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Expression And Regulation Of Homeobox-Like Maize Root-Specific Gene

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EXPRESSION AND REGULATION OF HOMEOBOX-LIKE MAIZE ROOT SPECIFIC GENE

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Zeung-Keun Cho

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APPROVAL SHEET

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ABSTRACT

Homeobox genes are known to code for transcription factors regulating developmental programs that are universally represented in animals and plants. Among three α-helices and a more flexible fourth helix, helices 3 and 4 recognize specific binding sites of DNA. Amino acid sequences, WFQNRR, are well conserved among both animals and plants. With primers corresponding to these amino acids, putative homeobox genes (Mrx series) are isolated from maize roots through the PCR technique. The translated amino acids are only conserved 17% comparing atypical homeodomains. Based on ectopic expression in Nicotiana tabacum, it is an atypical homeodomain that is expressed in the root epidermis near the root cap at the early embryoid stage. After two or three weeks, regenerated roots possess a drastically changed phenotype.

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INTRODUCTION

The plant life cycle has two generations, one that is dominant sporophytic for producing the diploid plant, and one that is gametophytic for developing pollen and the embryo sac (Goldberg et al., 1989). In general, the overall principles of developmental processes in plants are similar to those in animals, but certain aspects of plant development differ considerably from animal development. Among these differences are that plant cells lack cell movement during embryogenesis, plant tissues retain totipotency following differentiation, organogenesis is continuous during development, and plant development is directly linked to environmental signals (Steeves and Sussex, 1989). The different aspects of plants versus animals in development make very significant developmental fates.

In general, plant embryogenesis is classified into three stages: globular, heart, and torpedo. At the first stage most cells are equivalent morphologically except the outer most layer, which is differentiated as dermatogen that produces and secretes waxes characteristic of epidermal cells. The rapid cell divisions at the heart stage lead to formation of two cotyledon primordia and establish the shoot

and root apical meristems. Then the hypocotyl and radicle are recognized and vascular tissue begins to differentiate within those organs. Also at this stage the embryo begins chloroplast development. The embryo contains two primary organ systems - the axis and cotyledon that have distinct developmental goals and are both composed of three basic, or primordial, tissue layers - protoderm, procambium, and ground meristem, which will become the epidermal, vascular, and parenchyma tissues of the young seedling (Goldberg et al., 1994). In plants, embryonic development is identical for a given plant species (Jurgens, 1995), and meristematic activity is regulated by physiological and environmental signals. During embryogenesis the developmental programs are restricted to two regions at the apical and basal poles of the embryo, the shoot and root apical meristems. The plant body plan is determined by the growth of a number of meristems including apical meristem, leaf meristem and cambium layers, and depends primarily on the proper regulation of cell division versus cell differentiation at the meristems. In plant development, cell division is the basic process that 1) initiates organ development, 2) determines the plane in which a cell divides and often is a highly significant factor in determining what derivatives will become, and finally, 3) plays a role in the initiation and mainteinance of cell differentiation.

The vegetative shoot apical meristems can be transformed into floral meristems that exhibit terminal growth; they produce a set number of organs and grow no more. The root apical meristem is not terminal, and does not produce lateral organs (Kerk and Feldman, 1995).

Plant development is mostly postembryonic, but postembryonic development is greatly influenced by environmental factors and exogeneous signals (Lindsey and Topping, 1993). The process of plant development is virtually dependent on the basic signal-generating system for vegetative programming of the meristem. This system is located within the meristem itself. Meristem function is very sensitive to signals received from the environment and from the plant itself. Responses are adapted to these signals (Goldberg et al., 1989).

The plant growth regulator, indole-3-acetic acid (IAA, auxin), is synthesized and transported in a polarized, basipetal direction along the embryonic axis during embryogenesis (Goldberg et al., 1994). During the globular stage, auxin reaches the highest levels, and is distributed unequally which contributes to the establishment of bilateral symmetry (Bandurski and Schulze, 1985).

The plant growth regulator, cytokinin, plays an important role in the control of protein biosynthesis. Cytokinins are generally recognised as stimulators of cell

division, cell expansion, and for their ability to delay leaf senescence (Horgan, 1984). Also cytokinins interact with auxin in determining the direction of differentiation for cells (Wareing and Phillips, 1970). At the molecular level, cytokinins induce an increased rate of RNA synthesis and of post-transcriptional modification (Kulaeva, 1981). The Arabidopsis root mutant STP 1 is less sensitive to cytokinin, but it is sensitive to other growth hormones in a similar way to the wild type. STP 1 is a root protein that promotes elongation. Cytokinin down-regulates STP 1 activity.

The control of root growth and development is a complex program. Many processes are controlled by the interaction of multiple hormones. Both auxin and ethylene play a complicated and confusing role in signalling pathways for root hair initiation, position and growth. Auxin and cytokinin initiate morphogenesis in many callus cultures and regulate organogenesis in some cultured tissues through the balance between these two hormones (Reinert and Backs, 1968). IAA also initiates lateral root formation and is required for processes in lateral root development that initiate cell division in the pericycle and promote cell division and maintain cell viability in the developing lateral root (Celenza et al., 1995).

The complex pattern of cell divisions in the embryo gives rise to the various initials within root promeristem. Part of the root is not derived from initials but from a separate tier of embryonic cells. After germination, a fixed pattern of cells perpetually divides in the meristematic zone.

The primary root originates from the embryo, but lateral roots are derived from groups of pericycle cells in the mature root (Steeves and Sussex, 1989). At an early stage there are different developmental processes for the primary and lateral roots, but later phases of lateral and primary root development use the same processes and components. Root growth is initiated from analogous apical and cambial meristems. In addition, axillary meristems may occur in response to positional information set up by the action of opposing morphogenetic gradients (Talbert et al., 1995). Cells within root meristems are located at the distal end of continuous cell files extending into the mature root. The root radial pattern is consistent with the radial arrangement of root meristem initials at the heart stage of embryogenesis (Scheres et al., 1994).

There are no distinct cell lineages for root hypocotyl fate, but derivatives of hypophyseal cells do appear to be restricted to the columella and central cell region of the root (Scheres et al., 1994).

The mature root epidermis is composed of two cell types: root hair cells derived from trichoblasts, and non-hair cells derived from atrichoblasts. At the level of the root, the radial pattern is further differentiated to include lateral root cap, cortex, endodermis and pericycle, in addition to epidermis and vascular tissue. The differentiation of root epidermis requires signals that are 1) a Raf-like protein kinase playing a role in pattern formation/differentiation in the root epidermis and 2) ethylene as a diffusible signal involved in specifying pattern in the root epidermis (Dolan et al., 1994). Cell-type specific transcription in the root tip increases transcription in the vascular cylinder of anaerobically-treated maize roots (Maas et al., 1990).

Genes control major developmental events in plants including the transition between vegetative and reproductive growth (Aeschbacher and Benfey, 1992). During embryogenesis, gene expression is characterized by quantitative changes but not by a reduction in number of genes. Some of the embryo-specific genes are stage-specific (Mayer et al., 1991). Developmental signals control transcriptional programs and their activation either directly or indirectly. These signals drive the transition from G1 to S phase in the stereotypic embryonic pattern. The cell cycle is regulated by signals from other cells

(Duronio and O'Farrell, 1994). This gene expression is critical to regulate meristem functions that are related to cell proliferation through control of the cell cycle. The genes regulating the cell cycle are called cyclin genes. Cyclin genes are involved in determining cell fate through transcription factors and in determining function of tissue or organ structures through tissue-specific or organ-specific gene products. In eukaryotes, cell cycle progression is controlled by heterodimeric protein kinase complexes of a cell cycle-dependent, kinase-related subunit (Cdc2) and a cyclin subunit. Cyclins in higher plants form three distinct structural groups that have been conserved in both monocotyledonous and dicotyledonous species. Cyclins from all three groups have been shown to be present within a single plant species (Renaudin et al., 1994). Significant differences exist between plants and animals in regulation of the cell cycle. For instance, the Arabidopsis cyclin gene, cycl At, is expressed almost exclusively in dividing cells during very early events. The cell-specific pattern of cycl At expression is confined to root meristems (Ferreira et al., 1994).

In developmental systems, the responses of cells are directly related to changing patterns of gene expression. This gene expression is governed by a number of transcription factors which are growth and developmental

regulators in plants as well as in animals. The key mechanism in gene expression appears to be transcriptional regulation activated through sequence-specific DNA-binding proteins. These DNA-binding proteins recognize cis-acting elements located in the promoter and enhancer regions of the corresponding gene sequences. Plants have many types of DNA-protein and protein-protein interactions. Some of these motifs are unique to plants, others are similar to yeast and animal transcription regulatory gene mechanisms.

In animal systems transcription factors have various types of activation domains which include the TFIII genes and the TFII group with an array of regions that bind proteins that lead to initiation of RNA polymerase II (Guarente, 1992; Kim et al., 1993; Kim et al., 1993; Lu et al., 1992). For plant transcription factors a limited number of activation domains have been discovered. These include the acidic regions of the maize C1, tomato HSF, and rice OSH protein (Goff et al., 1991; Treuter et al., 1993; Tamaoki et al., 1995) that forms an α helix in the activation domain (Paz-Ares et al., 1990) and the Pro-rich region of G-box-binding factors (GBFs) which are potential activation domain characterized by a large number of aromatic residues and only a few charged residues. This implies that transactivation mechanisms are probably different in animal and plant Pro-rich regions.

In regard to the basic transcription factors, significant information has been accumulated concerning numerous factors and organisms in the animal kingdom. Knowledge of basic transcription factors (TBFs) is minimal for the plant kingdom (Vogel et al., 1993; Frohnmeyer et al., 1994).

Homeotic genes, one class of these transcription factors, are clustered in several chromosomal regions; the Antennapedia (ANT-C) and Bithorax (BX-C) complexes specify segment identity in Drosophila. Homeotic transformations are most conspicuous in the epidermal structures of animals even though homeotic genes are also involved in development of internal organs, such as the nervous system and muscles (Teugels and Ghysen, 1985). The Antennapedia (Antp) gene is composed of two promoters, eight exons, and two polyA regions (Schneuwly et al., 1986). The near exon of the 3' end of the genes, close to the intron/exon boundary, is a homeobox. This homeobox is a 180-bp DNA segment conserved by most homeotic genes (McGinnis et al., 1984; Scott and Weiner, 1984). Homeobox genes constitute a class of regulatory genes that control many important aspects of development, such as the time and place in which major cell types differentiate and the identity of tissues and organs that are to be formed. These genes initiate the expression of other genes, including the effector genes whose protein

products are necessary for the unique functions of organs. Recently, the homeobox genes have been referred to as HOX genes as similar sequences were discovered in mice and humans. Presently, four HOX gene complexes are known in humans Hox A, B, C, and D.

The homeotic genes often act in an antagonistic fashion (Mizukami and Ma, 1992) during organ development. The first step in organ development is the transition of an inflorescence meristem into an organ meristem. Early-acting genes determine the identity of the organ meristem, and late-acting genes determine organ identity (Mandel et al., 1992). Even though homeotic genes exist in plants, the expression of these genes seems to be tissue-specific. These genes appear to act as transcriptional mechanisms in control of higher plant development. Additionally, these genes are probably combinatorially regulated.

The DNA sequence motifs of homeobox genes code for about 61-amino-acid sequences. The proteins associated with these contain DNA-binding regions called homeodomains. Homeodomains bind to DNA sections associated with the transcription of proteins that have a member of regulatory roles. These include 1) key control along development, 2) specification of the body plan, 3) pattern formation, 4) determination of cell fate, and 5) several other basic developmental processes (McGinnis and Krumlauf, 1992) of

animals as well as plants. Homeodomains include a region consisting of three α-helices. The three-dimensional structure of this contains a helix-turn-helix motif that is highly conserved among otherwise very diverse species. The DNA-binding specificity is dependent not only on its own binding capability, but also on its association with other transcription factors (Johnson, 1992). Helices numer 2 and 3 organize a helix-turn-helix motif. Of the three α-helices, helix number 3 has the most conserved homeodomain sequence (WFXNRR). Post-transcriptional modification is very important in regulating activity of transcription factors, such as DNA binding, nuclear transport, and activation/deactivation through protein-protein interactions. In animals, the transcription factors (homeodomains) are phosphorylated on serine and threonine residues; all isoforms of these transcription factors are similarly phosphorylated at specific phases throughout embryogenesis (Gavis and Hogness, 1991). There is clear evidence that transcription factors are major targets for various kinds of signal transduction pathways; many kinases and phosphatases are involved in control of gene expression. These facts imply that promoter repression is generally insensitive to deletions and phosphorylation, but promoter activation is differentially sensitive to deletions through

multiple activating sequences spread throughout the N-constant region. Also,the expression pattern is modified in a complex manner by endogenous chemical regulators such as retinoic acid (RA) (Corte et al., 1993). In plants, there are many reports concerning regulation by phosphorylation of DNA-binding activity in nuclear extracts (Despres et al., 1995; Sun et al., 1993; Sarokin and Chua, 1992); it is implied that this activity is controlled at the step of nuclear transport (Varagona et al., 1992).

Recent studies show that ectopic expression or overexpression of homeobox genes causes alteration of cell fates; the homeobox-containing gene enhances expression during somatic embryo development (Smith et al.,1992; Sinha et al., 1993; Ma et al., 1994). There are two classes of plant homeotic genes that code for the typical homeodomain and the homeodomain/leucine zipper motif.

The first class is the typical homeodomain motif. The Knotted-1 (Kn1) homeobox gene was the first isolated from maize seedlings. Kn1 controls leaf development (Sinha et al., 1993). Kn1 is expressed in shoot apical meristems and immature shoot axes, but not root meristems. OSH1, a KN1 homolog in rice, is involved in rice development (Matsuoka et al.,1993). The Hooded mutation of barley is caused by a homeobox gene intron that is a homolog to the Knotted-1 (Kn1) gene of maize. The function of its homeobox gene is

similar to Kn1; it is expressed in meristematic regions and the duplication of the intron enhances gene expression in mono- and dicotyledonous plants (Clancy et al., 1994).

The second class of homeotic gene coding is the homeodomain/leucine zipper motif (HD-Zip). This motif has been isolated only from Arabidopsis (Sessa et al., 1993). The HD-Zip factors are specific only to plants, responding to environmental signals during development. These factors play a role in protein-protein interaction or DNA-protein interaction (Schindler et al., 1993). Recently, the Arabidopsis GLABRA2 gene was identified as a homeobox gene found in specific epidermal progenitor cells which differentiate into trichomes (Rerie et al., 1994). This gene is similar to the selector genes frequently associated with homeobox genes in Drosophila.

Maize is a higher plant that is readily accessible and is well characterized genetically and physiologically. Since McClintock, considerable effort has been made to elucidate development processes in view of genetics. This effort has yielded massive information related to the construction of mutant plants. The mutants have uncommon genetic variability that reveal factors which control plant developmental processes.

Recently a novel maize homeobox gene (Zmhox 1a) was isolated that interacts with the Shrunken (Sh) feedback

control element (Bellmann and Werr, 1992). The maize Shrunken (Sh) gene encodes the enzyme sucrose synthase. Zmhox 1a is dominantly expressed in sink organs; transcription is regulated by sucrose concentration and UDP-glucose. The Zmhox 1a is a single-copy gene that is transcribed in different maize tissues (root, leaf, shoot, kernel, and suspension cells). In Western blot experiments, no protein is detectable in root nuclei. The expressions of the open reading frames of both Zmhox 1a and Zmhox 1b show identical phenotypic alterations. These alterations include size reduction, formation of adventitious shoots, and homeotic floral transformations. In all five maize homeobox genes, the motif PYP is conserved in the loop between helices 1 and 2. Transgenic expressions of these two genes show evidence that maize homeobox gene products not only affect the development of the vegetative plant body but also alter the floral developmental program (Uberlacker et al., 1996).

Production of transgenic plants has traditionally presented problems to researchers. Plant roots have high levels of DNase activity (Jones and Boffey, 1984) that reduce the transformation efficiency. Fortunately, the intact Cauliflower Mosaic Virus 35S promoter furnishes tissue-specific constitutive expression of heterologous genes in most plants. This promoter confers a high amount

of expression in most plant cells and is independent of viral trans-acting factors (Benfey and Chua, 1990). Thus, a valuable tool is available for introducing new gene constructs into plants.

Based on reports of animal and plant homeotic genes, discovery and understanding of homeobox genes in maize may provide insight into the complex processes of root development. The localization and histochemical analysis of gene expression will help us better understand the molecular mechanisms regulating plant root development.

MATERIALS AND METHODS

PLANT MATERIALS

Maize caryopses (Zea mays, L. Pioneer 3343) are soaked overnight in running tap water, Grains are placed on wet paper towels in opaque plastic trays, covered with paper towels. The trays are placed in a vertical position at 24°C in Environmental Growth Chamber (Chagrin Falls, Ohio) for germination. Roots and shoots are selected at 2-3 cm in length. The apical 1.5 cm of the shoots and roots are collected using a razor blade. The excised tissue is washed with distilled water to remove mucilage. Tissue is immediately used for isolation of genomic DNA or total RNA. Excess tissue is kept in the ultrafreezer for future use.

Nicotiana tabacum is obtained from Carolina Biological Supply company (Burlington, North Carolina) and planted in a greenhouse for three weeks. The healthy, young, unblemished leaves are harvested. The tissue is sterilized in bleach solution for 15 to 20 min using gentle agitation. The tissue is rinsed 3 or 4 times with distilled sterile water. The tissue is cut into small squares to produce wound edges. The sterlized explants are precultured for 1 or 2 days on Murashige and Skoog's (MS) medium containing 1 g/L of Casein hydrolysate and 0.2 mg/L of Kinetin (Sigma, St. Louis, MO).

ISOLATION OF MAIZE GENOMIC DNA

Approximately 10 g each of maize roots and shoots are ground in a pre-cooled mortar/pestle under liquid nitrogen. The frozen powder is transferred to 15-ml centrifuge tubes and immediately mixed with SSET buffer (8% sucrose, 10% Sarkosyl, 50 mM EDTA, and 50 mM Tris-HCl, pH 8.0). The homogenate is incubated for 1 to 2 hours at 55°C. The homogenate is cooled to 37°C and the lysates are homogenized with 1 ml of isoamyl alcohol:Chloroform (1:24), 1 ml of 5 M NaCl, and 1 ml of hot TE (10 mM Tris. Cl, 1 mM EDTA, pH 7.6)-saturated Phenol (37°C). The supernatant is transferred to new tubes and centrifuged for 10 min in a Beckman JA-20.1 rotor at 5000 x g, 4°C. Ice-cold 100% ethyl alcohol (EtOH) is added. The mixture is incubated in a -20°C freezer for 20 min. The pellets are collected and washed with 70% EtOH. The resuspended pellet is centrifuged for 15 min in a Beckman JA-20.1 rotor at 8500 x g (4°C). The pellet is resuspended with TE buffer (10 mM Tris. Cl, 1 mM EDTA, pH 7.6) and treated with 1.5 µl (30 units) of RNase for 1 hour at 37°C. The pellet is repeatedly extracted with phenol/chloroform (1:1). After precipitation with 100% ethylalcohol, The pellets are resuspended in TE buffer. The resuspended DNA is mixed with 5 M NaCl (final concentration, 3M). Two volumes of ice-cold 100% ethyl alcohol is added.

The suspension is placed in a -20°C freezer for 20 min. The pellets are collected by centrifugation as described above and washed with ice-cold 70% ethyl alcohol. The pellet is resuspended in TE buffer and stored in a -20°C or ultrafreezer for future use.

ISOLATION OF TOTAL RNA

Before total RNA isolation, all components including distilled water, glassware, and any buffers except TE buffer are treated with DEPC (Diethylpyrocarbonate) to inhibit Rnase activity. Maize root and shoot tissue are ground and transferred to a 100 ml beaker. Homogenization buffer consists of 25 ml TLE (0.2 M Tris, 0.1 M LiCl, 5 mM EDTA, pH 8.2)-equilibrated with phenol. Twenty-five ml of chloroform is added to the slurry. The slurry is transferred to 15-ml cenrifuge tubes and incubated for 20 min in a 50°C waterbath. The mixture is centrifuged for 30 min at 10,000 rpm in a JA-20.1 rotor(4 C). The supernantant is drained off to remove as much of the aqueous layer as possible. The supernatant is transferred to a clean 15-ml centrifuge tube. Five ml of TLE-equilibrated phenol is added to the aqueous phase, mixed with 5 ml chloroform, and centrifuged for 20 min at 10,000 rpm (4°C), in a JA-20.1 rotor. The aqueous layer is collected, TLE-equilibrated phenol and chloroform are added in the previous manner, and recentrifuged. These

steps are repeated until no interface is visible. The aqueous phase is then extracted three times with chloroform.

For removal of contaminating DNA, the aqueous phase is transferred to 50-ml centrifuge tubes. Eight M LiCl (1/3 vol) is added to bring the solution to a final concentration of 2 M LiCl. The mixture is precipitated overnight at 4°C. Pellets are collected by centifugation for 20 min at 10,000 rpm, 4°C, in a JA-20.1 rotor. Pellets are rinsed with 5 ml of 2 M LiCl, and resuspended in 5 ml DEPC-treated water. The selective precipitation steps are repeated three times and the total RNA is dissolved in 2 ml of water by adding 200 ul of 3 M sodium acetate and 6 ml 100 ethanol. The mixture is allowed to precipitate at -70 C for 30 min. RNA is recovered by centrifugation for 15 min at 10,000 rpm, 4°C, in a JA-20.1 rotor. RNA is resuspended in 1 ml water. The total RNA is stored in an ultrafreezer for future use.

CONSTRUCTION OF cDNA LIBRARY

A. Primer Sequences used in Reverse Transcripion and Polymerase Chain Reactions

Three primers are designed and purchased (IDT, Coralville, IA) based on the sequence information and purpose. The first primer (HB13) is a degenerate primer based on the typical homeodomain $\alpha 3$ region amino-acid sequences (WFENRR). The second and the third primers are

OligodG$_{12}$ and OligodT$_{12}$ (described below). Two additional primers (zh3, zh5) are used in the reverse transcription (RT) and polymerase chain reactions (PCR) throughout this research (Bellmann and Werr, 1992).

1. HB13---5' (TCGA)C(TG) (TCGA)C(TG) (GA)TT (TC)TG (GA)AA CCA 3'
2. OligodG$_{12}$---GGG GGG GGG GGG GGG GGG
3. OligodT$_{12}$----TTT TTT TTT TTT TTT TTT
4. zh3----ACACATCTTCTGCAGTACAC
5. zh5----GGGACGATGAAGTTTGTAG

B. Reverse Transcription to Synthesize First-Strand cDNA

The first strand of cDNA is synthesized by the RT (Reverse Transcription) method with two primers (HB13, OligodT): 5 µg of total RNA in 14 µl of water is heated at 80°C for 3 min and cooled rapidly on ice. Meanwhile, reverse transcription components are prepared on ice in 5x reverse transcription buffer (250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM dithioerythritol, pH 8.5), 2 µl of dNTPs, 0.25 µl (10 units) of RNasin (Promega, Madison, WI), and 1 µl of primer (100 ng/µl). After mixing reverse transcription components and total RNA, 1 µl (200 units) of MMLV reverse transcriptase (Promega, Madison, WI) is added and the mixture incubated for 2 hour at 37 C. The reaction

is stopped by adding 1 ml of TE. Unincorporated primer is removed using a Microcon-100 (Amicon, Beverly, MA). The Microcon-100 unit is spun for 15 min at 500 x g, 25°C, in a microcentrifuge (Baxter, Osterode, Germany). The filtrate is transferred to a new microtube and stored at 4°C.

C. Addition of Poly(C) Tail to cDNA

The poly(C) tail is added to the first single strand cDNA for polymerase chain reaction. Five µl of cDNA is placed in a microtube, with 2 µl of 5x TdT (Terminal deoxynucleotidyltransferase) buffer (500 mM cacodylate buffer, pH 6.8, 5 mM $CoCl_2$, 0.5 mM DTT), 1 µl of 15mM $CoCl_2$ (1.5 mM final), 1 µl of 1 mM dCTP (100 µmol final). Ten µl (30 units) of terminal transferase is added (Promega, Madison, WI). This mixture is incubated for 30 min at 37°C. The reaction is inactivated by heating 2 min at 65°C. Purification is performed using Microcon-100 as described above. The final pellet is dried.

D. Amplification of Maize Root cDNA by Polymerase Chain Reaction

The dried pellet is resuspended in 15 µl water. Three µl of single strand cDNA is transferred into a clean

microtube. PCR reaction buffer is prepared: 10x reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.4, and 1.0% Triton X-100), 1.5 M dNTP, 10% DMSO, and 25 pmol each of primers (HB13, OligodG, zh3, and zh5). The aliquot of cDNA is added to 100 µl of PCR reaction components. The mixture is heated in a PTC-150 MiniCycler (MJ Research, Watertown, MA) for 5 min at 98°C to denature the first strand products. To avoid mis-priming or dimerization of primers in the crucial first cycle of the PCR, a "hot-start" procedure is routinely used (Chou et al., 1992). After cooling to 75 C, 2.5 units of Taq polymerase is added. Thirty µl of mineral oil is overlayered. The first cycle temperature program (95°C, 3 min; temperature gradient from 95 C to 52 C, 0.1 C/sec; 72 C, 3 min) is carried out for 30 cycles of amplication. The second cycle program is performed (95°C, 1 min; 52°C, 1 min; 72°C, 3 min). The final extension cycle is 15 min at 72°C. The reaction mixture is cooled to room temperature. The aliquots of the PCR reaction products are analyzed on 1% agarose gels. Photographs are made of gel-separated PCR products.

E. Ligation of cDNA into Vector and Transformation for Cloning PCR Products in the TA Cloning System

For cloning PCR products, with the TA Cloning System (Invitrogen, San Diego, CA) is used per manufacturer's

direction. Two vials of pCR II vectors are centrifuged to collect all the liquid in the bottom of the vials. Two 2 µl (25 ng/µl) of pCR II vectors are allocated to two clean microtubes. Five µl of water, 1 µl of 10x ligation buffer (60 mM Tris-HCl, pH 7.5; 60 mM $MgCl_2$; 50 mM NaCl; 1 mg/ml bovine serum albumin; 70 mM β-mercaptoethanol; 1 mM ATP; 20 mM dithiothreitol; 10 mM spermidine), 1 µl of fresh PCR product (cDNA), and 1 µl of T4 DNA ligase is added to each tube. The reaction mixture is incubated at 14°C overnight. The reaction mixture is centrifuged briefly and placed on ice. Two vials of competent cells (INVαF') are thawed on ice. Two µl of 0.5 M β-mercaptoethanol is pipetted into each vial of competent cells. The mixture is stirred gently with a pipette. Two µl of each ligation mixture is transferred into vials containing cells and mixed gently. The cells are incubated on ice for 30 min. The cells are heat shocked for 30 seconds in a 42°C water bath and placed on ice for 2 min. Four-hundred and fifty µl of SOC medium (2% Tryptone; 0.5% Yeast Extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) is added to each vial. The vials are incubated at 37°C on a shaker at 225 rpm for 1 hour. Fifty µl of transformed cells are spread on LB plates with 50 µg/ml kanamycin and X-Gal (5-bromo-4-

chloro-3-indolyl-β-D-galactoside). The plates are incubated overnight at 37°C. The plates are then refrigerated for 2-3 hours for color development. Positive colonies are designated Pmrx1, 2, 3, 4.

SCREENING OF cDNA LIBRARY

Plasmid is isolated for enzyme restriction and PCR analysis (Ausubel et al. 1992). At least 20 white colonies are selected and cultured overnight in 5 ml LB broth containing 50 μg/ml of kanamycin (Sigma, St. Louis, MO). One-and-a-half ml of cultured cells are transferred to 1.5 ml microtubes and microcentrifuged for 1 min. The pellet is resuspended in 100 μl GTE (50 mM glucose, 25 mM Tris-Cl, pH 8.0; 10 mM EDTA) solution and incubated for 5 min at room temperature. Cell suspension is lysed in 200 μl NaOH/SDS solution. The lysed suspension is placed on ice for 5 min. One-hundred fifty μl of 5 M potassium acetate solution is added. The mixture is placed on ice for 5 min and microcentrifuged for 5 min. The supernatant is transferred to new tubes and the plasmid is precipitated with 100% ice-cold ethanol. Plasmid is collected by microcentrifuging the mixture for 5 min. The pellet is washed with 70% ethanol and dried under vacuum. The pellets are resuspended and collected in one tube (total volume: 100 μl). The plasmid

is stored in a -20 C freezer. Five µl of plasmid pool is used for EcoRI digestion. One µl of plasimd is used as a template for PCR screening with three primer sets (OligodT, zh3; HB13, OligodG; zh5, HB13) (Bellmann and Werr, 1992).

ANALYSIS OF THE GENOMIC DNA

Genomic DNAs isolated from maize root and shoot are digested with three restriction enzymes (Hind III, EcoR I, BamH I) and run in the 0.8% agarose gel, 80V, for 90 min. Gels are photographed on a UV illuminater. The DNAs in the gel are depurinated in 0.25 N HCl for 10 min, denatured in 0.5 M NaOH/1.5 M NaCl and neutralized in 1.5 M NaCl/0.5 M Tris (pH 7.4). The gel is placed on Zeta-Probe GT membrane (Bio-Rad, Richmond, CA). The Zeta-Probe GT membrane is placed on a thin stack of Whatman 3 MM filter paper. DNA is transferred under vacuum to the nylon membrane. The DNA is UV cross-linked to the nylon filter using the UV Stratalinker 1800 (Stratagene, La Jolla, CA) with 120,000 µjoules. The filter is pre-hybridized for one hour in a sealed bag containing hybridization buffer (7% SDS, 1% Bovine serum albumin, 0.5 M $NaHPO_4$, pH 7.2, 1 mM Na_2EDTA). The DNA is hybridized with random labelled and denatured maize root cDNA fragment, overnight at 65 C. The filter is washed twice in 100 ml of 2x SSC (300 mM NaCl, 30 mM sodium

citrate) at room temperature (5 min, with agitation). The filter is washed twice in 200 ml of 1% SDS in 2x SSC (65°C for 30 min), with agitation. Finally the filter is washed twice in 100 ml of 0.1x SSC (room temperature for 30 min) with agitation. The filter is blot dried and exposed to x-ray film at -70°C for a week prior to development.

EXPRESSION ANALYSIS OF cDNA IN ROOT AND SHOOT OF MAIZE

Each 20 µg aliquot of total RNAs isolated from maize root and shoot is mixed with Northern reagents (5x MOPS buffer, 3.5 µl of Formaldehyde, 10 µl of Formamide), heated in 55°C water bath for 15 min, and cooled on ice. One gram of agarose is melted in 31 ml of DEPC-treated water, cooled to 55°C in water bath, and mixed with 9 ml of formaldehyde (Sigma, St. Louis, MO) and 10 ml of 5x MOPS buffer (0.2 M MOPS, pH 7.0; 0.05 M Na acetate; 0.005 M EDTA). The agarose solution is poured into a gel casting box and the agarose is cooled under a fume hood. The RNA size marker (Life Technologies, Gaithersburg, MD) and The samples are mixed with 2 µl of loading buffer and 1 µl of ethidium bromide (1 mg/ml). The markers and samples are loaded on the gel. The gel is run for about 10 min, and the back of the gel is cut off to remove excess ethidium bromide. The gel is run for an additional 2-3 hours at approximately 80 volts. The gel

is photographed with a ruler placed next to the gel on the UV illuminater. The gel is washed in several changes of water to remove formaldehyde. The gel is placed on four sheets of Whatman 3MM paper in an inverted gel casting tray. One of the sheets of filter paper overhangs the bottom of the gel tray. The 3MM paper is saturated with 0.4 M NaOH. NaOH is poured into a deep dish so that the 3MM wick ends are immersed in NaOH. The Zeta-probe GT membrane prewetted with distilled water is lowered onto the gel surface. Bubbles trapped between gel and membrane are removed. The two pieces of 3MM papers are placed onto the Zeta-probe GT membrane. A stack of pre-cut paper towels is placed on the 3MM/membrane/gel stack. A glass plate is placed on top of the paper towels. A thick catalog is placed on the top of the glass plate to maintain pressure. Capillary transfer is continued overnight. The membrane and gel are separated. The membrane is rinsed in 2x SSC. The nylon membrane is UV cross-linked, prehybridized immediately in a plastic bag containing prehybridization solution (50% formamide, 0.12 M Na_2HPO_4, pH 7.2, 0.25 M NaCl, 7% SDS) at 43°C for 10 min. Hybridization is performed by adding random primer-labeled maize root cDNA fragments (overnight at 45°C). The filter is washed twice in 2x SSC/0.1% SDS for 10 min at room temperature, once in 0.5x SSC/0.1% SDS at room temperature for 15 min, and once for 15 min in 0.1x SSC/0.1% SDS at

65°C. The filter is exposed to x-ray film at -70°C for a week.

PARTIAL SEQUENCING OF MAIZE ROOT cDNA

Ten µl of plasmid is denatured using the alkaline method (Ausubel et al., 1992) for double-stranded sequencing. The plasmid DNA is denatured by adding 0.1 volumes of 2 M NaOH/2 mM EDTA, mixed by pipette, and incubated 5 min at 37°C. The mixture is neutralized by adding 0.1 volumes of 3 M sodium acetate. The neutralized mixture is purified by centrifugation in a Micron-100 filter. Double-stranded plasmid DNA is sequenced using the dideoxy chain termination method (Sanger et al., 1977) and Sequenase version 2.0 (United States Biochemical, Cleveland, OH). The denatured plasmid DNA is annealed with M13 (-40) forward primer (5'-GTTTTCCCAGTCACGAC-3') (Sanger et al., 1977) by heating 2 min, 65°C, then cooling slowly to <35°C. Termination mixture (G, A, T, and C) is prepared according to the manual supplied by the manufacturer. The labeling reaction is carried out by adding labeling reaction mixture (DTT, 0.1 M; Diluted labeling Mix; [^{35}S]dATP; Sequenase) to the ice-cold annealed DNA mixture. The reaction solution is mixed, and incubated at room temperature 2-5 min. The labeling reaction solutions are transferred to termination tubes (G, A, T, and C), mixed, and incubated at 37°C for 5

min. The termination reaction is quenched with stop solution (95% Formamide; 20 mM EDTA; 0.05% Bromophenol Blue; 0.05% Xylene Cyanol FF). The quenched mixture is heated at 75°C for 2 min. Samples are immediately loaded onto 6% acrylamide/8 M urea sequencing gel. The gel is run for 1 hour for initial movement of samples from the wells into the gel. The loaded gel is run at 200 amps for 5-6 hours. Subsequently, the gel is soaked in 5% acetic acid/15% methanol to remove the urea. The gel is dried at 80°C for 2 hours, exposed to Kodak XAR-5 film at -70°C, frozen for 2 days, and developed. The DNA sequencing data obtained is analyzed using National Center for Biotechnology Information (NCBI) BLAST software.

EXPRESSION OF MAIZE ROOT cDNA IN *Nicotiana tobacuum*

A. Subcloning Maize Root cDNA into the GUS Gene Fusion Binary Vector pBI 121

Maize root cDNA fragment that was EcoR I digested from the library is collected and purified as described previously. For ligation of this cDNA into pBI 121 binary vector (Clontech, Palo Alto, CA), two linkers are converted into the adapter which has two restriction sites (EcoR I, BamH I). Two 16 mers (EI: 5'-AAT TCG AAC CCC TTC G-3', BI: 5'-GAT CCG AAG GGG TTC G-3') are purchased from commercial vender (IDT, Coraville, IA). The conversion adapters to

clone maize root cDNA into pBI 121 are modified from the original procedure (Stover et al., 1987). Two oligonucleotides are mixed in equimolar portions (EI and BI), heated to 95°C for 5 min, and slowly cooled to room temperature. Converted adapter mixtures are added to 2.5 µg of pBI 121 digested with BamH I in ligation buffer (60 mM Tris-HCl, pH 7.5, 60 mM MgCl2, 50 mM NaCl, 1 mg/ml Bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol, 10 mM spermidine). One µl (4 units) of T4 DNA ligase (Invitrogen, San Diego, CA) is added prior to incubation at 16°C for overnight. Unincorporated adapters are removed with Microcon-100 filter. Ligation of maize root cDNA containing EcoR I overhang and pBI 121 vector containing EcoR I adapter is performed in 1 µl of ligation buffer with 1 µl of T4 ligase (4 units) at 16°C for overnight. To confirm ligation, the clone is digested with EcoR I or BamH I and run on 0.8% agrose gel for 90 min at 80 volts. The gel is stained with ethidium bromide and photographed on the UV illuminater.

B. Transformation of Recombinant Maize Root DNA into Agrobacterium

Electroporation is used for transforming of the plasmid into Agrobacterium. Commercial Agrobacterium tumefaciens

LBA4404 (Life Technologies, Gaithersburg, MD) containing disarmed Ti plasmid pAL 4404 with only the *vir* and *ori* region of the Ti plasmid (Hoekema et al., 1983) is engineered for electroporation. The recombinant DNAs are transferred from Agrobacteriuim tumefaciens cells into plant cells using the plasmid pAL 4404 (Jefferson et al, 1987). Recombinant maize root DNAs are purified several times using Microcon-100 to achieve maximal transformation efficiency. The purified samples are stored on ice. Four ElectroMax A. tumefaciens LBA4404 samples are removed from the -70°C freezer and thawed on wet ice. Electroporator Proporator II (Hoefer, San Francisco. CA) is set at 18kv/cm, 1 msec, 5 bursts. DNA and Agrobacterium cells are placed into the electroporator chamber. Electroporation is carried out on ice. The samples are placed into culture tubes containing 1 ml YM medium (0.04% Yeast extract, 1.0% Mannitol, 1.7 mM NaCl, 0.8 mM $MgSO_4.7H_2O$, 2.2 mM $K_2HPO_4.3H_2O$). The cells are cultured at 30°C for 3 hours, shaking at 225 rpm. One-hundred µl of the cultured cells are spread on YM plates containing 100 µg/ml streptomycin and 50 µg/ml kanamycin (Sigma, St. Louis, MO). The plates are incubated 48-56 hours at 30 C. Selected colonies are cultured as described above for long-term storage (-70°C) and streaked on plates containing appropriate antibiotics.

C. Leaf Disc Transformation

The procedure used for transformation is the Horsh method (Horsh et al., 1985). The healthy, unblemished leaves from greenhouse-grown Nicotiana tabacum are collected and sterilized in 50% bleach (Chlorox) solution for 10 to 15 min with gentle agitation. Tissue is rinsed several times with sterile distilled water. The sterilized leaves are chopped into small pieces (about 1 cm x 1 cm). Tissue sections are placed in 100-mm petri dishes containing 5 ml of callus induction medium (Murashige and Skoog's basal medium, 2 mg/L of IAA, 0.2 mg/L of Kinetin and 1 g/L Casein hydrolysate; Sigma, St. Louis, MO). After 1 or 2 days, five pieces of leaf sections per petri dish are inoculated with 50 µl of Agrobacterium culture. The Agrobacterium culture was grown overnight in YM medium containing 25 µg/ml of streptomycin and 50 µg/ml of kanamycin. The petri dishes are incubated for 2 to 4 days at 28°C in the dark. The plant tissue is washed several times with MS medium containing 250 µg/ml of cefotaxime (Sigma, St. Louis, MO). The tissue is transferred to selective shoot inducing media (0.5 mg/L Kinetin, MS basal medium) containing 1 mg/ml of carbenicillin and 50 µg/ml of kanamycin sulfate (Sigma, St. Louis, MO). After 3 or 4 weeks, the regenerated plants

including embryoids are collected (Gill and Saxena, 1993) for immediate histochemical analysis and determination of growth regulator responses.

HISTOCHEMICAL ANALYSIS

The basic method (Jefferson, 1987) is modified for convenience. The regenerated plants are fixed in 0.05 M sodium phosphate buffer (pH 7.0) containing 2-3 glutaraldehyde (LADD Research Industries, Burlington, VT) for 30 min. Fixed tissue is rinsed with phosphate buffer. GUS (β-Glucuronidase) assay solution is prepared with 100 mM sodium phosphate (pH 7.0), 100 mM potassium ferricyanide, 100 mM potassium ferrocyanide (Mallinckrodt, Paris, KY); the solution is stored in the refrigerator (4 C). The substrate, 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GLUC, Sigma, St. Louis, MO), is dissolved in dimethyl formamide (Sigma, St. Loius, MO) at 100 mg/ml. The final concentration of the substrate solution is 10 mM phosphate buffer, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1 mM X-GLUC. The plant materials are incubated in the substrate solution at 37°C for overnight. When the tissues exhibit an intense blue color, the reaction is terminated by replacing the substrate with sodium phosphate buffer for 5 min. The tissues are stored in 100

ethanol at -20°C. Stained tissues are photographed with an automatic camera (Olympus PM-C358, Japan) and attached microscope (Leica Wild M10, Swiss). Root tissues which express the GUS gene are slowly warmed to room temperature, placed in toluene for two hours, and then transferred to molten paraffin, (Ameraffin; America Scientific Products, McGaw Park, IL) for overnight. Jung Histocut (Leica 820-II, German) is used for sectioning the roots. Sectioned tissues are photographed with the automatic camera (Olympus PM-C358, Japan) mounted on a microscope (Olympus BX-40, Japan).

ANALYSIS OF HORMONAL RESPONSES

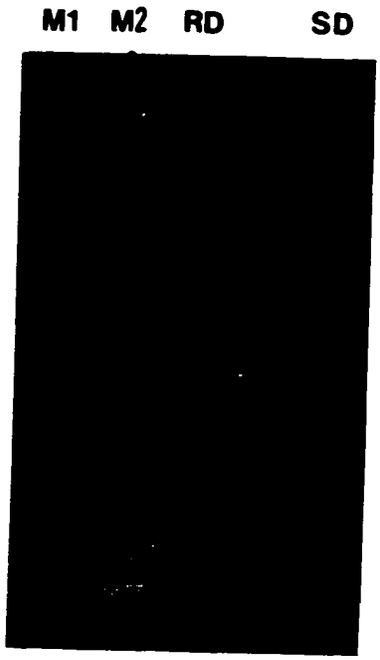
Regenerated plants which have been cultured for at least 3-4 weeks are transferred to the MS basal medium. Embryoids and leaves are excised and placed on MS medium containing IAA (2 mg/L) or Kinetin (0.5 mg/L) for 24 hr. The explants are fixed, stained, and photographed as described above.

RESULTS

ISOLATION OF GENOMIC DNA FROM MAIZE ROOTS AND SHOOTS

Isolation and purification of plant DNA is difficult because of the presence of a high concentrations of polysaccharide. Genomic DNA isolation from maize seedling roots is especially difficult due to high concentrations of nuclease (Jones and Boffey, 1984) as well as polysaccharide. Additionally, roots contain less DNA when compared with other tissues. A modified method is used for isolation of genomic DNA from maize roots and shoots (Riven et al., 1982; Dellaporta et al., 1983; Guidet et al., 1990; Do and Adams, 1991; Ausubel et al., 1992; Luro and Laigret, 1995). The products are analyzed on an 0.8% agarose gel. Genomic DNA fragments, approximately 35 kb, are isolated from both tissues (Fig. 1). The nuclear genome of corn is 5.5 to 6.0 pg per haploid genome based on the reassociation kinetics of single-copy DNA (Hake and Walbot, 1980). This is of medium size in comparison to other plants and about twice the size of mammalian genomes. In units of base pairs the corn genome is about 5 X 10^{8} kilobase pairs and around 20% of the genome is highly repeated sequences. This indicates that a reasonable chance exists for recovery of an individual recombinant gene (Maniatis et al., 1982).

Figure 1. Isolation of Genomic DNA from Maize Roots and Shoots: Lane M1, M2-molecular size markers, Lane RD-Maize Root genomic DNA, Lane SD-Maize shoot genomic DNA. 32kb genomic DNAs are isolated from both root and shoot.



ISOLATION OF TOTAL RNA FROM MAIZE ROOTS AND SHOOTS

Total RNA for maize seedling roots and shoots is isolated by following current protocols in molecular biology (Ausubel et al., 1992) and run on formaldehyde 1% agarose gel. The results are shown in Figure 2. Yields of total RNAs are suitable for use in Northern blot assays.

AMPLIFICATION OF THE cDNA FRAGMENT BY RT-PCR

Total RNA from maize roots is reverse transcribed with homeobox α3 region primer HB-13 or OligodT$_{12}$. These reactions are modified with terminal deoxynucleotidyltransferase (adding dCTP to 5' end), and directly used as the templates for PCR reactions with specific primers, OligodG$_{12}$, HB-13, Zh3, and Zh5. The "hot start" method improves the sensitivity and specificity of the PCR (Sparkman, 1992), However, when amplifying extensively-degraded target DNA the amplification is less efficient. Negative controls, repetition of tests, and partial sequence analysis are applied for control of contamination. The products of PCR are analyzed on a 1% agarose gel. Two different fragments are amplified and one is amplified non-specifically as designated (Fig. 3).

Figure 2. Isolation of Total RNA from Maize Roots and Shoots: Lane M-RNA marker (range-0.36-9.49kb), Lane Rr-total RNA of Maize roots, Lane Sr-total RNA of Maize shoots. First bands are 28S (4.4kb) and second bands are 18S (2.37kb).

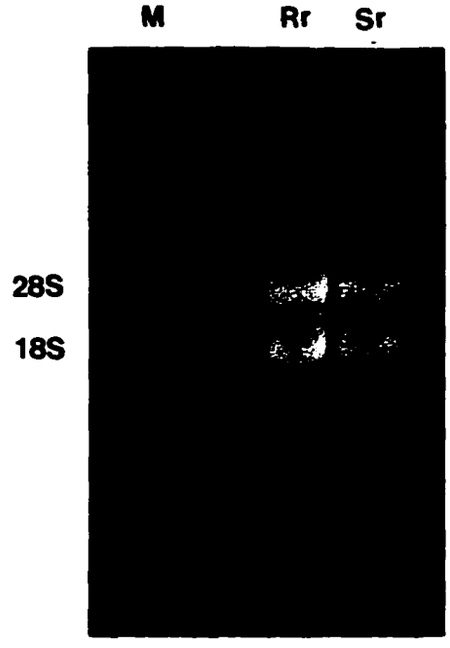
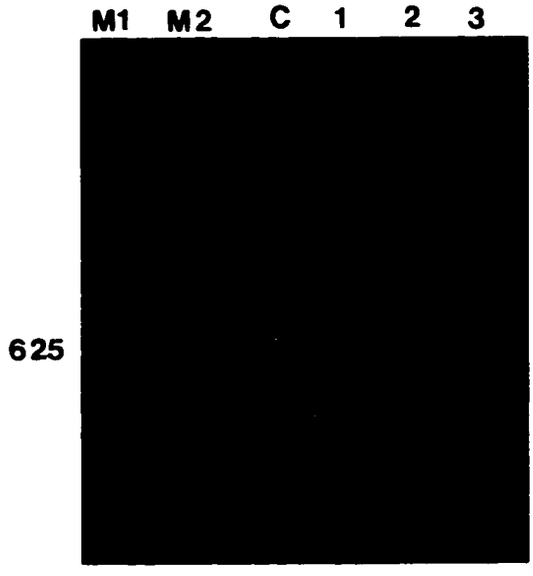


Figure 3. Amplification of the cDNA Fragment by RT-PCR: Lane M1-Lambda DNA/Hind III marker for the large size molecule, M2-pBR322 DNA/Msp I marker for the small size molecule, Lane C-negative control, Lane 1-non-specific amplification with primer zh3 and $OligodT_{12}$, Lane 2-specific amplification with primer HB13 and $OligodG_{12}$ (625 bp), Lane 3-specific amplification with primer zh5 and $OligodG_{12}$.



ANALYSIS OF THE MAIZE GENOMIC DNA

Plasmid DNA from a positive colony is isolated using alkaline lysis. The inserted DNA is digested as previously described and analyzed by restriction enzyme digestion and PCR. The insert is 630-bp cDNA. PCR amplified a similar size cDNA. The genomic DNAs of roots and shoots are digested with restriction enzymes, run on the agarose gel and transferred to Zeta-Probe GT filter paper. Prehybridization is performed; hybridization is followed with random labeling of denatured maize root cDNA as previously described. Hybridization of the genomic DNAs of roots and shoots shows that the cDNA is a single-copy gene (Fig. 4).

EXPRESSION ANALYSIS OF MAIZE cDNA IN ROOT AND SHOOT OF MAIZE

Total RNAs from maize roots and shoots are isolated, run on the formaldehyde gel, and transferred to the Zeta-Probe GT membrane. After hybridization, the filter is exposed to x-ray film and developed. The root total RNA is expressed as two distinct bands. The shoot total RNA is expressed as one band which is the same size as one of root total RNA bands (Fig. 5). This result shows that the cDNA is root specific and expressed in two distinct RNAs.

Figure 4. Southern Blot Analysis of the Maize Genomic DNA: Lane SH and RH-Maize genomic DNA from shoots (SH) and roots (RH) digested with Hind III, Lane SE and RE-digested with EcoR I, Lane SB and RB-digested with BamH I. Each side shows DNA marker-Lambda DNA/EcoR I + Hind III. Maize root cDNAs (Mrxs) are hybridized to maize root and shoot genomic DNA at high stringency and analyzed by autoradiography. Each lane shows a single copy gene.

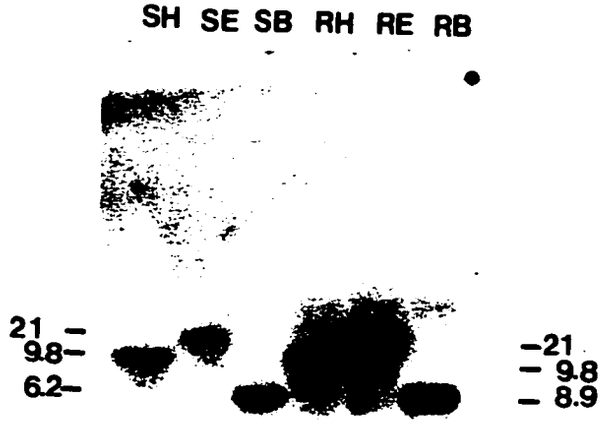
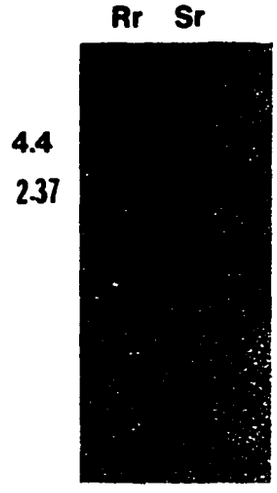


Figure 5. Expression Analysis of Maize cDNA in Roots and Shoots of Maize: Total RNA is extracted from maize roots and shoots. Each twenty microgram sample of total RNA is run on 1 formaldehyde agarose gel and transferred to the Zeta-probe GT membrane. The membrane is hybridized with maize root cDNA probe and exposed to x-ray film. Lane Rr-shows two different bands, Lane Sr-shows only one band corresponding to the first band of Rr.



CLONING AND PARTIAL SEQUENCING OF THE MAIZE ROOT cDNA FRAGMENT

The 630-bp cDNA fragment obtained from maize root RNA RT-PCR amplification is ligated directly with the pCR II vector system and transformed into competent cells (INVαF'). Positive colonies are selected by antibiotic (Kanamycin) and X-Gal. Even though the efficiency of transformation is low, several positive colonies are selected. Minipreps of DNA are prepared by alkaline lysis and analyzed by EcoRI (Fig. 6). Double-stranded DNA is isolated from positive plasmids using alkaline lysis and sequenced using the Sanger method (Sanger et al., 1977). DNA sequence information obtained is compared with other sequences using the BLAST software program from NCBI. The sequence of maize root RT-PCR product is not homologous to other homeobox gene sequences, but comparison of amino acids translated from the gene sequence indicates that the sequence is homologous to atypical homeodomains for a small percentage of each helix (Fig.7).

SUB-CLONING AND TRANSFORMATION OF MAIZE ROOT GENE

Maize root cDNA is ligated with binary vector pBI 121 using the conversion adapters, and transformed into commercial Agrobacterium using electroporation. After

Figure 6. Restriction Enzyme (EcoR I) Analysis of Maize Root cDNA: Lane M-DNA size marker, Each lane is run with miniprep cDNA digested with EcoR I. Ten randomly selected, positive colonies show cDNA insertion. The first, second, and eight lanes show exact size inserts (630 bp).

M

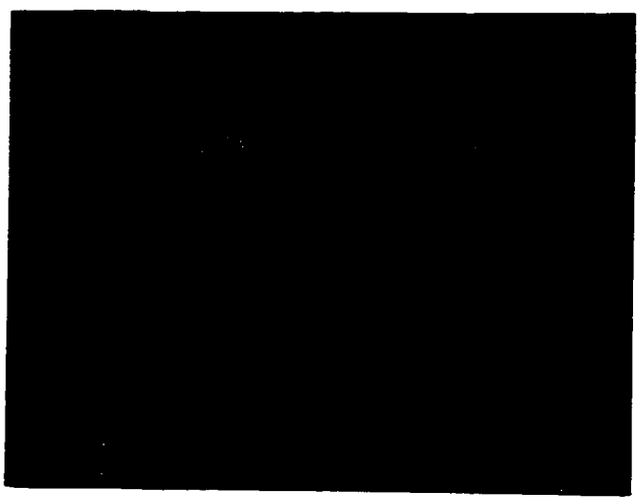
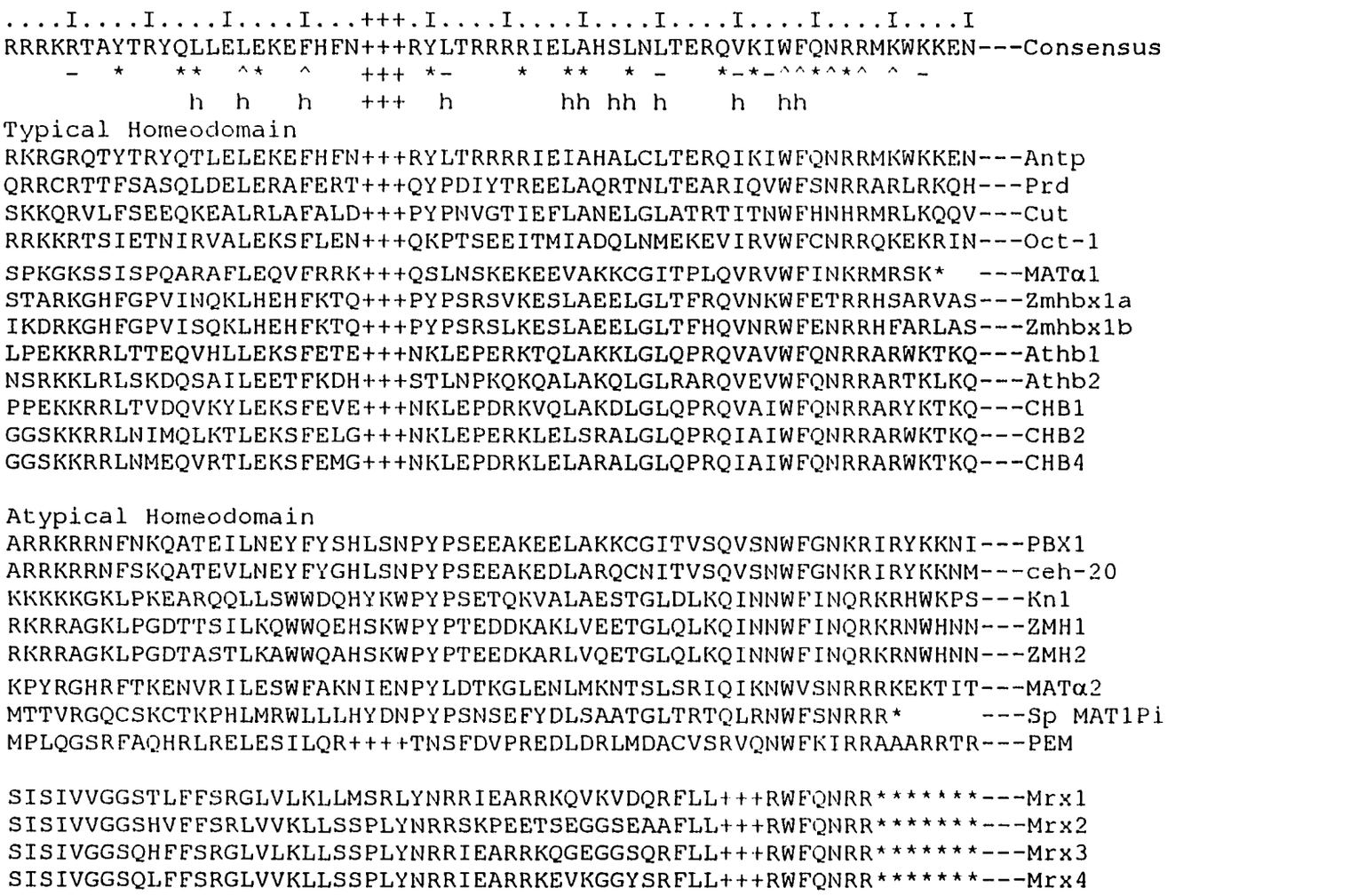


Figure 7. Alignment of Atypical and Typical Homeodomain Sequences: Symbol (+) represents the gaps introduced for optimal sequence alignment. Symbol (h) represents hydrophobic core that is responsible for the tertiary structure of the homeodomain. Symbol (^) is used for the most conserved amino acids. Symbol (-) is for highly conserved regions. Symbol (*) is for conserved positions. Compared with atypical homeodomains, the Mrx series cDNA show around 17 identities.



antibiotic screening the positive colonies are confirmed with the PCR (Fig. 8).

LEAF DISC TRANSFORMATION

Leaf disc transformations are carried out using Nicotiana tabacum sterilized with bleach. Control Nicotiana tabacum grows well and shows typical leaf and root shapes (Fig. 9). Embryoids develop from leaf segments (Fig.10); two leaves and adventitious roots differentiate from embryoids within two weeks.

Several experiments are performed to examine cells infected with Agrobacteria carrying maize root cDNA. Transformed Nicotiana tabacum leaves are screened with carbenicilin and kanamycin. Leaf tissue is grown under conditions required to induced embryoids. Once callus appears on leaf segments, enhanced root development occurs. One or two roots develop per leaf segment. Shoot development is severely inhibited or delayed. After two weeks, roots undergo homeotic changes which result in a long linear shaped mass between the meristematic region (Fig. 11) and root cap. Shoots develop leaves with altered shapes, sizes, and numbers (Fig. 12).

Figure 8. PCR Screening of Maize Root cDNA Transformants: with two primers, HB13 and OligodG[illegible], isolated supernatant from transformants is amplified by the PCR method. A total of ten samples were run on the 1[illegible] agarose gel and Ethidium Bromide stained, then photographed. Lane 4 is the strongest candidate for the next experiment.

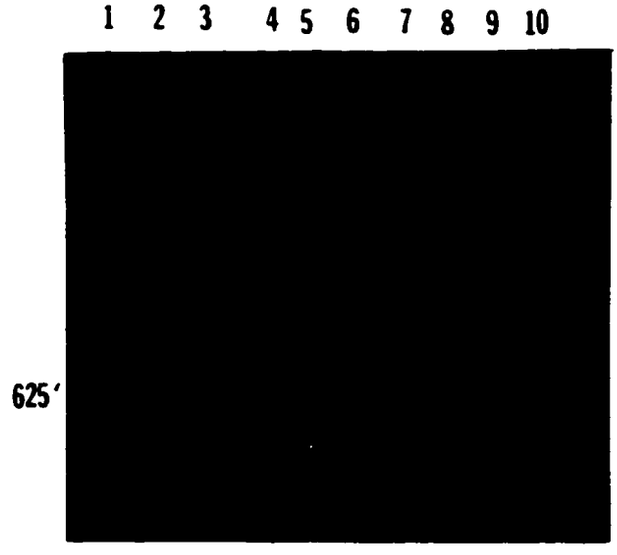


Figure 9. Regenerated *Nicotiana tabacum*: Control tobacco plants are treated with callus induction medium. The regenerated plants show typical leaf and root morphology.

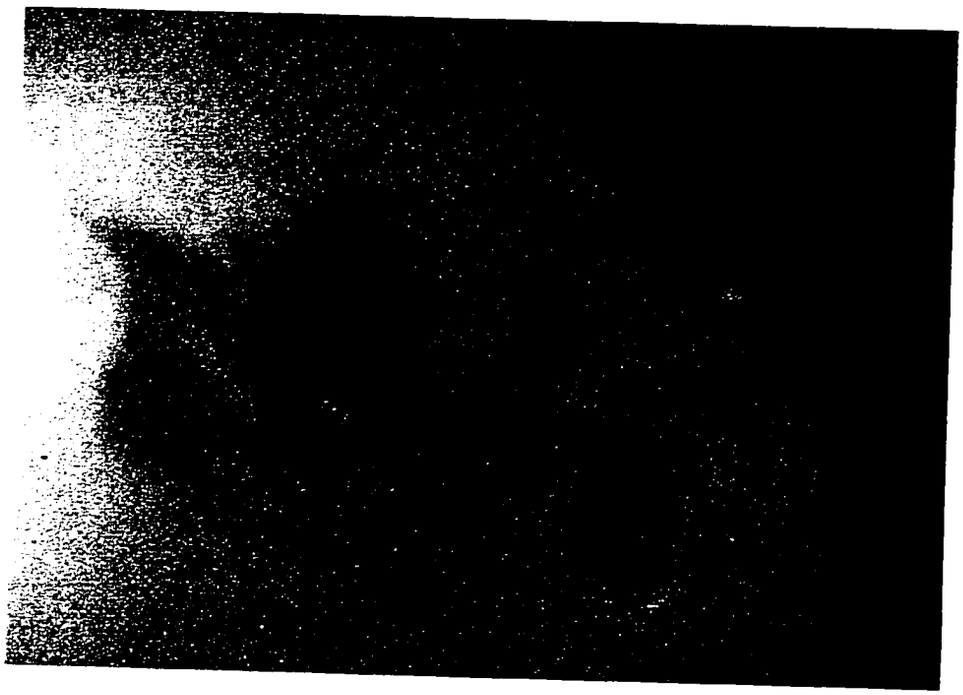


Figure 10. Typical Embryoid Regenerated from Tobacco Tissue: Compared with a recent report of somatic embryogenesis in Nicotiana tabacum (Gill and Saxena, 1993), it has basic embryoid morphology.

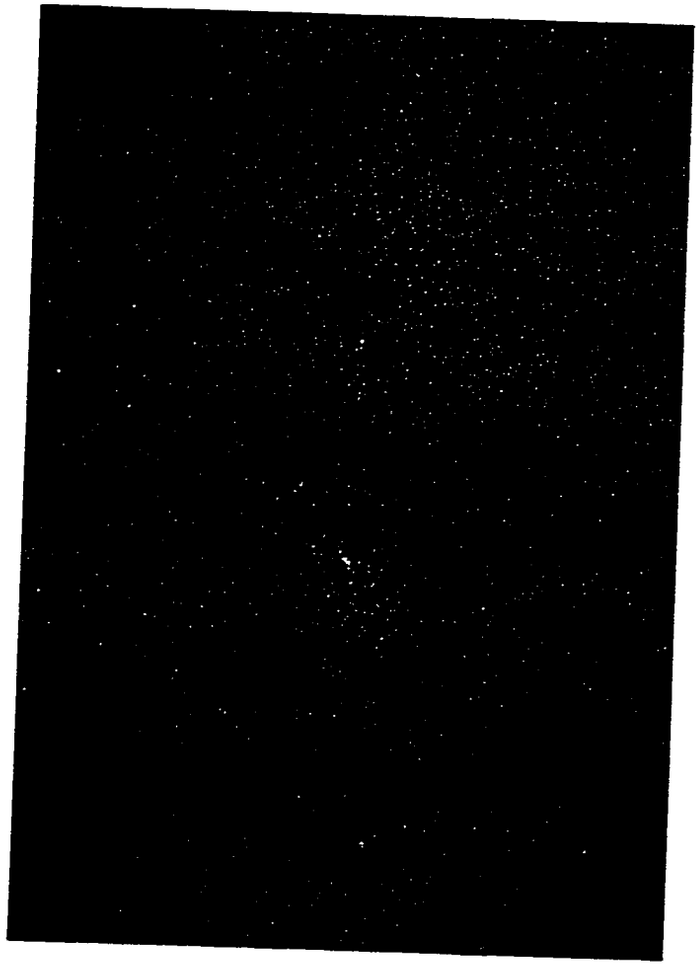


Figure 11. Close-Up Tobacco Root Development: This photograph illustrates a drastic morphological change between the meristematic zone and root cap. Gus is expressed only in the meristematic region.

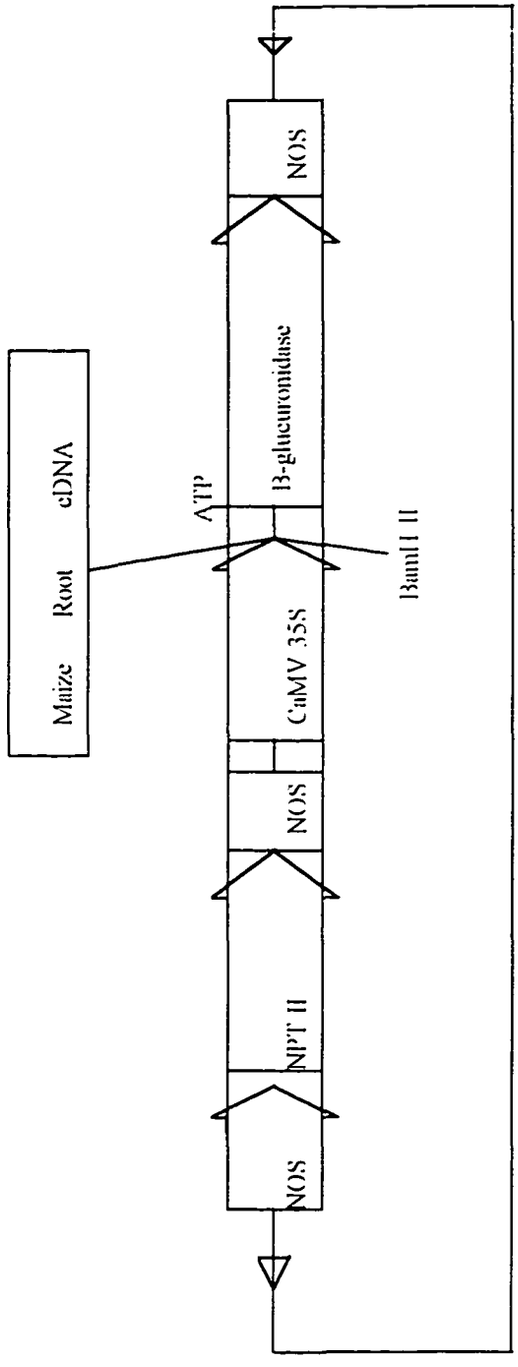
Figure 12. Variety of Regenerated Tobacco Plants: All three pictures show plants with modified morphology; overgrown roots, jar-type leaves, and different numbers of tissues or organs.

HISTOCHEMICAL ANALYSIS OF GENE EXPRESSION

Many higher plants, including tobacco, have endogenous β-glucuronidase activity which is below the limits of detection (Jefferson et al., 1987). It is possible to use the GUS gene fusion system as a general purpose vector by ligating the coding region of GUS 5' of the nopaline synthase polyadenylation site to the polylinker site of the pBIN19 (Bevan, 1984). This vector, pBI121 (Fig. 13), is very similar to pBI101 except it contains the 35S promoter from Cauliflower Mosaic virus (CaMV). The pBI121 vector contains unique restriction sites for HindIII, SphI, and PstI, upstream of the CaMV 35S promoter. XbaI, BamHI, and SmaI are downstream of the CaMV 35S promoter. CaMV 35S is an 800-bp promoter fragment.

The maize root cDNA is fused to pBI121. Nicotiana tabacum plants are transformed with Agrobacterium binary vectors containing maize root cDNA. Several kanamycin-resistant plants are regenerated from each transformation. Four transformants are chosen for further study. All parts and organs of plants from transformation are fixed. X-GLUC is the preferred substrate for histochemical staining of β-glucuronidase. X-GLUC is readily detected at low concentrations. The final cleavage product (ClBr-indigo) exhibits aqueous stability (insolubility). During the reaction 5-bromo-4-chloro-3-indoly-β-D-glucuronide is

Figure 13. Map of pBI121 and cDNA Insertion: It contains 35S promoter from Cauliflower Moasic Virus (CaMV) and β-Glucuronidase (GUS). NOS-Nopaline synthase gene. NPT II-Neomycin phosphotransferase II.



cleaved into two final products. One of the products is oxidized for dimerization (dichloro-dibromoindigo) and precipitation (Fig. 14). Precipitation of the final product is a very useful tool for cellular localization of enzymatic activity. Roots which express GUS genes are embedded in paraffin, sectioned, and photographed.

This analysis shows that the gene is specific for roots. Controls do not show any colored reaction product (Fig. 15). Transformed tissue shows localized, intense colors in the root area (Fig. 16). GUS activity is found in areas where meristematic cells are actively dividing. Sectioned roots provide more details of the tissues. Only the root epidermis is colorized near the root cap (Fig. 17). The root cap is not colorized (Fig. 18). Differentiated tissues in older regions of the root show color as a band (Fig. 19), even though some parts of shoot and leaf show isolated spots of color, tissues which express GUS do not show active shoot development (Fig. 20). This may indicate that expression of a root-specific gene inhibits further development of shoots.

ANALYSIS OF HORMONAL RESPONSES

After three weeks, embryoids and fully-developed leaves from transgenic tobacco are collected. These explants are treated on MS basal medium containing IAA or Kinetin for

twenty-four hours. The plant materials are fixed and photographed (Fig. 21). Embryoids treated with IAA exhibit inhibition of GUS gene expression, but trichomes exhibit GUS reaction products (Fig. 22). Kinetin treatment does not inhibit GUS gene expression, but enhances expression (very clear blue color) throughout the whole tissue. Trichomes are not formed with this treatment (Fig. 23). These results indicate that at an early stage of development, high concentrations of IAA cause inhibition of root gene expression. IAA plays a role in expansion of cells during early stages of development; this expansion may regulate morphogenesis of the shoot and radicle. Kinetin triggers cell division and polarity. The control culture exhibits GUS expression in leaf vein area exhibiting the most meristematic acivity (Fig. 24).

Figure 14. The Procedure of Cleavage of X-GULC (5-bromo-4-chloro-3-indolyl-β-D-glucuronide): The substrate (X-GLUC) breaks down to glucuronic acid and ClBr-indigo. At the final step insoluble blue dichloro-dibromoindigo (ClBr-indigo) forms through the oxidative dimerization (Stomp, 1992).

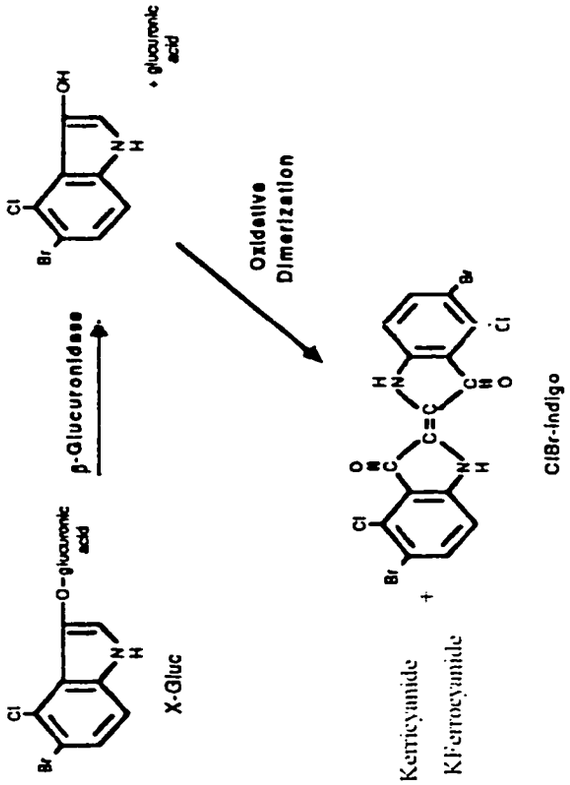


Figure 15. Control of Regenerated Tobacco Plants: There is no GUS expression and no endogenous GUS activity.

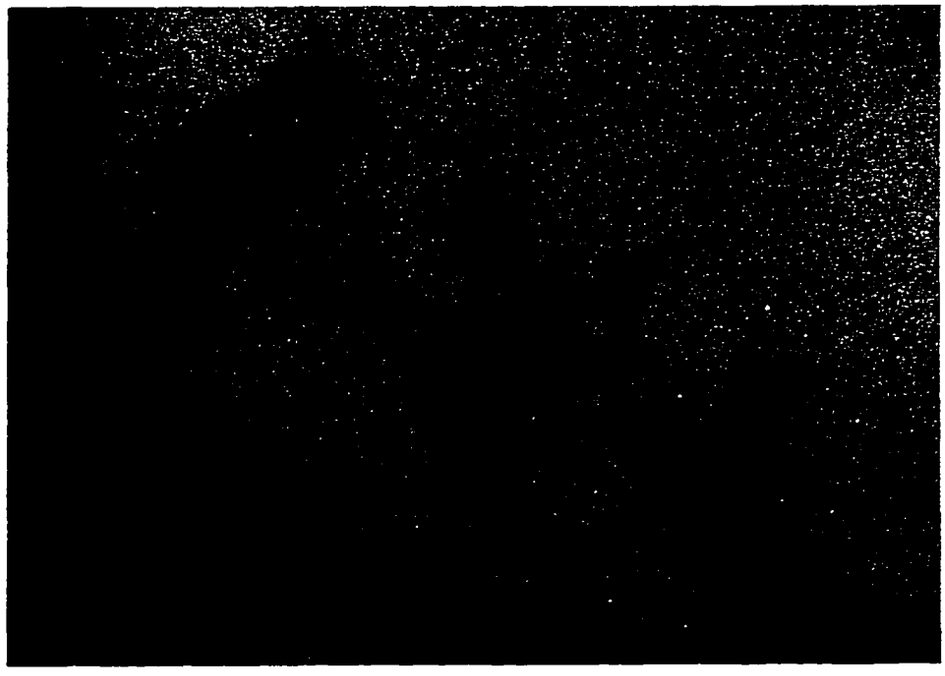


Figure 16. Root Specific GUS Expression: Blue staining is found only in the root area. The other area (leaf segment) exhibits no GUS expression.

Figure 17. The Cross-Section of Root Meristematic Region: Even though it does not clearly show root structure it does show the root epidermis (blue color).

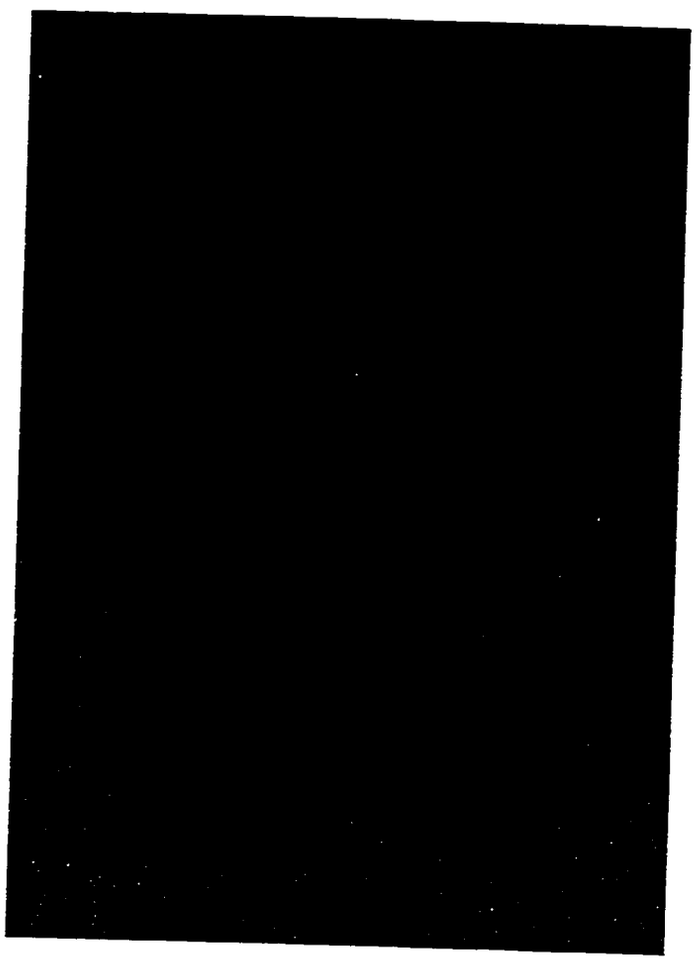


Figure 18. Cross-Section Near The Root Cap: This section shows tissues such as protoxylem, but no GUS expression.

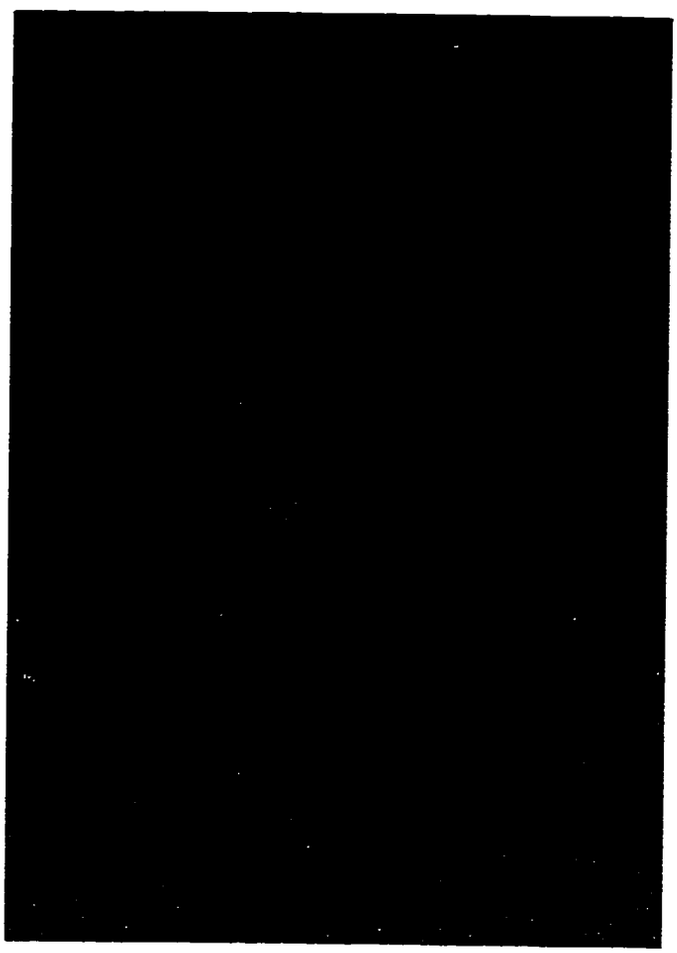


Figure 19. Close-Up of Tobacco Root: GUS expression is localized as a blue band on the root.

Figure 20. GUS Expression on Putative Shoot Meristematic Area: Tissue in this illustration is primarily root. Putative shoot meristem is inhibited and exhibits GUS expression as a blue dot.

Figure 21. The Transgenic Tobacco on MS Basal Medium: This photo illustrates regeneration and GUS expression on leaf vein area.

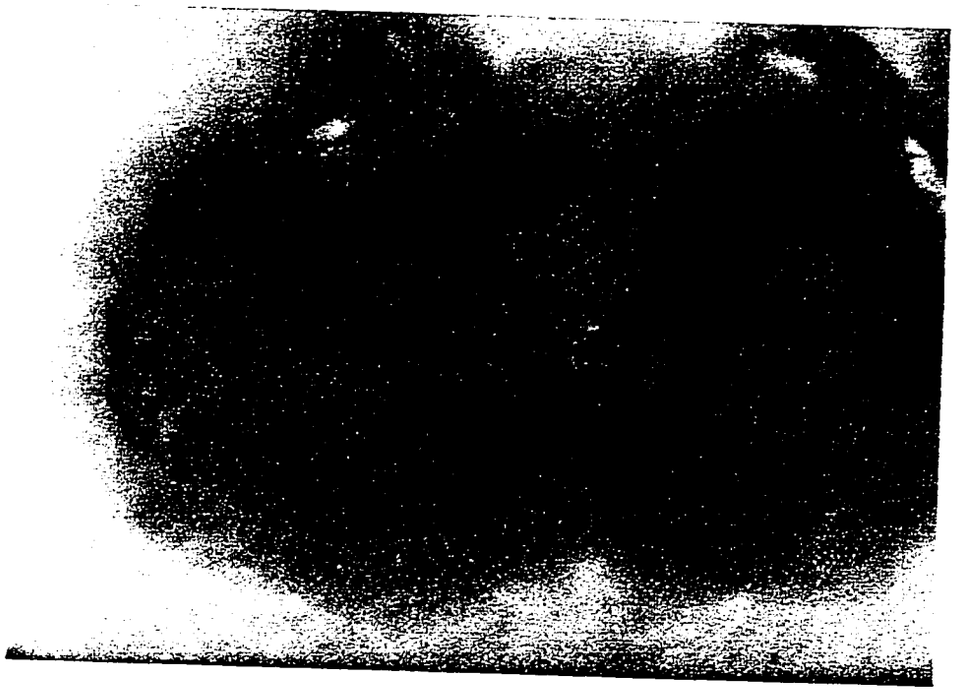


Figure 22. Tobacco Embryoid Treated with IAA: There is no GUS expression on embryoid, but trichomes exhibit GUS reaction products.

Figure 23. Tobacco Embryoid Treated with Kinetin: This plant growth regulator activates intense GUS expression on embryoid, but no trichomes are formed.

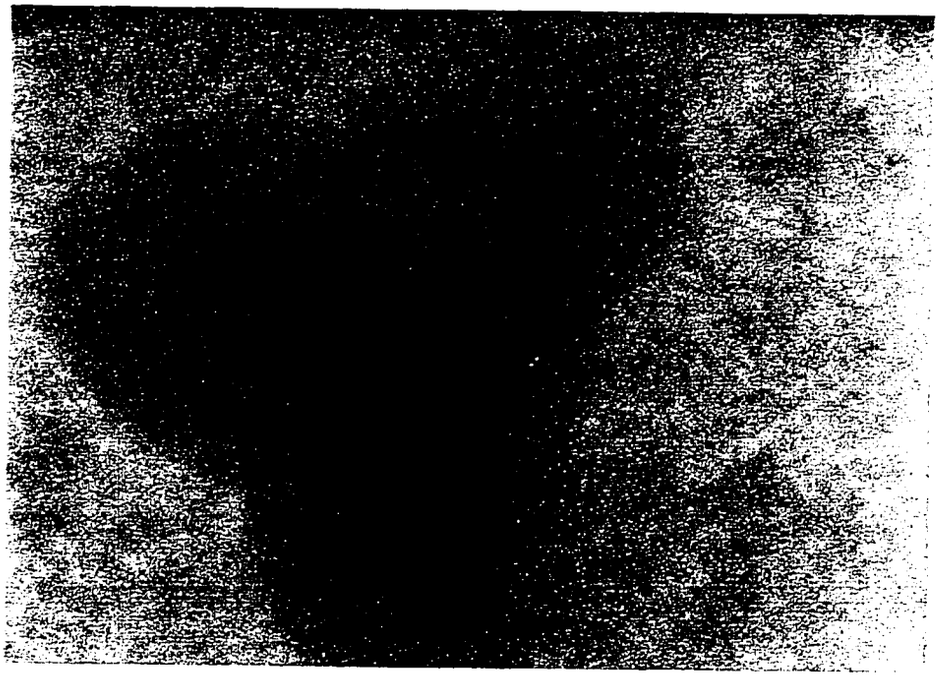


Figure 24. Regenerated Leaf Segment on MS Basal Medium: GUS expression is found mainly on leaf vein areas.

DISCUSSION

PRESENCE OF A ROOT SPECIFIC HOMEOBOX GENE

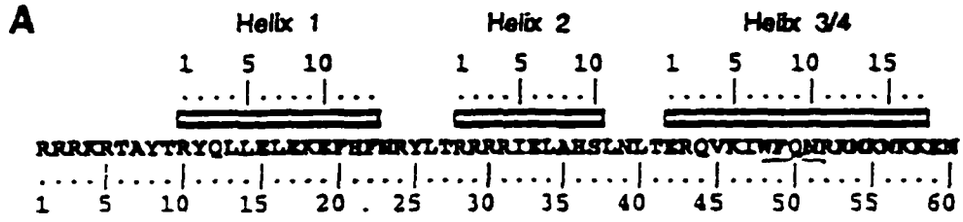
Total RNA analysis shows that maize root cDNAs are expressed in two mRNAs, one of 4.4 Kb and an other of 2.37 Kb. In shoots, total RNA maize root cDNAs are expressed in a single band at 4.4 Kb. This suggests that the 4.4 Kb size mRNA shares genetic information in the root and shoot, but the 2.37 Kb size mRNA is expressed only in the root (root-specific). This is an unusual expression pattern compared to other plant homeobox genes, which are expressed in almost all plant organs. In genomic analysis of the maize root, the cDNAs appear to be single-copies of genes. Maize roots have high levels of Zmhox1a mRNA but no protein (Bellmann and Werr, 1992); this leads to incomplete processing or differential splicing of maize root Zmhox1a mRNA. Zmhox1a protein levels are controlled translationally.

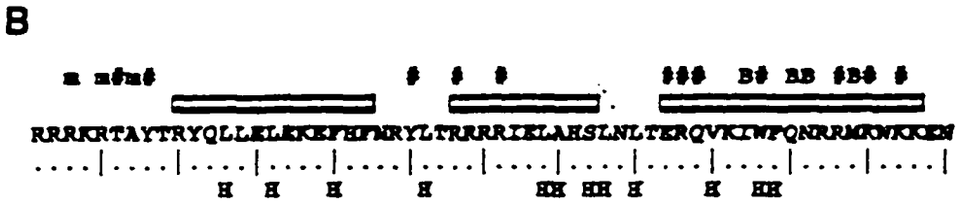
STRUCTURE AND FUNCTION OF THE HOMEODOMAIN

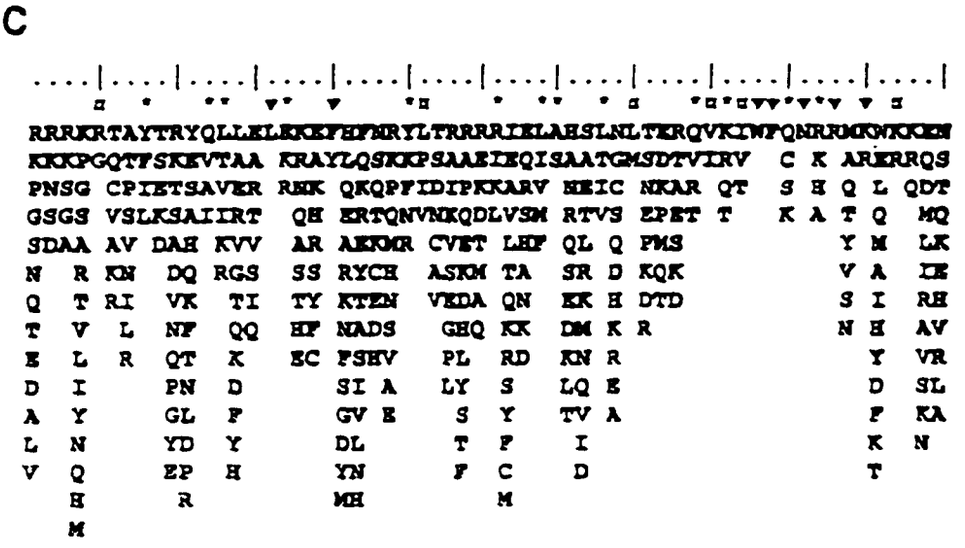
Homeotic genes encode transcription factors of the regulatory proteins that bind to specific cis-regulatory sequences in their target genes (Cho et al., 1988; Beachy et al., 1988). NMR spectroscopy analysis of some protein

homeodomains indicate three well-defined α-helices and a more flexible fourth helix (Otting et al., 1990). Helices two and three form a helix-turn-helix motif as found in various prokaryotic repressors, but the recognition helix 3 is extended to helix 4. The recognition helix contacts the DNA in the major groove, and there are additional contacts between the loop preceding the helix-turn-helix motif and the DNA backbone in the minor groove (Percival-Smith et al., 1990). The amino acid at position 9 of the recognition helix (amino acid 50) has an effect on the binding specificity (Hanes and Brent, 1989; Treisman et al., 1989). Certain positions of amino acids in homeodomains are significantly more conserved than others; they are consistent with the structural studies in which they form part of the hydrophobic core, or residues critical for homeodomain-DNA interactions (Fig. 25). Homeobox genes are considered to be coordinate genes at the top of a regulatory hierarchy and are first expressed maternally during oogenesis (Nusslein-Volhard, 1991). Subsequently, they appear to behave as morphogens that regulate development of early structures by different concentration levels of their protein products (Wang and Lelmann, 1991). These proteins may be produced in a gradient such as early in Drosophila development.

Figure 25. Homeodomain Consensus Sequence: (A) Amino acid consensus sequence based on 346 homeodomains. The three helices and their numbering is shown above the consensus sequence. (B) The area of intramolecular and protein-DNA contacted by individual amino acids of the homeodomain; H-hydrophobic core, B-contact bases in the major groove, m-contact bases in the minor groove, #-contact the sugar-phosphate backbone. (C) Amino acids most frequent at a given position in the homeodomain (Scott et al., 1989).



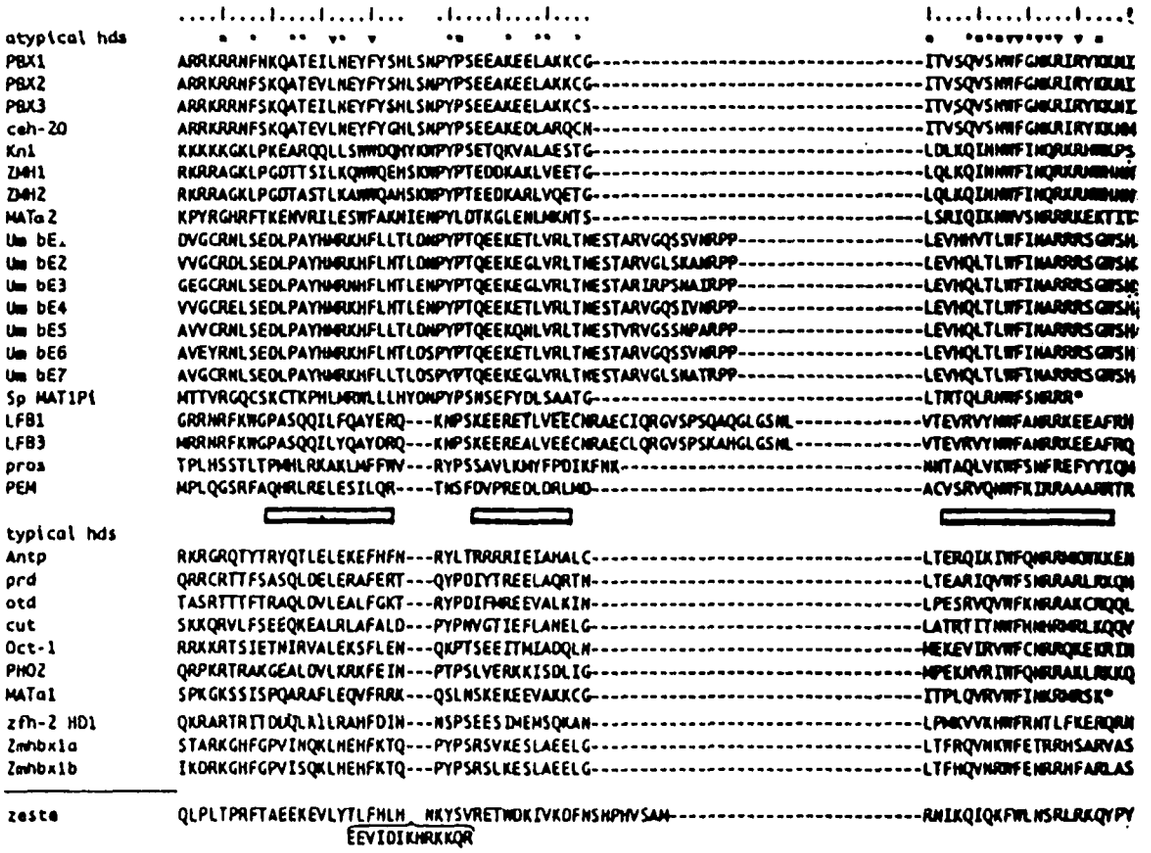




No. of amino acids encountered: ▾ 1-2; ¤ 3-5; * 6-9

Homeodomains may be classified as typical homeodomain and atypical homeodomain. Atypical homeodomains have seven highly-conserved positions, two in helix 1, and five in helix 3. The tryptophan residue at position 48 and the asparagine residue at position 51 are invariant except in two plant homeodomains (Fig. 26). The position 16 leucine may be substituted by methionine; position 20 and 49, phenylalanine, can be substituted by tyrosine. Position 53 is almost invariably an arginine except in two homeodomains (Fortini et al., 1991; Morinaga et al., 1991; Lundell and Hirsh, 1992). The position 57 is either an arginine or a lysine. In order to be considered a homeodomain, most of the sequence should be matched to these conservatively. Residues in the recognition region of helix 3 are highly conserved and are a token for the homeodomain. Members of different classes have, in general, 45-57% homology among the specific sequences of homeodomains. Some homeodomain sequences require insertions and/or deletions to obtain optimal alignment; 3 amino acids are looped out between helix 1 and helix 2. Extra amino acids are inserted in several genes between helix 2 and helix 3 (Finney, 1990). In general, atypical homeodomains have quite divergent sequences from other classical or typical homeodomains, only 15-28% identities to typical homeodomains. The position 16 leucine is replaced by proline. Also, the Drosophila gene

Figure 26. Alignment of Atypical and Typical Homeodomain Sequences: References; PBX1, PBX2, and PBX3--(Kamps et al., 1990; Monica et al., 1991; Nourse et al., 1990), ceh-20--(Burglin and Ruvkun, 1992), MATα1 and MATα2--(Astell et al., 1981; Miller, 1984), Kn1, ZMH1, and ZMH2--(Vollbrecht et al., 1991), Um bE--(Kronstad and Leong, 1990; Schulz et al., 1990), Sp MATPi--(Kelly et al., 1988), pros--(Chu-Lagraff et al., 1991; Matsuzaki et al., 1992; Vaessin et al., 1991), PEM--(Sasaki et al., 1991).



(pros) has no significant similarity to homeobox genes (Chu-Lagraff et al., 1991; Matsuzaki et al., 1992), but conserves a sufficient number of key residues. With respect to the intracellular location of pros and its function, it is an atypical homeobox gene.

The sequences of Maize root cDNAs (Mrx1, Mrx2, Mrx3, Mrx4) show that they are atypical homeobox genes. First, almost all hydrophobic cores responsible for the tertiary structure of homeodomains are consistent with other homeodomains. Among the seven highly-conserved positions, one in helix 1 and four in helix 3 are consistent with the basic homeodomain. Position 48 and 51 have tryptophan and asparagine as found in typical homeodomains. Position 53 is invariably an arginine, but there are three amino acid inserted between helix 1 and helix 2. Interestingly, there are three amino acid deletions in helix 3. Finally, the amino acid at position 9 of the recognition helix (amino acid 50) having a strong influence on binding specificity and is glutamine. The consensus binding sites for glutamine are (CC/AATTA) similar to Antennapedia and Fushi tarazu, Drosophila genes. Maize root homeodomains show 17% identities to 349 classical homeodomains.

Recently, five genes with atypical homeodomains (Kn1, Zmh1, Zmh2, Sbh1; Vollbrecht et al., 1991; Bellmann and Werr, 1992; Ma et al., 1994) and eight genes with typical

homeodomains were isolated from Arabidopsis, Maize, Carrot, and Lycopersicon (Athb1, Athb2, Athb3, HAT22, Zmhbx1a, Zmhbx1b, CHB1, Lp hbx7; Schena and Davis, 1992; Bellmann and Werr, 1992; Kawahara et al., 1995). The Arabidopsis genes have a conserved region containing a leucine zipper following the homeodomain. Zmhbx1a has a POU-specific domain. Although typical homeodomains do not all contain leucine zipper or POU-specific domains, they are very specific, but not significant. Maize homeodomains exhibit a high degree of diversities when compared to C. elegans Ceh-5 protein (Buerglin et al., 1989), but helix 3, the DNA recognition helix, is well conserved among the Mrx series (Fig. 7).

OVEREXPRESSION OF MAIZE ROOT cDNA

The maize root cDNAs (630bp) driven by the CaMV 35S promoter, the β-glucuronidase gene, and terminated by nopaline polyadenylation region termination sequences (35SMrx-GUS-NOS) are introduced into tobacco leaf discs. After three or four weeks, the Mrx transformants are regenerated and show striking morphology when compared to wild-type tobacco. The phenotypes are variable. Some leaves develop one or two jar-type or fused forms. There are three types of regeneration processes. In the first type, leaves are regenerated into wild-type tobacco forms

except for phenotypes including the number of leaves. In the second type, only one or two roots are regenerated without any leaves. In the third type, leaves and roots are regenerated normally except for shapes and numbers. In general, root development is enhanced compared to the wild-type; leaf development is inhibited or delayed. Most regeneration occurs in leaf vein areas. Root development shows striking differences in shape and number; roots are thicker and longer than the wild-type. Only one or two roots form on each tobacco tissue section. Microscopic analysis of the root shows the homeotic change. This change is characterized by a long and thin span between root meristematic region and root cap. The loss of apical dominance appears association with the expression of this maize gene.

The morphological changes observed can be attributed to auxin and cytokinin effects. The ratio of auxin and cytokinin is crucial for the production of shoots and roots in plant tissue culture (Skoog and Miller, 1957). The altered morphology of transgenic plants suggest deviation from the general notion. These findings suggest that interaction between plant growth regulators and homeobox genes may play a key role in the regulation of plant growth and development.

HISTOCHEMICAL AND HORMONAL ANALYSIS OF Mrx GENE EXPRESSION

Many higher plants have endogenous β-glucuronidase activity that is too low for detection of transferred GUS gene. The binary vector, pBI121 is similar to pBI101 except that it contains an 800-bp fragment 35S CaMV promoter that gives a high level of GUS upon transformation of tobacco (Jefferson et al., 1987). After fixation of regenerated tobacco plant, tissues are treated with GUS assay solution and substrate (X-GLUC) for overnight. The blue color is intensified in the tissues expressing the gene.

Gene expression is localized in the putative meristematic regions where the root or radicle is developing. No color is found in the leaf or shoot regions. During the early stages of tobacco regeneration, the primary root or radicle shows very high GUS expression, but after three or four weeks, GUS expression is only localized at the root meristematic region.

Microscopic examination reveals specific sites and tissues where GUS is expressed. Surprisingly, only the root epidermis adjacent to the meristematic region shows color. The root cap exhibits no colored reaction product.

In the hormonal response experiments, regenerated leaf segments exhibit GUS expression at the leaf vein. Embryoids treated with IAA (2 mg/L) do not exhibit GUS expression but do exhibit tricome development. Embryoids treated with

kinetin exhibit strong GUS expression. These data suggest that at an early developmental stage, IAA controls homeobox gene expression. During embryogenesis IAA is synthesized and transported in a basipetal direction along the embryo axis (Goldberg et al., 1994). During the globular stage, IAA reaches the highest levels. These levels of IAA induce cell expansion and coordinate cell division through interaction with cytokinin.

In animals, some homeobox genes are coordinate genes expressed maternally during oogenesis. Homeobox genes play the role of morphogens that regulate the morphological structure by resulting in differential protein gradient in animals during later stages of development (Wang and Lelmann, 1991). In plants, homeobox genes may coordinate plant growth regulators and play the role of morphogens that are expressed and localized in specific structures.

In conclusion, these Mrx cDNA series are expressed as two different transcriptional products; one is a 4.4-Kb product transcribed in the roots and shoots, and the second is a 2.37-Kb product that is expressed only in the root. Based on partial sequencing, the cDNAs are novel atypical homeobox genes. GUS expressions demonstrate that the genes are root-specific genes. Microscopic examinations suggest they are root epidermis genes. In the future, full-length cDNA isolation will provide greater opportunity for analysis

of the genome structure and function. This may reveal the homeodomains result in proteins that exert their influence on early development stages of plants or may reveal the genes as morphogens.

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