AN ABSTRACT OF THE THESIS OF

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John Fowler

Regulation of cell division and expansion are critical for plant development. The mechanisms that control these processes are not clearly understood in higher plants. In other eukaryotic models it has been shown that the highly conserved Rho family GTPases play a crucial role in these mechanisms. One distinct subset of the Rho GTPases, designated Rop, is present in higher plants and may play a role in cellular morphogenesis. I have conducted an initial molecular characterization of genes encoding ROPs in the monocot in *Zea mays* (maize), and also isolated mutant alleles in two of these genes using TUSC methodology (in collaboration with Pioneer Hi-Bred International, Inc.). The molecular analysis revealed phylogenetically-related subsets within the *rop* gene family, and multiplex RT-PCR assays indicated differing relative expression levels for the different genes in different plant tissues. These data thus indicated the potential for unique functions for each maize ROP, and provided

clues to assist in phenotypic characterization of mutations in each gene. My initial characterization of heritable *Mu* insertion alleles in the *rop*6 and *rop*7 genes was assisted by the development of a rapid PCR-genotyping assay to identify single and double mutant homozygotes. Likely null alleles for both *rop*6 and *rop*7, based on RT-PCR analysis, were identified; however, no mutant phenotype could be identified in either single or double mutant homozygotes. These results suggest that although these genes may serve specialized roles in plant signaling, there is also some degree of redundancy providing functional overlap for these monomeric signaling proteins.

Identification and Characterization of Seven ROP GTPases in *Zea mays*

by

Todd M. Christensen

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Chair of the Department of Botany and Plant Pathology

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Dean of Graduate School

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Todd M. Christensen, Author

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Chapter 1

Introduction

Todd M. Christensen

The control of cell division and expansion is critical for plant development [Clark S.E. et al. 1997; Laux T. et al. 1996]. However, the mechanisms by which these processes are regulated are not clearly understood in higher plants. In other eukaryotic organisms (e.g., mammalian cells, the yeast S. cerevisiae) it has been shown that Rho family GTPases play an active role in these mechanisms [Cabib et al. 1998; Drubin et al. 1996; Fu et al. 2001]. Utilizing techniques to alter the activity of specific Rho proteins *in-vivo* (e.g., microinjection of mutagenized proteins, protein overexpression, and mutant analysis), members of this family of GTPases have been shown to influence a number of pathways important for cell division and expansion. For example, the Cdc42p protein in yeast is required for developing cell polarity and for growth of the daughter cell bud immediately preceding asymmetric cell division [Johnson et al. 1999]. Among the cellular processes known to be affected by the Rho family in diverse non-plant eukaryotes are actin cytoskeletal regulation, cell cycle control, membrane trafficking, formation of focal adhesions, NADPH oxidase activity and cell wall synthesis [Yang Z. et al. 1997; Hall et al. 1998; Van Aelst et al. 1997]. These results suggest that Rho family proteins are conserved players in the regulation and control of cellular mechanisms involved in morphogenesis, and suggest that Rho family homologs from higher plants might have similar roles. In mammalian and fungal cells, Rho family GTPases have been grouped into three major subfamilies, based on sequence and function: Rac, Rho and Cdc42 [Mackay et al. 1998; Hall et al., 1998]. Although no true Rac, Rho or Cdc42 orthologs have yet been found in plants, a higher plant-specific group of the Rho family, designated ROP (Rho of plants), has

been isolated. The ROP family is most closely related to the mammalian Rac subgroup [Winge et al. 1997; Valster et al. 2000; Li et al. 1998; Lin et al. 1996]. Recent results indicate that Rops affect morphogenesis in diverse plant cells [Yang et al. 1998; Valster et al. 2000; Zheng et al. 2000; Fu et al. 2001].

Rop GTPases are small monomeric GTPases that are thought to act in a manner similar to that of the well-characterized Ras GTPase: they serve as molecular switches or rheostats by cycling between an active, GTP-bound form, and an inactive, GDP-bound form [Zheng et al. 2000; Valster et al. 2000] (Figure 1). ROP activation occurs following exchange of GDP for GTP, altering ROP conformation and allowing it to activate downstream "effector" molecules through protein-protein interactions [Wu et al. 2001]. The inherent GTPase activity of ROP hydrolyzes GTP to GDP, thus inactivating ROP. In mammalian and fungal cells, a host of accessory proteins have been shown to affect small GTPase activity, and homologous plant proteins could affect ROP. For example, GTPase activating proteins (GAPs) are negative regulators, and ROPGAPs have been isolated from Lotus japonicus and Arabidopsis thaliana [Borg et al. 1999; Wu et al. 2000]. Although no plant homologs have been identified to date, guanine nucleotide exhange factors (GEFs) catalyze GDP > GTP exchange on small GTPases, and thus are potential ROP activators. Finally, GDP dissociation inhibitors (GDIs) are thought to associate with GDP-bound GTPases, and negatively regulate their signaling activity by inhibiting nucleotide exchange. A GDI from

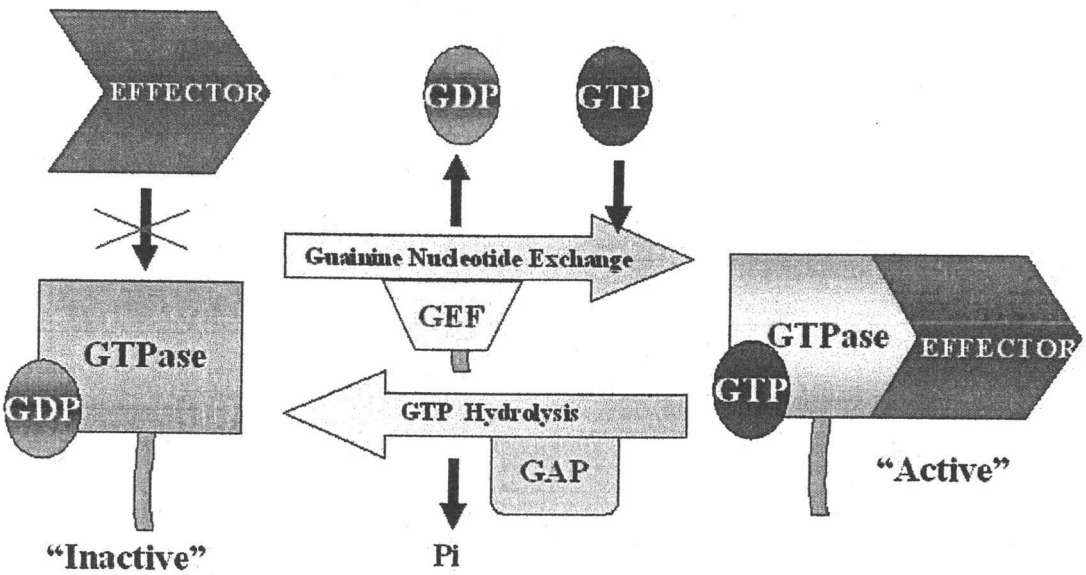

Figure.1 General model for regulation of Rho GTPase activity. A GEF (guanine nucleotide exchange factor) mediates the displacement of GDP for GTP. A GAP (GTPase activating protein) increases the Rho GTPase's intrinsic hydrolytic activity. The change in conformation between inactive and active GTPase is controlled by the association of GDP or GTP, respectively, and this serves as the molecular switch for the association of downstream effectors.

A. thaliana (AtRhoGDI1) binds to at least two *A. thaliana* ROPs [Bischoff et al. 2000]. Upstream signaling factors thus can mediate regulation of the GTPase switch by affecting the activity of GAPs, GEFs and GDIs.

Association with specific target membranes also influences *in-vivo* activity of Rho proteins (Figure 2). The Rho family proteins carry sequence motifs (e.g., the CaaX box) that influence GTPase targeting to specific membranes [Glomset et al. 1994; Magee et al. 1999]. These motifs, in an area designated the hypervariable region (Figure 3), are located at the protein C-terminus and influence post-translational attachment of lipid groups, which help associate the GTPase with membranes through hydrophobic interactions. One of the inhibitory activities of GDIs is thought to derive from their ability to bind to either farnesyl or geranylgeranyl lipid groups on Rho GTPases, thus preventing their association with membranes [Bischoff et al. 2000]. Distinct sequences in the hypervariable region lead to the attachment of distinct lipid groups, which in turn target the GTPase to distinct membrane regions of the cell., for example, AtROP1 to the plasma membrane at the tip of the pollen tube [Kost et al. 1999] and maize ROP7 uniformly throughout the entire plasma membrane [Ivanchenko et al. 2000]. Mutation of these motifs in the hypervariable region has been shown to inhibit membrane localization of ROPs, in both maize and other plants [Kost et al. 1999; Ivanchenko et al. 2000]. Subcellular localization is a key parameter in regulating the cellular responses to Rho activations, as Rho proteins induce localized changes in some downstream processes, for example, membrane-associated

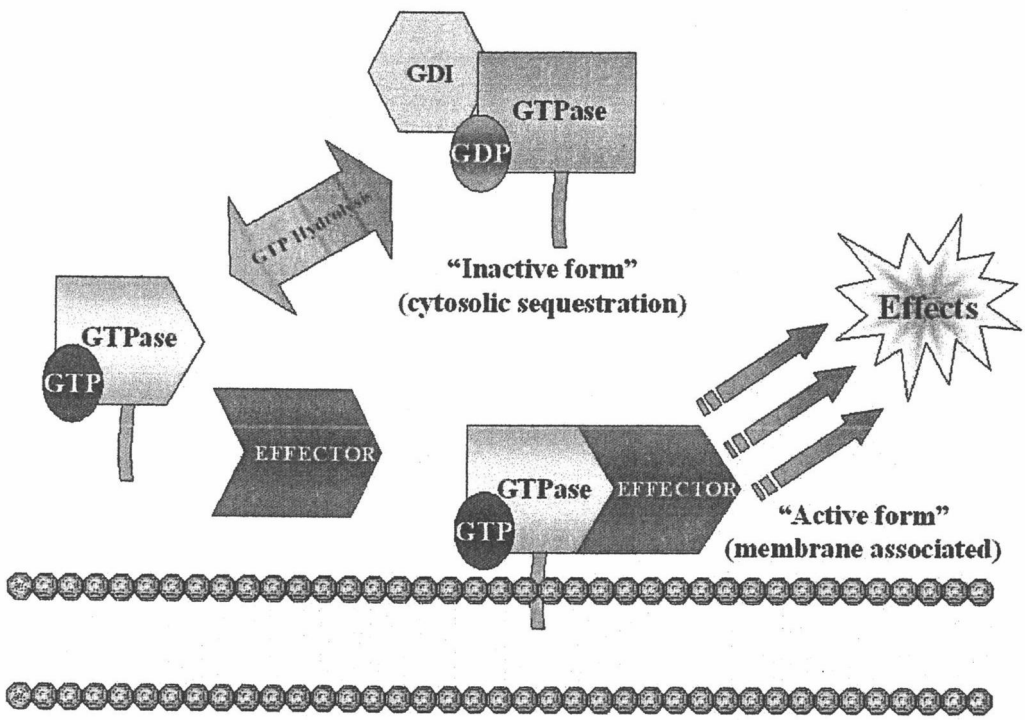

Figure 2. Membrane association provides a second level of regulation in vivo. GDI (guanine disassociation inhibitors) are thought to serve as upstream regulators by sequestering inactive (GDP-associated) GTPases in the cytosol. The green bar attached to the GTPase represents either a prenyl group and/or palmitic acid group attached via post-translational modification. These lipid groups have been shown to mediate localization to specific cellular membranes or to localized regions within membranes.

Figure 3. General protein sturucture of a small GTPase. Small GTPases contain 6 general domains. There are 4 GTP binding domains (GTP1-4), an effector domain which assist in binding to downstream effectors, and a C-terminal region designated the HVR (hypervariable region) that undergoes post-translational modification. The folded structure of this protein creates a GTP-binding pocket with the four GTP domains, whereas the effector domain and the HVR are thought to remain on the surface of the folded protein.

polymerization of actin filaments in animal cells [Welch et al. 1999].

The plant-specific ROP subgroup of Rho GTPases, containing genes designated ROP [Zheng et al. 2000], RAC [Winge et al. 2000], or At-Rac [Kost et al. 1999] by different groups of investigators, may influence several signaling pathways in plant cells, including those that influence cellular morphogenesis, cytoskeletal regulation, and programmed cell death [Valster et al. 2000]. ROPs have been most definitively linked to the regulation of polarized cell growth. Localization studies have shown several ROP proteins are found at the site of polar growth in pollen tubes, and antibody inhibition studies and expression of dominant mutant ROPs provide strong evidence that at least some of these GTPases play a role in regulating pollen tube tip growth [Yang et al. 1997; Zheng et al. 2000]. For example, transient expression of a constituitively-active At-Rac2 (also known as AtROP5) in tobacco pollen has been shown to cause delocalized, 'balloon-like' growth in pollen tubes, whereas expression of a dominant negative At-Rac2 severely inhibits pollen tube growth, at least in part by disrupting actin cytoskeletal organization [Kost et al. 1999].

However, other *in-vivo* activities have also been linked to ROPs. Rac13 in cotton is highly expressed in cotton fibers and is thought to be involved in regulating the differentiation of fiber secondary cell walls. Expression of Rac13 is also correlated with higher levels of hydrogen peroxide production, which may be a signaling intermediate between Rac13 and cell wall synthesis [Delmer et al. 1995; Potikha et al. 1999]. In addition, the influence of these GTPases on production of hydrogen peroxide and other reactive oxygen species (ROS) may regulate certain

mechanisms involved in disease resistance and the hypersensitive response (HR). The rice *rop* OsRac1 has been linked to production of ROS, apoptosis and pathogen-induced HR in transgenic rice [Kawasaki et al. 1999]. An unidentified *rop* (or *rops*) also appears to be associated with the CLV1 signaling pathway that affects the balance of cell proliferation and cell differentiation in the meristem [Trouchard et al. 1999]. The CLAVATA1 receptor-like kinase, a part of the meristem-signaling pathway in *A. thaliana,* coprecipitates with a ROP protein in immunoprecipitation experiments [Trouchard et al., 1999]. Finally, a recent analysis of a mutant allele of AtROP10 indicates that this ROP inhibits signaling by the phytohormone ABA [Zheng et al. 2002].

Due to their implied importance in plant signaling, these GTPases are prime targets for mutational analysis. Such an analysis would provide a method to test a specific *rop* gene for functions in any of the previously-mentioned processes, by determining whether specific mutant phenotypes (e.g., a reduction in polarized cell growth, changes in meristem cell proliferation) are associated with a mutated *rop* gene. However, there are very few published characterizations of mutant alleles of any *rop* gene, in any plant [Zheng et al. 2002; Arthur et al. 2003]. Most of the published data linking ROPs to specific functions has been derived from studies using overexpression of dominant mutant forms of the proteins to test for mutant phenotypes [Zheng et al. 2000]. While these dominant mutant *rop*s provide a useful tool to generate clues to *rop* function, they lack the capacity to provide an unequivocal view of the functions of particular *rops.* At least two problems are inherent to the

'dominant overexpression' approach. First, overexpression of a dominant mutant form of a gene is likely to have a more drastic influence than simply eliminating the two genomic copies of the same gene, as it may affect not only processes downstream of the wild type gene, but also downstream processes of closely-related homologs. Although this can help to circumvent difficulties due to genetic redundancy, it can also obscure specific functions of particular gene products. Second, overexpression of a dominant mutant protein may overload or otherwise affect a signaling cascade that the wild-type gene product and its homologs do not.

Analysis of mutations that eliminate the function of a single gene (a 'knockout' mutation) allows for a more subtle approach to study gene function and signal transduction. When a knockout mutation is associated with a phenotype, it provides more definitive information as to the function of the associated gene, compared to a phenotype produced by dominant overexpression, that may be unrelated to the *in-vivo* function of the wild-type gene product. Thus, whereas analysis of a dominant mutation can provide generalized clues, analysis of a knockout can more precisely reveal wild-type gene function.

However, a knockout mutational analysis can be hindered by genetic redundancy, in which loss of a gene function due to mutation is compensated for by the presence of another, related gene. There is precedent for redundancy within Rho family GTPases (e.g, *RHO3/RHO4* in *Saccharomyces cerevisiae* [Matsui et al. 1992], *Rac1/Rac2/Mtl* in *Drosophila melanogaster* [Hakeda-Suzuki et al. 2002]). In plants, *rop*s are present in a gene family, with anywhere from seven (rice – [Christensen et al.

2003]) to eleven (*A. thaliana* -[Winge et al. 2000]) members in a genome. Certain pairs of these genes appear to be ancient duplicates, yet, interestingly, they have been retained in modern plant genomes as complete, highly-conserved genes (Figure 4). The high degree of conservation suggests that they serve some role that constrains these *rop* genes from evolutionary change. For example, although these ROPs are highly conserved, they are no longer identical, suggesting that they could play similar, but slightly distinct, roles within the organism. These roles may be as subtle as localizing to a different position within the cell, interacting with a slightly modified downstream effector protein, or being expressed in a different cell or tissue type. In this scenario, two duplicated genes share many redundant functions, but each has additional distinct functions that may exert only minor effects on the organism. Thus, over evolutionary time, by duplication and mutation, an organism builds on the machinery (the gene families) that it already has and "slightly modifies" (by spontaneous mutation) one or more components to do a similar, but slightly different job. Many of the genes within such families overlap in their function on some level, but can retain a specialized role as well. However, the loss of only one such gene in a family by knockout can produce very minor phenotypic effects.

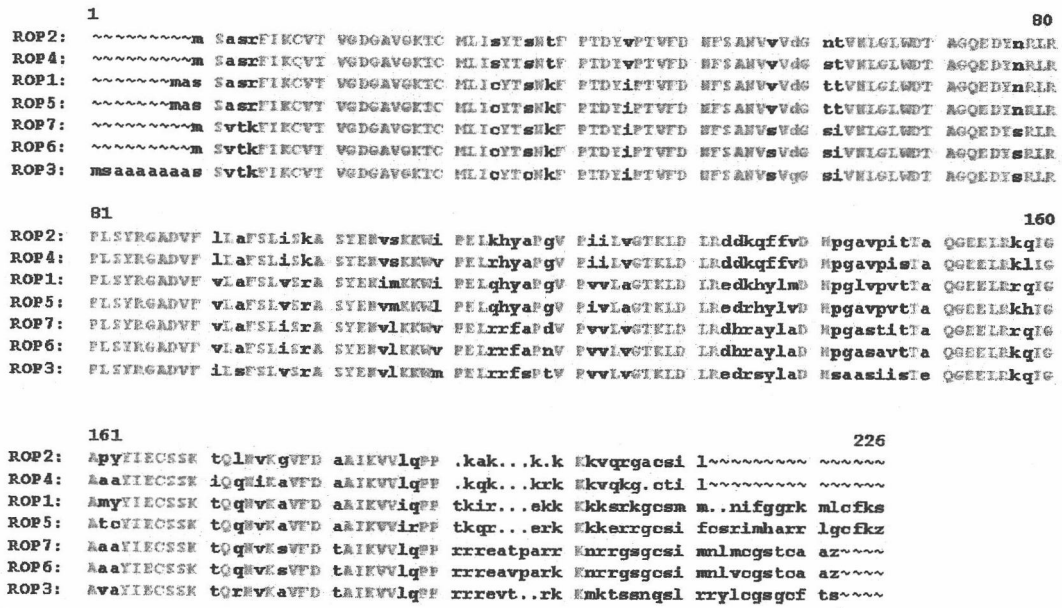

Figure 4. Amino acid alignment showing a high level of conservation among the members of the maize ROP family. The sequences of maize ROP1 through ROP7 are shown. The areas in green represent 100% amino acid identity between all seven ROP sequences, where as areas in black identify amino acids that do not share complete identity with all seven ROP sequences. ~ represents the absence of a corresponding amino acid.

My work has revealed a total of seven ROP GTPases in our model organism, *Zea mays*. (Two additional maize ROPs have subsequently been identified [Christensen et al. 2003]). Through the use of phylogenetic analysis, determination of mRNA expression patterns, and characterization of knockout mutations, we are attempting to determine the function of these maize *rop* genes. My first goal was to elucidate the expression patterns of these genes in various maize tissues, and during different developmental stages, to help determine their possible roles in maize morphogenesis and development. For this purpose, I used an RT-PCR approach relying on multiplex PCR reactions and a dilution scheme to determine relative expression levels of seven *rop* genes within different tissues of the wild-type (W22 inbred) plant. In conjunction with this expression analysis, I have also characterized six different mutant alleles in two *rop* genes, *rop6* and *rop7*. I have characterized the molecular phenotypes of these mutations (i.e., can they produce viable mRNA transcripts?), and have also examined homozygous mutant plants to determine whether their development is altered by loss of ROP function. Our results lead us to hypothesize that while our knockout mutants inhibit the production of viable mRNA transcripts, this molecular inhibition does not translate into a visualized phenotype due to functional overlap and gene redundancy in this family of GTPases.

Chapter 2

Phylogenetic and RNA Expression Analysis of Seven *rop* Genes in Maize

Todd M. Christensen

Introduction:

Rho family GTPases are well-characterized regulators of cellular morphogenesis in fungal, insect, and mammalian cells [Valster et al. 2000]. Rho proteins appear to exert this control, at least in part, through evolutionarily conserved functions in directly controlling distribution of the actin cytoskeleton. Because the actin cytoskeleton is also a crucial component in plant cell morphogenesis [Fowler et al. 1997; Kost et al. 1999], Rho homologs might also be expected to play important roles in plant development. Recent studies provide evidence that the higher plant-specific *rop* subfamily of Rho GTPases is involved in regulating pollen tube growth, cell wall synthesis, and cell proliferation in the meristem [Valster et al., 2000]. These proteins may also have significant roles in signaling pathways induced by environmental stimuli. For example, the generation of reactive oxygen species (ROS) and cell death in response to pathogen infection has also been correlated with overexpression of OsRac1, a member of the ROP family in rice [Kawasaki et al., 1999]. However, a clear picture of ROP function in higher plants is lacking, in part because ROP proteins form a multi-gene family in all plants characterized to date. Thus, it is unclear whether certain ROPs have unique functions in specific developmental processes, whether specific ROP intracellular localization influences each protein's activity, or whether ROPs performs shared, redundant functions with other members of the family. Most of the current approaches to understanding ROP function focus on specific model processes in cultured cells (e.g., pollen tube growth),

and do not directly address the roles of ROP throughout the plant life cycle. We have therefore undertaken a complete molecular description of the genes encoding ROP proteins in the model genetic species Zea mays (maize) as a prelude to dissecting their function using a genetic approach.

Maize is an excellent model to use for molecular, genetic and developmental analyses. Development of the plant has been well characterized, and the genome has been defined by a large number of mutations and molecular markers. With the recent advances in transposon-mediated mutagenesis [Das et al. 1995], functional genomics [Gai et al. 2000], and transient transformation techniques [Ivanchenko et al. 2000], maize can serve as a model to investigate gene function using approaches that combine both Mendelian genetics with cell and molecular biology. Maize offers the ability to generate mutants in any identified and sequenced gene, and then examine these mutants, from field studies down to the cellular level, to characterize the effect of loss of specific gene functions. In addition, data from maize serves as an important complement to data obtained from the dicot model plant *Arabidopsis thaliana.* The world's most important crop species are monocots, and the ancient divergence of monocots and dicots suggest that functional data from *A. thaliana* may not always be directly applicable to crop species. Information gleaned about ROP function in maize may be more directly applicable to agricultural goals, not only to maize but also to other grass species (e.g., rice, wheat). Future genetic modification of these key regulatory genes could increase crop productivity, as ROPs may influence not only

growth and development, but also plant defense responses to pathogens [Valster et al. 2000].

In collaboration with Pioneer Hi-Bred International, we have used both library screening and EST database searches to identify cDNA clones for seven unique *rop* genes in maize, and have determined their sequences. Analysis of gene structure and comparison of maize *rop* cDNA sequences to other higher plant *rop* gene sequences was used to develop insight the evolutionary relationships between plant *rop*s. By generating phylogenetic trees based on both *rop* nucleotide and predicted amino acid sequences, we can also begin to address whether similar *rop* mRNA expression patterns correlate with close phylogenetic relationships. These phylogenetic trees will guide future testing of predictions for putative ROP functions, by providing a framework for assessing whether closely related ROPs have similar functions in different species.

As a first step in understanding the functions of ROP GTPases *in-vivo*, we have also determined mRNA expression profiles for the maize *rop*s at several distinct developmental stages. To do this we have optimized an assay utilizing multiplex RT-PCR and gene specific primers (GSPs) to get a first estimation of the relative mRNA expression levels of these seven maize *rop*s. The technique uses an internal *actin1* standard and two sets of *rop* GSPs, each set specific for four of the *rop* transcripts. Through the use of plasmid controls we have optimized this method to determine the relative quantity of each of the *rop* transcripts in the original template cDNA. A survey of mRNA levels from tissues at various stages of development indicates that

maize *rops* are differentially expressed, and that their highest expression levels correlate with tissues undergoing active cell division and expansion.

Materials and Methods

Plasmids and Bacterial Strains

Plasmids with inserts corresponding to the maize *rop5*, *rop6* and *rop7* genes were isolated from a Lambda ZAP maize shoot apical meristem cDNA library (provided by S. Hake and L. Smith, USDA-PGEC, Albany, CA) using standard low-stringency radioactive screening methods [Sambrook et al. 1989; Fu et al. 2002]. The probe used to recover these clones was amplified from the highly conserved region (corresponding to amino acids 14-69 of maize ROP1) of two rice EST clones (Genbank Acc. # D23963 and D41794) identified by the Rice Genome Research Program (RGP) of the Japanese Ministry of Agriculture. One *rop5*, one *rop6* and two independent *rop7* cDNA clones were isolated and sequenced using standard automated protocols at either the UNC DNA Sequencing Facility or the OSU CGRB Central Services Lab. cDNA clones for *rop1*, *rop2*, *rop3*, and *rop4* (also known as *ropA-D*) were a gift from Pioneer Hi-Bred International, Inc., and were identified by BLAST searches of their large proprietary EST database [Hassanain et al. 2000]. In addition, three rice EST clones identified by the RGP, corresponding to the undescribed rice genes designated *Osrop4* (Genbank Acc. # C26233, AU077893) and *Osrop5* (Genbank Acc # C74803), were requested and sequenced in full to provide additional monocot *rop* sequences. Sequences were analyzed using the GCG package

(Madison, WI) and web-based BLAST programs [Altschul et al. 1997; Fu et al. 2002] at the National Center for Biotechnology Information of the US NIH.

Genomic clones for *rop6* and *rop7* were isolated by PCR amplification using gene-specific primers from the B73 inbred line. Amplified fragments were cloned into pPCR-Script Amp SK (+) using the manufacturer's protocol (Stratagene) and sequenced. Intron/exon junctions were determined by comparing cDNA and genomic sequence, assisted by the Splice Predictor application [Usuka et al. 2000].

Phylogenetic Analysis

Phylogenetic trees were generated from nucleotide and predicted amino acid sequences utilizing the PAUP 4.0 software included in the GCG package. Sequences were aligned using GCG Pileup and then converted to Nexus format. Bootstrap analyses were carried out using parsimony as an optimizing criterion with a heuristic search algorithm, and 100 replicates of bootstrap resampling. The HsRAC1 sequence was designated as an outgroup; and bootstap values of >50 are shown. Data files generated by GCG were then imported into Treeview [Page, 1996] on a Macintosh for publication.

Plant material, *in-vitro* pollen germination, and RNA isolation

Corn seeds (*Zea mays* W22 inbred) were surface sterilized in a 10% bleach solution for five minutes followed by two ten minute water rinses. Seeds were grown in Pyrex dishes on moist paper towels for two weeks at room temperature. Extracted

tissues consisted of: 1) root tip, the last five millimeters of the primary and adventitious seminal root tips; 2) root, including the zone of elongation basal to the proximal meristem having few or no visible root hairs; 3) shoot apex, excision of the internal 5 millimeter region of the proximal side of the primary node, including the apical meristem, but lacking the coleoptile and leaf sheath; 4) mature leaf, consisting of either fully mature leaf tissue from adult leaves or from juvenile leaves of two week old seedlings in a region 10 millimeters from the proximal leaf tip (Figure 5). Seedlings were dissected into approx. 100 mg of the desired tissue portions and immediately placed in 1.5ml microfuge tubes and frozen with liquid nitrogen.

Germinated pollen was obtained by collecting pollen from greenhouse-grown W22 inbred plants and placing it onto an isotonic sucrose/agar media [Walden et al. 1994]. Germinated pollen was harvested by after 18 hours of growth at room temperature.

Plant material was ground using RNAse-free pestles, and RNA was extracted from homogenized tissues using Trizol® reagent (GibcoBRL) in accordance with the manufacturer's protocol, and RNA concentrations were determined by spectrophotometry.

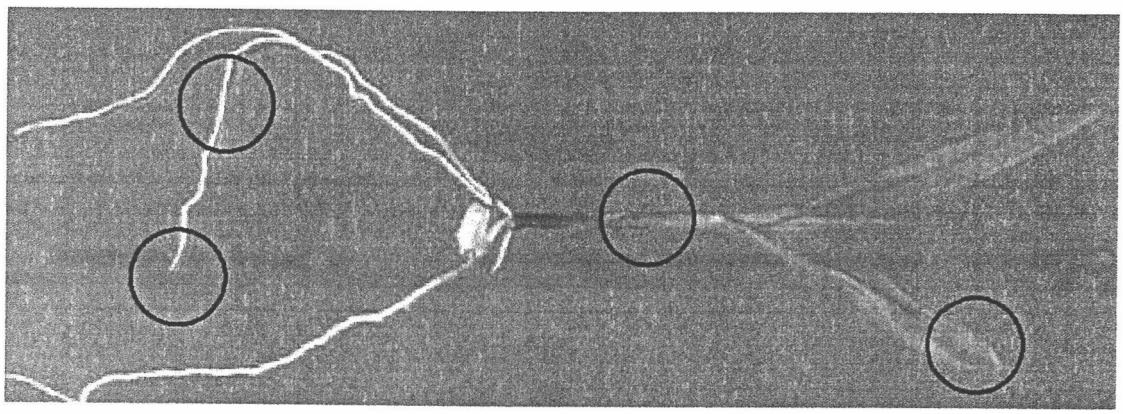

Figure 5. 2-week-old seedling showing tissues isolated and assayed in the expression analysis. Extracted tissues, identified within circles, consisted of: root tip; root; shoot apex (within the surrounding leaf sheath); and mature leaf.

Gene-specific Primers and Multiplex RT-PCR

PCR primers specific to each maize *rop* gene were designed to minimize the chances of cross-amplification and competitive inhibition by selecting sequences that were in diverged regions of the genes. cDNA sequences for the seven *rop* genes were aligned and compared using the GCG10 pileup and pretty programs in order to determine the regions of highest divergence. All gene specific primers (GSPs) were designed with a Tm of approximately 70°C, and were selected to bridge intron/exon junctions when possible to prevent amplification of genomic DNA. The primers generate amplified fragments that range in size from 300bp to 600bp, to minimize the potential for preferential amplification of shorter (vs. longer) fragments, and variability associated with the extension phase of PCR. An additional primer set was designed to amplify maize *actin1* mRNA transcripts to provide an expression reference between tissues *(MAc*1; GenBank accession no.J01238).

Two multiplex assays were developed to visualize the relative expression levels of these genes. The first multiplex reaction consisted of primer sets for *rop6, rop7, rop5, rop1,* and *actin.* The second assay included primer sets for *rop1*, *rop2, rop3, rop4,* and *actin.* PCR reaction parameters and primer concentrations were optimized and standardized for each multiplex reaction using and equimolar mix of *rop* cDNA plasmids. *rop1* primers and *actin1* primers were common to both assays to serve as an internal control between different tissues as well as between the two multiplex reactions.

Gene	Primer Name	Sequence 5'-3'
Mu Transposon	Mu	AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC
	MUEND-2	GASAGRAVASTRATAHCTGTTWAVRBA
actin	ZMACT-1	CACTGGAATGGTCAAGGCCGGTTTC
	ZMACT-2	AACCGTGTGGCTCACACCATCACCT
	ZMACT-3	AATCTCCCGCTCAGCGCTCGT
rop1	ZMROPA-F	TTGTTTTGGCAGGCACAAAATTGGAT
	ZMROPA-R	CAAACTCGGGGTCACACTCGTGGTAT
rop2	ZMROPB-F	GACCCAACTAAACGTGAAGGGCGTCT
	ZMROPB-R	CAAACATATCGCTCAATGCCTCGACA
	ZMROPB-F3	GGGCCACCACCACCCACTCTAC
	ZMROPB/D-R4*	TCAACCACAAC(AG)TTGGCACTGAAGT
	ZMROPB-F2	CGAATGCAGCTCGAAGACCCAACTAA
	ZMROPB-R2	TCTTTTGCTCAAACACGGGAAATACCTTCT
rop3	ZMROPC-F	AGCTTCGCCGATTTTCACCTACTGTTC
	ZMROPC-R	AACGCAAAAGAGCACTCTATCGGAAGG
rop4	ZMROPD-R2	CAGCCAGACTGTTGCCCTCTACCG
	ZMROPD-F2	CCAGCCACCAAAGCAAAAGAAGAGG
	ZMROPD-R3	CCATCTGCAGTGCTAGTTTTGTCTTGTCC
	ZMROPD-F	TGGGACAAAACTTGATCTGCGTGATG
	ZMROPD-R	CCCGGTGGTCTACACAACACAGTAGGA
	ZMROPD-F3	CGGTGGTCAACTTGTGTCCTGACCT
rop5	ZMRAC9-R	GCAACGAGCACTTTGGAACATGGAGACA
	ZMROP9-R	CGAGCACTTTGGAACATGGAGACAAAC
	ZMROP9-R2	TGAGCCAATGGTACATAAACGAAGTGCT
	ZMROP9-R3	TGGTCAACTAAGTAGTGCCTGTCTTCACG
	ZMROP9-F2	ACTACTTAGTTGACCATCCTGGTGCGGTA
	ZMROP9-F	TGTTGGCCGGAACTAAATTGGATCTTC
rop6	ZMROP6-F	AAGACCTGCATGCTCATCTGCTACACCA
	ZMRAC6-R	TCAGCCCTTGAAGAGCCTGACGAACT
	ZMROP6-R2	ACGATCAGCTAGTAAACGGCGGGAGA
	ZMROP6-F2	TCCGCGACCACAGAGCCTACCTC
rop7	ZMRAC1-F	AGCAACAAGTTCCCCACGGATTACATCC
	ZMRAC1-R	TTCACCTTCACCTCCTTCCAGATCAGTGT
	ZMROP1-F2	TCCGTGACCACAGGGCCTACCTT
	ZMROP1-F3	ATCAATGCGTTCCCTTCCTTCTCTGTT
	ZMROP1-R2	CCGTGGCCGAGATTGAAATGATAGAC

Table 1. Primers used for MTRP, 3'RACE, and genotyping of maize *rops*. Some primer designations were created from original gene name designations that have since changed to the current nomenclature of *rop1* to *rop7*.

Before PCR could be performed, cDNA was generated using 5ug of total RNA from each tissue sample using oligo-d(T) primers with MMLV reverse transcriptase (SuperScript™ cDNA Synthesis kit) in accordance with manufacturer's instructions. These cDNA samples were used in subsequent multiplex PCR reactions to determine relative expression levels of the *rop* genes. In order to assess relative expression levels serial dilutions (1:4) of each cDNA sample were prepared and tested [Nebenfuhr et al, 1998]. PCR products from the multiplex reactions were visualized on 2% agarose or 3% Nusieve® GTG® agarose gels. RT-PCR multiplex reactions for each tissue were performed in triplicate, starting with RNA isolation from three independently frozen aliquots of tissue, and the expression data was compared between these experiments.

Primers located in the final introns of the *rop6* and *rop7* genes were used in RT-PCR reactions to test for the presence of alternatively spliced or alternatively polyadenylated *rop6* and *rop7* transcripts. For 3' RACE (Rapid Amplification of cDNA Ends [Frohman et al. 1993] of the putative alternatively polyadenylated *rop6* transcript, parameters were optimized for amplication of cDNA fragments utilizing a modified oligo-d(T) primer and ZMROP-6F2 (1mM $MgCl_2$ and 46° annealing). The resulting PCR products were gel purified and directly sequenced.

Results

Phylogenetic analyses place ROPs within distinct subgroups. In conjunction with expression analysis, we examined the gene structure and looked at phylogenetic

relationships between ROP GTPases in higher plants. Following the development of a multiple sequence alignment of ROP GTPase amino acid sequences, we used UPGMA analysis to construct a phylogenetic tree based on sequence data for ROPs in *Arabidopsis thaliana*, *Oryza sativa* (rice), and *Zea mays* (Figure 6). This tree helps place Rops within distinct subgroups of more closely-related proteins, which could have closely related functions across species boundaries. From this tree, we also compared the grouping data to the expression data we obtained in our study.

Due to the high degree of sequence conservation among the ROP gene family (72% to 97% identity at the nucleotide level), analyzing the expression patterns of specific family members by northern-blot hybridization appeared likely to be problematic. We were able to obtain (through EST database searching, sequencing of cDNA clones, and through our collaboration with Pioneer Hi-Bred) cDNA sequence data for the seven known ROP GTPases in maize. From these sequences I generated gene specific primers (GSPs) that could specifically amplify each of the seven ROP genes I was characterizing. I used these GSPs in a set of Multiplex Titration RT-PCR (MTRP – [Nebenführ et al. 1998]) experiments to determine the relative expression level of each *rop* in a variety of maize tissues. MTRP allows estimation of relative transcript abundance by determining the step in a serial diluation of cDNA in which a specific template (i.e., a reverse-transcribed mRNA for a particular gene) becomes limiting for amplification (Figure 7). The more highly a given *rop* is expressed, the more dilute its cDNA template can be made while still allowing amplification and

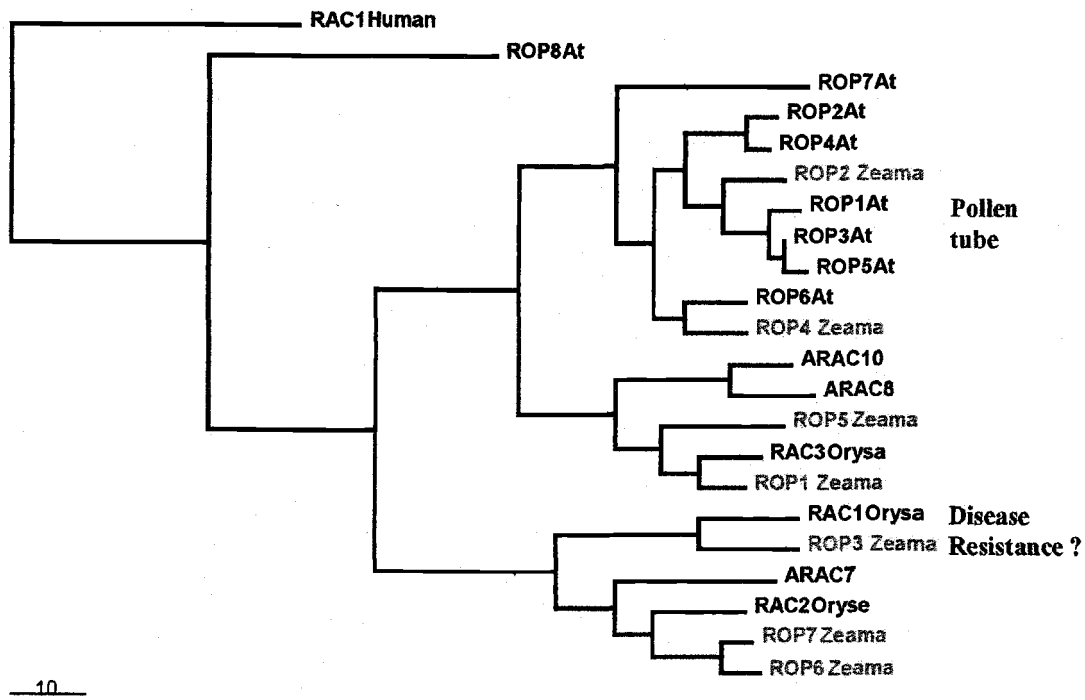

Figure 6. Phylogenetic tree based on analysis of several ROP amino acid sequences from higher plants. Maize sequences are in green. Two possible functional groupings, based on evidence from other models, are shown in yellow to the right.

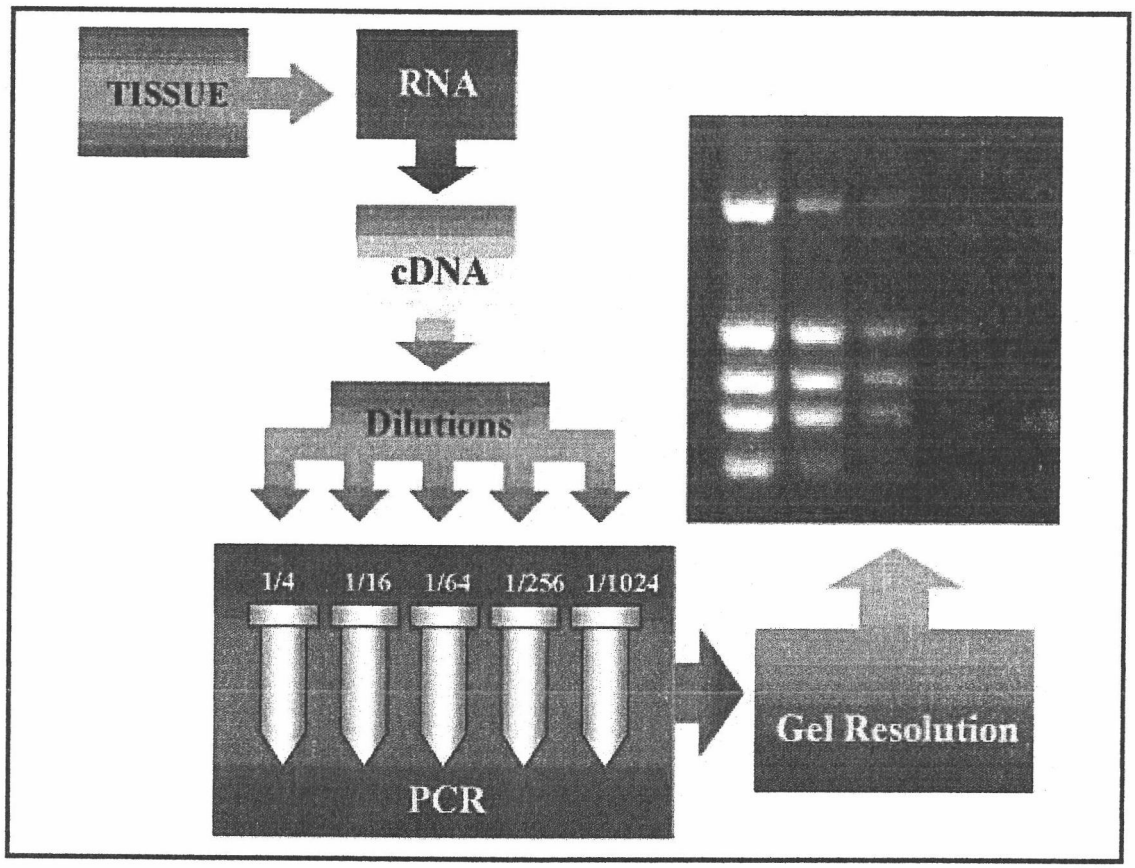

Figure 7. An overview of the MTRP assay for determining relative expression levels for multiple distinct mRNA species. Total RNA was extracted from particular maize tissues. Oligo-dT primers were then used to generate cDNA for each RNA sample, and these cDNA samples were used in subsequent multiplex PCR reactions to determine relative expression levels of the *rop* genes. To assess relative expression levels, serial dilutions (1:4) of each cDNA sample were prepared, and each was subjected to a PCR reaction using sets of *rop* gene specific primer pairs. The resulting amplified fragments were run in adjacent lanes on a gel, allowing estimation of relative transcript abundance by determining the step in the serial dilution in which a specific template (i.e., a specific *rop* gene) became limiting for amplification.

visualization of the band. This technique avoids some concerns regarding the potentially differential amplification efficiencies of distinct primer pairs, because the determination of relative expression levels depends only on the dilution step at which a band is no longer visible, and not on the intensity of particular bands. In addition, using primer pairs in multiplex allows the simultaneous assaying of expression levels of several genes, and which helps to provide internal controls for comparing among reactions.

To insure the accuracy and specificity of our Multiplex Titration RT-PCR assay (MTRP), it was necessary to perform several experiments utilizing *rop* plasmid DNA in known concentrations. First, due to possible interactions between multiple primer sets and similar sequences in the highly related *rop* gene family it was necessary to optimize this assay on an individual basis for each gene. These experiments allowed me to obtain an identical set of parameters that could be used for all multiplex experiments to determine the wild type expression patterns of the *rop* genes in question. Second, to test for the specificity of each primer pair for one particular *rop*, primer pairs were tested individually on an equimolar plasmid mix containing all seven *rop* clones. Each primer pair produced only a single band of the appropriate size, corresponding to the specific target for amplification (Example: *rop1* and *rop2* - Figure 8). Furthermore, PCR reactions on a set of four-fold dilutions of this plasmid mix demonstrated that the ability to amplify a target sequence by individual primer pairs did not vary significantly from the ability of the same primer

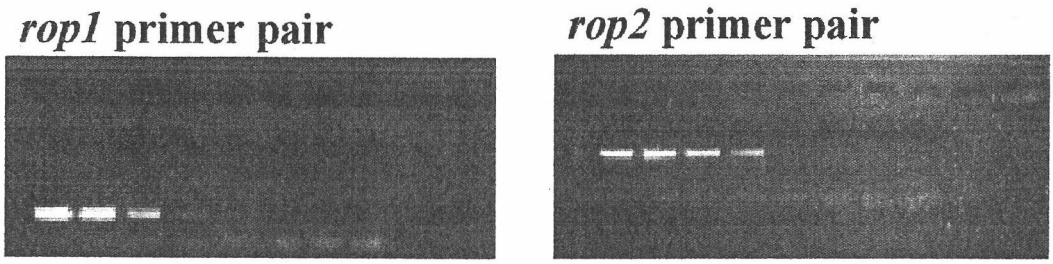

Figure 8. Specific pairs of GSPs only amplify their specific target cDNA sequence from a set of serial dilutions of an equimolar *rop* cDNA plasmid mix. Only results from the *rop1* and *rop2* primer pairs are shown. The level of amplification between the two primer sets is comparable, each producing visible bands in the fourth dilution for each reaction set, but none in the fifth dilution. Similar results were seen for all seven *rop* primer pairs, using the same equimolar plasmid mix (data not shown).

pairs in a multiplex reaction mix, using the same plasmid controls. Third, to eliminate possible amplification of genomic DNA, several primer sets were designed to bridge exon/intron junctions. However, my initial experiments (data not shown) indicated that genomic contamination was not a significant source of error and thereafter, exon/intron junction spanning primers, although preferred, was no longer deemed necessary.

After testing the primer sets on an individual basis, we then integrated the primer pairs to form two MTRP assays that allowed me to assay seven of the maize *rop* transcripts in two sets of reactions. The PCR cycling parameters for both assays were identical, with only the composition of the primer sets being altered between the two. The first assay (MTRP-1) consisted of primer pairs for *rop1, rop5, rop6*, and *rop7*, as well as for the *actin1* internal control. The second assay (MTRP-2) contained primers to amplify the *actin1* control, as well as *rop1, rop2, rop3* and *rop4*. Primers for *rop*1 were included in both reaction sets, as the gene appeared to be expressed in all of the tissues examined, and thus, along with actin1, served as a good baseline value for which to compare results between the two assays. A set of four-fold dilutions of an equimolar plasmid mix was again used as a control for the MTRP assay. Because the amplification efficiency differed among the seven distinct primer pairs, it was necessary to alter the concentration of certain primer pairs to insure even amplification of target sequences, based on the equimolar *rop* mix in our plasmid control (Figure 9). This empirical optimization of the two primer sets achieved a nearly identical

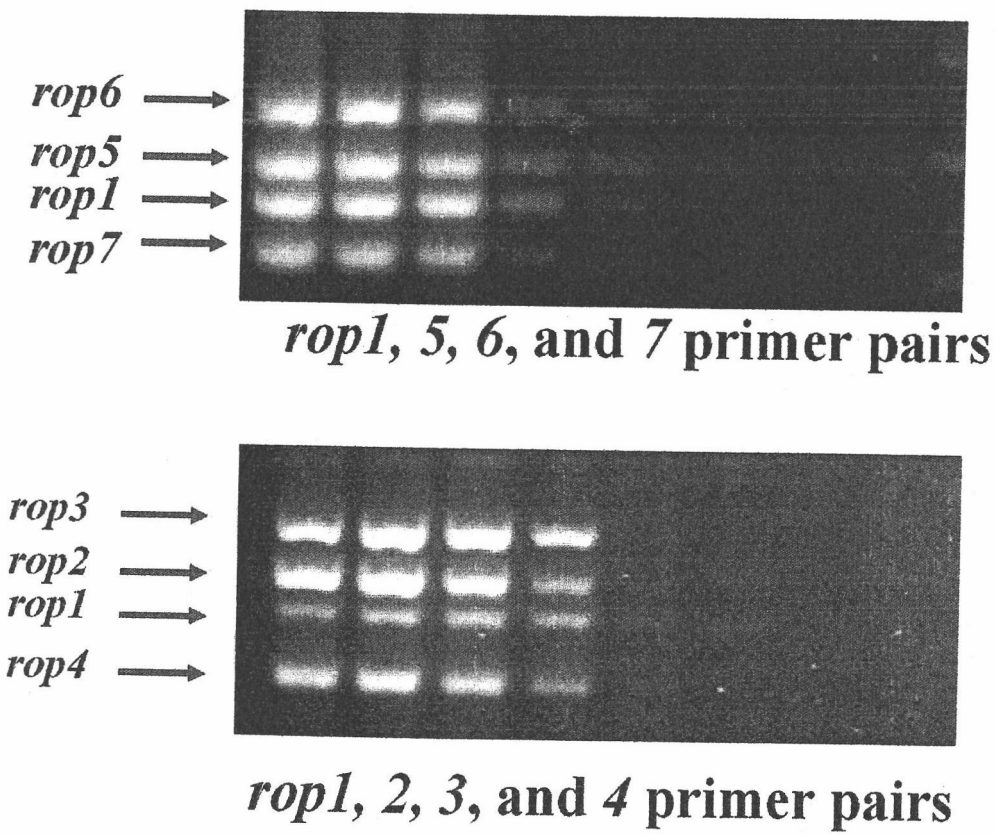

Figure 9. Calibration and specificity of the MTRP-1 (*rop1, 5, 6,* and 7) and MTRP-2 (*rop1, 2, 3,* and *4*) assays were established using a set of serial dilutions of an equimolar *rop* cDNA plasmid mix. The amplification of visible products from a particular dilution for each *rop* primer pair is comparable within and between the two assays, showing that the assays are calibrated to insure all PCR products amplify with equal efficiency if the cDNA template levels are at equimolar concentrations.

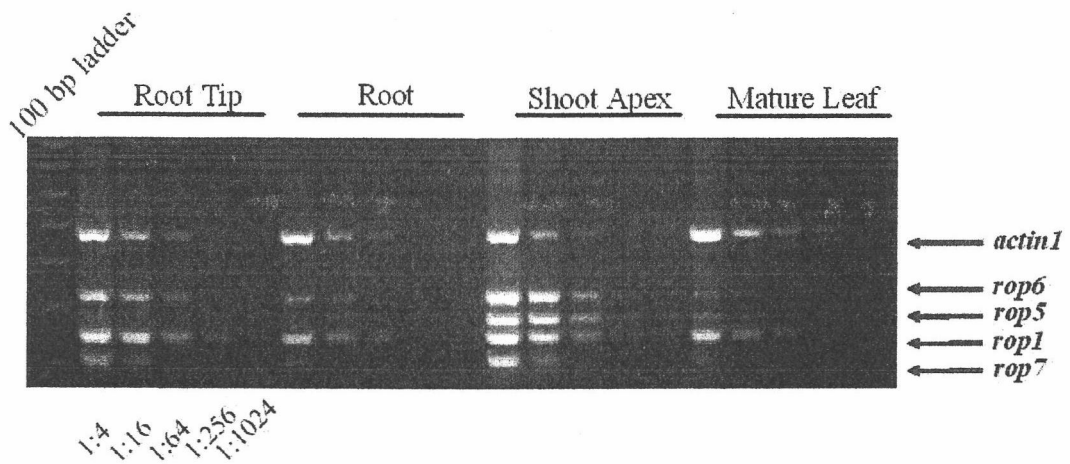

Figure 10. Results from one of the MTRP-1 experiments showing the relative expression levels of four *rops* in four maize tissues. Note the generally high expression levels in the shoot apex sample. Each dilution represents a four-fold decrease in the level of cDNA template; therefore the *rop6* transcript in the shoot apex is expressed at a level of approximately 4 times that of *rop6* in root or root tip, and 16 to 64 times that of *rop6* in mature leaf.

"amplification response" (i.e. the inability to amplify a product at a specific dilution step) for each *rop*.

Both MTRP-1 and MTRP-2 were run on RNA samples from five different maize tissues (Figure 10). The results of the MTRP experiments, repeated in triplicate, are summarized in Table 2. As can be seen from the differential "amplification response" for the different *rops*, there are differential expression patterns for the genes in the various maize tissues. In general, it appears that tissues from areas undergoing cell division and expansion, as seen in the root tips and the shoot apex, show the highest levels of *rop* mRNA transcripts. The relatively high expression levels of all seven *rops* in these two tissues is particularly notable when compared to the low levels in the mature leaf, in which cells are no longer actively dividing or expanding. Only *rop1* appears to show no significant reduction in expression in the mature leaf. Other differential expression patterns were also revealed by these experiments; for example, *rop5* is at low levels in root tissue, compared to its level in the shoot apex. However, perhaps the most striking example of differential expression is evident in the germinated pollen. These experiments indicate that *rop2* is the only GTPase of the seven that I tested that is highly expressed in this cell type. Phylogenetic analysis shows this GTPase to be one of the most closely related to the *A. thaliana* gene *Arac5*/AtROP1, a ROP expressed in *Arabidopsis* pollen, and important for pollen tube growth [Li et al. 1999].

Tissue / mRNA	Root Tip	Root	Shoot Apex	Mature Leaf	Germinated Pollen
rop7	++++	+++	++++	+	-
rop6	++++	+++	+++++	+	-
rop5	+	+	++++	+	-
rop1	+++++	+++++	+++++	+++++	+
rop2	++++	++++	+++++	+	+++++
rop3	++	++	+++	-	-
rop4	++++	+++	++++	+	-

Table 2. A summary of the results from the MTRP experiments reveals Rop GTPase expression levels in different tissues. Each + corresponds to the presence of a band in each dilution reaction. A difference of one "+" thus represents an approximately four-fold difference in the relative expression level. For example, *rop1* in the shoot apex is expressed at a level of 256 times that of *rop1* in germinated pollen.

Another example of the differentiation in these genes was evident upon close inspection of the genomic sequences of *rop6* and *rop7*. Analysis of genomic sequence raised the possibility that these genes produced alternative transcripts, which encoded ROP proteins with distinct C-terminal HVR sequences. In the final intron of both genes was a stop codon followed by putative polyadenylation site (Figure 11). Although both sequencing of *rop6* and *rop7* cDNAs and RT-PCR experiments (data not shown) indicated that this intron was spliced out in the majority of transcripts from these genes, an unspliced and truncated transcript was still a possibility. If there is an alternate transcript that is produced, then the protein it encodes is a Rop variant that may function in a different role and/or location in the cell. The ROP6/ROP7 final exon sequence contains HVR motifs that serve as post-translational modification sites critical for protein targeting, and the truncated sequence has been shown to alter the subcellular targeting of a GFP-ROP7 fusion [Ivanchenko et al, 2000].

To determine if these genes were alternatively spliced and polyadenlyated, primers specific to the region immediately upstream of the predicted polyadenylation site were developed for *rop6* and *rop7*. Initial RT-PCR experiments of extracted cDNA from wild-type plants suggested that *rop6* might be alternatively spliced, whereas *rop7* is not (Figure 12). An intron-specific primer for *rop6* (6R2 – see Figure 11 for primer locations), located downstream of the putative intron stop codon, was able to amplify a band of the predicted size for the unspliced variant from Shoot Apex cDNA, but not from Mature Leaf cDNA, when used in combination with a forward

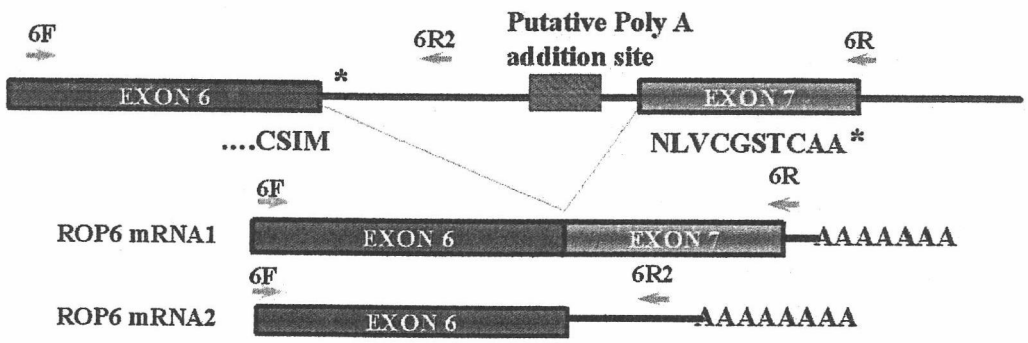

Figure 11. Sequence analysis reveals that alternative splicing and polyadenylation may produce an additional ROP6mRNA, encoding a protein with a truncated HVR. Graphical representation of exon 6 and 7 of *rop6*, showing the putative polyadenlyation site as well as the final four amino acids for the predicted truncated transcript. These final four amino acids (corresponding to the CaaX box motif, a target for prenylation) could serve as an alternate site for posttranslational modification. Primers 6F, 6R, and 6R2 were designed to test for the presence of this predicted alternatively-spliced transcript. The 6R2 primer annealing site provides specificity for the selective amplification of the alternate transcript only.

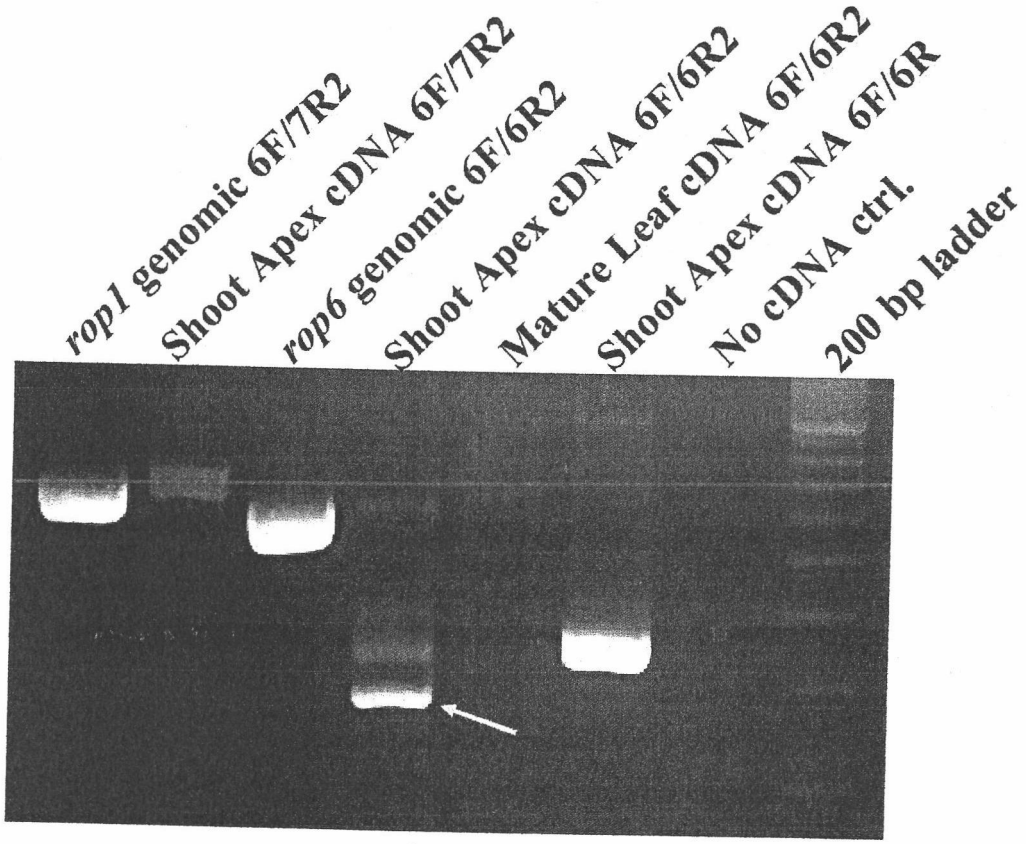

Figure 12. The maize *rop6* gene may produce two transcripts. A band corresponding to an alternative *rop6* transcript containing the final intron was amplified from Shoot Apex cDNA using the 6F and 6R2 primers (arrow). The originally-described *rop6* transcript lacking this intron was detected in the lane labeled Shoot Apex cDNA by PCR amplification using 6F and 6R. See Figure 11 for primer positions and experimental design.

primer (6F). Because the cDNA was generated using a poly-dT primer, this result suggests that the final *rop6* intron can be found in a polyadenylated transcript. Although the 6F/6R2 band is not as strong as the band amplified from the fully-spliced transcript (6F/6R), the unspliced transcript makes up a significant, detectable portion of the *rop6* mRNA. The putative unspliced *rop6* band was purified and sequenced, confirming that its sequence corresponds to the predicted alternative transcript sequence. This contrasts with *rop7*, which did not produce similar results using a *rop7*-specific intron primer (1R2), even though both *rop6* and *rop7* primer pairs amplify genomic DNA, a positive control (Figure 12).

Two methods were used to try and confirm the existence of this alternative transcript. First, an oligo-d(T) primer was used with a *rop6*-specific primer in a technique known as 3' RACE (Rapid Amplification of cDNA Ends), to amplify the entire 3' end of the *rop6* transcript adjacent to the poly-A tail. This experiment produced two fragments that corresponded in size to the shorter, fully-spliced transcript, and to a longer, alternatively spliced fragment that still retained the final intron (data not shown). Thus, this experiment did not support the existence of the predicted alternate polyadenlyation site in the final intron (Figure 11), raising the possibility that the initial RT-PCR experiment had detected an immature transcript (a splicing intermediate). However, attempts to use a second approach to confirm the existence of this alternative transcript by northern blotting and probing of oligo-dT purified mRNA were unsuccessful (data not shown), and thus it remains unclear whether the putative *rop6* alternative transcript is a mature, translatable transcript.

Conclusions

This work focuses on the characterization of seven *rop* genes in maize; subsequent experiments have identified two more maize *rops* (*rop8* and *rop9* – [Christensen et al. 2003]). Of the seven in my analysis, two appear to be a pair derived from an ancient whole genome duplication event: *rop6* and *rop7*. The two newly identified genes also form pairs (*rop8* with *rop1*, and *rop9* with *rop2*) that are also likely derived from this same whole genome duplication event that occurred approximately 11 million years ago [Gaut et al. 1997; Christensen et al. 2003]. Thus, duplicates for the *rop3*, *rop4* and *rop5* genes have not been identified, despite extensive maize genome and EST sequencing, and may have been eliminated from the maize genome, suggesting a total of nine maize *rops*. This is a number comparable to the seven *rop* genes in *Oryza sativa* (rice), and the eleven *ROP* genes in *A. thaliana* [Yang et al. 2002]. The phylogenetic tree generated by analysis of ROP protein sequence (Figure 6) suggests that some of the genes in *A. thaliana* (e.g., *Rop2At* and *Rop4At*) are probable ancient duplicates as well; this hypothesis has been supported by other published work [Winge et al. 2000].

To determine expression patterns for members of this family of GTPases, it was critical to design and evaluate a method that could provide rapid, reproducible, and semi-quantitative measurements of transcript levels in different tissues. The use of Multiplex Titration RT-PCR (MTRP) provided a method for evaluating multiple

gene transcripts with a single PCR reaction. This served several roles: it allowed for the comparison of multiple gene transcripts with an *actin1* standard in each reaction; it provided a method to compare overall mRNA levels between distinct tissues; and it facilitated the rapid accumulation of data from multiple tissues. Establishing the MTRP assays did require a significant amount of optimization and quality control testing. However, through the use of rop cDNA clones, we were able to develop an artificial, standardized control template to examine and discount several potential complications associated with MTRP.

However, the subsequent discovery of two additional rops in maize (*rop8*, an ancient duplicate for *rop1*, and *rop9*, an ancient duplicate for *rop2*) [Christensen et al. 2003] raises some concerns about certain of these MTRP results, in retrospect. Comparison of the sequences of *rop8* and *rop9* to the primers used for the MTRP detection of *rop1* and *rop2* raises the likely possibility that my experiments amplified both duplicates along with their intended targets. This "duplicate amplification" may explain why more recent MTRP experiments differentiating between *rop1* and *rop8* detect *rop8* in pollen, but not *rop1* [Christensen et al. 2003].

Although several of the characterized genes are derived from of the ancient maize duplication event, and all of the genes in the family are highly conserved at the protein level, it appears that at least some of the maize ROPs play different roles in the developing plant. Our expression data has provided an initial insight into where and when these genes are expressed, and revealed differences among ROP family members. Consistent with a role for this family in cell division and expansion,

expression levels for all seven ROPs were highest in the shoot apex and (except for *rop5*) and the root tip, tissues undergoing active cell division and expansion. This expression pattern is also consistent with a role for Rop GTPases in signaling mechanisms in the meristem (e.g., in CLV1 receptor-like kinase pathways - [Trotochaud et al 1999]). However, the data suggest that *rop5* may have little role in root development.

At a ROP family-wide level, both germinated pollen and mature leaf tissue exhibited decreased levels of ROP mRNA. However, particular ROPs are expressed at relatively high levels in these samples, perhaps indicating particular functions for these genes (Table 2). For example, *rop1* is the only ROP tested that is highly expressed in the mature leaf (Figure 10). In fact, *rop1* is highly expressed in every tissue tested, raising the possibility that this gene is necessary in some constitutive, 'housekeeping' capacity. One hypothesis is that *rop1* could have a role in disease resistance and programmed cell death, similar to that suggested for the closely-related rice *OsRac1* gene [Kawasaki et al 1999]. Although *rop3* is the most closely related maize gene to *OsRac1* (Figure 6), this ROP exhibits a low expression level in most tissues. This raises the possibility that maize *rop1* could play a more important role in disease resistance in maize, perhaps sharing this function with other closely-related maize rops identified in the phylogenetic tree, including *rop3*, *rop5*, *rop6* and *rop7*. Exploring these hypotheses will require the development of pathogenicity assays to determine if mutant alleles in these *rops* influence disease resistance *in planta*.

Although the germinated pollen tube is a structure that is associated with a

high rate of cell elongation, it is also highly specialized, such that the elongation remains polarized at the pollen tube tip [Yang et al. 2002]. Its incredible rate of elongation may in part be enabled by restricting the cell such that it only expresses components necessary for pollen tube growth and polar development. The MTRP results indicating a high expression level for *rop2* (and, in other results, its ancient duplicate, *rop9* - [Arthur et al. 2003]) in pollen, compared to low levels for the other tested ROPs, raise the possibility that the nearly-identical GTPases encoded by these genes have a unique role to regulate pollen tube growth. The phylogenetic studies also are consistent with this possibility, as *rop2* is grouped with the *A. thaliana* genes *Rop1At*, *Rop3At*, and *Rop5At*. Both *Rop1At* and *Rop5At* are highly expressed in *A. thaliana* pollen, and have been shown to influence the polar growth of the pollen tube [Yang et al. 2002]. A recent characterization of mutant alleles of the maize *rop2* gene indicates that these mutations negatively affect pollen function, strongly supporting this hypothesis [Arthur et al. 2003].

Finally, a primary goal of this analysis was to determine expression patterns for the *rop6* and *rop7* genes, in order to assist in the identification of possible mutant phenotypes associated with several *rop6* and *rop7* mutations (Chapter 3). Before this analysis, there was no information regarding ROP expression patterns in maize, and one hope was to identify tissues expressing *rop6* and/or *rop7* alone, reducing the possibility of functional redundancy among ROP GTPases, and increasing the chances of identifying a mutant phenotype. However, based on these data, it appears that mutational strategies targeting other ROPs (e.g., *rop1*, *rop2*) are more likely to provide

a more evident phenotype. Both *rop6* and *rop7* show very similar expression patterns, perhaps reflecting their evolutionary past as duplicate genes. Furthermore, tissues in which they are highly expressed (e.g., the shoot apex) are also sites of expression of several other family members, increasing the likelihood of genetic redundancy.

Chapter 3

Mutational Analysis of Two ROP GTPases

Todd M. Christensen

Introduction

Rho family GTPases have been well studied in several eukaryotic organisms, but they have not been well characterized in higher plants. In murine and yeast models, these GTPases have been shown to influence cell morphogenesis, apoptosis, polar growth, and cell proliferation [Gao et al. 2004, Moon et al. 2003]. Although studies of these homologs provide clues to the potential function of these proteins in higher plants, it is necessary to provide direct evidence to definitively determine their roles. To characterize the functions of the plant-specific Rho GTPases known as ROP GTPases in higher plants, I employed a genetic approach utilizing maize as a model system.

A common method to characterize gene function is by examining the phenotype of individuals that carry mutant alleles of the gene being studied. Traditionally one would characterize a phenotype or function and then determine the gene or genes responsible for the change ("forward genetics"). Work in the Fowler lab (Chapter 2), however, had already identified several maize ROPs, and determined their sequences. To approach the problem of determining ROP gene function from the opposite direction ("reverse genetics"), it was necessary to identify and characterize mutations in these genes, and determine whether they were associated with a mutant phenotype that provides clues about the gene function.

To carry out this approach, a collaboration with Pioneer Hi-Bred Intenational, Inc. was established to utilize their Trait Utility System for Corn (TUSC) technology [Benson et al. 1995; Mena et al. 1996]. This methodology involves using the *Mutator* (*Mu*) transposon system, which randomly inserts into the maize genome, as a

mutagen. The insertion of *Mu* transposons within a gene of interest can produce heritable mutant alleles, which can then produce mutant phenotypes for characterization. To generate a library of such *Mu*-induced alleles throughout the maize genome, plants from a *Mu*-active line were outcrossed to produce a large number of mutagenized progeny plants. The progeny were both self-fertilized (to preserve the new alleles) and extracted for DNA (to provide material for identification of mutations of interest). In a TUSC screen, these individuals are screened by PCR, using a primer specific for the gene of interest along with one to the *Mu* inverted repeat. Individuals with a *Mu* insertion near the target gene test positive by producing a PCR product, and progeny from these individuals are then further tested to determine if the insertion is heritable and within the target gene. After confirming heritability, new alleles can then be sequentially backcrossed into inbred lines to dilute out any other new, unlinked mutations, and to produce populations for phenotypic analysis.

Materials and Methods

Mutant Alleles

Six *Mu* insertion alleles of the *rop6* and *rop7* genes (three for each gene) were obtained in a TUSC screen conducted by Pioneer Hi-Bred International, Inc.. From these initial mutant individuals, families were generated carrying the insertion alleles in four different inbred backgrounds: W22, W23, H99, and A188. Utilizing four different backgrounds also provided the possibility of revealing a mutant phenotype

that was masked in one inbred line due to genetic background effects. The mutant alleles were first crossed to *Mu* inactive lines (based on the *bz1-mum9 Mu* activity reporter) to prevent the generation of any new insertion events, and to keep the ROP mutant alleles stable. *Mu* inactive lines were subjected to backcrosses in all four inbred backgrounds. To date these backcrosses range from 3 to 5 generations depending on the particular inbred background. All mutant plants were identified using PCR-based genotyping (see below), labeled and crossed using standard maize genetic procedures.

PCR Genotyping

The mutant alleles were sequenced and the *Mu* insertion sites were determined. Alleles containing a *Mu* insertion site within an exon were given priority in phenotypic analyses. Gene sequences were also used to generate primers that were specific for each gene for determination of plant genotype by PCR.

For PCR genotyping reactions, approximately 50 mg of leaf tissue was used for DNA extraction. DNA extraction yields were higher on young leaf tissue, but all stages of leaf development were adequate for providing template DNA. The protocol (detailed in [Arthur et al 2003]) consisted of a modified CAPS extraction. Individuals were screened using primer pairs for the *Mu* terminal inverted repeats, *rop6*, and *rop7* mutant alleles, depending on the genotypes segregating in the family. Since corn is a diploid, families were subjected to dual PCR assays to determine the individual plant genotype. The first PCR experiment screened for individuals carrying the mutant

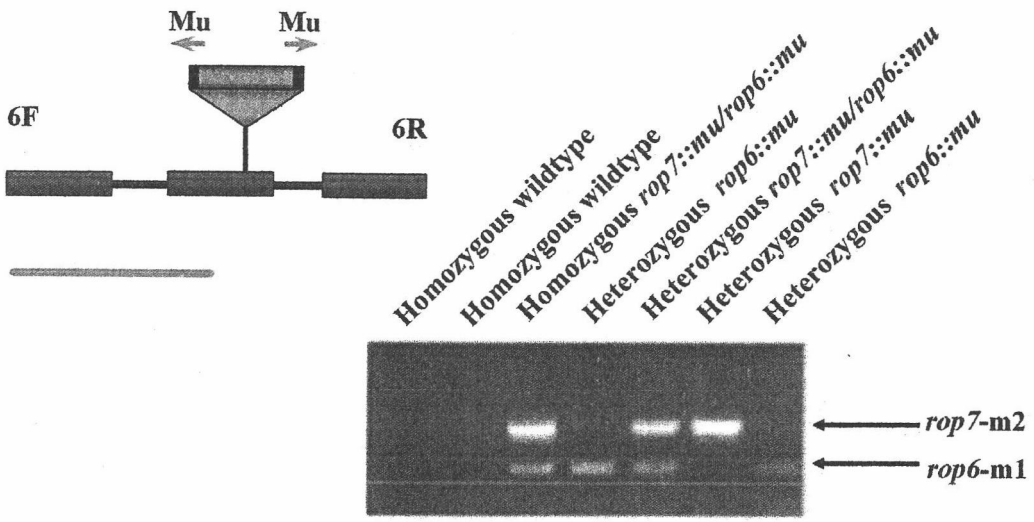

Figure 13. A two-step multiplex PCR genotyping assay is an efficient method for identifying mutant individuals. The gel above only shows the first step PCR reaction. A primer specific for *Mu* and another specific for the gene of interest (e.g., 6F in this diagram) are used together to test for the presence of mutant alleles. Plants that carry one or more copies of a mutant allele generate bands of predicted sizes, depending on the insertion site of *Mu* into the corresponding gene. The second step PCR reaction (not shown) employs only gene specific primers (e.g. 6F and 6R in this diagram). The presence of a *Mu* element prevents PCR amplification across the entire mutant allele; thus, mutant homozygotes are recognizable by the absence of a wild-type band.

alleles, and the second screened for individuals carrying the wild type alleles (Figure 13). In addition to screening the individual plants, this dual PCR experiment served as an internally controlled experiment to determine the suitability of each DNA prep for PCR, as all genotypes should allow amplification of at least one band.

RT-PCR of Mutants

To determine if the wild-type transcript was diminished or absent in our mutants, RT-PCR was used to amplify products from the ROP transcript from each gene. The procedure utilized was identical to that used to generate the ROP expression data from wild-type plants (Chapter 2). RNA was extracted from the shoot apices of a pool of homozygous mutant seedlings in a segregating family to help control for background effects on transcript levels.

Phenotype Detection

Plants were subjected to differing environmental conditions within the lab, including; high salt, cold, and dark-induced etiolation. Additional laboratory observations included microscopic examination of pollen, germinated pollen, dissected meristems, immature leaves, mature leaf epidermal cell shapes and sizes, mature leaf cross sections, roots, and root hairs. Seedlings from mutant segregating families were grown in pyrex dishes for two weeks and plant morphology was examined. Roots, root hairs, leaves, coleoptile, and other structures were compared.

Greenhouse and field studies involved examining full-grown plants and characteristics within and between families, time to flowering, and plant morphology.

Results

Sequencing of the genomic DNA near each *Mu* insertion indicated the exact insertion site for each transposon in either the *rop6* or the *rop7* gene, and helped identify the alleles most likely to interfere with gene function - the exon insertions (Figure 14). The alleles for *rop6* were located both within and downstream of the coding region: *rop6-m1* is within the penultimate exon, *rop6-m2* is within the final intron, and *rop6-m3* is downstream of the stop codon, in the 3' untranslated region (UTR). For the *rop7* mutant alleles, *rop7-m2* and *rop7-m3* are located within exons, whereas *rop7-m1* is located within the third intron.

Because not all *Mu* insertions (particularly those in introns) eliminate the production of wild-type transcripts from a gene, I used RT-PCR to determine the effect of each insertion on the gene in question. Multiplex RT-PCR experiments included primers to *rop6*, *rop7* and *actin1* as an internal control, and tested RNA from each mutant homozygote singly, as well as a *rop6*-m1; *rop7*-m2 double mutant homozygote (Figure 15). This experiment showed that the wild-type transcript is either eliminated or severely reduced to undetectable levels by four of the mutations: *rop6*-m1 and -m3, and *rop7*-m2 and -m3. These four mutants include all three exon

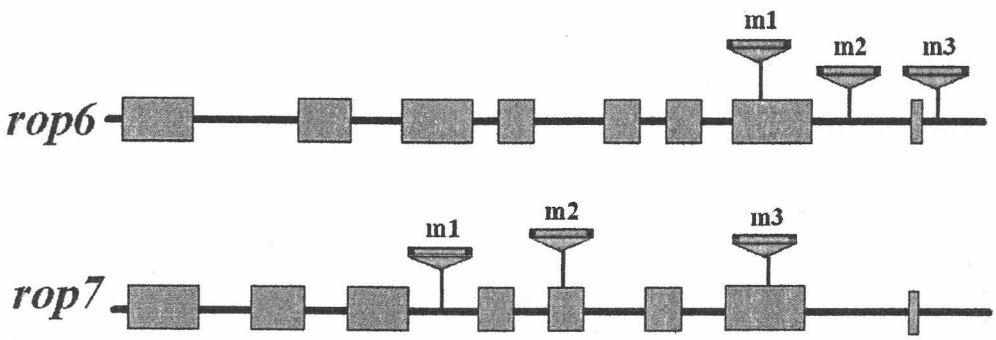

Figure 14. Location of *Mu* insertion sites for *rop6* and *rop7* mutant alleles. These six different mutant alleles (designated *m1*, *m2* and *m3* at the transposon insertion site, for each gene) were obtained using Pioneer Hi-Bred's TUSC methodology. Insertion sites were determined by sequencing PCR products containing the Mu-rop junctions from each allele.

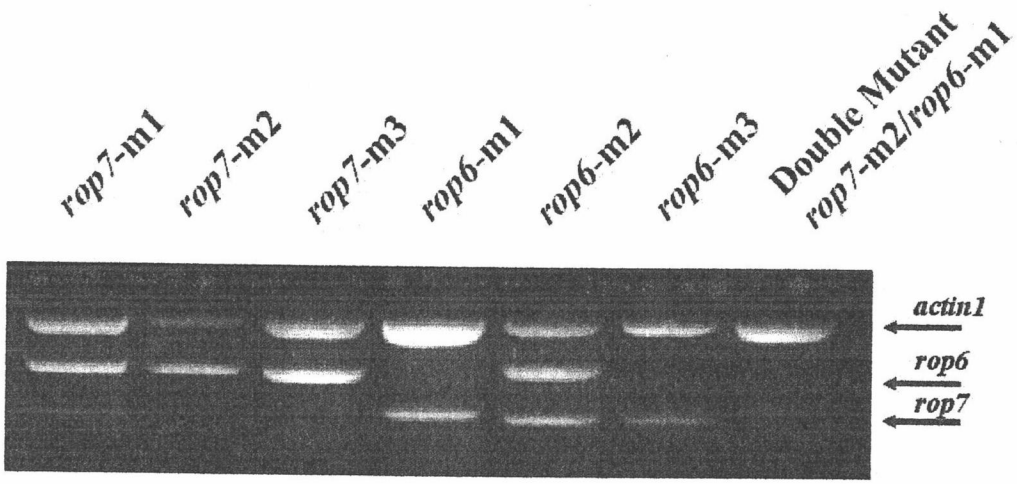

Figure 15. Certain *rop6* and *rop7* insertion alleles affect the production of ROP transcripts. Exon insertions (*rop6-m1*, *rop7-m2* and *rop7-m3*) and 3' UTR insertion (*rop6-m3*) inhibit normal transcript production. However, the intron insertions (*rop6-m2* and *rop7-m1*) appear to be spliced out, allowing for the production of a transcript similar to the wild-type size.

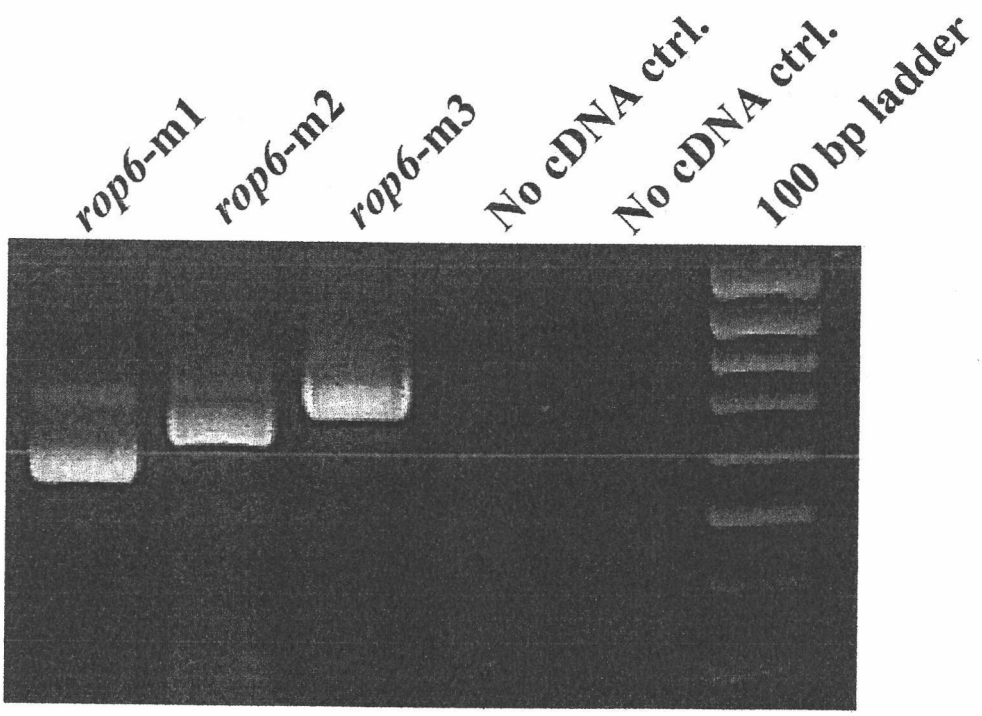

Figure 16. Mutant *rop6* alleles produce modified transcripts containing *Mu* transposon sequences. A *Mu* primer along with a *rop6* primer (located 5' of the first transposon insertion site) indicates that all three alleles produce *rop6* transcripts containing *Mu* element sequences.

insertion alleles, as well as the *rop6* insertion in the 3' UTR. In addition, this confirms that the double mutant homozygote does not produce detectable wild-type transcripts for either gene. The two additional mutant insertions, *rop6*-m2 and *rop7*-m1, are located within introns and do not appear eliminate the wild-type ROP transcript (although the *rop7*-m1 mutation may reduce the transcript level), suggesting that the splicing machinery can remove the *Mu* element transcribed into the pre-mRNA.

I also tested, using a *Mu* inverted repeat primer, whether the *rop6::Mu* alleles produced detectable *rop6* transcripts containing the *Mu* transposon. Using a *Mu* primer along with a *rop6* primer indicates that all three alleles produce *rop6* transcripts containing *Mu* element sequences (Figure 16). Although the *rop6*-m3 allele did not produce a wild-type transcript (Figure 15), these data suggest that it produces a modified transcript containing the *Mu* sequence downstream of the mRNA's coding region. Because the inserted *Mu* sequence in this transcript is located downstream of the coding region, it may not interfere with translation of a full-length, wild-type ROP6 protein from this allele. However, because the GTPase C-terminus is crucial for membrane association and correct protein function [Magee et al. 1999], it is likely that, even if translated, the three exon insertion mutants (*rop6*-m1, *rop7*-m2 and *rop7*-m3) will eliminate production of a functional ROP GTPase. However, no work on ROP6 or ROP7 protein levels in these mutants has yet been attempted.

After determining that these three exon-insertion alleles inhibit expression of a wild-type transcript, it was important to set up experiments that would examine the impact of the alleles on plant growth and development. By starting with mutant lines

backcrossed into standard inbreds, I was able to produce maize populations segregating the *rop6* and *rop7* mutations that minimized genetic background effects and variability, to help in identifying subtle phenotypes. PCR-based genotyping (see Materials and Methods) was used to select plants for comparison of homozygous mutants to wild-type and heterozygous siblings within a population. In addition to making homozygous mutants for the three exon insertion alleles, I also generated double mutant families to eliminate the function of both *rop6* and *rop7* in the same plant, hoping to eliminate any redundancy between the two ancient duplicates. After examining several developmental parameters in these families (see Materials and Methods), I found no strong correlation between any mutant phenotype and the individual's genotype, including the double mutant homozygotes. These experiments were conducted in four different backgrounds, and still there was no definite phenotype associated with these mutants besides the loss of a detectable wild-type transcript. To determine if these genes are under environmental control and only influence plant development in response to some signal, I also conducted initial experiments to examine growth responses under varying environmental conditions. Stress conditions used included; high salt, cold, and darkness. Again, none of these factors revealed a phenotypic difference between wild-type and ROP mutant plants.

Conclusions

I carried out a series of experiments designed to reveal the functions of two maize *rop* genes (*rop6* and *rop7*) through a "reverse genetic" mutational analysis. I was able to confirm that certain of our mutant alleles eliminated or strongly reduced the presence of the corresponding wild-type *rop* transcript, and therefore were likely to strongly effect the corresponding *rop* function. However, despite examining mutant plants carefully, no phenotypic difference between wild-type and mutants were discovered. Thus, although a great deal of work has been completed on these *rop* mutants, these experiments have not yet generated information that explains how *rop*s function in maize. The fact that I was also able to generate *rop6*; *rop7* double mutants, thus eliminating both ancient duplicates, and still observe no mutant phenotype suggests several hypotheses. These genes may serve no critical role, or function in a pathway not normally induced under standard growing conditions. Other possibilities are that the mutant alleles may not completely block production of their corresponding ROP protein, or the genes may have homologs in the maize genome that compensate for their functions when absent (functional redundancy).

The hypothesis that these genes serve no important role in the plant seems unlikely. The strongest evidence against this possibility is that both of these *rop*s were duplicated in a genome-wide event approximately 11 million years ago [Gaut et al. 1997], and then retained as highly conserved, expressed genes (93 % identity between the two genes in the coding region) suggests they play some role that provides a selective advantage. To select against spontaneous mutation and subsequent loss of

these genes requires that *rop6* and *rop7* positively influence the fitness of the organism. Examining the roles of similar mammalian proteins, such as Ras and Rho, show these GTPases often serve in critical signaling pathways [Gao et al. 2004; Moon et al. 2003], also arguing that maize *rop*s may play similar, important roles. Finally, despite being highly conserved in the ROP coding region, different members of the maize *rop* gene family show differential expression patterns (Chapter 2), suggesting that several of maize *rop*s have distinct and specific roles.

The second possibility explaining the lack of an evident phenotype is the potential for these molecular switches to function in a pathway that must be induced by some environmental factor. In such a scenario, a mutant phenotype in a *rop6*; *rop7* mutant would only be expressed when subjected to this particular environmental condition. Although my initial studies, subjecting the *rop* mutants to several environmental stresses, have not revealed such an induced phenotype, it is quite possible that these limited studies failed to subject the mutant plants to the "correct" condition. Mutations in large gene families in *A. thaliana* have been screened using a large-scale "gauntlet" of different environmental stresses to reveal mutant phenotypes and characterize gene function [Krysan et al., 1999; Fu, 2001]. This technique has been very successful in *A. thaliana* at revealing gene function for otherwise "silent" mutations, and may also be useful in maize. One specific possibility worth testing is that the maize ROPs may regulate pathways for disease resistance, including programmed cell death. This possibility is particularly relevant given the recent work on the rice *OsRac1* gene, a closely-related homolog of *rop6* and *rop7* (Chapter 2),

which has shown this gene to modulate the hypersensitive response to pathogen infection in rice [Kawasaki et al, 1999]. There are several other pathways (e.g., phytohormone response signaling) that have yet to be explored. Collaborative efforts with other maize genetics lab, including ones with expertise in plant pathology, may increase the scope of the maize *rop* mutant "gauntlet" beyond my initial work.

Another explanation for our lack of a phenotype would be that our mutant alleles do not completely block production of the wild-type transcript, or the wild-type protein. Although the RT-PCR experiments (Figure 15) showed the absence of a wild-type transcript, it is possible that a mutant transcripts could produce a functional protein ,or that there may be a small amount of wild-type transcript (below the detection threshold for RT-PCR) that is translated. Although these possibilities seem unlikely, due to the sensitivity of RT-PCR, and the importance of the GTPase C-terminus for correct protein function, they have not been excluded. To date we have done little in the way of protein analysis, as it has proved difficult to obtain an antibody that is specific for each ROP, given the high amino acid identity among family members.

Finally, my expression analysis (Chapter 2) indicates that several other maize *rop*s, including the closely-related *rop3*, are expressed in the same tissues as *rop6* and *rop7*. These homologous *rop*s may compensate for the loss of *rop6* and *rop7* function due to mutation. It is clear that although the *Mu* insertion alleles provided by the TUSC method have been a valuable tool for identifying the function of certain genes [Benson, 1995; Mena 1996], this approach has been less successful with genes in large

families [J. Fowler, personal communication]. This functional redundancy among gene family members may thus be found throughout a genome (such as maize) derived from a relatively recent duplication event. To address this possibility, either additional mutant alleles in the *rop* family will need to be isolated and combined with the *rop6* and *rop7* mutants, or a strategy (such as the use of RNA interference) to eliminate multiple *rop* functions simultaneously, will need to be pursued.

Chapter 4

Summary

Todd M. Christensen

A great deal of work has been focused on the ROP GTPase families in plants [Valster et al. 2000; Yang 2002], but there is still a great deal of information needed before we can conceptualize exactly how these proteins function in plants. Although there have only been nine ROP GTPases identified in maize [Christensen et al., 2003], these nine genes are thought to interact with multiple signaling pathways and cellular processes, providing a high level of functional diversity. However, the mechanisms by which this functional diversity is generated and regulated to produce specific responses in plants is not understood.

My work showed that expression levels for seven maize ROP genes were highest in tissues undergoing active cell division and/or elongation. As a result, it is perhaps not surprising that the single and double mutants I characterized did not produce a visible phenotype. The decreased expression from the mutant alleles could have been compensated for by the presence of other closely-related and highly-expressed ROP GTPases. Although I examined the relative expression level of the maize ROPs in wild-type plants, I did not closely examine expression levels across the entire ROP family in the mutant plants. ROPs have been shown to function as rheostats in signaling during anoxia [Baxter-Burrell et al 2002], and it would be interesting to see if these GTPases have some method for auto-regulation, possibly to influence the expression of the other ROPs within the family. Such auto-regulation might provide an additional method to compensate for the loss of *rop6* and *rop7* function.

Although these GTPases appear to function as switches for several cellular

processes [Yang 2002], they also appear to regulate specialized functions as well. For example, my experiments indicated that germinated pollen and mature leaf tissue exhibited decreased levels of ROP mRNA when considered on a family-wide scale. However, these two tissue/cell types also showed unique high levels of expression of particular ROPs. Germinated pollen showed the specific high expression of *rop2* (and its duplicate gene *rop9* [Arthur et al. 2003]), and no other ROP. My phylogenetic analysis shows that ROP2 is grouped with the *A. thaliana* ROP1At, ROP3At, and ROP5At proteins, which are expressed in pollen tubes and can influence the polar growth of these structures. Follow-up studies involving knockout mutants for *rop2* have shown that *rop2* mutant alleles confer a competitive disadvantage to the maize male gametophyte [Arthur et al. 2003], thus helping to validate one of my speculative hypotheses.

Another interesting expression pattern can be attributed to *rop1*. Only *rop1* appears to show no significant reduction in expression in the mature leaf. This high level of *rop1* mRNA might be attributed to a functional role in response to environmental changes or disease resistance. It is therefore perhaps not surprising that ROP1 is grouped with the *Arabidopsis* ROP10At protein, which has been recently identified as a negative regulator of abscisic acid (ABA) responses in *A. thaliana* [Zheng et al. 2002]. This specific expression of *rop1* in mature leaf tissue, where other ROPs are less-highly expressed, as well as its phylogenetic relationship to ROP10At, suggests that this gene may influence the ABA signal response as well. Thus, *rop1* is a good target for further mutational analysis.

Utilizing phylogenetic analysis and relative expression profiles, I have developed insights into where the maize ROPs are expressed and suggested hypotheses for what functions they may perform *in vivo*. This information is very helpful for identifying optimal gene candidates for further knockout mutational analysis. Already the knockout mutants in *rop2* have shown the value of this approach [Arthur et al. 2003]. Without knowing where *rop2* is expressed, it might have been much more difficult to identify the subtle phenotype associated with the male gametophyte in mutant *rop2* maize plants. Thus, a genetic approach paired with future experiments examining this gene family at the protein level may ultimately provide a comprehensive picture of how ROP GTPases function in maize.

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