura and Goldberg, 1989). Furthermore, quantitative as well as qualitative changes in gene expression play an important role in the establishment and maintenance of the unique structure and function of each plant organ and its tissue types. The expression of organ-specific genes can

be regulated in several ways. These include regulation at the transcriptional, post-transcriptional, translational and post-translational levels. Each of these processes plays an important role in establishing the expressed state of a gene, the functional protein product. For example, analyses of steady-state nuclear mRNA and cytoplasmic mRNA populations indicate that both transcriptional and post-transcriptional regulatory processes determine the pattern of gene expression in leaves. Anther-specific and root-specific genes are not detectably transcribed in the leaf indicating regulation at the transcriptional level. Stem-specific structural genes on the other hand are transcribed, but are not detectable on leaf polysomes indicating post-transcriptional control (Okamura and Goldberg, 1989).

Root-preferential gene expression

Many plant genes are expressed in a highly regulated manner. Genes can be regulated during growth and development of plants or in response to environmental stimuli and stresses, in an organ-specific or tissue-specific manner. Well known examples of organ-preferential expression include seed storage proteins such as zeins in maize, which are expressed only in the embryo and endosperm (Heidecker and Messing, 1986), genes expressed during flower development such as anther-specific genes (Koltunow et al., 1990), genes expressed during root nodule development such as leghemoglobins (Marker et al., 1984) and light regulated genes expressed predominantly in green tissues

such as RUBP carboxylase (Rubisco) and Chlorophyll a binding protein (CAB) (Gallagher and Ellis, 1982).

To isolate genes involved in root development, two major approaches have been taken. The genetic approach involves generation and analysis of root mutants (Schiefelbein and Benfy, 1991; Scheres et al., 1995). The other approach involves analysis of root development at the molecular level by isolation and characterization of genes expressed in the root. Most of the genes thus identified have been isolated by differential screening of root cDNA libraries (Montoliu et al, 1989; Conkling et al., 1990; John et al., 1992; Collazo et al., 1992; de Pater and Schilperoort, 1992; Michalowski and Bohnert, 1992; Choi et al., 1996). Differential screening has permitted isolation of genes whose function is known as well as genes whose biological function is not known.

A number of root-preferentially expressed genes whose function is known have been isolated. For example, leghemoglobin and nodulin genes are known to be specifically expressed in root nodules of soybean (Marker et al., 1984; Miao and Verma, 1993). Also from soybean, a cytosolic glutamine synthetase gene whose expression is detected in both roots and root nodules has been identified (Miao et al., 1991). Similarly, a gene encoding a cell wall hydroxyproline rich glycoprotein expressed during lateral root initiation (Keller and Lamb, 1989) and a root-specific gene encoding a membrane channel protein have been isolated from tobacco (Yamamoto et al., 1991). Other root-preferential genes whose function is known include a root-specific barley lectin gene (Lerner and Raikhal, 1989), two α-tubulin genes expressed

preferentially in radicular tissues of maize (Montoliu et al., 1989), a root epidermis-specific gene from pea which shows homology to a pathogenesis-related gene (Mylona et al., 1994), and an ACC synthase gene which shows root-predominant expression in wheat (Subramanium et al., 1996). In addition to these genes, several O-methyltranferase (OMT) genes have been isolated which are expressed in a root-preferential manner. Collazo et al. (1992) isolated a gene coding for a caffeic acid OMT involved in lignin biosynthesis from maize which is expressed at the highest level in the elongation zone of roots. A chalcone OMT gene involved in the synthesis of a flavanoid compound that induces nodulation genes in *Rhizobium* has been isolated by Maxwell et al. (1993) from alfalfa roots. Another OMT gene (*zrp*4) that is thought to be involved in suberin biosynthesis has been isolated from maize roots (Held et al., 1993). Few genes have been identified from roots whose function is at least partly known due to the conditions under which they were isolated. For example, Cleas et al. (1990) isolated a root-preferential gene (SalT) from rice that is induced under salt stress. Similarly Michalowski and Bohnert (1992) have isolated a germin-like protein from salt stressed roots of ice plant.

In contrast to the genes mentioned above, several genes have been described whose functional significance is not known. For example, John et al. (1992) isolated a gene (*zrp*3) whose mRNA accumulates specifically within the inner cortex of roots in maize seedlings. Homologs of *zrp*3 have been subsequently described from other species. Dietrich et al. (1992) have described a gene (AX92) which is expressed in a

similar fashion from oilseed rape. Two more homologs of *zrp*3 have been isolated from rice (Xu et al., 1995). Recently, another homolog, whose mRNA shows an expression pattern similar to *zrp*3, has been isolated from roots of bean (Choi et al., 1996). Additionally, two genes (cos6 and cos9) of unknown function have been described from rice that are highly expressed in seedling and mature plant roots (de Pater and Schilperoort, 1992). In addition to these genes, a maize gene (*zrp*2) whose mRNA accumulates preferentially in the cortex of roots has been isolated previously in our laboratory (John, 1991). The function of this gene remains to be elucidated since it does not show any sequence similarity to known genes. However, based on its sequence characteristics, we currently believe ZRP2 to be a structural or a storage protein.

In addition to investigating the function of root-preferentially expressed genes, mechanisms by which the mRNA accumulation is regulated in these root-preferential genes has been the focus of several investigations (Conkling et al., 1990; Yamamoto et al., 1991; Miao et al., 1991; Dietrich et al., 1992; Miao and Verma, 1993; Xu et al., 1995; Capellades et al., 1996).

O-methyltransferases

O-methyltransferases (OMT)s carry out transmethylation reactions. These reactions involve the transfer of a methyl group from an S-adenosyl-L-methionine (SAM) to an oxygen of a suitable acceptor

molecule. O-methyltransferases are widely distributed in nature from prokaryotes to eukaryotes. For example, the OMT, L-isoaspartyl methyltransferase involved in the repair of altered aspartyl residues in proteins, is found in *E. coli*, animals including mammals, and plants (Mudgett and Clarke, 1996). In addition to methylation of DNA, RNA, and proteins, OMTs are also involved in methylation of secondary metabolites in plants. The occurence of a vast multitude of secondary plant products possessing one or more O-methyl groups in their structure provides an indication of the importance of transmethylation in plant metabolism (Poulton, 1981). Methylation fulfills many physiological functions in plants. There is increasing evidence that transmethylation may play a crucial role in directing intermediates towards specific biosynthetic pathways (Poulton, 1981). The secondary plant products that are methylated include lignin precursors, phenolics, coumarins, flavonoids and alkaloids.

Several OMTs have been cloned from plants to date. Caffeic acid-3-O-methytransferase (COMT) which is involved in the synthesis of lignin monomers has been cloned from alfalfa (Gowri et al., 1991), maize (Collazo et al., 1992), aspen (Bugos et al., 1991), poplar (Dumas et al., 1992) tobacco (Jeack et al., 1992, Jeack et al., 1993, Pellagrini et al., 1993) and *Zinnia* (Ye and Varner, 1995). Production of lignin monomers involves methylation of caffeic acid to ferulic acid by COMTs. In dicots, COMT is bispecific and can also catalyze methylation of 5-hydroxy ferulic acid to sinapic acid (Capellades et al., 1996). Some of these COMTs have been shown to be induced in response to plant-pathogen

interactions (Gowri et al., 1991; Jeack et al., 1992; Pellagrini et al., 1993). Recently it has been shown that brown midrib mutation in maize is due to a non- functional maize COMT gene (Vignols et al., 1995). In addition to COMTs, a few other OMTs have been described. A myoinositol OMT which catalyzes the first step in the biosynthesis of the cyclic sugar pinitol which accumulates in the salt-stressed ice plant has been described by Vernon and Bohnert (1992). Maxwell et al. (1993) described an alfalfa chalcone OMT involved in the induction of *nod* genes in *Rhizobium*. An OMT from barley that is induced in response to fungal pathogens and UV light has been identified (Gregersen et al., 1994). Two caffeoyl-coenzymeA 3-O-methytransferases (CCoAMT), one that is involved in an alternative pathway of lignin biosynthesis in *Zinnia* (Ye et al., 1994) and another that is induced by fungal elicitors in parsley (Schmitt et al., 1991) have been described.

*zrp*4, a gene encoding an OMT has been previously isolated in our laboratory (Held et al., 1993). ZRP4 mRNA has been shown to accumulate preferentially in the endodermis and exodermis of young maize roots. *zrp*4 appears to be distinct from other known plant OMTs since it shows little similarity with respect to sequence identity and localization within root. For example, ZRP4 amino acid sequence shares about 50% similarity (30% identity) with COMTs isolated from aspen (Bugos et al., 1991), alfalfa (Gowri et al., 1991) and maize (Collazo et al., 1992). These COMTs would be expected to be localized principally in the xylem tissue since COMTs are involved lignin biosynthesis. In contrast, ZRP4 mRNA levels accumulate to the highest level in the

endodermis and at lower levels in the exodermis during the time when these regions are in the process of forming the Casparian strip (Held et al., 1993). A major O-methylated plant metabolite known to accumulate specifically in the endodermis and exodermis at the time of deposition of Casparian strips is suberin (Held et al., 1993). Therefore, it has been proposed that ZRP4 may be an OMT involved in suberin biosynthesis.

Suberin biosynthesis

Suberin is an insoluble polymeric material attached to the cell walls of certain tissues such as periderm (including wound periderms), Casparian strips in the endodermis and exodermis of roots, and bundle sheaths of grasses (Kolattakudy, 1981).

Suberin is composed of aliphatic monomers and phenolic components. The most common aliphatic components are fatty acids, fatty alcohols, ω-hydroxy fatty acids and dicarboxylic acids (Kolattakudy, 1981). The phenolic components appear to be derived mainly from hydroxy cinnamic acids (Bernards et al., 1995). Based on available evidence, the structure of suberin is thought to be composed of a phenolic matrix similar to lignin, attached to the cell walls, and the aliphatic components appear to be covalently attached to this phenolic matrix (Kolattakudy, 1981). The suberin phenyl propanoid subunits are synthesized in the cytoplasm, deposited and polymerized extracellularly in a manner analogous to lignin (Kolattakudy, 1987). The O-methyltransferase required for biosynthesis of suberin could be a

caffeic acid OMT (COMT) and, is thought to be distinct from the bifunctional caffeic acid/5-hydroxyferulic acid OMT involved in lignin biosynthesis (Held et al., 1993).

Generation of antibodies

Antibodies are an essential tool employed in the characterization of gene expression at the protein level. A complete understanding of gene regulation and expression cannot be gained at the RNA level alone since expression of a gene is ultimately dependent upon the availability of the protein product. Furthermore, any kind of translational or post-translational control can only be detected at the protein level. Once antibodies against the protein of interest has been made, it becomes possible to study protein abundance at the organ, tissue and sub-cellular levels. In addition, antibodies can be used in the isolation and purification of the protein from the experimental organism initially and subsequently, in the characterization of the purified protein. Antigen necessary for the production of antibodies can be obtained in several ways. These include the use of purified native protein from the experimental organism, the use of a synthetic oligopeptide based on the cDNA sequence, and the use of *E. coli* expressed protein. This latter method has been used frequently when the cDNA sequence of the gene of interest is available and when a purification strategy from the source has not been available.

Expression of proteins in *E. coli* involves cloning of the cDNA of interest into an expression vector followed by induction of the host cells containing the vector by IPTG (isopropyl-B-D-galactopyrano- side). IPTG induces the transcription promoter, which can produce large amounts of mRNA from the cloned gene. (Ausubel et al., 1994). In the absence of the inducer IPTG, the target gene is transcribed at a very low level. This is important since certain gene products can be toxic to host bacteria. The expressed protein may be a fusion protein depending whether the coding region includes vector sequences in addition to the sequences derived from the cDNA. Most expression vectors currently available also contain a sequence for affinity purification of the expressed protein (eg., histidine tag). In the host cells, the expressed protein may accumulate mainly in the soluble fraction or the insoluble fraction of the *E. coli* protein extract. Accumulation of the protein in the soluble fraction is more desirable since the protein is more likely be active in the soluble undenatured form. Thus it becomes possible to assay the activity of expressed enzymes. However, accumulation of the expressed proteins as insoluble inclusion bodies is more commonly observed (Ausubel et al., 1994). In such instances the protein is found in the denatured form and is useless in activity assays. Activity of these proteins may be recovered by solubilization in denaturents followed by careful renaturation (Marston et al., 1990). For the purpose of antibody production however, either form of the protein is usually equally effective. Once the target protein is purified, using a method

such as affinity purification or gel purification, the purified protein can be then used as the antigen to inject animals to produce antibodies.

Dissertation organization

This dissertation is organized as two main chapters which are written as manuscripts that will be submitted for publication. The first manuscript describes the detection and distribution of ZRP2 protein and the second manuscript describes detection and distribution of ZRP4 protein in maize. These chapters are preceded by a general introduction and followed by three chapters that describe experiments and results which are not included in the manuscripts to be submitted for publication, and general conclusions. References cited throughout the dissertation are included in the literature cited section.

MAIZE ZRP2 PROTEIN ACCUMULATION IS ROOT-PREFERENTIAL AND TRANSIENTLY DOWN-REGULATED BY ABSCISIC ACID

A paper to be submitted to Plant Molecular Biology

Lathika Moragoda and James T. Colbert

Abstract

The *zrp2* gene encodes an mRNA that accumulates preferentially in roots of maize. To study *zrp2* gene expression at the protein level, an antibody was raised against an *E. coli*-produced ZRP2 protein. Western analyses using this antibody revealed an approximately 100 kDa protein in seedling roots. This size was about 30 kDa larger than the predicted molecular weight. Within the seedling root, low levels of ZRP2 protein were detected in the root tip and high levels were detected throughout rest of the root. In mature maize plants, ZRP2 protein was present at high levels in roots and stems. Other organs of mature plants lacked detectable amounts of this protein. *In vitro* translation of ZRP2 mRNA resulted in the production of a 100 kDa polypeptide indicating that the observed size of ZRP2 protein is unlikely to be due to a post-translational modification. When maize roots were subjected to various external stimuli, *zrp2* expression was observed to be transiently down-regulated by abscisic acid and down-regulated under high salinity.

Key Words

ABA, root-preferential, western analysis, ZRP2 protein, *Zea mays*

Introduction

Roots serve many important functions in plants. These include anchorage, water and mineral absorption, transport, storage, synthesis of hormones, and interaction with soil microbes. The root apical meristem remains fairly uniform throughout the life cycle of a plant in contrast to the shoot apex which can change from a vegetative to a floral meristem developmentally.

As a first step towards understanding the underlying molecular mechanisms regulating the root development and function we have identified and characterized genes that are preferentially expressed in maize roots. Relatively few genes have been described that are expressed in roots (Evans et al.,1988; Keller and Lamb, 1989; Conkling et al., 1990; Collazo et al., 1992; Dietrich et al., 1992; Held et al., 1993; Xu et al., 1995; Choi et al., 1996). Few of these genes have been isolated from maize (Collazo et al. 1992; Held et al., 1993).

We have described three *Zea* Root Preferential (ZRP) clones to date ZRP2 (John, 1991), ZRP3 (John et al., 1992) and ZRP4 (Held et al., 1993). The ZRP2 cDNA clone corresponds to an mRNA that accumulates in roots and in stems of the maize plant. Within the root, ZRP2 mRNA level is lowest at the root tip and is found at high levels in the remainder of the

root. *In situ* hybridization studies indicate that ZRP2 mRNA accumulates in the cortical cells of the root (Wang, 1994). The predicted amino acid sequence of ZRP2 codes for a 67 kDa protein which is largely hydrophilic in nature. The amino acid sequence also contains the conserved motif 'RKATTSYG' that is repeated 16 times throughout the polypeptide. This amino acid sequence does not show any significant homology to any known protein.

The purpose of this study was to characterize ZRP2 gene at the protein level. Antibodies raised against a ZRP2 fusion protein recognized an approximately 100 kDa protein in the roots and stems of the maize plant. The ZRP2 protein distribution pattern was similar to that observed for the ZRP2 mRNA accumulation pattern. The observed size of ZRP2 protein is about 30 kDa larger than the predicted molecular weight and this larger size does not appear be due to a post-translational modification. We also show that this gene is transiently down-regulated in response to abscisic acid (ABA) treatment.

Materials and Methods

Plant material

To obtain seedling tissue, maize seeds (*Zea mays* L. cv NKH31) were grown on germination paper. Germination paper bundles were placed in plastic containers half filled with distilled water. The containers were covered with a similar container to provide high humidity. The seedlings were grown for 5-7 days in a growth chamber

at 30°C/16 h light and 28°C/8 h dark cycle. Seedling roots up to 10 cm in length were harvested for protein isolation. In some experiments, root tips, defined as the first centimeter from the root tip, were excised and processed seperately. Organs from mature plants were harvested from green house grown plants at pollination.

ZRP2 expression, purification, and antibody production

An Xho1 digested 1.36 kbp fragment from the 1.8 kbp coding region of the ZRP2 cDNA was cloned in frame into the pET28c expression vector (Novagen, WI) digested with Xho1. This 1.36 kbp fragment does not include the 5' and 3' most ends of the coding region of the ZRP2 cDNA. The resulting pET28.Z2 construct allowed the production of a fusion protein containing a histidine tag at the N-terminal.

E. coli host strain BL21(DE3) (Novagen) containing pET28.Z2 was induced with 1 mM IPTG at an O.D. of about 1.0 and grown for 7 h at 37°C before harvesting for protein extraction. The protein was purified under denaturing conditions, using 6M urea according to the His-Tag purification protocol (Novagen). The affinity purified protein was concentrated using 30 kDa filters (Amicon) and electrophoresed on 7.5% SDS-PAGE preparative gels. The protein eluted from the gel, was used to inject rabbits. Approximately, 100 μg of the antigen was used to inject each of two New Zealand white rabbits subcutineaously. The rabbits were injected twice before final bleeds were collected. Injection of rabbits and collection of sera were carried out by the Iowa State University Cell and Hybridoma Facility.

Protein extraction and Western analysis

Various organs from seedlings and mature plants were frozen in liquid nitrogen and stored at -80°C. Approximately 0.5 g of plant material was ground with sand in liquid nitrogen and suspended in extraction buffer (100 mM Tris-HCl pH 8.0, 5 mM EDTA, 5 mM DTT 100 mM NaCl, 15% glycerol) containing a protease inhibitor cocktail (Boerhinger-Mannhiem). The extracts were centrifuged at 12,000 rpm in a microcentrifuge for 5 minutes. The supernatants were collected and stored at -80°C until used. Protein concentration of each sample was determined by the Bradford assay using BSA as the standard (Bio-Rad).

Proteins were separated by SDS-PAGE using 7.5% gels, and electroblotted onto nitrocellulose (Schleicher & Schuell) using semi-dry transfer (Bio-Rad). The membranes were blocked with 5% BSA in Phosphate Buffered Saline containing 0.1% Tween-20 (PBS-T) overnight at 4°C. The primary antibody was used at a 1:3,000 dilution and the HRP-conjugated secondary antibody was used at a 1:10,000 dilution in PBS-T. Blots were developed using enhanced chemiluminescence (ECL, Amersham).

***In vitro* translation and Immunoprecipitation**

In vitro translation was carried out using a rabbit reticulocyte lysate system and ^{35}S labeled methionine as described by the manufacturer (Promega). One μg of poly(A)$^+$ RNA from seedling roots was used in 50 μl of translation reaction. After incubation at 30°C for

1 h, proteins were immunoprecipitated with ZRP2 antibodies as follows. The reaction was first incubated with 1-2 µl of antiserum for 1 h at 4°C followed by incubation with 30 µl of 1:1 slurry of protein-A sepharose (Sigma) and dilution buffer (0.01 Tris-HCl pH 8.0, 0.14 M NaCl, 0.025% NaN_3, 0.1% Triton X-100) for another hour at 4°C. The pellet was washed once with dilution buffer and twice with dilution buffer without Triton X-100. A final wash of the pellet was carried out with 0.05 M Tris-HCl (pH 6.8). Translated proteins were analyzed by SDS-PAGE, and visualized by fluorography.

RNA isolation and Northern analysis

Total RNA from ABA treated seedling roots were isolated using the aurin tricarboxylic acid method (Wadsworth et al., 1988). Poly(A)$^+$ RNA was isolated from total RNA using poly(U) sephadex columns according to Lissemore et al., (1987). RNA was fractionated on 1% agarose gels containing 3% formaldehyde. To ensure equal loading, gels were photographed under UV light to verify equal loading of rRNA, prior to transfer to membrane (Gene Screen, DuPont). Northern analysis was performed as described by Cotton et al. (1990). To synthesize an antisense RNA probe, pZRP2 was linearized with HindIII and transcribed with T3 RNA polymerase. Hybridization and washing conditions were as described (Cotton et al., 1990) except that low stringency washes were done at 65°C instead of at room temperature.

ABA treatment of maize seedlings

Maize seeds were germinated on germination paper in distilled water. Each bundle containing germinated seedlings was subsequently transferred to an 80 μM ABA solution (Sigma). The treatment time in the ABA solution varied from 5 h to 3 days. Once the seedlings were transfered, they remained in the ABA solution until samples were collected. Samples from different ABA time treatments were collected at the same time six days later so that the seedlings were the same age. Root segments up to 10 cm in length were harvested and frozen in liquid nitrogen and stored at -80°C.

Stress treatments of maize seedlings

To induce salt stress, seeds were grown on germination paper for 3 d in distilled water and transferred to a 100 or 400 mM NaCl solution. Root samples were collected after 1 and 2 d of salt treatment. For wounding studies, longitudinal incisions were made along the length of the roots of 5-d-old seedlings using a razor blade. Root samples were harvested afer 6, 12, and 24 h of wounding. Desiccation treatments were carried out by transferring germination paper bundles containing 5-d-old seedlings into empty containers. Seedlings were allowed to dehydrate for 6, 12 and 24 h and samples collected.

Results

Generation of antibodies

To generate antibodies against the ZRP2 protein, ZRP2 cDNA was expressed in *E. coli*. Specifically, a 1.36 kbp fragment of the 1.8 kbp ORF of the ZRP2 cDNA was cloned into the expression vector pET28c to produce pET28.Z2. The cloned region represents approximately 75% of the coding region. This 1.36 kbp fragment encodes 13 of the 16 repeated amino acid sequence motifs (RKATTSYG). The functional or structural significance of this sequence is not known. The vector sequences supplied both the initiation and the termination codons of the fusion protein (Fig. 1). The histidine tag present in the vector allowed affinity purification of the 58 kDa fusion protein (Fig. 2, lanes 3 and 4). The protein was purified under denaturing conditions since the protein was found mostly in the insoluble fraction of *E. coli*. (data not shown). Following affinity purification the protein was further gel purified (lane 5) and injected into rabbits. The fusion protein was specifically recognized by the antiserum and not by the preimmune serum (Fig. 3).

Western analysis of ZRP2

To investigate the distribution of ZRP2 in the organs of maize seedlings, western analyses were performed (Fig. 4). The preimmune sera did not cross-react with any proteins from the seedling root and leaf extracts (Fig 4). With ZRP2 antiserum, an approximately 100 kDa protein was detected in the roots which was not detected in the

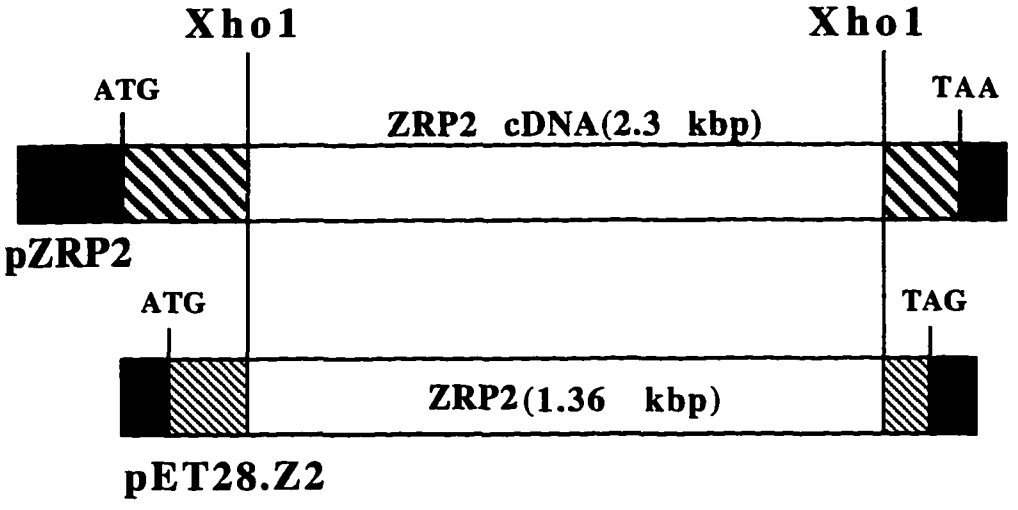

Figure 1. Construction of pET28.Z2
A Xho1 digested 1.36 kbp fragment of the ZRP2 cDNA was ligated into the Xho1 site of the pET28b vector. The translation start and termination codons of the ZRP2 cDNA and pET28.Z2 are indicated. The shaded regions 5' and 3' to the Xho1 sites include the vector sequences that are included in the ORF of pET28.Z2.

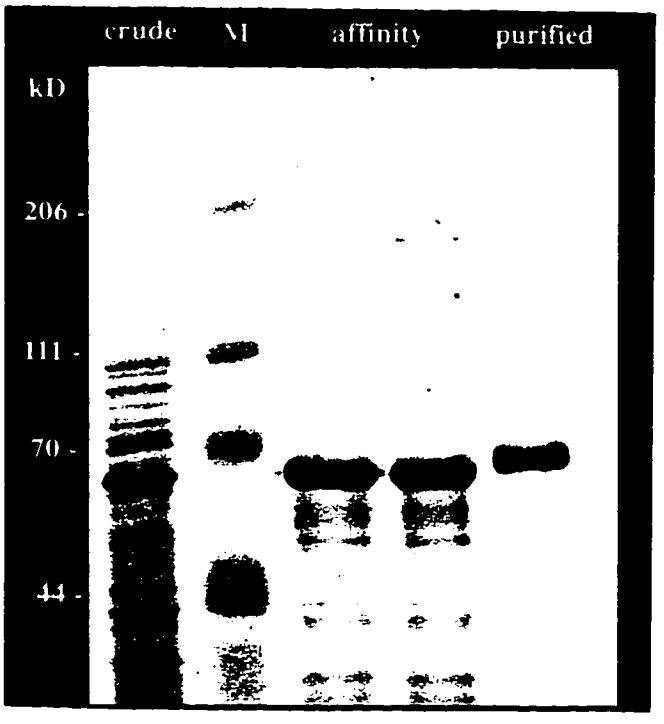

Figure 2. Expression and purification of ZRP2 fusion protein. Protein extracts from IPTG-induced (crude) cultures of *E. coli* cells containing pET28.Z2 were subjected to electrophoresis on 7.5% SDS-PAGE gels. The induced ZRP2 fusion protein was purified using affinity purification (affinity) and subsequent gel purification (purified). Lane 2 contains molecular weight markers (M).

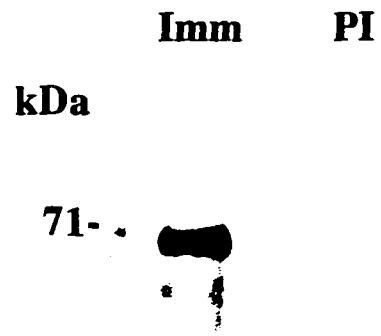

Figure 3. Specificity of ZRP2 antiserum.
Purified ZRP2 fusion protein was electrophoresed on 7.5% SDS-PAGE gels, electroblotted onto nitrocellulose, the blots were incubated with either ZRP2 antiserum (Imm) or preimmune serum (PI). Primary antibody was detected with HRP-conjugated secondary antibody using chemiluminescence.

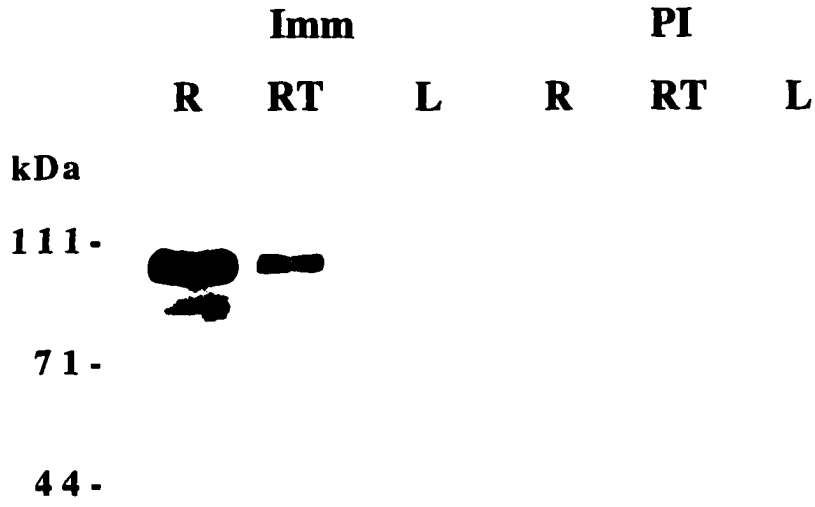

Figure 4. Immunodetection of ZRP2 protein in maize seedling organs. Proteins were extracted from whole roots (R), root tips including the first cm of the root (RT), and leaves (L) of seedlings. Protein extracts (30 µg each) were electrophoresed on 7.5% SDS-PAGE gels and electroblotted onto nitrocellulose and incubated with either preimmune serum (PI) or immune serum (Imm) as the primary antibody. The primary antibody was detected with HRP-conjugated secondary antibody using chemiluminescence.

leaves. Within seedling roots, this protein was detected at a lower level in the first cm of the root than in the remainder of the root (Fig. 4, lane 2). ZRP2 protein accumulation in the organs of the mature maize plant was also investigated (Fig. 5). High levels of ZRP2 protein was detected both in mature root systems and young prop roots (lanes MR, YR). In addition, ZRP2 was also detected in stems at high levels. These levels were comparable to seedling root levels of the protein. However, ZRP2 was not detected in the other organs of the plant including leaves, ears at pollination, ears 10 days after pollination, silks and tassels (lanes L, EP, 10E, Si and T).

In vitro translation of ZRP2 protein

The observed size of 100 kDa for the ZRP2 protein is larger than the expected size of 67 kDa calculated from the full length cDNA clone (John, 1991). A possible explanation for this size difference in the observed molecular weight based on SDS-PAGE and the predicted size based on the cDNA would be a post-translational modification of the polypeptide. The amino acid sequence derived from the ZRP2 cDNA contains two potential N-glycosylation sites (John, 1991). Furthermore, ZRP2 is a serine/threonine rich protein and these amino acids are arranged in a repeated motif consisting of 'RKATTSYG' which could potentially be O-glycosylated. Therefore, in order to study whether this size disparity could be due to a post-translational modification, *in vitro* translation was carried out.

Poly(A)$^+$ RNA from maize seedling roots was translated in a rabbit

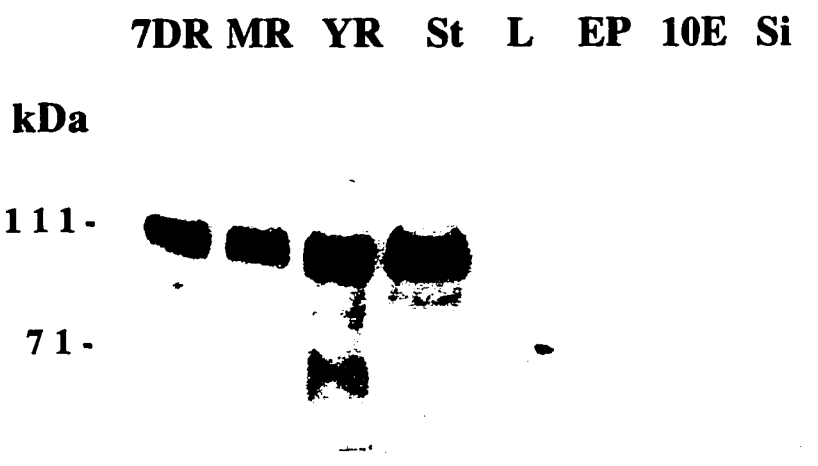

Figure 5. Immunodetection of ZRP2 protein in the organs of the mature plants. Western analysis was carried out as described in Materials and Methods. Proteins were extracted from various organs of mature maize plants at pollination including mature roots (MR), young prop roots (YR), stems (St), leaves (L), ears at pollination (EP), ears 10 days after pollination (10E), silks (Si), and tassels (T). Proteins extracted from 7-D-old seedling roots (7DR) was included for comparison. 30 μg of total protein extracts were loaded per lane.

reticulocyte lysate in the presence of ^{35}S methionine. The translated products were immunoprecipitated with ZRP2 antibodies. This experiment revealed a translation product of approximately 100 kDa in the immunoprecipitate of the reaction containing RNA. In the control reaction (lacking RNA) no proteins were visible (Fig. 6).

Effect of ABA on *zrp2* expression

Investigation of factors that influence ZRP2 expression, such as enviromental or hormonal effects, may provide an insight into the function of the *zrp2* gene. It has been shown previously that ZRP2 mRNA is more abundant in etiolated seedlings than in light grown seedlings (Held, 1993). In this study, the effect of exogenous application of growth regulators was studied. Specifically, the effect of ABA on *zrp2* gene expression was looked at since ABA is known to be induced during adverse environmental conditions such as drought, high salinity, cold and wounding. Furthermore, ABA is a known inhibitor of root growth (Pilet et al., 1981; Raymond et al., 1987; Leung et al., 1994). ABA is known to interfere with mitotic phases of cell division in the root meristem thus inhibiting cell division (Leung et al., 1994; Hemerly et al., 1993). These effects were seen in ABA-treated maize seedling roots in the form of decreased root length of the primary roots and by the absence of lateral roots compared to untreated controls.

Investigation of *zrp2* gene expression at the protein level shows that ZRP2 protein level starts to decrease after 10 h of ABA treatment and reaches a minimum level after 18 h (Fig. 7A). Thereafter, it

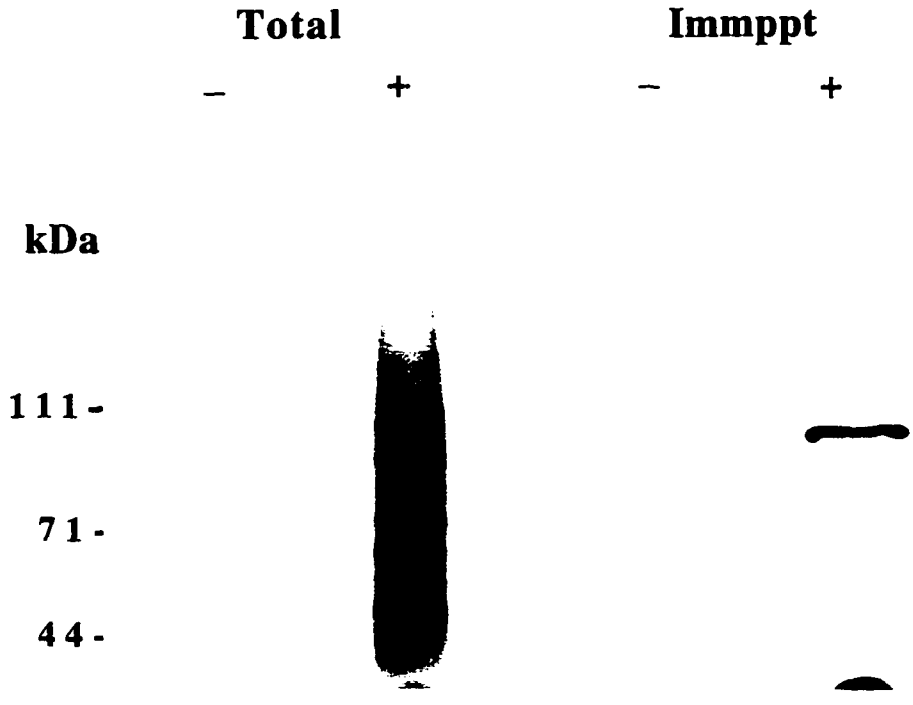

Figure 6. *In vitro* translation of ZRP2.
Poly(A)$^+$ RNA isolated from maize seedling roots was *in vitro* translated with ^{35}S-labeled methionine using rabbit reticulocyte lysate. The *in vitro* translated proteins were immunoprecipitated using ZRP2 antiserum. Total, Total reaction after translation with (+) and without (-) added RNA. Immppt, Immunoprecipitate of the reactions after translation with (+) and without (-) RNA.

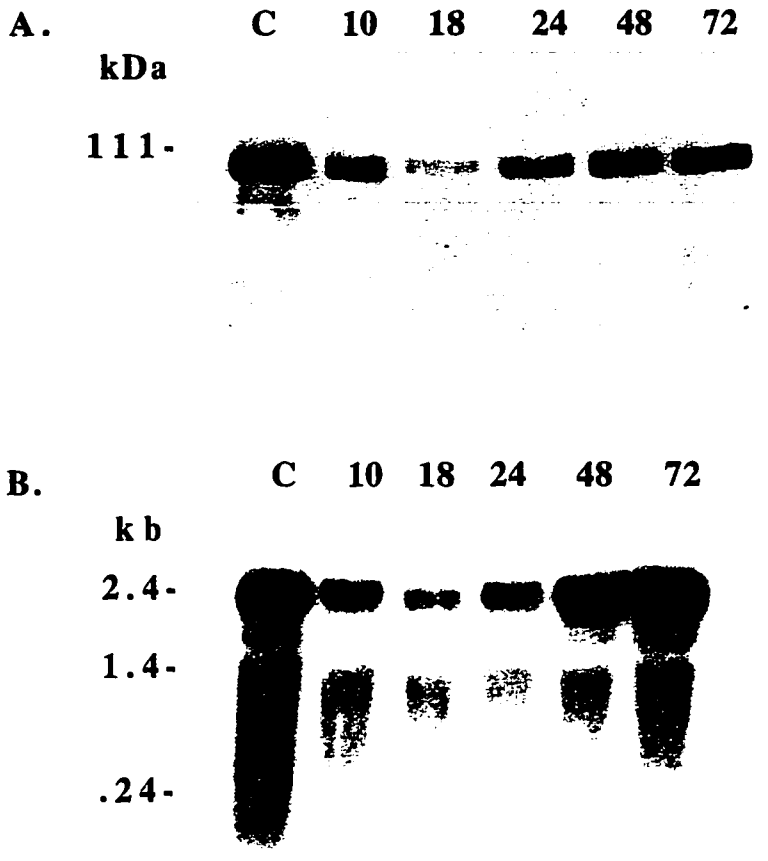

Figure 7. Effect of ABA treatment on *zrp2* expression.
3-d-old seedlings were subjected to 10, 18, 24, 48 and 72 h of 80 µm ABA treatment and accumulation of ZRP2 mRNA and protein in response to exogenous ABA was studied. Sample C corresponds to untreated control. **A.** Protein accumulation. Proteins extracted from roots of ABA-treated seedlings were electrophoresed on 7.5% SDS-PAGE gels (20 µg each) and electroblotted onto nitrocelulose. Western analysis was carried out as described in Materials and Methods.
B. mRNA accumulation. Total RNA from roots of ABA-treated seedlings was isolated and 5 µg of total RNA was electrophoresed on 3% formaldehyde/1% agarose gels, blotted onto a nylon membrane and hybridized with ^{32}P-labeled antisense RNA probe.

gradually increases to control levels by the third day of ABA treatment. Coomassie stained general protein profiles of these treatments appear similar to the control (data not shown). This observation suggests that the observed decrease in ZRP2 expression is most likely not due to an inhibition of protein accumulation in the root in response to ABA. In order to investigate whether this down-regulation extends to the RNA level, northern analysis was performed using total RNA isolated from ABA treated roots (Fig. 7B). The ZRP2 mRNA accumulation was similar to protein accumulation pattern with lowest levels detected after 18 h. The RNA fragments associated with the ZRP2 band has been previously observed and may indicate either *in vitro* or *in vivo* instability of this mRNA (Held, 1993).

Effect of salt, desiccation, and wounding on *zrp2* expression

Since *zrp2* gene expression appears to be regulated by ABA, it was of interest to see whether ZRP2 protein levels can be affected by environmental stress conditions, such as osmotic stress, desiccation and wounding. ABA levels are known to increase in tissues and organs subjected to such stresses (Godoy et al., 1984; Zhao et al., 1991). Under these conditions, specific genes are expressed that can be induced in unstressed plants by the application of exogenous ABA (Skriver and Mundy, 1990). High salinity was chosen as the osmotic stress since maize is a salt-sensitive species (Fortmeier and Schubert, 1995). The results of the salt stress study show that ZRP2 protein levels decline dramatically after 2 d of high salt treatment (Fig. 8A). However, at the

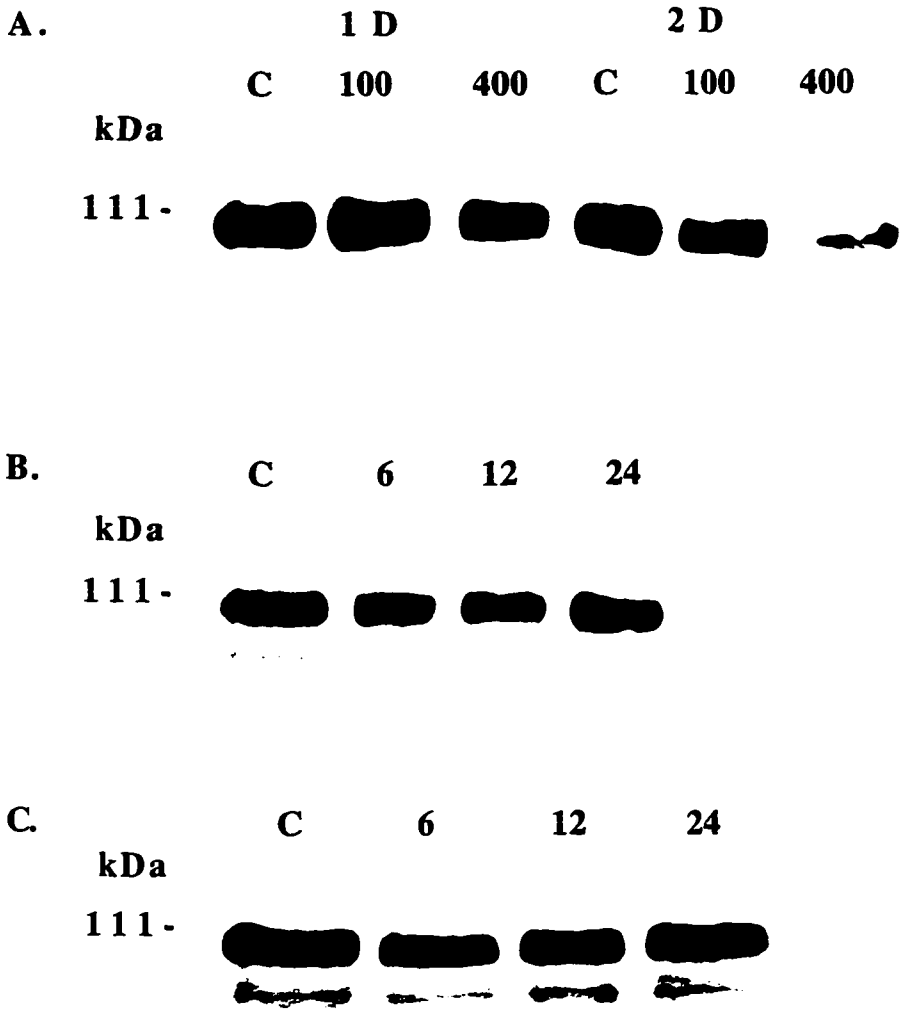

Figure 8. Effect of salt, desiccation and wounding on ZRP2 protein level. **A**. 3-d-old maize seedlings were subjected to either 100 or 400 mM NaCl for 1 or 2 d. **B**. 5-d-old seedlings were subjected to desiccation for 6, 12 and 24 h. **C**. 5-d-old seedling roots were subjected to wounding and harvested after 6, 12 and 24 h. Sample C corresponds to untreated control. Protein extraction and western analysis was carried out as described in Materials and Methods. 30 μg of protein extract was loaded in each lane.

low salt concentration, ZRP2 protein levels remained unchanged after two days. Coomassie blue stained protein profiles of the control and salt treated roots appeared similar to each other (data not shown). Hence, the observed decrease in ZRP2 protein level appears to be specific and not due to secondary effects of salt stess. In contrast to the results observed with salt treatments, wounding and desiccation of maize roots did not result in a decrease in the ZRP2 protein level, but rather remained unchanged (Fig. 8, B and C).

Discussion

Western analysis of ZRP2 protein distribution along the length of the seedling roots shows that it is expressed at a high level in the root except at the tip where it is detected at a much lower level. These data are consistent with the ZRP2 mRNA accumulation pattern in which high levels of the mRNA were detected in the root except at the tip where it was undetectable (Held, 1993). In the mature plants, ZRP2 protein was detected at a high level in roots and stems but not detected in other organs. These observations are also similar to previous RNA data which showed that ZRP2 mRNA was detectable only in the roots and stems of the mature plant. These data support the hypothesis that ZRP2 gene is indeed expressed and functions in the roots and stems of maize. Comparison of western analysis data with *in vivo* transcription studies conducted previously (Held, 1993), which indicated transcription in root nuclei but not in leaf nuclei, provides

support for a transcriptional level of control for the regulation of *zrp2* gene expression.

The results of *in vitro* translation studies show that the observed and calculated differences in the ZRP2 protein size are probably not due to a post-translational modification. The most likely explanation is an aberrant electrophoretic behavior of the protein on SDS-PAGE. Similar differences in apparent molecular mass based on the nucleotide sequence and the SDS-PAGE mobility have been observed with other proteins (John and Keller, 1995; Parsey et al., 1994; Kieliszewsky et al., 1990). For example, John and Keller described a proline rich protein from cotton with an estimated size of 21 kDa and an observed size of 55 kDa on SDS-PAGE. These anomalous behaviors have been attributed to the presence of charged amino acids that contribute to the overall shape of the molecule. ZRP2 amino acid sequence contains a high amount of arginine (10%), which could contribute to the net charge of the molecule. In an attempt to determine the size of ZRP2 protein by another approach we have conducted gel filtration studies (data not shown). Preliminary results from these experiments have shown a size of approximately 130 kDa for ZRP2. Therefore, ZRP2 protein appears to show an aberrrant chromatographic behavior as well.

We have shown that ZRP2 protein levels can be transiently down-regulated in seedlings treated with ABA. The observation that this effect also seen at the RNA level suggests that ABA acts through repression of ZRP2 transcription. Over 150 genes have been described that are inducible by exogenous ABA (Giradaut et al., 1994). In

contrast, only a few genes are known that are down-regulated by ABA. For example, the α-amylase genes of barley are known to be transcriptionally repressed by ABA (Skriver et al., 1991). The GA responsive elements (GARE) found in the promoters of these genes are also repressible by ABA. Study of the ZRP2 promoter region has not revealed such elements (Held, 1993). However, ZRP2 promoter does contain a tandem repeat of the core sequence ACGT at -113 (Held et al., in preparation) which has been found in the upstream regions of some ABA-response genes (Marcotte et al., 1989, Mundy et al.,1990). Whether this core sequence plays a role in ABA response in the *zrp2* gene is not known at present. It is possible that ZRP2 promoter may contain novel elements that are involved in the down-regulation by ABA.

zrp2 gene expression appears to be down-regulated at the protein level in response to high salt. Examples of genes that are down-regulated in response to salt stress are known. (Cleas et al., 1990, Michalowski and Bohnert, 1992). This response may be a response to ABA that is produced during salt stress, although the pattern of down-regulation by ABA is different from the pattern observed during salt stress. The observation that salt-stress induced repression of ZRP2 protein level is a delayed response may indicate that it may not be a primary response to salt stress. Induction or down-regulation within hours of salt treatment have been noted with other salt response genes (Cleas et al, 1990). The functional significance of this response is not clear since the function of this gene is not known at present.

Acknowledgements

We would like to thank Dr. Eve S. Wurtele for her advice and helpful comments. We would also like to thank the past and present members of our laboratory for their assistance and helpful comments regarding this work. This work was initially supported by a grant from Northrup King/Sandoz Company and subsequently by a grant from USDA (95-37304-2296).

MAIZE ZRP4 PROTEIN IS ROOT-PREFERENTIAL AND IS DEVELOPMENTALLY REGULATED

A paper to be submitted to Plant Molecular Biology

Lathika Moragoda, Eve S. Wurtele and James T. Colbert

Abstract

The maize *zrp4* gene codes for a putative O-methyltransferase whose mRNA has been shown to accumulate preferentially in the endodermis of maize roots and at low levels in other organs. In the present study, distribution of ZRP4 protein was investigated using antibodies generated against an *E. coli*-expressed ZRP4 protein. In 7-d-old seedlings, ZRP4 protein was detected at high levels in the roots but was not detected in leaves. Within the seedling root, this protein was detected at low levels in root tips and at high levels in the remainder of the root. In mature plants, high levels of the protein was detected in young prop roots whereas low levels were found in the predominantly mature roots. The ZRP4 protein was not detected in leaves, stems, ears and tassels of the mature plant. These results suggest that ZRP4 protein distribution closely follows the mRNA accumulation pattern and that *zrp4* gene expression is developmentally regulated in the maize root.

Key words

root-preferential, ZRP4 protein, western analysis, *Zea mays*

Introduction

Roots serve important functions of anchorage, absorption, conduction and storage in plants. The root apical meristem continues to produce new cells throughout the life cycle of a plant which results in a series of developmental stages being present along the length of the root.

Two major approaches have been undertaken to study root development. One approach has involved genetic analysis of various mutants in root development (Benfey et al., 1993; Scheres et al., 1995). A second approach involves isolation and characterization of genes that are specifically or preferentially expressed in roots (Conkling et al., 1990; Collazo et al., 1992; John et al., 1992; Xu et al., 1995; Choi et al., 1996). One such gene, *zrp*4, corresponds to an mRNA that accumulates preferentially in young roots and is located in the endodermis and exodermis of the maize root (Held et al., 1993). Within the seedling root this mRNA is developmentally regulated with lowest levels in the first cm of the root and high levels in the remainder of the root. This mRNA appears to code for an O-methyltransferase (OMT) that has been proposed to be involved in suberin biosynthesis (Held et al., 1993).

OMTs are involved in O-methylation of DNA, RNA and proteins using S-adenosyl-L-methionine (SAM) as the methyl donor in bacteria, animals and plants (Bugos et al., 1991). In plants OMTs are also involved in the transmethylation of secondary plant metabolites. These include lignin precursors, isoflavonoids and flavonoids and defense related plant metabolites such as phytoalexins (Greisbach et al., 1981). cDNA clones corresponding to OMTs have been isolated from several plant species (Bugos et al., 1991; Gowri et al., 1991; Collazo et al., 1992; Dumas et al., 1992; Jaeck et al., 1993; Vernon & Bohnert, 1992; Gregersen et al., 1994; Ye and Varner, 1995). The ZRP4 polypeptide shares sequence similarity with these OMTs with respect to functional domains. However, these clones show little similarity to ZRP4 with respect to localization in the plant. ZRP4 is the only OMT described thus far that is implicated in suberin biosynthesis.

In this study, we have characterized ZRP4 at the protein level. Using antibodies generated against an *E. coli* synthesized ZRP4 polypeptide, we describe the ZRP4 protein distribution pattern in the maize plant.

Materials and Methods

Plant material

To obtain seedling organs, maize seeds (*Zea mays* L. cv NKH31) were grown on germination paper. Germination paper bundles were placed in a plastic container half filled with distilled water. Another

plastic container was placed on top to provide high humidity. The seedlings were grown for 7 days in a growth chamber under a 30°C/16 hr light and 28°C/8 h dark cycle. Seedling roots up to 10 cm in length were harvested for protein isolation. In some experiments, root tips defined as the first cm from the root apex, were excised and processed seperately. Organs from mature plants were harvested from plants at pollination, grown under green house conditions.

***E. coli* expression of ZRP4**

pZRP4 containing the full length ZRP4 cDNA (Held et al. 1993) was digested with BamHI and the cDNA insert was isolated. The ZRP4 insert was further digested with SalI to produce a 0.58 kbp 5'- terminal fragment of ZRP4. This 0.58 kbp fragment was ligated, in frame, into BamHI and SalI digested pET28b vector (Novagen, WI) to produce pET28.Z4 (Fig. 1). This construct contained a histidine tag downstream of translation start site of the vector. The 0.58 kbp ZRP4 insert includes 16 nucleotides of a polylinker and 20 nucleotides of the 5' UTR upstream of the translation start site. The coding region represents approximately 50% of the ORF of ZRP4 cDNA.

The *E.coli* host strain BL21(DE3) (Novagen) containing pET.Z4 was grown at 37°C to an optical density of about 1.0 and induced with 1 mM IPTG. The cells were harvested after 7 h of induction. The expressed fusion protein was found to accumulate mainly in the insoluble protein fraction of *E. coli*. A 100 ml culture of induced cells were harvested by centrifugation at 5,000 x g for 5 min and suspended in 40 ml binding

buffer (5 mM imidazole, 1M NaCl, 20 mM Tris-HCL pH 7.9). The suspension was sonicated to resuspend pellet completely and centrifuged at 20,000 x g for 15 min to collect the inclusion bodies and cell debris. The pellet was washed twice with 20 ml of binding buffer and resuspended in 20 ml of binding buffer containing 6 M urea, and incubated on ice for 1 h to dissolve the protein. Insoluble material was removed by centrifugation at 39,000 x g for 20 min, and the supernatant was used to load the affinity column. Column preparation and elution of the protein was carried out according to manufacturer's instructions (Novagen). The affinity purified extract was concentrated and electrophoresed on 15% SDS-PAGE preparative gels as a further purification step. The ZRP4 fusion protein was eluted from the gel and used to inject two New Zealand white rabbits. The rabbits were injected subcutineaously with approximately 70 µg of the protein and injected twice more before the terminal bleeds. Injection of rabbits and collection of sera were carried out by the Iowa State University Cell and Hybridoma Facility.

Protein extraction and Western analysis

The plant material harvested for protein extraction was frozen in liquid nitrogen and stored at -80°C. Approximately 0.5 g portions of plant material were ground in liquid nitrogen and suspended in 300 µl of extraction buffer (100 mM Tris-HCl pH 8.0, 5 mM EDTA, 5 mM DTT, 100 mM NaCl, 15% glycerol) containing a protease inhibitor cocktail (Boerhinger-Mannhiem). The extracts were centrifuged at 12,000 rpm

in a microcentrifuge for 5 min and supernatants were collected and stored at -80°C until use. The protein concentration of each extract was determined by a Bradford assay with bovine serum albumin (BSA) as the standard (Bio-Rad). The proteins were separated on 10% SDS-PAGE gels and electroblotted onto nitrocellulose (Schleicher & Schuell) using semi-dry transfer (Fischer Scientific). The membrane was blocked using 5% BSA in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) overnight at 4°C. The ZRP4 primary antibody was added at a dilution of 1:2,500 and the horse radish peroxidase (HRP)-conjugated secondary antibody was added at a dilution of 1:10,000 in PBS-T. ZRP4 protein was detected using enhanced chemiluminescence (ECL, Amersham).

In vitro translation and immunoprecipitation

In vitro translation was carried out using a rabbit reticulocyte lysate system and ^{35}S-labeled methionine as described by the manufacturer (Promega). Total RNA and poly $(A)^+$ RNA was isolated from seedling roots as decribed by Held et al., 1993. One µg of poly$(A)^+$ RNA was used in 50 µl of translation reaction. The reaction was incubated at 30°C for 1 h, and the *in vitro* translated ZRP4 protein was immunoprecipitated as follows. The reaction was first incubated with 1-2 µl of ZRP4 antiserum for 1 h at 4°C followed by incubation with 30 µl of a 1:1 slurry of protein-A Sepharose (Sigma) and dilution buffer (0.01 M Tris-HCl (pH 8.0), 0.14 M NaCl, 0.025% NaN_3, 0.1% Triton X-100 (v/v)) for another hour at 4°C. The pellet was washed once with dilution

buffer and twice with dilution buffer without Triton X-100 and finally with 0.05 M Tris-HCl (pH 6.8). Translated proteins were analyzed by SDS-PAGE. The ^{35}S-labeled proteins were visualized by fluorography.

Results

Generation of antibodies against ZRP4 protein

To study the distribution of ZRP4 protein in the maize plant, generation of antibodies against ZRP4 was undertaken. As a first step, the ZRP4 cDNA was expressed in *E.coli*. A 0.58 kbp 5'-terminal fragment of the 1.4 kbp full length ZRP4 cDNA was cloned into the pET28 expression vector (Fig. 1). This fragment includes 50% of the ORF of the ZRP4 cDNA. The C-terminal half of the pZRP4 protein was intentionally not included since this region contains the sequence encoding the putative SAM binding site of ZRP4. This binding site is conserved among other O-methyltransferases (Bugos et al., 1991). Seventy-six percent of the fusion protein consists of amino acids encoded by the ZRP4 gene. The remaining 24% of the fusion protein consisted of amino acids derived from the pBluescript polylinker and ZRP4 5' untranslated region (5%) and pET28 vector sequence (19%). A protein of the expected size (27 kDa) was expressed when *E. coli* cells were induced with IPTG (Fig. 2A). The expressed protein accumulated mainly in the insoluble protein fraction of *E. coli* (data not shown). The protein was therefore purified under denaturing conditions as described in Materials and Methods. The purified protein was injected into rabbits. The resulting antiserum

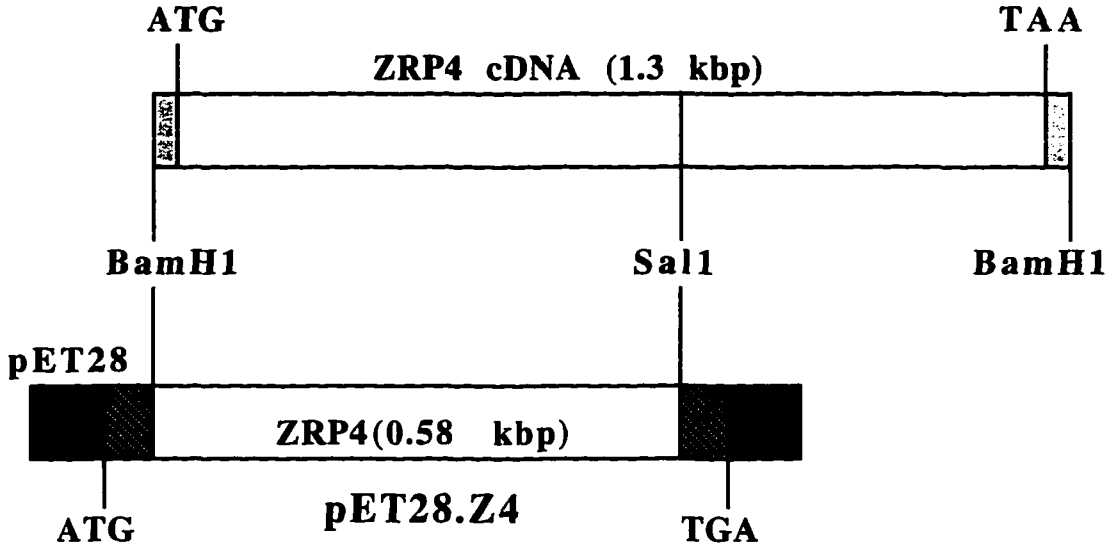

Figure 1. Construction of pET28.Z4.
A BamH1 and Sal1 digested 0.58 kbp fragment of the ZRP4 cDNA was ligated into the multiple cloning site of the pET28b vector digested with the same restriction enzymes. The translation start and termination codons of the ZRP4 cDNA and pET28.Z4 are indicated. The stippled region 5' to the ATG codon of ZRP4 cDNA includes the polylinker and the 5' UTR of the pZRP4. The shaded regions 5' to the Bam site and 3' to the Sal site includes the vector sequences that are included in the ORF of pET28.Z4.

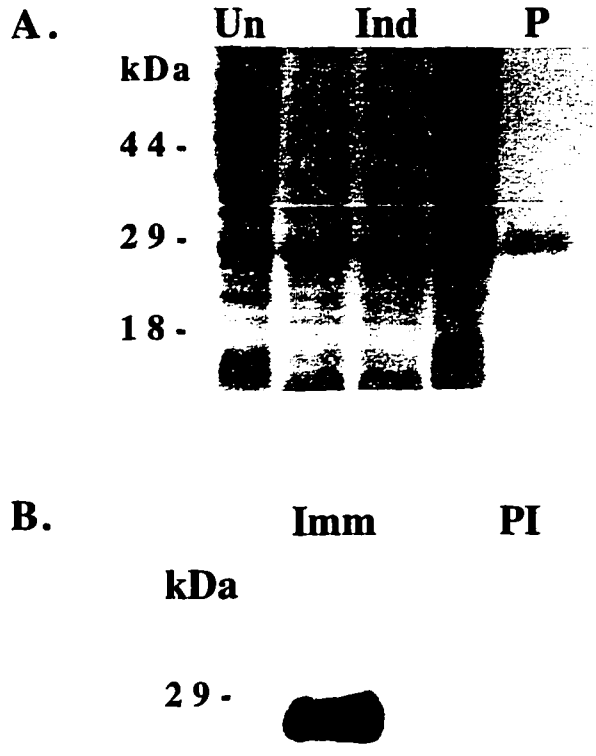

Figure 2. Generation of antibodies against ZRP4 fusion protein. **A**. Expression and purification. Protein extracts from uninduced (Un) and IPTG-induced (Ind) cultures of *E. coli* cells containing pET28.Z4 were prepared and subjected to electrophoresis on 15% SDS-PAGE gels. The induced ZRP4 fusion protein was purified using affinity purification (P). **B**. Specificity of ZRP4 antiserum. Purified ZRP4 fusion protein was electrophoresed on 15% SDS-PAGE gels, electroblotted onto nitrocellulose, the blots were incubated with either ZRP4 antiserum (Imm) or preimmune serum (PI). Primary antibody was detected with HRP-conjugated secondary antibody using chemiluminescence.

recognized a 27 kDa protein in *E. coli* that had been induced by IPTG (Fig. 2B). This protein was not detected by preimmune serum.

In vitro translation of ZRP4

In order to test the specificity of the antibodies generated against one half of the ZRP4 coding sequence, *in vitro* translation studies were conducted with poly(A)$^+$ RNA isolated from maize seedlings. The *in vitro* translated ZRP4 protein was immunoprecipitated with ZRP4 antiserum. A single translation product of approximately 40 kDa was immunoprecipitated with ZRP4 antiserum indicating the antibody is specific (Fig. 3). The control reactions without RNA added to the *in vitro* translation reactions did not show any protein synthesis. This experiment suggests that the ZRP4 antiserum generated against the ZRP4 fusion protein is able to recognize and bind to the full-length form of the ZRP4 protein.

Immunodetection of ZRP4 protein in maize organs

ZRP4 protein distribution was investigated in 7-d-old maize seedlings using western analysis with the ZRP4 antiserum. A prominant protein band corresponding to the expected size of 40 kDa was observed in entire root systems (Fig. 4). In root tips, this 40 kDa band was detected at a much lower level (lane 5). This protein was not detected in leaves. No cross-reacting bands were detected with preimmune serum in either root or leaf extracts. In addition to the major 40 kDa band, a few relatively faint bands were detected by the ZRP4 antiserum in the root extracts but not in leaf extracts. A 27 kDa

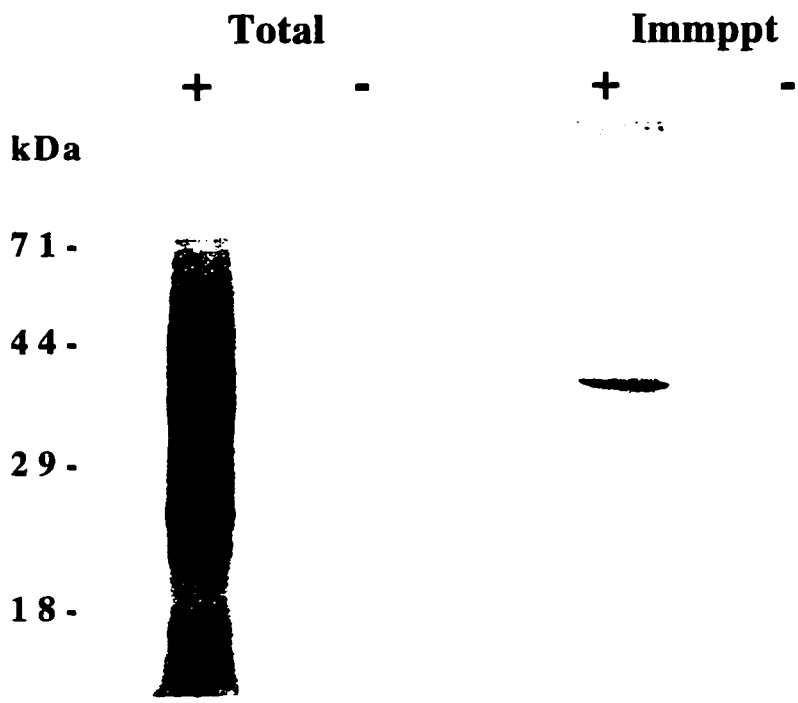

Figure 3. *In vitro* translation of ZRP4.
Poly(A)$^+$ RNA isolated from maize seedling roots was *in vitro* translated with ^{35}S-labeled methionine using rabbit reticulocyte lysate. The *in vitro* translated proteins were immunoprecipitated using ZRP4 antiserum. Total, Total reaction after translation with (+) and without (-) added RNA. Immppt, Immunoprecipitate of the reactions after translation with (+) and without (-) RNA.

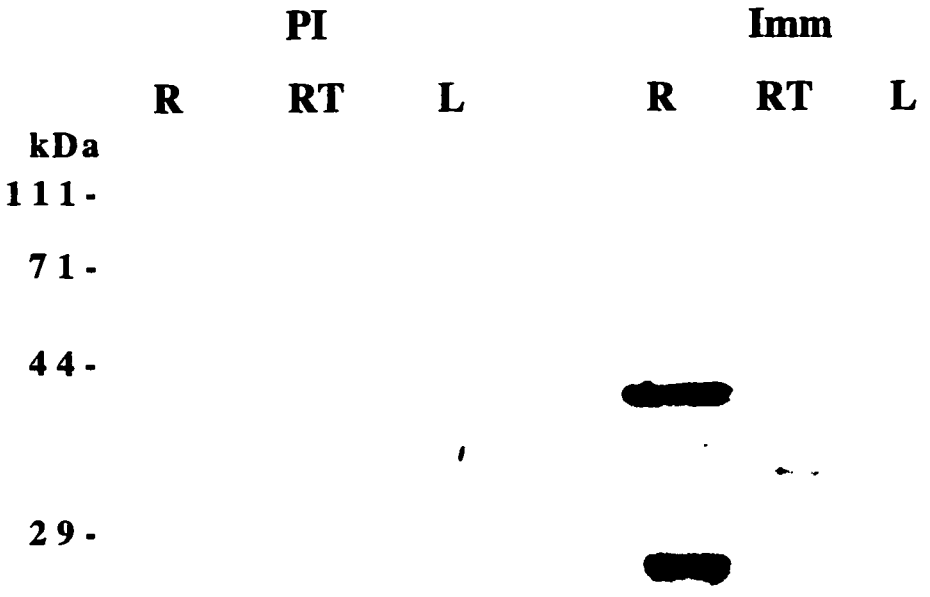

Figure 4. Immunodetection of ZRP4 protein in maize seedling organs. Proteins were extracted from whole roots (R), root tips including the first cm of the root (RT), and leaves (L) of seedlings. Protein extracts (40 µg each) were electrophoresed on 10% SDS-PAGE gels and electroblotted onto nitrocellulose and incubated with either preimmune serum (PI) or immune serum (Imm) as the primary antibody. The primary antibody was detected with HRP-conjugated secondary antibody using chemiluminescence.

protein was consistently observed in seedling roots. In the root tips a band representing a polypeptide of about 35 kDa was detected, this band was approximately equal in intensity to the ZRP4 polypeptide. This 35 kDa protein was also detected in the whole root extract although at a much lower level. In addition to these low molecular weight bands, a high molecular weight band around 100 kDa was also observed at a very low level in the root extracts.

ZRP4 protein distribution in the organs of mature plants at pollination was investigated (Fig. 5). The 40 kDa ZRP4 protein was detected in mature roots growing in soil and young prop roots. The mature roots appear to have lower levels of the ZRP4 protein compared to prop roots and 7-d-old roots from seedlings. The prop roots used in this experiment were young adventitious roots on mature plants that had not yet entered soil. Therefore, they actually represent an early stage of development. In the mature leaves, ZRP4 protein was not detected (Fig. 5, lane ML), however, a faint, slightly smaller band of about 35 kDa in size was detected in the leaf extract. The ZRP4 protein was not detected in the other organs tested including stems, ears, silks and tassels (Fig 5, lanes St, EP, 10E, Si, T). The 100 kDa high molecular weight protein observed in seedling roots was also observed at a very low level in the roots and stems of the mature plant.

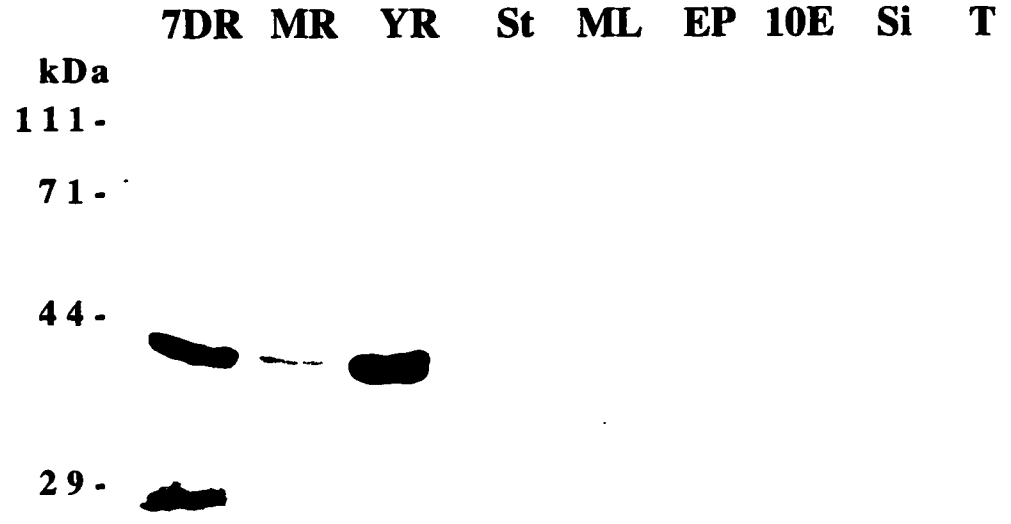

Figure 5. Immunodetection of ZRP4 protein in the organs of the mature plants. Western analysis was carried out as described in Materials and Methods. Proteins were extracted from various organs of mature maize plants at pollination including mature roots (MR), young prop roots (YR), stems (St), leaves (L), ears at pollination (EP), ears 10 days after pollination (10E), silks (Si), and tassels (T). Proteins extracted from 7-D-old seedling roots (7DR) was included for comparison. 40 μg of total protein extracts were loaded per lane.

Discussion

zrp4 is a gene whose mRNA accumulates preferentially in young roots of maize. In an attempt to further characterize this gene at the protein level, antibodies were generated against the N-terminal half of the ZRP4 protein. The specificity of the resulting antiserum was verified by its ability to immunoprecipitate the *in vitro* translated ZRP4 protein. The observed 40 kDa size of the ZRP4 protein in the root extracts agrees with the expected size as determined from the ZRP4 cDNA (Held et al., 1993) and from the results of our *in vitro* translation studies. Detection of other proteins in addition to the ZRP4 protein may indicate that these proteins share some epitope similarity with ZRP4. These proteins include a 100 kDa protein that was observed in roots and stems, 35 and 27 kDa proteins detected in the seedling roots and a 35 kDa protein observed in leaves of mature maize. These proteins may represent gene products of related members of this gene family. Alternatively, it is possible that the 35 and 27 kDa proteins detected in the roots may be degradation products of the ZRP4 protein.

The pattern of ZRP4 protein distribution in young maize plants is similar to the pattern of ZRP4 mRNA accumulation. Specifically, ZRP4 protein is abundant in young roots but, is not detected in young leaves. Within the root, ZRP4 protein is present at low levels in the root tip and at higher levels in the remainder of the root, indicating that this gene is developmentally regulated within the root. *In situ* hybridization studies have shown previously that ZRP4 mRNA is localized in the

endodermis and exodermis of the maize roots (Held et al., 1993). Attempts to immunolocalize ZRP4 protein using tissue print techniques have been unsuccessful to date.

In the mature plant, ZRP4 protein accumulates to low levels in older roots. These data are consistent with the RNA blot data where very low levels of the ZRP4 mRNA are detected in the mature roots (Held et al., 1993). However, young rapidly growing prop roots of the same plant showed high levels of the protein. These results indicate that the expression of ZRP4 protein is indeed developmentally regulated. Past a certain stage in root development ZRP4 protein seems to be required at a much lower level which could be a result of the roots reaching a certain level of maturity. The exact stage at which this occurs remains to be determined. ZRP4 protein however, was not detected in the aerial organs of the mature plant. These results are in contrast with northen analyses data which showed low levels of ZRP4 mRNA in leaves, stems, ears, silks and tassels (Held et al., 1993). The reason for this disparity is not clear, although these results may be explained by levels of ZRP4 protein below the limit of detection in these organs or by translation or post-translational control of *zrp4* gene expression.

To determine whether *zrp4* gene expression could be induced under certain environmental conditions, we have tested *zrp4* gene expression in the presence of exogenous ABA, wounding, and salt stress (data not shown). Results of these experiments indicate that ZRP4 protein levels are unaffected by these stimuli.

Acknowledgments

This work was supported by a grant from USDA (95-37304-2296). We would like to thank Victor Nunez for his assistance in growing plants in the green house.

DEVELOPMENT OF A PCR BASED METHOD FOR IDENTIFYING ZRP4 ANTISENSE MUTANTS

Introduction

ZRP4 mRNA is known to accumulate in the endodermis and exodermis of maize roots (Held et al., 1993). *zrp4* codes for an O-methyltransferase thought to be involved in suberin biosynthesis. An important goal in the study of ZRP4 was to obtain mutants in order to better understand the function of this gene. To achieve this goal, two approaches have been taken. One approach has been to obtain mutants through the production of transgenic maize plants containing antisense constructs of ZRP4. The other approach involves screening progeny of a mutator(mu)-tagged maize population (provided by Pioneer-Hybrid). It is possible to employ PCR (Polymerase Chain Reaction) (Ausubel et al., 1994) based methods in the initial screening of potential mutants. For example, in analyzing mu-tagged plants it is possible to screen for mutants by the use of primers based on the mutator and target gene sequence together. In the case of analyzing antisense plants, it is possible to amplify the introduced gene by the use of 5' and 3' primers of the introduced gene sequence. In this chapter, the development of a PCR-based technique to analyze putative antisense plants is described.

Materials and Methods

Two oligonucleotide primers were initially designed from the untranslated region (UTR) of the cDNA sequence of ZRP4 (Held et al., 1993). The upstream primer 5'Z4P1 (5'-GCG GGA TCC GTA GCA GTA TCA GCA TGG-3"), extended from nt 7 to 24. The downstream primer 3'Z4P1 (5'-CCG GGA TCC CTG AAT AGA TAT CTC GAC-3'), extended from nt 1231 to 1248. The subsequent primer set synthesized consisted of primers based on the coding region of the ZRP4 cDNA sequence. The upstream primer 5'Z4P2 (5'-CAT GGC GCT CAA GTC CGC AAT ACA C-3') extended from nt 110 to 134. The downstream sequence 3'Z4P2 (5'-AGC TTC GGA GAA AAT CTT GCT CCA CTC CT-3') extended from nt 1021 to 1049. The PCR mixture contained 0.6 µm of each primer, 2.5 units of *Taq* DNA Polymerase (Gibco, BRL), 200 µm of each dNTP, 2 mM of $MgCl_2$, reaction buffer (without $MgCl_2$) and template DNA (10 ng pZRP4 plasmid or 500 ng Genomic DNA) in a total of 50 µl. The amplification program consisted of 30 cycles: Cycle 1 consisted of denaturation at 94°C (1.5 min), annealing at 60°C (1 min) and extension at 72°C (1 min). Cycles 2-29, 94°C (1 min), 60°C (1 min) and 72°C (1 min). Cycle 30; 94°C (1 min), 60°C (1 min) and 72°C (7 min). For analysis, 10-20 µl of amplified product was electrophoresed on a 1% agarose gel. DNA from maize leaves was isolated as described by Dellaporta et al., 1984.

Results

To use PCR as a means of screening for ZRP4 antisense mutants containing full length form of the ZRP4 cDNA, primers based on the cDNA sequence were synthesized. These primers were based on the 5' and 3' UTRs of the cDNA sequence. Since certain regions of the coding sequence are conserved among OMTs, the coding region was not chosen initially for primer synthesis to avoid potential amplification of related OMTs. The 5' primer included the translation start codon and nucleotides immediately upstream of translation start site. The 3' primer included 18 nucleotides close to the poly(A) tail. The primers were PCR tested using pZRP4 plasmid (as a positive control) and genomic DNA from the genotype NKH31 as templates. A band of the expected size, 1.2 kbp, was observed with plasmid DNA (Fig. 1). With NKH31 DNA, a slightly larger band of approximately 1.3 kbp was observed (Fig. 1) indicating the presence of a small intron or introns. Next, PCR amplification was carried out with genotype HiII. This was necessary since the antisense gene was introduced into the HiII background. However, no amplification was detected with HiII DNA. Furthermore, when two other genotypes, B73 and A188 were tested no amplification was observed (data not shown). Therefore, a second set of primers spanning the coding region of pZRP4 was synthesized. PCR analysis using these primers produced a 0.9 kbp band of the expected size with plasmid DNA and a slightly larger, 1.0 kbp band was observed with NKH31 DNA (Fig. 2). When genotypes HiII, B73 and A188 were

Figure 1. PCR amplification of ZRP4 from NKH31 genomic DNA (lane 1) and pZRP4 (lane 2, lower band) using primers spanning the 5' and 3' UTRs of ZRP4 cDNA. Lane 2 shows a mixture of two seperate pZRP4 and NKH31 PCR amplification reactions. Lane 3 indicates molecular weight markers. PCR conditions were as described in Materials and Methods. 10 µl of PCR reaction was electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

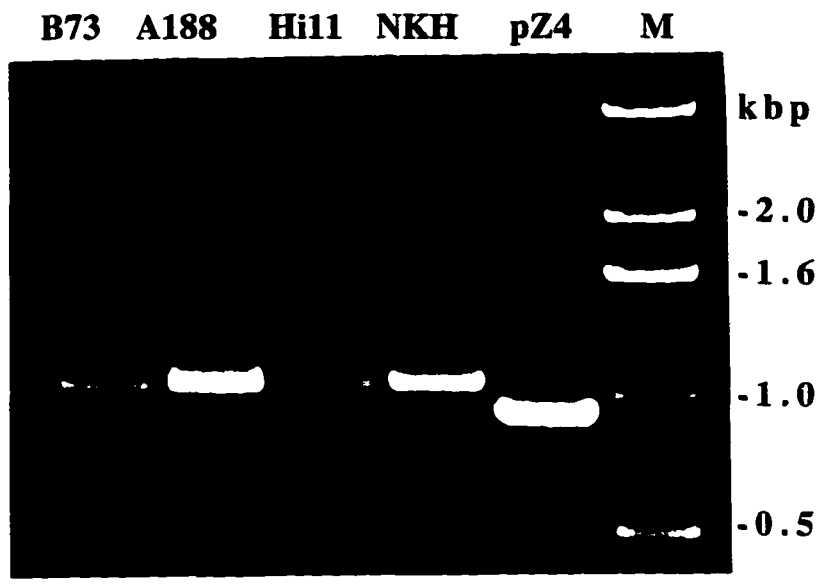

Figure 2. PCR amplification of ZRP4 from plasmid DNA and maize genomic DNA using primers spanning the coding region of ZRP4 cDNA. Lanes1-4. amplified product using DNA from genotypes B73, A188, Hi11, and NKH31 as template. Lane 5. amplified product using pZRP4 as template. Molecular weight markers (M) are indicated in lane 6.

tested, a similar 1.0 kbp band was observed after PCR amplification. This result was similar to results obtained with the previous experiment in that the band observed with genomic DNA was about 100 bp larger than the band observed with ZRP4 cDNA. Since such a size difference can be seen as separate bands on a gel, it was predicted that a transgenic plant carrying an antisense copy of the gene should produce two bands of 0.9 and 1.0 kbp bands corresponding to the introduced cDNA and the genomic copy of ZRP4, upon PCR amplification. This was indeed what was observed when transgenic plants were tested (Fig. 3).

Discussion

A PCR-based method was developed to screen for ZRP4 antisense mutants. This type of PCR-based method of analysis provides a relatively easy and rapid method of initial screening of putative transformants. Once the putative antisense plants are selected, the presence of the antisense gene can be verified by more time consuming methods such as Southern analysis. Development of a PCR based method was possible because of the small size of the genomic copy of ZRP4, which can hence be easily amplified. The 100 bp difference between the genomic copy and the cDNA copy indicates that *zrp4* possesses a small intron(s) that does not exceed 100 bp in length. The observation that the primers spanning the 5' and 3' UTRs failed to amplify *zrp4* gene from genotypes other than NKH31 indicates that these regions may be less conserved among maize genotypes. On the other hand, the coding

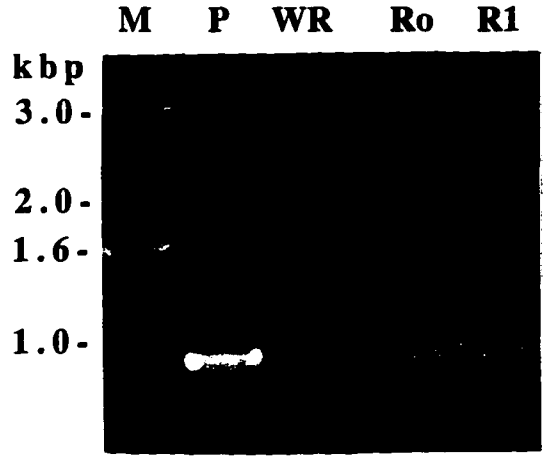

Figure 3. Analysis of putative ZRP4 antisense mutants using PCR amplification. DNA from transgenic plants was isolated and used as template for PCR amplification under conditions described in Materials and Methods. P. pZRP4, WR. untransformed regenerant, Ro. transformed regenerant, R1. an F1 plant from regenerant Ro.

region appears to be more conserved since the use of primers spanning the coding region resulted in the amplification of *zrp4* gene from all four maize genotypes tested.

PARTIAL PURIFICATION AND DETERMINATION OF THE MOLECULAR WEIGHT OF NATIVE ZRP2 PROTEIN

Introduction

ZRP2 is a root-preferentially expressed protein in maize (chapter 2). The ZRP2 cDNA codes for a protein of 67 kDa in size (John, 1991). However, ZRP2 protein is detected as a 100 kDa band when subjected to SDS-PAGE (chapter 2). Therefore, it was of interest to attempt to determine ZRP2 molecular weight by employing an alternative method. Furthermore, it was of interest to study the native conformation of ZRP2 protein. In an attempt to address these questions, gel filtration studies were conducted.

Material and Methods

Protein extraction

40 g of 7-d-old maize seedling roots (cvNKH31) were ground in liquid nitrogen to a powder and suspended in 10 ml of protein extraction buffer without DTT (chapter 2). The protein extract was vortexed and centrifuged at 10,000 x g for 15 min at 4^0C and the supernatant collected.

Ammonium sulfate precipitation

The supernatant containing soluble proteins was subjected to ammonium sulfate precipitation. The solid salt was added to 25% saturation and the pH adjusted to 7.0. The proteins were allowed to precipitate overnight at 0^0C, and centrifuged at 25,000 x g for 30 min. The resulting pellet was dissolved in 1 ml of extraction buffer and desalted using a G-50 Sephadex (Sigma) 1.5 x 43 cm column (flow rate, 1ml/5 min) equilibrated with 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1 mM PMSF. The peak fractions were concentrated using Centricon filters (Amicon) and analyzed by western analysis.

Gel filtration studies

Partially purified protein samples were loaded onto a Sephacryl S-200 (Sigma) 1.5 x 46 cm column. The column was calibrated using high molecular weight markers from Sigma (MW-GF-200). Proteins were eluted with the same buffer used for desalting at a flow rate of 1 ml/5 min. The fractions collected were concentrated and ZRP2 was detected by western analysis.

Western analysis

SDS-PAGE and western analysis of ZRP2 protein was carried out as described in chapter 2.

Results

Prior to conducting gel filtration studies, the crude protein extract from maize roots was partially fractionated using ammonium sulfate precipitation at 25% saturation. Sequential salt fractionation experiments showed that ZRP2 precipitates at low concentrations of salt (Fig. 1). Precipitation of ZRP2 was not observed above 35% saturation. Following salting out, the partially purified protein extract was desalted and fractionated further using a desalting column. Fractions collected were analyzed by immunoblotting and ZRP2 was found to elute as a broad peak (data not shown). Pooled eluate from two separate desalting experiments was loaded onto a Sephacryl-200 column calibrated with molecular weight standards from 12.4 kDa to 200 kDa. Relative to the protein standards, the molecular weight of ZRP2 protein was estimated to be approximately 132 kDa based on the peak fraction (Fig. 2).

Discussion

The observation that ZRP2 precipitated at low salt concentrations was unexpected since ZRP2 is known to be a very hydrophilic protein based on the amino acid sequence (John, 1991). Proteins that precipitate below 25% saturation are known to be generally preaggregated or have very high molecular weights (Scopes, 1982). Detection of ZRP2 at low levels in fractions corresponding to molecular masses larger than 132 kDa may indicate that this protein may preaggregate in solution.

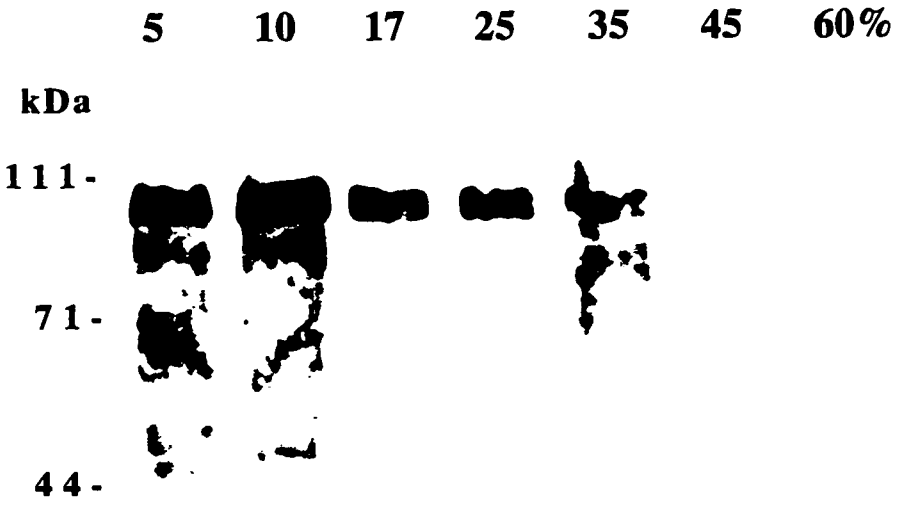

Figure 1. Ammonium sulfate precipitation of ZRP2 protein.
A crude protein extract was subjected sequential precipitation at increasing salt concentrations from 5-60%. The precipitate at each salt concentration was collected and analyzed by western analysis.

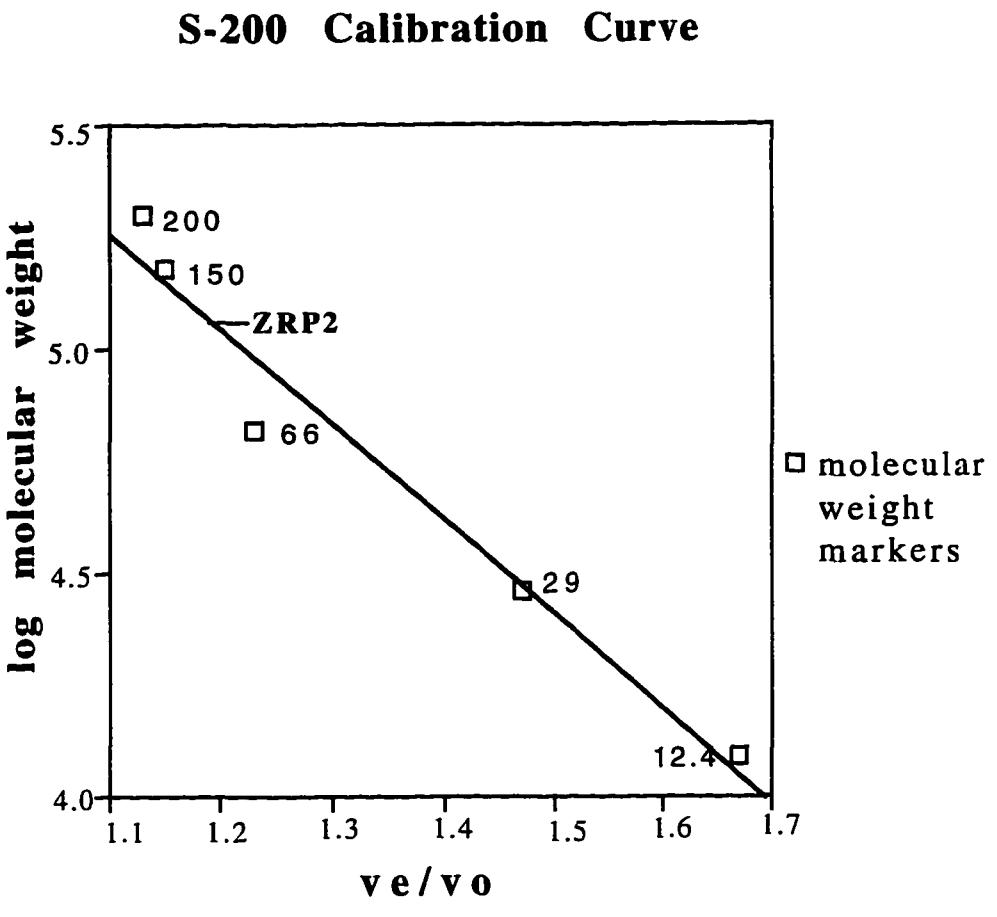

Figure 2. Estimation of molecular mass of ZRP2.
A Sephacryl S-200 column was calibrated with Blue dextran (2000 kDa), Cytochrome C (12.4 kDa), Carbonic anhydrase (29 kDa), Bovine Serum Albumin (66 kDa), Alcohol Dehydrogenase (150 kDa), and β-amylase (200 kDa). Ammonium sulfate precipitated, desalted protein extract was loaded onto the column. Fractions collected were analyzed by immunoblotting.

levels in fractions corresponding to molecular masses larger than 132 kDa may indicate that this protein may preaggregate in solution. The estimated 132 kDa size for ZRP2 from gel filtration studies is close to the 100 kDa size observed with SDS-PAGE. Therefore, the ZRP2 protein appears to exhibit an aberrant chromatographic behavior as well. However, the possibility that the observed size corresponds to a dimer of 67 kDa cannot be ruled out, although a peak corresponding to a 67 kDa monomer has never been observed during gel filtration. Such discrepencies between the estimated size from cDNA sequence, SDS-PAGE and column chromatography are known for proteins such as proline-rich proteins (John and Keller, 1995). This aberrant electrophoretic and chromatographic behavior has been attributed to the presence of a proline-rich pentapeptide motif. This motif is found repeated throughout the protein and is thought to contribute to the rod-like conformation of these molecules. Similarly, ZRP2 contains a serine/threonine-rich oligopeptide that is repeated 16 times throughout the polypeptide. Whether this motif contributes to the observed aberrant electrophoretic and chromatograpic behavior of ZRP2 protein remains to be determined.

ATTEMPTS TO IMMUNOLOCALIZE ZRP2 PROTEIN USING THE TISSUE PRINT TECHNIQUE

Introduction

ZRP2 is a protein that has been shown to preferentially accumulate in maize roots and stems (chapter 2). The mRNA accumulation of this gene has been studied previously (Held, 1993). *In situ* hybridization studies have shown that ZRP2 mRNA accumulates in the root cortex of seedlings (Wang, 1994). In an initial attempt to study the ZRP2 protein accumulation at the cellular level, tissue print immunoblotting was undertaken. Tissue print immunoblotting was selected since this method is relatively easy to perform and since this technique has been used successfully to immunolocalize proteins at the cellular level (Cassab and Varner, 1987, Ye and Varner, 1991).

Materials and Methods

Tissue print Immunoblotting

Tissue prints were made according to Cassab and Varner (1991). Briefly, 1-2 mm thick root or stem sections were cut and blotted to remove excess fluid and pressed onto supported nitrocellulose (Schleicher and Schuell) for 20-40 s. The membrane was air-dried and blocked in Phosphate Buffered Saline (PBS) containing 0.5% Tween-20 and 5% BSA for 1 h at room temperature or overnight at 4°C. The

membrane was washed twice for 10 min in PBS containing 0.1% Tween-20 (PBS-T). The primary antibody was used at a 1:2,000 to 1:5,000 dilution in PBS-T. After three 10 min washes with PBS-T the membrane was incubated with alkaline phosphatase-conjugated anti rabbit IgG antibody (Sigma) at a 1:10,000 dilution for 1 h at room temperature. After washing, the membrane was incubated in a NBT-BCIP (Sigma, B5655) substrate solution for 10 min prepared according to the manufacturer's instructions. After development of color, the membrane was rinsed in distilled water and stored in the dark.

Presorption of ZRP2 antiserum

0.5 g of maize leaves were ground in liquid nitrogen and the ground material was transferred to a microfuge tube containing 300 µl of protein extraction buffer. The tube was vortexed and centrifuged at 12,000 rpm for 5 min. The supernatant was added to 5 ml of PBS-T containing ZRP2 antiserum at a 1:2,000 dilution. The ZRP2 antiserum was presorbed for 1 h at room temperature. At the end of this time period the blocked membrane was introduced and incubated for another hour. Washing, incubation with secondary antibody and immunodetection was as described above.

Purification of the IgG fraction

10 ml of ZRP2 antiserum (Rabbit 2553, exsanguinate serum) was used in affinity purification of IgG fraction. The antiserum was passed through a DEAE Affi-Gel column (Bio-Rad) regenerated with 1.5 M

NaSCN solution to remove serum proteases. The serum was eluted with PBS. The affigel eluate was loaded onto a Protein A column (Sigma). The column was washed with PBS. The bound IgG fraction was eluted using 0.5% acetic acid (pH 3.0), 0.85% NaCl. The peak fractions were collected and neutralized with NaOH. The concentration of IgG was calculated by measuring absorbance at 280 nm.

Affinity purification of ZRP2 antibodies

Proteins were extracted from maize roots as described above and subjected to SDS-PAGE on 7.5% preparative gels. The proteins were electroblotted onto nitrocellulose. The 100 kDa band corresponding to ZRP2 was cut out and blocked with PBS-T containing 5% BSA for 2 h. The membrane was next incubated with ZRP2 antiserum for 2 h, washed for 1 h with PBS-T, and antibodies eluted with glycine buffer (0.1 M glycine pH 2.5, 1% BSA w/v, 0.2% v/v Tween-20). The eluate was neutralized and concentrated.

Results

Prior to performing tissue print immunoblots, control experiments were conducted using either primary or secondary antibodies alone. These experiments indicated that neither primary nor secondary antibodies alone contribute to non-specific signals. Furthermore, when tissue prints were directly incubated in the substrate solution (without

prior incubation with antibodies) no signal was observed indicating that substrate did not produce a non-specific signal either.

Immunoblotting was conducted using ZRP2 antiserum as the primary antibody (1:2,000 dilution). An immune signal was observed in all tissues of root and stem with more intense signal detected in the vascular tissue (Figs. 1A and 2A). However, when a control reaction was carried out using preimmune serum, tissue print signals observed were identical to that observed with immune serum (Figs. 1B and 2B). A specific signal, in addition to the non-specific signal observed in both, was not observed with ZRP2 antiserum. Attempts were subsequently made to reduce this non-specific signal by several means such as increasing the amount of blocking agent, use of different blocking agents (e.g. milk powder), increase of the blocking time up to overnight and use of higher dilutions of primary and secondary antibodies (e.g. primary; 1:5,000, secondary; 1:10,000). However, these treatments did not result in elimination of non-specific signal observed with both preimmune and immune sera, although the overall intensity of the signal was reduced (data not shown). The non-specific nature of the background signal was confirmed by the detection of this signal in tissue prints of maize leaves (data not shown).

In an effort to reduce this non-specfic signal, the IgG fraction of ZRP2 antiserum was purified to eliminate contaminating serum proteins, etc. Development of tissue prints using the purified IgG as the primary antibody produced no reduction in the non-specific signal (data not shown) and were in fact similar to results observed using crude serum.

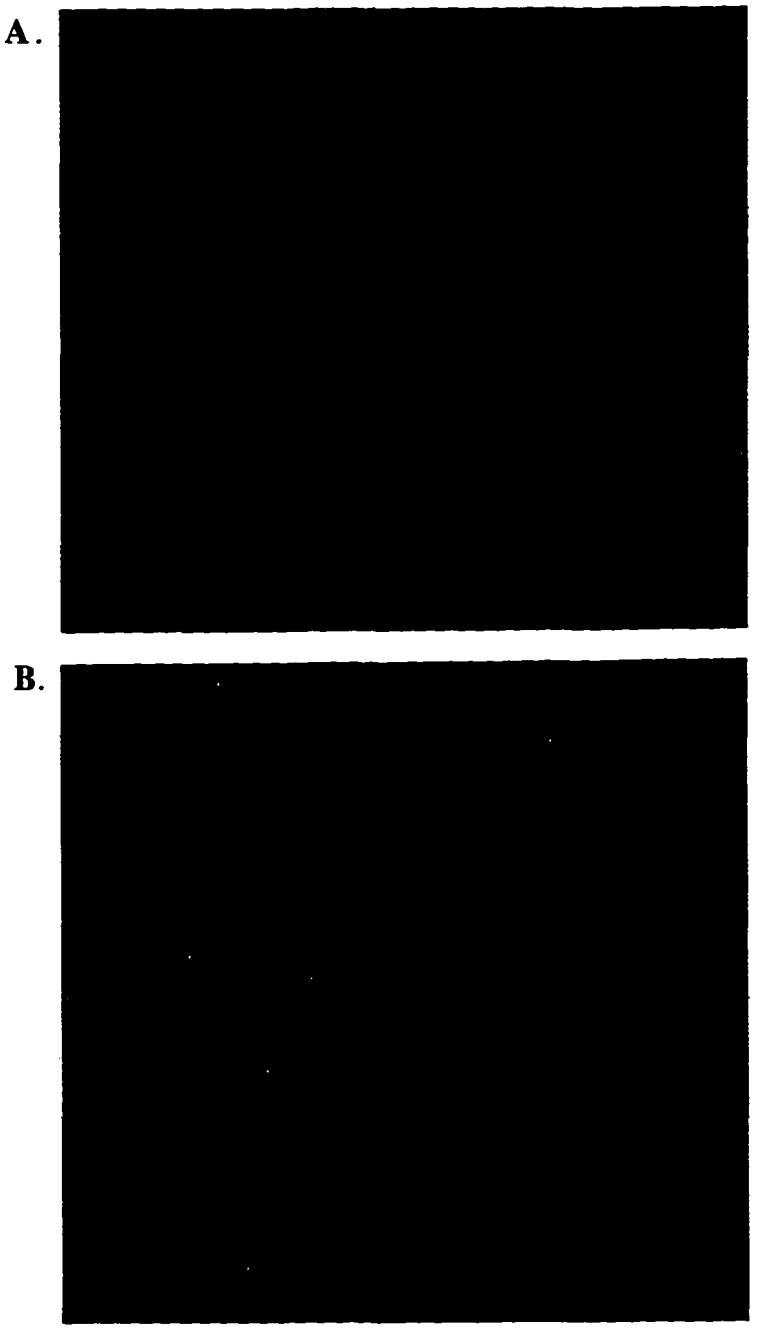

Figure 1. Tissue print immunolocalization of ZRP2. The tissue prints of maize roots were prepared as described in Materials and Methods and incubated with either immune (A) or preimmune (B) serum and detected with alkaline phosphatase-conjugated secondary antibody.

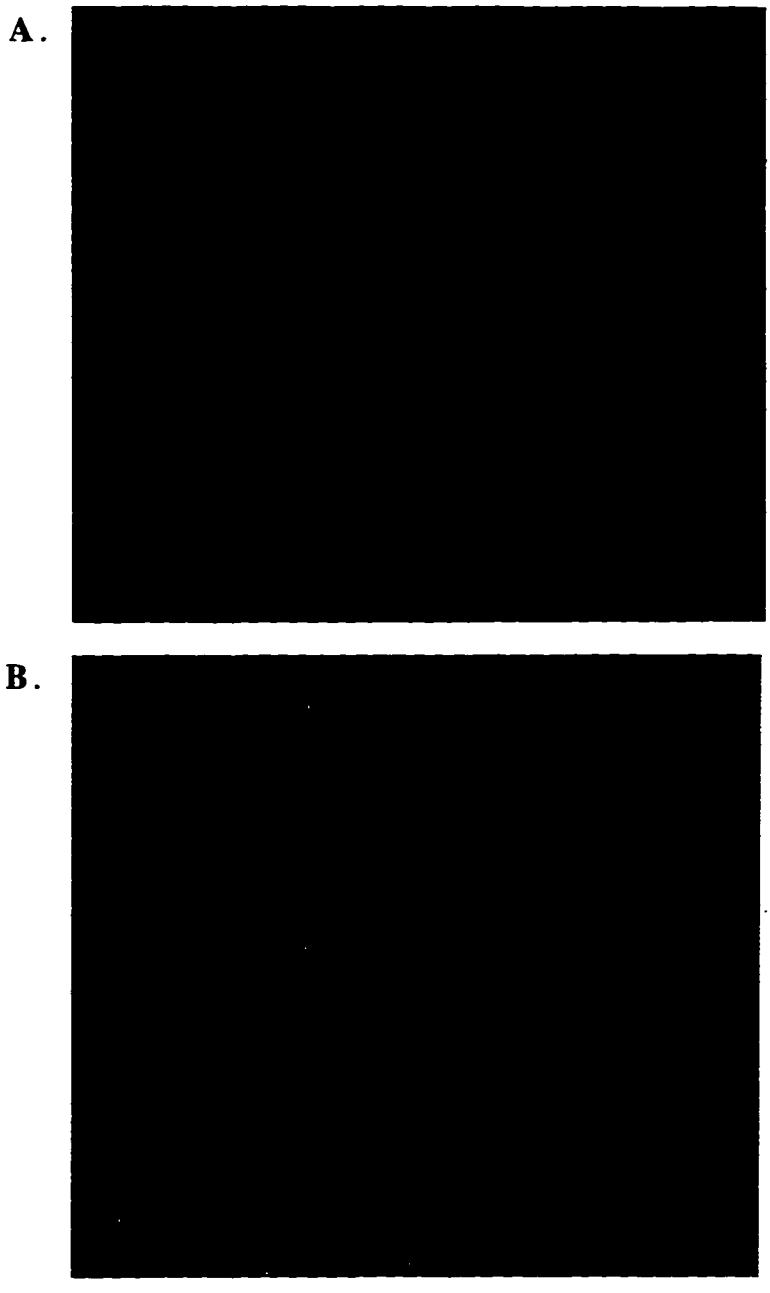

Figure 2. Tissue print immunolocalization of ZRP2. The tissue prints of maize stems were prepared as described in Materials and Methods and incubated with either immune (A) or preimmune (B) serum and detected with alkaline phosphatase-conjugated secondary antibody.

Therefore, attempts were made to remove non-specifically binding antibodies present in the crude serum by incubating the ZRP2 antiserum with a maize leaf extract first. This presorbed antiserum was used to perform tissue print immunoblots subsequently. The results did not show any significant reduction in the background signal. Furthermore, a ZRP2-specific signal was not observed (Fig. 3A).

Since neither purification of the IgG fraction nor presorption of ZRP2 antisera proved to be successful in getting rid of the non-specific signal and improving specificity, ZRP2 antiserum was affinity purified as a final attempt. The affinity purified antibodies were used in the immunodetection of tissue prints. However, results observed were similar to previous results. Although the non-specific signal appeared to be much reduced, the specific signal expected was not observed (Fig. 3B).

Discussion

In order to gain an idea of the cellular distribution of ZRP2 protein, immunolocalization studies were undertaken using the polyclonal antibodies raised against ZRP2 protein. Specifically, the tissue print technique was employed as an initial attempt to immunolocalize ZRP2 protein at the cellular level before undertaking more time-consuming immunolocalization techniques at the sub-cellular level.

Based on the *in situ* hybridization studies conducted previously (Wang, 1994), ZRP2 protein was expected to be localized in the cortex of

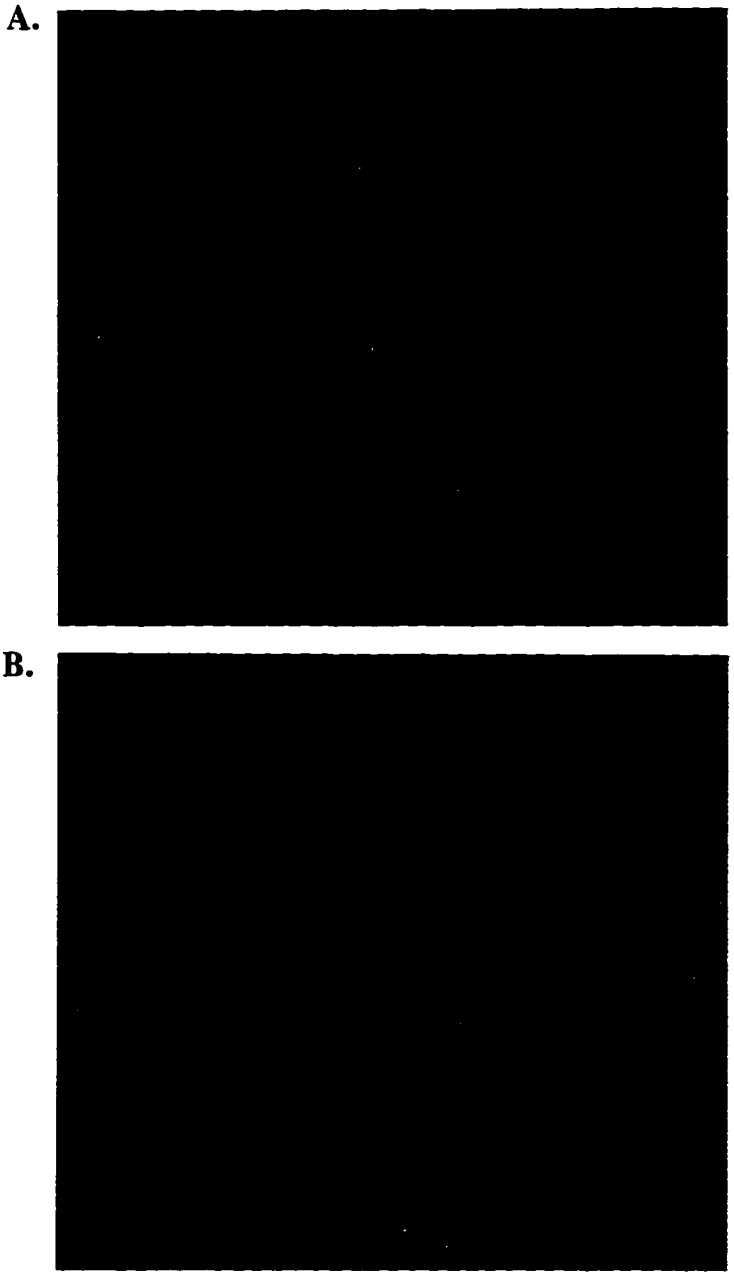

Figure 3. Attempts to immunolocalize ZRP2 in roots using (A), presorbed ZRP2 antiserum and (B), affinity purified ZRP2 antibodies. Preparation and immunodetection of tissue prints was carried out as described in Materials and Methods.

the maize root. In the stem, ZRP2 protein was expected to be localized in the ground tissue. However, tissue print experiments failed to produce a similar localization signal. Instead, a non-specific signal was observed throughout the entire root section with both preimmune and immune sera. When tissue print experiments were conducted with antisera available against another maize protein, ZRP4, the non-specific signal detected with ZRP2 antibodies was again observed and a ZRP4-specific signal was not detected. These observations show that inability to obtain a specific signal is not due to a problem associated with ZRP2 antibodies but may rather be due to the presence of non-specifically binding antibodies against maize proteins in rabbit serum in general. However, it is difficult to explain the lack of a specific signal with ZRP2 antibodies even after affinity purification. The presence of low background after affinity purification could be due to contamination with crude antiserum due to incomplete removal during affinity purification. The reasons for failure to obtain a specific tissue print signal are not clear. Polyclonal antibodies raised in rabbits may not be of sufficient quality to produce a specific signal with tissue prints. Use of a better affinity purifcation method than the one used in this study, such as the use of an affinity column immobilized with either an *E. coli* produced ZRP2 protein or the native protein purified from roots may substantially improve the specificity of the signal. Alternatively, the use of monoclonal antibodies raised against ZRP2 protein should produce a specific signal. Although this antiserum appears unsuitable for tissue print immunoblotting, it may prove to suitable for other

immunolocalization techniques which involve fixing, embedding and sectioning of tissue prior to immunodetection either at the cellular or sub-cellular level.

GENERAL CONCLUSIONS

Chapter 2 describes detection and distribution of ZRP2 protein in maize. ZRP2 protein is found at high levels in roots and stems and it is not detected in other organs. Within seedling roots, ZRP2 protein is detected at a low level in the first cm of the root, but is detected at high levels in the remainder of the root. The pattern of ZRP2 protein accumulation is similar to the mRNA accumulation pattern observed previously (Held, 1993). Therefore, this study confirms the root-preferntial nature of *zrp2* expression and points to a regulation of *zrp2* expression at the RNA level. The ZRP2 protein is observed as a 100 kDa protein on SDS-PAGE although the predicted size of this protein is 30 kDa smaller based on the ZRP2 cDNA sequence. This apparent size difference appears unlikely be due to a post tranlational modification, but rather due to an aberrent electrophoretic behavior. This conclusion is further supported by gel filtration studies (chapter 5). In addition, *zrp2* gene expression appears to be transiently down-regulated by abscisic acid. Since the function of this gene is not known, the significance of this observation is not clear. Sub-cellular localization studies of ZRP2 protein may aid in attaining this objective. However, these studies may require production of a new ZRP2 antibody since initial attempts to immunolocalize this protein using tissue print technique have been unsuccessful (chapter 6). In addition, production of ZRP2 mutants showing reduced levels of ZRP2 protein may facilitate the understanding of function of ZRP2 protein. The promoter region of

this gene has already been isolated (Held, 1993). Histochemical analysis of stable maize transformants containing ZRP2 promoter-GUS fusion constructs have confirmed the root-preferential nature of ZRP2 promoter (unpublished observations).

Chapter 3 describes the detection and distribution of ZRP4 protein. mRNA of ZRP4 has been shown previously to accumulate preferentially in maize roots (Held et al., 1993). ZRP4 protein is detected as a 40 kDa protein (which corresponds to the expected size estimated from the ZRP4 cDNA) in roots. This protein is not detected in other organs of maize. ZRP4 protein accumulation appears to be developmentally regulated with high levels in young roots and low levels in mature roots. Within the seedling roots, very low levels are detected in the first cm of the root, but high levels are detected in the remainder of the root. Pattern of ZRP4 protein accumulation is similar to ZRP4 mRNA accumulation described previously (Held et al., 1993).

Since *zrp4* is known to code for an O-methyltransferase it would be of interest to assay the activity of ZRP4 enzyme *in vitro*, and to determine whether ZRP4 is actually involved in suberin biosynthesis. Furthermore, the availability of ZRP4 mutants should help to further elucidate the function of this enzyme in maize. Towards this objective, ZRP4 antisense mutants have already been obtained and analysis of these mutants are underway (chapter 4). Additionally, isolation of the promoter region of *zrp4* may lead to obtaining of an endodermis and exodermis-specific promoter which would be useful in genetic engineering of crop plants against root pathogens.

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ACKNOWLEDGMENTS

I would like to express my deep appreciation to my major adviser Dr. James Colbert for his guidance, encouragement, and for always making me see the brighter side of things. He was a good friend and a pleasure to work with.

I also wish to thank my committee members, Drs. Eve Wurtele, Steven Rodermal, Jonathan Wendel, and Patrick Schanable for their input and valuable time. My thanks also goes to Dr. Martin Spalding for graciously agreeing to serve as a committee member for my final exam and to Dr. John Hill for his help and advice.

I would like to sincerely acknowledge past and present members of our laboratory, Dennis Byrne, Theresa Tirimanne, David Higgs, Victor Nunez, Jim Holtz and Yongwen Wu, for their help both in and outside of lab. I also thank fellow graduate students, Cyril Roberts and Jhuwan Eom for their help and advice.

My deepest gratitude goes to my parents for their love, patience, and for giving me every opportunity and encouragement during my studies. I dedicate this dissertation to them.

Finally, I would like to thank my husband Saroj, for all his love, support, advice, and for helping me to keep things in perspective.