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Expression patterns and effects of stress stimuli on nodulin 26-like proteins of Arabidopsis thaliana

Rana Ferrebee

To the Graduate Council:

I am submitting herewith a thesis written by Rana Ferrebee entitled "Expression patterns and effects of stress stimuli on nodulin 26-like proteins of Arabidopsis thaliana." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Daniel M. Roberts, Major Professor

We have read this thesis and recommend its acceptance:

Elizabeth Howell, Albrecht von Arnim

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Expression Patterns and Effects of Stress stimuli on Nodulin 26-like Proteins of *Arabidopsis thaliana*

A Thesis Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Rana Ferrebee

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DEDICATION

This thesis is dedicated to

my parents

Mr. Robert Ferrebee

and Mrs. Carol Ferrebee

whose support and love made this possible.

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I want to express my deepest thanks to my parents who were always there to give me love and support when I needed it the most. If it hadn't been for their unconditional love, wisdom and encouragement I wouldn't have made it this far in life. Finally, I would like to thank other graduate students in the department who have been my friends and family during these last three years.

ABSTRACT

The transport of water across lipid bilayers is regulated in part by aquaporins. Aquaporins are integral membrane proteins that belong to the ancient major intrinsic protein (MIP) superfamily. From sequence analysis and biochemical localization studies plant aquaporins can be divided into three groups. PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins) and nodulin 26-like proteins (NLMs). Nodulin 26 is a major protein of the symbiosome membrane of nitrogen-fixing nodules of soybeans where it functions as an aquaglyceroporin, mediating the transport of water as well as uncharged solutes. The biological function of nodulin 26 in the symbiosome membrane remains elusive, but a role in osmoregulation as well as metabolite transport has been proposed. Until recently, it was thought that nodulin 26 was a unique MIP with a dedicated function on the symbiosome membrane. However, it has recently become clear that other "nodulin 26-like" MIPs (NLMs) are present, not only in other legumes, but also in nonlegumonous plants and in tissues besides nodules. With the completion of the sequencing of the *Arabidopsis* genome we now know that there are 10 NLMs in *Arabidopsis* ranging from 32.1% to 62.4% identity to nodulin 26. In addition, sequence analysis suggests that many of these proteins will be aquaglyceroporins and also be targets for calcium dependent protein kinase (CDPK). In the present study we have investigated: 1) The tissue specific expression patterns of three NLMs, At-NLM1, At-NLM2, and At-NLM3 in *Arabidopsis thaliana*; 2) Effects of various environmental

stimuli on the expression patterns of these NLMs; 3) The functions of one At-NLM3 by using transferred (T-DNA) mutagenesis technology.

To help elucidate the specific function of NLMs in *A. thaliana* their tissue distribution patterns were evaluated. The results of RT-PCR show that At-NLM1 is specifically expressed in two organs, the flowers and the roots. The expression in the roots is slightly higher than that in the flower. In contrast, At-NLM3 was found to be expressed in all organs tested, but the expression levels between tissues were different. At-NLM3 was expressed at the highest levels in roots and the stems and was expressed at lower levels in the flowers and leaves, and was nearly undetectable in the siliques. Lastly, At-NLM2 was expressed nearly evenly in all organs tested but at higher levels than At-NLM1 and At-NLM3.

To determine the potential involvement of At-NLMs in stress biology the analysis of At-NLM1 and At-NLM3 transcript levels upon challenge with various environmental signals was performed. At-NLM3 levels showed a differential response to the applied stresses. At-NLM3 levels showed little sensitivity to mannitol, and showed a slight decrease with cold temperature and NaCl. However, At-NLM3 showed a higher sensitivity to drought, with a 2.5 fold reduction in At-NLM3 levels observed over 2.5 hours. In addition, the transcript level of At-NLM3 showed a 2.5 fold decrease with the application of the stress hormone abscissic acid (ABA). Lastly, At-NLM3 shows a substantial up regulation of 7-fold upon growth in darkness.

Similar to At-NLM3, the application of 0.1 mM ABA decreased the amount of At-NLM1 mRNA by four fold. However, in contrast to At-NLM3, transcript levels of At-NLM1 exhibited a higher sensitivity to NaCl and decreased temperature, showing a 2-

fold reduction for both treatments. The transcript levels of At-NLM1 respond rapidly to NaCl treatment, showing a decrease by 30 minutes. The transcript levels began to decrease within a 30 minutes and eventually reach a four-fold reduction at the 34 hour time point. The levels of At-NLM1 mRNA did not seem to be greatly affected by drought or mannitol. In addition, At-NLM1 expression showed diurnal variation in expression with highest transcript levels during the light period of the photocycle and lowest levels within the dark part of the photocycle.

The regulation of AtNLM1 and At-NLM3 by stress/environmental stimuli suggest they play a role in adaptation to changing osmotic conditions and also are developmentally controlled by light stimuli. However, the role of these putative channel proteins in stress biology and membrane function remains unclear. To gain insight into its role in stress physiology, a T-DNA knockout mutant of At-NLM3 has been generated. Preliminary phenotype analyses were done on *A. thaliana* plants homozygous for the At-NLM3 knockout. Two reproducible growth defects were observed, one with green plants and one observed with etiolated plants. Preliminary analysis of the knockout mutant suggests some developmental defects including reduced stem and leaf growth, reduced stem thickness and stability, increase in the timing of bolting and developemental defects in etiolated palnts. Overall, the data show that the NLMs in *Arabidopsis* are differentially expressed and are regulated at the transcript level by osmotic and stress stimuli.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Plant-Water Relations

Water is the universal solvent and the most abundant molecule in all living tissue (Johansson et al., 2000). In common with other organisms, life in plants takes place in an aqueous medium. For many, water comprises 90% or more of their weight. Water is essential for the structural integrity of biological molecules and hence the integrity of cells, tissues and the organism as a whole. It serves to dissolve and bring in minerals from the soil, it is the solvent for sugars from photosynthesis being carried throughout the plant, it maintains the turgor pressure in the cells, and it cools the leaves as it evaporates.

Rooted plants depend on a supply of water for growth and development and must maintain a tight control over water balance (Maurel, 1997). In plants, water moves through the living tissue in response to hydrostatic and osmotic forces (Boyer, 1985). In actively growing plants there is a transpiration stream that begins as water is absorbed through the roots and ends as water exits the plant as vapor through the stomatal pores of the leaves. Long-distance transport is achieved by bulk flow through the vascular tissue, the xylem and phloem, where membrane barriers are not a major problem (Johansson et al., 2000). In contrast, there are many physiological processes that require short-distance

water flow, such as cell expansion and osmoregulation, where water must travel across membranes (Maurel, 1997).

Water transverses biological membranes by two distinct mechanisms, simple diffusion through the lipid bilayer or by facilitated transport through proteinaceous water channels (King and Agre, 1996). Two terms that are often used to describe membrane permeability are the osmotic permeability coefficient (P_f) and the diffusion permeability coefficient (P_d). P_d and P_f can be used to differentiate between lipid and protein channel-mediated water flow. P_f represents the water permeability exhibited in the presence of an osmotic gradient whereas P_d reflects the diffusive permeability of membranes under isoosmotic conditions (Finkelstein, 1987). The ratio of these two biophysical parameters is used as a measurement of whether water flow is facilitated. In a bare lipid bilayer P_f/P_d is approximately unity, whereas if transport is through a facilitated path, P_f exceeds P_d (Finkelstein, 1987; Tyerman et al., 1999). Simple diffusion through a lipid bilayer is constrained by membrane lipid organization and fluidity, and therefore shows an acute dependence on temperature and exhibits a high Arrhenius activation energy ($E_a > 10$ kcal/mol) (King and Agre, 1996). In contrast, channel-mediated transport occurs in aqueous pores and therefore exhibits a lower Arrhenius activation energy ($E_a < 5$ kcal/mol) nearly characteristic of diffusion of water in an aqueous environment (King and Agre, 1996). The more rapid rate and lower energy cost of channel-mediated transport helps to explain multiple physiological processes in plants, such as cell elongation and stomatal movement that require rapid translocation of large volumes of water across membranes (Johansson et al., 2000).

Major Intrinsic Protein (MIP) Family

Our understanding of the molecular nature of facilitated water transport has been revolutionized by the discovery of water-selective transport channel proteins known as aquaporins (Preston et al., 1992). The ubiquitous major intrinsic protein (MIP) family of transmembrane channel proteins is a very old family of proteins, dating back perhaps 2.5 to 3 billion years in evolutionary time (Park and Saier, 1996). It has been suggested that all MIP family members have evolved from two bacterial paralogs, an aquaporin (AQPZ) and a glycerol facilitator (GlpF) (Johansson et al., 2000). The MIP family was named after MIP26 protein of the bovine lens fiber cell membrane, the first member that was sequenced and characterized (Gorin et al., 1984). Since the discovery of bovine lens MIP, proteins with similar structures have been found widely distributed in microorganisms, plants, and animals. The size of the MIP family continues to grow at an extraordinary rate, with more than 200 family members identified (reviewed in Calamita, 2000). The array of MIP family members is particularly complex in higher organisms, which contain many isoforms that are expressed in different tissues, at different developmental stages, or in response to changing environmental conditions (reviewed in Johansson et al., 2000).

All MIP family members contain a similar topology with conserved structural hallmarks. For example, the MIP family members generally have a similar molecular weight of 23-31kDa, and possess six putative membrane spanning regions, and cytosolic,

hydrophilic amino and carboxyl terminal regions (Agre et al., 1998; Borgnia et al., 1999). The topology of soybean nodulin 26 is shown in Figure 1, illustrating the typical structural characteristics of the family. The six transmembrane regions show an obverse symmetry with the first half of the molecule related by a two-fold axis with the second half of the molecule. Two highly conserved loops, B and E contain the family's signature sequences: Ser-Gly-X-His-X-Asn-Pro-Ala-Val-Thr (where X=any amino acid) and Asn-Pro-Ala-Arg, respectively. Each contain a canonical NPA (Asn-Pro-Ala) box (Hohmann et al., 2000). These two relatively hydrophobic loops dip into the membrane bilayer, forming a continuous solute channel (the so called hourglass), lined by a chain of hydrogen-bonding amino acids (Jung et al., 1994; Hohmann et al., 2000). The hourglass model was recently verified with the crystallization of the *Escherichia coli* glycerol facilitator (GlpF) (Fu et al., 2000), the structure of which is described below.

In addition to their structural similarity, members of the MIP family show similar patterns of function. The pioneering study that confirmed the role of MIPs in water transport was conducted with CHIP28 (channel-forming integral protein of 28 kDa) now named aquaporin-1, AQP1 (Preston et al., 1992). *Xenopus* oocytes expressing AQP1 showed a substantial increase in osmotic water permeability (P_f) ($P_f \sim 200 \times 10^{-4}$ cm/sec), which was greater than ten times that of water injected oocytes (Preston et al., 1992). Further studies with proteoliposomes reconstituted with purified AQP1 also supported its role as a water channel (Zeidel et al., 1992; van Hoek and Verkman, 1992). Biophysical measurements show that AQP1 exhibits all the properties proposed for a facilitated water transport pathway including: a high osmotic water permeability (P_f), a low Arrhenius activation energy, an osmotic to diffusive water permeability ratio

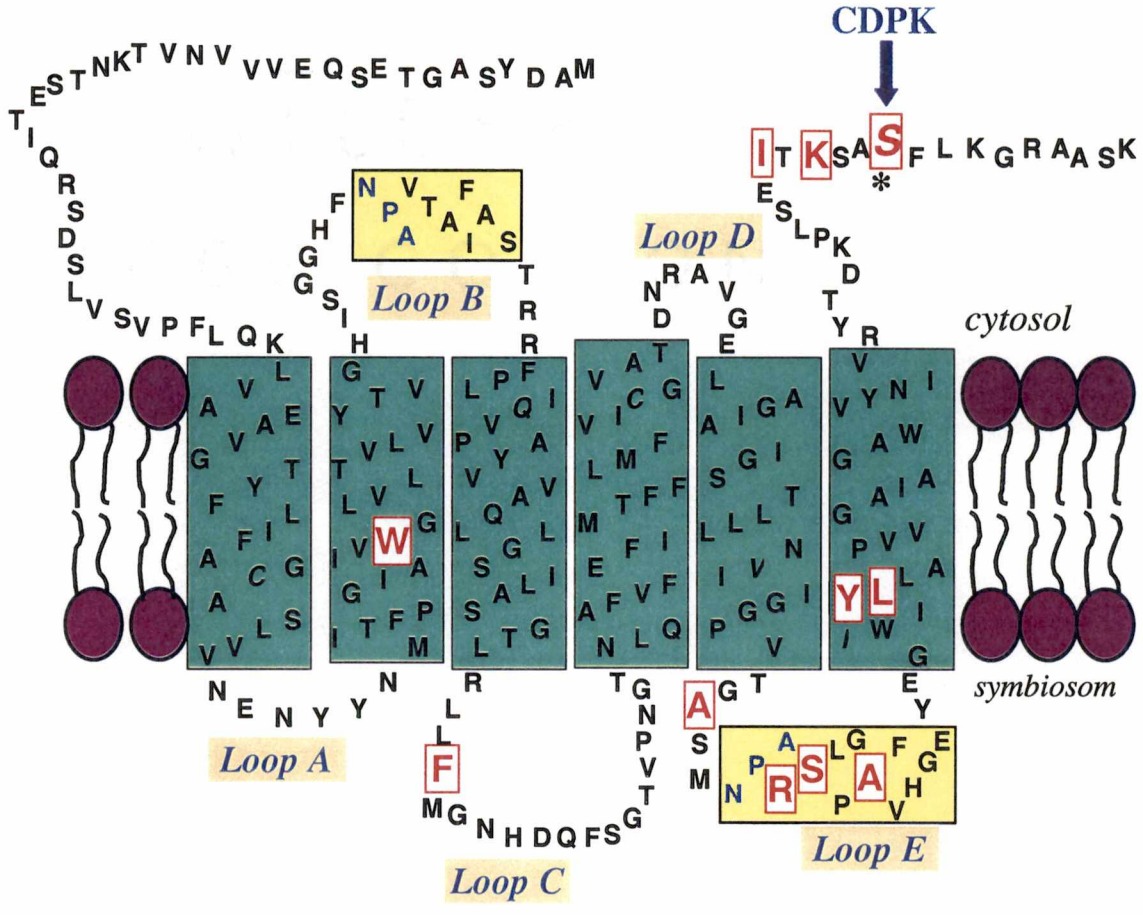


Figure 1: Nodulin 26 topology showing conserved structural and functional features. Green boxes represent six α-helical transmembrane domains. Yellow boxes represent helical regions in loops B and E that contain the two NPA signature sequence motifs. The calcium-dependent protein kinase phosphorylation (CDPK) site is highlighted in the carboxyl terminal domain (hydrophobic-X-basic-X-X-Ser) (Bachman et al., 1996). Residues boxed in red correspond to those proposed to be important for glycerol/water specificity (Froger et al., 1998; Fu et al., 2000).

(Pf/Pd) > 1, and inhibition by mercurial reagents (Finkelstein, 1987; Stein et al., 1990; Chrispeels and Agre, 1994; Brown et al., 1985; Agre et al., 1995; Maurel, 1997). AQP1 was shown to be highly specific for water and does not flux ions, protons or solutes (Zeidel et al., 1992). Following these studies, AQP1 and other proteins with similar functions were designated as aquaporins (the official name accepted by the Human Genome Nomenclature Committee) (Agre et al., 1993; Agre, 1997). Since the discovery and functional characterization of AQP1, the transport properties of many MIPs have been analyzed.

Transport studies using MIP channels expressed in *Xenopus* oocytes and detailed sequence comparisons have led to the classification of MIPs into two major categories: (1) aquaporins, which are highly specific for water and (2) glycerol facilitators, which transport glycerol and possibly other solutes in addition to, or even in preference to, water (Borgnia et al, 2000; Agre et al., 1998). However, classification of transport properties of MIP proteins may depend on the method used to establish its function. For example, when bovine lens MIP is expressed in *Xenopus* oocytes, it is clearly an aquaporin (Mulders et al., 1995). But when the protein is incorporated into the lipid bilayer, it behaves as an ion channel (Zampighi et al., 1985). Bacteria have MIPs that are glycerol channels (Maurel et al., 1994), and some mammalian MIPs have been reported to be bifunctional, transporting water and ions (Yool et al., 1996; Yasui et al., 1999) or glycerol, urea, and water (Ishibashi et al., 1994; Frigeri et al., 1995; Echevarria et al., 1996; Ishibashi et al, 1997). Recent studies have suggested that aquaporins may also be permeated by gases (Cooper and Boron, 1998; Prasad et al., 1998). Despite these varied and conflicting reports, biochemical and genetic evidence suggest that MIPs fall into one

of two transport classes: 1. water selective aquaporins and 2. glycerol transporters, including selective glyceroporins (e.g. the glycerol facilitator) or multifunctional aquaglyceroporins (Agre et al., 1998). Needless to say, however, there are other activities of MIP proteins that may occur under certain physiological conditions and merit further consideration.

Since the discovery of AQP1, nine additional mammalian homologs have been reported and are referred to as AQP0-AQP9 (reviewed in Agre et al., 1998). AQP1 is expressed in many tissues and cell types, including erythrocytes, kidney, lung, and eye (Borgnia et al., 2000). AQP1 is a constitutively active water-selective pore that permits osmotically driven water movement (Engel et al., 2000). AQP1 is structurally the most well studied aquaporin. AQP1 is a homotetramer containing four independent aqueous channels (Jung et al., 1994; Engel et al., 2000). The three-dimensional structure of AQP1 has been determined at 6Å resolution by cryo-electron microscopy. Each AQP1 monomer has six tilted, bilayer spanning α-helices, which form a right handled bundle surrounding a central density (Engel et al., 2000). The central density formed by the long loops B and E has recently been resolved in a higher resolution structure as two short helices projecting outwards from the center of the monomer which are connected to adjacent helices by loop regions, thus confirming the hourglass model (Simon et al., 2000). This has been further confirmed by the x-ray crystal structure of GlpF at 2.2 angstrom resolution which provides for the first time a structure of a MIP family member at atomic resolution (Fu et al., 2000). GlpF crystallizes as a symmetric arrangement of four channels with three glycerol molecules in each. Six transmembrane and two half-membrane-spanning α-helices form a right-handed helical bundle around each channel.

The channel achieves selectivity and conductance through an amphipathic pathway that closely matches successive CH-OH groups. Three key residues, Phe 200, Trp 48, and Arg 206 make up the selectivity filter at the narrowest region of the GlpF pore (Fu et al., 2000). The two aromatic rings, Trp 48 and Phe 200, form hydrophobic van der Waals forces with the hydrocarbon portion of the glycerol. Two conserved NPA motifs form the key interface between two-gene duplicated segments that encode three-and-one-half membrane spanning helices around each channel (Fu et al., 2000). The relative location of these domains/motifs are shown on the nodulin 26 sequence illustrated in Figure 1.

AQP2 is an aquaporin specifically expressed in the collecting duct of the kidney and is responsible for the increased permeability of the membrane in response to vasopressin via phosphorylation (Fushimi et al., 1997; Katsura et al., 1997; Kuwahara et al., 1995). The fourth aquaporin, AQP4, is located in the brain, residing in the perivascular membrane of astroglial cells where it probably aids in the removal of excess brain water preventing edema (Agre et al., 1998). Interestingly, a mutation of this protein in *Drosophila* leads to a phenotype known as "big brain " (Rao et al., 1990). The fifth aquaporin, AQP5, resides in apical membranes of type I alveolar pneumocytes as well as in a subset of salivary and lacrimal glands where it may participate in airway humidification and generation of saliva and tears (Nielsen et al., 1997). Three other water-selective homologs, AQP0, AQP6 and AQP8, have been identified and partially characterized. AQP 0 is the name given for the original member of the family, the MIP 26 protein of bovine lens (Mulders et al., 1995; Zampighi et al., 1995; Chandy et al., 1997). Both AQP0 and AQP6 have low water permeability and are found in the lens of the eye and the kidney, respectively. AQP 6 is functionally distinct from other

aquaporins. When expressed in *Xenopus* oocytes, AQP6 exhibits low basal water permeability. However, when treated with the known water channel inhibitor, Hg^{2+}, the water permeability of AQP6 oocytes rapidly rises up to tenfold and is accompanied by ion conductance (Yasui et al., 1999). In addition, at pH less than 5.5, anion conductance is rapidly and reversibly activated in AQP6 oocytes (Yasui et al., 1999). Lastly, the cDNA encoding AQP8 has recently been isolated from testis, pancreas, liver, and other tissues (Koyama et al., 1997).

AQP3 is an aquaglyceroporin that transports both water and glycerol and is genetically closer to the *Escherichia coli* glycerol transporter GlpF (Sasaki et al., 1998). AQP3 expressed in the basolateral membranes of principal cells in the collecting duct and in the airways, suggesting roles in renal water reabsorption and mucosal secretions and allergic rhinitis (Ecelbarger et al., 1995; Nielsen et al., 1997). The function of glycerol solute transport activity of AQP3 remains unresolved. Multiple other aquaglyceroporins are now being identified using cDNA cloning and searching expression sequence tagged libraries (Agre et al., 1998). Another relative of AQP3, known as AQP7 has been identified in rat testis and is thought to provide a port for water and glycerol as a carbon source and may permit the replacement of water with glycerol during cryopreservation of sperm (Ishibashi et al., 1997; reviewed in Borgnia et al., 1999). Kuriyama et al. (1997) recently identified a related cDNA, AQP7L, from adipose tissue that is thought to play a role in exporting glycerol during lipolysis. Thus, despite the high homology and common structural framework between aquaporins, each probably has special features that confer a unique functional or regulatory properties (Agre et al., 1998).

Regulation of MIP Transport Activity

Facilitated water transport through aquaporins enables fast and reversible changes in transcellular water flow, and it is becoming clear that aquaporins are regulated to modulate this flow. There are many different mechanisms by which the rate of water transport can be altered across membranes. For example, the cellular levels of several MIP proteins are regulated at the transcriptional level. Gene expression is regulated developmentally in a cell-specific manner, in response to hormones, and by environmental cues as diverse as nematode infection or drought (reviewed in Maurel and Chrispeels, 2001). In addition, post-translational modifications of aquaporins include phosphorylation, glycosylation, and proteolytic processing and some of these may also modulate MIP activity (reviewed in Johansson et al., 2000). Recent work done in animals have shown aquaporins to be modulated in response to pH, free calcium change or by phosphorylation (Németh-Cahalan and Hall, 2000; Yasui et al., 1999; Zeuthen and Klaerke, 1999; Han et al., 1998; Maurel et al., 1995; Johansson et al., 1998). In addition, controlled protein targeting of mammalian AQP2 to the plasma membrane is triggered by phosphorylation in response to hormonal signals (Nielsen et al., 1995).

In plants, nodulin 26, kidney bean α-TIP, and PM28A have been shown to be phosphorylated *in vivo* (reviewed in Johansson et al., 2000). Nodulin 26 (Weaver and Roberts, 1992; Weaver et al., 1991) and PM28A (Johansson et al., 1998) are phosphorylated in the C-terminal domain, at serine-262 and serine-274, respectively, while α-TIP is phosphorylated at serine-7 in the N-terminus (Maurel et al., 1995). Both

PM28A and α-TIP showed an increase in water transport after phosphorylation in *Xenopus* oocytes (Maurel et al., 1995; Johansson et al., 1998). Recently, phosphorylation has been shown to also enhance the activity of nodulin 26 upon expression in *Xenopus* oocytes (Roberts and Chanmanivone, 2000). In addition, by incubating spinach leaves in buffers with different osmolarities, the phosphorylation state of PM28 was shown to increase with increasing apoplastic water potential (Johansson et al., 1996; Johansson et al., 1998). The observation suggests that as water levels decline, the protein is less phosphorylated, and therefore less active. In contrast, when water potential is high, the protein would be phosphorylated, and the channel would be open. Johansson et at. (1998) proposed a model in which loss of turgor caused by water deficit results in the dephosphorylation of PM28A so as to decrease aquaporin activity and conserve water.

The characteristics, position and amino acid environment, of the serine-274 of PM28A seem to be a unique feature for the regulation of PIP2 subfamily aquaporins in response to osmotic signals (Johansson et al., 2000). Although the amino acid environment surrounding serine-262 of NOD26 differs from that of PM28A, they both contain one or two non-polar and a basic residue on the carboxy terminal side of the phosphoserine (Johansson et al., 2000). The serine-7 phosphorylation site of kidney bean α-TIP is surprisingly not conserved in putative orthologs, *Arabidopsis* α-TIP, and pumpkin MP23 and MP28 (Inoue et al., 1995; Höfte et al., 1992). There is, however, a highly conserved serine in the first cytoplasmic loop that is found in PIPs, TIPs, and mammalian aquaporins but is not conserved nodulin 26-like MIPs (Johansson et al., 2000).

The phosphorylation of NOD26, PM28A, and kidney bean α-TIP have all been shown to be dependent on the well known, intracellular messenger Ca^{2+} (Johansson et al., 1996; Weaver et al., 1991; Johnson and Chrispeels, 1992). The kinase responsible for this is likely CDPK (calcium-dependent protein kinase) (reviewed in Roberts and Harmon, 1992; Roberts, 1993; Harmon et al., 2000). CDPKs have been identified in higher plants, algae, and protists and seem to replace or complement protein kinase C and -Ca^{2+} /calmodulin-dependent protein kinase, which are Ca^{2+}-dependent protein kinases in animals (Johansson et al., 2000). CDPKs are dependent on micromolar or submicromolar concentrations of calcium and bind calcium directly without the involvement of effector molecules, such as phosphatidylserine and diacylglycerol or calmodulin (reviewed in Roberts and Harmon, 1992; Harmon et al., 2000). NOD26 is one of the few endogenous substrates identified for a purified CDPK (Weaver et al., 1991).

Plant Aquaporins

The existence of water channels in mammals was conceived by observations made several decades ago. For example, osmotically driven water movement across red blood cell membranes and renal proximal tubular epithelium was too rapid to be explained by simple diffusion through the membrane and could be reversibly inhibited by mercurial compounds (reviewed in King and Agre, 1996). The presence of aquaporins or water transporting molecules in plants, unlike animals, was not so easily accepted for the simple reason that the majority of plants do not posses organs or tissues where

comparable amounts of water are transported through living tissue (Eckert et al., 1999). Simple diffusion of water through the membranes was originally thought to be adequate for the requirements of plant cellular water movement.

There are two distinctive types of water movement across membranes in plant cells: (1) fluxes that are necessary to maintain size, turgor and osmolarity of an individual cell or (2) transcellular movement of water through non-vascular tissue (Johansson et al., 2000). Most mature plant cells have a single vacuole that may take up 50 to 90 percent of the cell's interior. This fluid filled organelle stores amino acids, sugars, ions, and toxic wastes (Mohr and Schopfer, 1995). The cytosolic compartment lies between the vacuolar membrane and plasma membrane and occupies a narrow, yet critical, space. The composition of the cytosol must be tightly regulated to maintain conditions required for various metabolic activities. Rapid water movement mediated by the plasma membrane and vacuolar membrane, in combination with the movement of ions and other osmolytes are key in maintaining the proper osmolarity of the cytosol (Johansson et al., 2000). In addition, water flow has been suggested to have crucial roles in extension growth and organ/cell movements. Plants continue to grow throughout their lifetime, and to sustain growth an influx of water into the expanding cells is necessary (Johansson et al., 2000). Guard cells and motor cells within the pulvinus rely on shrinking and swelling to control stomatal aperture and leaf movement, respectively. This shrinking and swelling behavior is accomplished by osmotic gradients established by ion fluxes, which then drives massive water fluxes across the plasma membranes of these cells (Johansson et al., 2000). These observations have led to considerable interest in the characterization of facilitated pathways for water flux. By the 1980's, plant genes

encoding MIPs were discovered because of their abundance, (reviewed in Maurel and Chrispeels, 2001), and the simplified view of water transport by simple diffusion was challenged.

The break through in plant MIP research came in 1993 when Maurel et al. (1993) showed that the tonoplast intrinsic protein γ-TIP formed an aquaporin channel when expressed in *Xenopus* oocytes. γ-TIP was isolated by homology cloning using the cDNA corresponding to α-TIP, a seed-specific tonoplast integral protein, which constitutes 2% of the total extractable protein of bean cotyledons and was identified due to its abundance (Johnson et al., 1989). The first plasma membrane aquaporins in plants were identified using mammalian COS cells as an expression system and efficient microtiter-based strategy for immunoselection (Kammerloher et al., 1994). COS cells were transfected with an *A. thaliana* root cDNA library constructed in a bacterial mammalian shuttle vector and screened with an antiserum raised against purified deglycosylated integral membrane proteins from *A. thaliana* roots (Kammerloher et al., 1994). Antibodies directed against a prominent 27kDa antigen led to the identification of five different genes. They compromised two subfamilies related to the MIP superfamily and were named plasma membrane intrinsic proteins, PIP1 and PIP2, due to their suspected subcellular locations (Kammerloher et al., 1994). These studies showed that plant MIPs are found on both cellular and organellar membranes and account for the elevated water permeabilites associated with these membranes.

Since the initial findings our knowledge base regarding plant MIPs and facilitated water flow has expanded greatly. Aquaporins have been found in nearly all living organisms but seem to be even more diverse and numerous in plants. This first came to

light in the study by Weig et al. (1997) that identified 23 expressed *A. thaliana* MIP genes by searching the expressed sequence tag database and by using polymerase chain reaction with oligonucleotides to conserved plant aquaporin domains. An examination of the completed *Arabidopsis* genome indicates 39 genes encoding MIP proteins which accounts for nearly half of the total channel proteins in the genome, and which is at least 10-fold higher than the number of MIPs found in the sequenced genomes of *C. elegans*, *Drosophila*, or yeast (The *Arabidopsis* Genome Initiative, 2001). This underscores the importance and complexity of water relations in higher plants compared to other terrestrial organisms. Plant aquaporins can be grouped into three structurally and functionally homologous subfamilies (Weig et al., 1997). Two of these groups, the tonoplast intrinsic proteins (TIPs) and the plasma membrane intrinsic proteins (PIPs), have distinct subcellular locations on the tonoplast membrane of the vacuole or on the plasma membrane, respectively. The third group is the nodulin 26-like MIPs, NLMs (Weig et al., 1997). Members of this subfamily are homologous to the soybean protein, nodulin 26, which is a major component of the symbiosome membrane that encloses the nitrogen fixing rhizobium bacteroid of legume nodules (Fortin et al., 1987; Weaver et al., 1991). The features of this subfamily are discussed further below.

Since the initial work of Maurel et al. (1993) on γ-TIP, functional studies show that TIP and PIP proteins are aquaporins. For example, water transport activity has been demonstrated for several PIPs and TIPs in *Arabidopsis* (seven PIPs and three TIPs), tobacco (one PIP and two TIPs), ice plant (two PIPs), kidney bean (one TIP), sunflower (two TIPs), spinach (one PIP), and maize (one TIP) (reviewed in Johansson et al., 2000). As discussed further in detail below, nodulin 26 and nodulin 26-like MIP, NLM1, also

have aquaporin activity, but unlike the TIP and PIP proteins discussed above, they also are able to transport uncharged solutes.

Besides primary sequence similarities and functional properties that segregate plant MIPs into subclasses, the analysis of their physiochemical properties has yielded some interesting patterns. For example, the TIPs tend to be smaller and more acidic in their COOH-terminal regions than both the PIPs and the NLMs. Also, as discussed further below, the NLMs generally have a conserved phosphorylation site in this region. This finding raises the possibility that the conservation of putative phosphorylation sites or sorting, trafficking signals in the COOH-terminal regions may be essential for targeting or regulation within these subfamilies (reviewed in Johansson et al., 2000).

Besides the phylogenetic diversity of the MIP family in plants, another characteristic feature is the tendency for their expression to be regulated at the transcriptional level. Alterations in MIP gene expression have come to light in many studies investigating plant adaptation to drought or osmotic stress. Some aquaporins have been identified by differential hybridization performed in order to isolate stress induced genes. Clone 7a from *Pisum satium*, which belongs to the PIP1 subgroup, was shown to accumulate in response to a loss of turgor in wilted pea shoots (Guerrero et al., 1990). RD28, an *Arabidopsis* aquaporin belonging to the PIP2 subgroup was also shown to be up regulated in response to desiccation in *Arabidopsis thaliana* (Yamaguchi-Shinozaki et al., 1992). Desiccation and osmotic stress was shown to increase the mRNA abundance of the tonoplast aquaporin BobTIP26-1 in cauliflower cells (Barrieu et al., 1999) and the gene corresponding to TRAMP, a PIP1 homolog in tomato (Fray et al., 1994). Many of these genes that are induced by water deficit are also induced by the

plant hormone abscisic acid (ABA) (Yamaguchi-Shinozaki and Shinozaki, 1993). Thus, plant water deficit triggers the production of ABA, which in turn induces the expression of various genes. The products of these genes are thought to function in protecting the plant from dehydration (Dure et al., 1989; Bohnert et al., 1995).

The expression patterns and effects of salt- and drought-stress for three MIPs, NeMip1, NeMip2, and NeMip3 from *Nicotiana excelsior* have recently been demonstrated. NeMip1 was shown to be expressed in the roots and reproductive organs. In contrast, both NeMip2 and NeMip3 were expressed in all tissue examined (Yamada et al., 1997). All three transcripts, however, were shown to be up-regulated under salt- and drought-stress (Yamada et al., 1997). In addition, homologs to PIPs and TIPs were isolated from the desiccation-tolerant resurrection plant, *Craterostigma plantagineum*, by two approaches: firstly, a cDNA library constructed from RNA of dehydrated *C. plantagineum* leaves was screened with an *A. thaliana* Ath-PIP1b cDNA probe and secondly, a cDNA library was screened differentially to isolate early drought-induced transcripts (Mariaux et al., 1998). Transcripts of the clones isolated, Cp-PIPa, Cp-PIPc, and Cp-TIP, were shown to accumulate when exposed to abscisic acid (ABA) or dehydration (Mariaux et al., 1998).

Aquaporins expressed mainly or only during stress may exist in all plants, especially considering the large number of aquaporin and aquaporin homologs present in *Arabidopsis* and presumably in all plants (Johansson et al., 2000). In sunflower, two homologous tonoplast aquaporins, SunTIP7 and SunTIP20 are both expressed in guard cells but are differentially regulated. The transcript level of SunTIP7 accumulates during drought conditions and shows diurnal fluctuations, being low at dawn and dusk and high

at noon (Sarda et al., 1997). This diurnal pattern correlates well with stomatal movements. When the stomata start closing, the level of SunTIP7 transcript is high and at night when the stomata are closed, the transcript level is low, indicating a role for SunTIP7 in the rapid water efflux necessary during stomata movement (Sarda et al., 1997). The transcript level of SunTIP20, on the other hand, did not change during the day or in response to water deficit (Sarda et al., 1997).

Two other recent studies have shown diurnal fluctuations of aquaporin expression. Henzler et al. (1999) correlated diurnal variations in root hydraulic conductivity (L_{pr}) and root pressure to expressions of aquaporins in *Lotus japonicus*. The L_{pr} of excised roots was found to vary over a 5-fold range during a day/night cycle. This behavior was seen when L_{pr} was measured in roots exuding, either under root pressure (osmotic driving force), or under an applied hydrostatic pressure of 0.4 MPa which produced a rate of water flow similar to that in a transpiring plant (Henzler et al., 1999). When mRNAs from roots were probed with cDNA from *Arabidopsis thaliana* AthPIP1a gene, an abundant transcript was found to vary in abundance in accordance with the diurnal pattern of variation in root L_{pr} (Henzler et al., 1999). A protein of 31 kDa that cross reacted with the AthPIPa antibody was isolated. The second study correlated the reduction in L_{pr} after nutrient deficiencies and diurnal fluctuation of L_{pr} to fluctuations in the abundance of aquaporins in *Lotus japonicus*. N-, P-, and S-deficiencies results in major reduction of L_{pr} (Clarkson et al., 2000). In nutrient-sufficient wheat plants, root L_{pr} is extremely sensitive to brief treatments of $HgCl_2$; these effects are completely reversed when Hg is removed (Clarkson et al., 2000). The low values of L_{pr} in N- or P-deprived roots of wheat roots are not affected by Hg treatments. Gene

transcripts were found in the roots of *Lotus japonicus* that have high homology to *Arabidosis* PIP1 and PIP2 aquaporins (Clarkson et al., 2000). The fluctuations of these transcripts were shown to coincide with the fluctuations in L_{pr}. In addition, it is known that the aquaporin encoded by AtPIP1 has its water permeation blocked by the binding of Hg. Thus, it appears that the lowered root L_{pr} may be due to a decrease in either the activity of the water channels or their density in the plasma membrane (Clarkson et al., 2000).

Seawater-strength salt stress of the ice plant (*Mesembryanthemum crystallinum*) initially results in wilting, but full turgor is restored within two days. Analysis of changes in gene expression during this stress yielded three members of the MIP family that are thought to be aquaporins. One transcript, MipB, was found only in the root RNA, whereas two other transcripts, MipA and MipC, were detected in the roots and the leaves (Yamada et al., 1995). All transcripts declined intially during salt stress but later recovered to at least prestress levels (Yamada et al., 1995). The highest levels of MipA and MipC coincided with when the leaves regained turgor. Both MipA- and MipB-encoded proteins lead to an increase in water permeability when expressed in *Xenopus* oocytes (Yamada et al., 1995). In addition, Kirch et al. (2000) have recently characterized the cellular distribution and expression patterns of MIPs in *Mesembryanthemum crystallinum* using oligopeptide-based antibodies. The tonoplast MIP-F was found in all cells, while signature cell types identified different PM-MIPs: MIP-A predominantly in phloem-associated cells, MIP-B in xylem parenchyma, and MIP-C in the epidermis and endodermis of immature roots (Kirch et al., 2000).

Another interesting study was performed by Kaldenhoff et al. (1996) that showed the regulation of *Arabidopsis thaliana* aquaporin gene AthH2 (PIP1b) by light and phytohormones. In this study, transgenic plants containing constructs of the aquaporin AthH2 promoter and the coding region of beta-glucoronidase (GUS) were treated with varying light qualities and phytohormones. The activity of the AthH2 promoter was determined in situ using a specific GUS assay. The results showed that blue light (400-550nm), white light, gibberellic acid (GA) and abscisic acid (ABA) treatments activated the promoter. In contrast, red light and indole-3-acetic acid (IAA) had only minor effects on promoter activity (Kaldenhoff et al., 1996).

Gene expression has even been shown to be affected by parasitic invasions on plants. For example, root-knot nematodes are thought to affect expression of numerous plant genes. Root-knot nematodes are obligate plant parasites that induce development of an elaborate feeding site during root infection (Opperman et al., 1994). The tobacco root-specific gene, TobRB7, encodes a protein (pRB7) that is a member of a family of membrane proteins and has been shown to function as a water channel. Expression of TobRB7 is induced during the feeding site development (Opperman et al., 1994). It was also shown that the cis-acting sequences that mediate induction by the nematode are separate from those that control normal root-specific expression (Opperman et al., 1994).

The discovery of aquaporins in plants has provided a molecular explanation for the permeability of biological membranes to water. In plants, aquaporins are likely to be important both at the whole plant level and at the cellular level. Plants, more than other organisms, modify the water permeability of their membranes to accommodate changing osmotic conditions, or in response to environmental cues that indicate a need for a change

in water flow. Transmembrane water flow is necessary for many physiological processes, including the transpiration stream, phloem loading, stomatal opening, pulvinar movement, and osmotic adjustments (Weig et al. 1997). The abundance and activity of aquaporins must be regulated so the plant can keep a tight control over water fluxes into and out of the cells, as well as within the cell. From recent studies in the field we have gained insight into expression patterns, functionality, regulation, and structure of aquaporins in plants. PIPs and TIPs have been characterized to some extent in several species; however in comparison less is known about the nodulin 26-like MIPs. Below studies done on nodulin 26 and nodulin 26-like MIPs are discussed.

Nodulin 26

Nodulin 26 was first identified in soybean where it was found to be the product of a plant gene that is expressed specifically during the formation of nitrogen-fixing root nodules (Fortin et al., 1987). The membrane topology of nodulin 26 is shown in Figure 1. Nodules are formed upon infection of soybean roots by *Bradyrhizobium japonicus* bacteria. The formation of the root nodule represents a complex developmental pathway that involves signal exchange between the bacterium and legume culminating in the formation of the nodule (van Rhijn and Vanderleyden, 1995). During the formation of the nodule, the bacteria become encapsulated in a specialized organelle, known as the symbiosome, which is the major organellar component of the infected cells of the nodule (Roth et al., 1988). The bacteria are separated from the plant cell cytosol by the plant-derived symbiosome membrane (SM). The formation of nodules is marked by

the expression of nodule-specific plant genes that code for plant proteins termed nodulins (Legocki and Verma, 1980). Nodulins are involved in the development, structure, maintenance and overall metabolism of the nodule (Verma et al., 1986; Legocki and Verma, 1980; Franssen et al., 1992). A subset of nodulin proteins were discovered to be synthesized and targeted to the symbiosome membrane of the nodule (Fortin et al., 1985; Fortin et al., 1987; Mellor et al., 1989). Of the nodulins identified in the SM (Mellor et al., 1989), the best characterized is nodulin 26. Nodulin 26 was first identified as a symbiosome membrane nodulin by Fortin et al. (1987) by immunoprecipitating polysomes with antibodies against purified soybean symbiosome membranes. Its sequence similarity to other MIP proteins was first noted by Sandal and Marcker (1988) and Shiels et al. (1988). Nodulin 26 is specifically targeted to this membrane where it is the major proteinaceous component of the SM constituting 10-15% of the total protein of the SM (Weaver et al., 1991; Rivers et al., 1997).

The finding that nodulin 26 is the major proteinaceous component of the symbiosome membrane argues for an integral role in symbiosome function. Early in vitro work, similar to the work with bovine lens MIP (Zampighi et al., 1985; Ehring et al., 1990) suggested that nodulin 26 forms a large, nonselective ion pore (Weaver et al., 1994; Lee et al., 1995). However, subsequent work with isolated symbiosome membrane vesicles and heterologous expression in *Xenopus* shows that like other MIPs it forms a water channel (Rivers et al., 1997). However, it is clear nodulin 26 possesses unusual functional features that distinguish it from other aquaporins. For example, while the protein is an aquaporin, its single channel transport rate is almost 50-fold lower than that of other aquaporins, such as AQP1 (Rivers et al., 1997; Dean et al., 1999). In addition,

nodulin 26 is not specific for water, but transports glycerol and other small, uncharged solutes (Rivers et al., 1997; Dean et al., 1999). Thus, nodulin 26 belongs to the "aquaglyceroporin" class of MIPs (Agre et al., 1998), and was the first MIP to be demonstrated to be a member of this functional subclass. While its function in the symbiosome is not yet clear, a potential role for the primary function of nodulin 26 in osmoregulation or metabolite transport has been proposed (Rivers et al., 1997; Dean et al., 1999; Niemietz and Tyerman, 2000). Until recently, it was thought that nodulin 26 was a unique MIP with a dedicated function on the symbiosome membrane. However, it became clear that the nodulin 26 subclass is more widespread and other members of the family were discovered (Weig et al., 1997). Thus, nodulin 26 and its family members likely play a broader role in water relations and membrane transport than originally envisioned. Below the details of this MIP protein subfamily are discussed further.

Nodulin 26-like MIPs (NLMs)

From previous work it was clear that nodulin 26 is a nodule specific protein with functional properties distinct from other plants MIPs. However, it has recently become clear that other "nodulin 26-like" MIPs (NLMs) are present, not only in other legumes (Guenther and Roberts, 2000), but also in nonleguminous plants and in tissues besides nodules (Weig et al., 1997; Weig and Jakob, 2000). The first nodulin 26-like protein, At-NLM1, was identified by Weig et al. (1997) as an expressed sequence tag in *Arabidopsis thaliana*. Similar to soybean nodulin 26, At-NLM1 was shown to be an active aquaporin when expressed in *Xenopus* oocytes (Weig et al., 1997). The presence of At-NLM1 in *X.*

laevis oocytes increased the value of the osmotic permeability coefficient of the plasma membrane from 3.0 x 10^{-3} to 16.4 x 10^{-3} cm s^{-1} (Weig et al., 1997). Subsequent work with At-NLM1 and the closely related At-NLM2 shows that both can also transport glycerol upon expression in yeast (Weig and Jakob, 2000). Thus, these nodulin 26 related proteins also form aquaglyceroporin activities.

NLM proteins have been reported from six plant species: GmNOD26 from *G. max* (Fortin et al., 1987), LIMP2 from *Lotus japonicus* (Guenther and Roberts, 2000), OsrMIP from *Oryza sativa* (Liu et al., 1994), NaPIP28 from *Nicotiana alata* (GenBank accession number P49173), PsNLM1 from *Pisum sativum* (GenBank accession number AJ243308) and the AtNLMs from *A. thaliana* (Weig and Jakob, 2000). Within this subgroup the most detailed information is known about GmNOD26. The nodulin 26 ortholog, LIMP2 from *Lotus japonicus* has been shown to be expressed exclusively in nodules and to facilitate the flux of glycerol and water when expressed in *Xenopus* oocytes (Guenther and Roberts, 2000). The *N. alata* protein seems to be pollen specific, the *P. sativum* protein was found in seed coats, and the rice protein was expressed in the shoots (Weig and Jakob, 2000).

The finding of nodulin-26-like sequences in a nonlegume species such as *Arabidopsis* is intriguing and suggests that this protein family participates in additional biological functions beyond that of symbiosis. In addition, by understanding these other functions of the protein family we may be able to shed some light on the symbiotic role of nodulin 26, which has remained elusive. In the present study we wished to investigate: 1) The tissue expression patterns of the NLMs in *Arabidopsis thaliana*; 2) Effects of

various environmental stimuli on the expression patterns of the NLMs; 3) To deduce the functions of the NLMs by using transferred (T-DNA) mutagenesis technology.

CHAPTER II

MATERIALS AND METHODS

Identification of Nodulin 26-like Proteins (NLMs) in Arabidopsis

The Basic Local Alignment Search Tool (BLAST) program was used to search the Genbank database using the nucleotide sequence of nodulin 26 (NOD26). Analysis of the hits from the search resulted in the identification of ten putative Nodulin 26-like-proteins from the BAC clone F13C5 of *A. thaliana.* The similarity between the ten *A. thaliana* sequences and NOD 26 were then compared using the alignment program, MEGALIGN, from DNASTAR. A phylogenetic tree was then constructed using DNASTAR to compare the relatedness and clustering patterns for the ten Nodulin 26-like sequences in *A. thaliana*, soybean Nodulin 26, and LIMP2, an ortholog of Nod26 from *Lotus japonicus* (Guenther et al., 2000).

Amplification and Cloning of At-NLM Genomic Sequences

Genomic DNA was isolated from *A. thaliana* leaf tissue from 6-week old plants using the Promega Genomic Wizard Isolation Kit. Leaf tissue was frozen with liquid nitrogen and ground using a mortar and pestle. Leaf tissue (40 mg) was then transferred to a 1.5ml microcentrifuge tube and the cells and nuclei were lysed by vortexing tissue with 600µl Nuclei Lysis Solution and incubating at 65°C for 15 minutes. Next the

samples were subjected to a RNase digestion step by adding 3 µl of RNase A to the samples and inverting 25 times and then allowing to incubate at 37°C for 15 minutes. The cellular proteins in the extract were then removed by salt precipitation with the Protein Precipitation Solution, followed by a centrifugation step at 16,000g for three minutes. Finally, the supernatant containing the genomic DNA was removed and the DNA was concentrated by adding 600 µl room temperature isopropanol, inverting the tube several times and then centrifuging at 16, 000 x g for one minute at room temperature. The supernatant was carefully decanted and the pellet was washed with 600 µl of 70% ethanol by gently inverting the tube. The samples were then centrifuged at 16, 000 x g for one minute at room temperature and the ethanol was very carefully aspirated off with a Pasteur pipette. The samples were then allowed to air dry for 15 minutes and were then rehydrated by adding 100µl of DNA rehydration solution (10 mM Tris-HCl, pH 7.4/ 1 mM EDTA, pH 8.0) and incubated at 65°C for 1 hour. The purity and quantitiy of the DNA was determined spectrophotometrically by reading the absorbance at 260nm and 280 nm. The final sample was stored at -20°C.

Genomic DNA (300ng) was amplified by touchdown PCR with PCR primers designed against the known genomic sequence within exons at extreme 5' and 3' ends to increase specificity of primers for isolation of the desired genes. A list of all primers used for this study is shown in Table I. All oligonucleotide primers were designed and synthesized by Fisher Scientific. Oligonucleotides were reconstituted by dissolving in sterile water at a working concentration of 50 ng/µl. PCR was performed with a Perkin Elmer GeneAmp® PCR system 2400 with the following conditions: denaturation 94°C, 2

Table I: Primers used for the amplification of NLMs, RT-PCR, and T-DNA knockout screens.

Primers[a]	Forward	Reverse	Intron border
At-NLM1	GTGTTATAAACCCATGCACCCG	GGTATTCACCGCTTGTGCATCGG	CCCTCTCAAACAGGTTCCAGC
At-NLM2	CGGGAAACGGTGGTGATGCTAGA	GTCCTCTGTAGCAACTGTAGACC	CTCTTAAACAGGTTCCGGCTTA
At-NLM3	TATTTGCTGGCTGTGCCGCCATTGC	TCAAGGCCCCAGAGACGGCACCAAG	CTAGTTCCTCTGTAGTTCTCTTGG
TDNA/At-NLM1	CTTTAGAAATGAACCACTTTTAGTGATTT	ATCGCGGAGTTTCTTGGAACCTACTTTTT	
TDNA/At-NLM3	ATGGATGACATATCAGTGAGCAAAGCAA	TAGTTACAATGAGAGCAGCGAACAGATTT	
Actin2	GTTGGTGATGAAGCACAATCCAAG	CTGGAACAAGACTTCTGGGCATCT	
JL202[b]	CATTTTATAATAACGCTGCGGACATCTAC		

[a] Forward and reverse primers were used to amplify sequences from genomic DNA, intron border primers were used for RT-PCR when amplifying cDNA and T-DNA primers were used for screen knockout libraries. [b] The JL202 primer was designed against the left border of the T-DNA insert.

min: annealing 30 sec: extension 72°C, 2 min. The annealing temperature was decreased in two degree increments every third cycle from 54°C to 46°C, followed by fifteen cycles with an annealing temperature of 54°C. Molecular Bio-Products Easystart Micro 100 PCR ready tubes containing buffer

and dNTPs were used with Taq DNA polymerase (Eppendorf). The PCR products were resolved on a 1% w/v agarose gel in 1X Tris-acetate-EDTA buffer (TAE) and were visualized by staining with ethidium bromide (0.5 μg/ml) (Sambrook et al., 1989). Selected PCR fragments were cloned into the pCR2.1 vector by using the invitrogen TA Cloning Kit using the manufacture's protocol. Restriction enzyme digest analysis using *EcoR*I was performed to verify the cloned sequence was the correct molecular weight.

Plant Growth and Stress Treatments

Arabidopsis thaliana seeds were sterilized in 50% (v/v) ethanol for 1 min, and then in 50% (w/v) bleach and 0.1% (v/v) Tween 20 for 10 min. The seeds were washed five times by low speed centrifugation with sterile water and finally resuspended in sterile water. Seeds were germinated on Whatman Filter Paper that was laid on the Manshig Skoog (MS) agar. The seeds were vernalized at 4°C for 48 hours and then transferred to tissue culture growth chamber at 22°C with a 16-h light/ 8-h dark photoperiod. For hormone and stress treatments, the filter paper carrying the seedlings was transferred onto a new MS agar plate containing various treatments: 0.1 mM abscisic acid, 0.3 M NaCl, or 0.3M mannitol. The seedlings were grown an additional 24-h and were harvested, frozen in liquid nitrogen and stored at -80°C. For drought stress treatments the filter paper containing the plants was incubated on the bench for 2.5 h as

discussed in Weig et al., (1997). Diurnal treatments were performed by first entraining the plants with a 16h light/8h dark photoperiod. Seedlings were harvested at intervals beginning at 30 minutes after the light period commenced. Additionally, diurnal variations were distinguished from light- dependent effects by entraining the plants to a 16 h light/ 8 h dark cycle and then measuring diurnal cycling after lights in the absence of light. Etiolated plants were prepared by germinating seeds and growing in total darkness for 19-d. Light grown contols were grown under the standard 18h/6h light/dark photocycle for 19 days. Low light plants were grown under dim light conditions (partially etiolated) for 19 days. For greened tissues, plants were grown in the low light conditions for 14 days and then transferred to standard light conditions for 5 days. After the various treatments, all plants were then harvested, frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA Extraction

Arabidopsis thaliana ecotype Columbia plants were grown 5-6 weeks in soil with a 16-h light/ 8-h dark photoperiod at 22°C in a Percival growth chamber. To characterize the expression levels of the NLMs in different tissues, total RNA was isolated by the guanidine thiocyanate procedure of Chirgwin et al. (1979) from roots, stems, leaves, flowers, and siliques. Tissue (2 grams) was frozen and ground in liquid nitrogen using a mortar and pestle. Three milliliters per gram of tissue of guanidine thiocyanate buffer (4.2 M guanidine thiocyanate, 0.7% (v/v) β-mercaptoethanol, 20 mg/ml diethyldithiocarbamate, and 25 mM Tris-HCl, pH 7.5) were then added and the samples

briefly vortexed and were centrifuged at 10,000 rpm in a SA-600 rotor at 4°C for 15 minutes. The supernatant fraction was then transferred to a fresh Corex tube, and the nucleic acids were precipitated by adding 0.1 volumes of 3 M Na acetate (pH 5) and 2.5 volumes of ethanol. The mixture was incubated in a dry ice/ethanol bath for 15 minutes, and was centrifuged at 10,000 rpm in a SA-600 rotor at 4°C for 10 minutes. The supernatant fraction was discarded and the pellets were resuspended in 1 ml of resuspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.2% (w/v) SDS, and 20 mM diethyldithiocarbamate). Samples were then subjected to a phenol/chloroform extraction and then nucleic acids were precipitated by addition of 0.1 volumes sodium acetate (pH 5) and 0.7 volumes isopropanol, and were collected by centrifugation in a microcentrifuge for 10 minutes at 4°C. Pellets were then resuspended in 0.25 ml DEPC-treated water containing 0.1% (w/v) SDS and then precipitated overnight at -20°C by the addition of 83 µl of 8M LiCl. The samples were then centrifuged for 10 minutes at 4°C and were reprecipitated by the addition of 200 µl of 2 M LiCl. Pellets were redissolved in DEPC-treated water and the RNA was precipitated with ethanol. Pellets were washed with 80% ethanol and finally resuspended in DEPC-treated water and stored at -80°C.

Identification and Quantitation of NLM cDNAs by RT-PCR

Total RNA was isolated as described above and quantitated spectophotometrically by measuring the absorbance at 260nm and 280nm. Two micrograms of purified total RNA were used to synthesize first strand cDNA with a Gibco First Strand cDNA Synthesis Kit using reverse transcriptase and an oligo-dT primer. RNA, dNTPs, and oligo(dT) primer (0.5μg/μl) were combined and incubated at 65°C for 5 minutes and then placed on ice. Next 10X RT buffer, $MgCl_2$ (25mM), DTT (0.1M), and RNaseOUT Recombinant RNase Inhibitor were added to the samples. 50 units of reverse transcriptase SUPERSCRIPT II (Gibco) was then added to the samples and they were incubated at 42°C for 50 minutes. The reactions were terminated by incubating at 70°C for 15 minutes. The RNA template was then degraded by treatment with 1 μl of RNase H for 20 minutes at 37°C. The cDNAs corresponding to the NLMs were then amplified using gene specific primers designed to sequences flanking the intron borders (Table 1). This was done to lower the likelihood of amplifying genomic sequences. PCR reactions (50 μl) were performed with 1μl of the cDNA reaction, 4.2 μl of the gene specific primers (12 pmol/μl), 4μl of Ex Taq buffer, 4μl dNTPs (2.5 mM stock), sterile water and TaKaRa Ex Taq DNA polymerase (2.5 units) (Fisher Scientific). The internal control used to ensure amplification efficiency and uniform loading was actin 2 (50 ng/μl) due to the fact that it is expressed equally in all tissue (McKinney and Meagher, 1998).

Southern blot analysis was then performed using nick translated PCR products generated from amplification of genomic DNA as described in Sambrook et al., (1989).

PCR samples were first separated on a 1% (w/v) agarose gel in 1X TAE. The DNA was depurinated by washing in 0.25M HCl for 15 minutes, denatured by washing in 0.5 M NaOH/ 1M NaCl for 30 minutes and then neutralized with 1.5 M Tris-HCl, pH 7.4/ 3 M NaCl for 30 minutes. The gel was then blotted onto Zeta-Probe nylon membranes by either vacuum transfer or by capillary transfer. For vacuum blotting the BIORAD Model 785 vacuum blotter was used. Gels were blotted at 5 in. of Hg for 90 minutes using 10 X SSC as a transfer buffer. Gels that were transferred by gravity were set on a flat surface, with the gel inverted with a nylon membrane. Paper towels were placed on top and the nucleic acids were allowed to transfer to the membrane in 10 X SSC. The blotted nucleic acids were then crosslinked using a UV Stratalinker 2400 (Stratagene). The membranes were prehybridized with 0.5 M $NaPO_4$, pH 7.2, 7% (w/v) SDS for 10 minutes at 65°C. The membranes were then hybridized overnight at 65°C in 10 ml of the same solution containing 10^6 cpm/ml of various nick-translated radiolabeled probes generated by PCR. The membranes were washed twice at 65°C with 1 mM EDTA, 40 mM $NaPO_4$, pH 7.2, 5% (w/v) SDS for 30 minutes, followed by two additional 30 minute washes with 1 mM EDTA, 40 mM $NaPO_4$, pH 7.2, and 1% SDS (w/v). The membranes were air dried and then exposed to X ray film at -80°C.

Transferred (T-DNA) Knockouts

Gene specific primers were designed to At-NLM3, At-NLM1, and At-NLM2 following specific guidelines provided by the University of Wisconsin *Araidopsis*

Knockout Facility (Table I) (http://www.biotech.wisc.edu/Arabidopsis/). In addition, two control primers, Con-1A ad Con-1B, and the left border primer, JL202, were synthesized (Table I). Gene specific primers were designed against extreme 5' and 3' ends of the genes or slightly outside the coding region. The primers were designed to be exactly 29 bp in length and with either zero or one G or C at position 28 or 29 of the 3' end of the primer. Following the guidelines stated above the optimal Tm of the primers is 65°C. Control PCR reactions were performed to test the ability of the primers to amplify the genes from wild type *Arabidopsis thaliana* (ecotype Wassilewskija (WS)) genomic DNA. The four 50 µl control PCR reactions were set up up using Takara Ex Taq polymerase, 2 µl of WS genomic DNA (0.2 ng/µl), 1 µl each primer (12 pmoles/µl), 4 µl 10 X Ex Taq buffer, 4 µl dNTP mix, and water. The first control reaction uses the Con-1A and Con-1B primers to test the PCR conditions and provide a standard with which to judge the gene specific primers. The second control reaction uses Con-1A, Con-1B, and JL-202 primers to test the compatibility of Con-1A and Con-1B with the left T-DNA border primer. The third control reaction contains only the 5' and 3' gene specific primers. The last reaction contains both your 5' and 3' gene specific primers with the JL-202 primer to test the compatibility of the gene specific primers with the left T-DNA border primer. The following PCR conditions were used for all reactions: hold at 96°C for five minutes, followed by a 94°C denaturation step for 15 seconds, 65°C annealing temperature for 3 seconds and a 72°C extension temperature for 2 minutes for 36 cycles, and then a hold step at 72°C for 4 minutes. The reactions were separated by electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide and photographed. The two At-NLM3

gene specific primers were then used by the University of Wisconsin Knockout Facility for round one PCR using 30 DNA super pools of 60,480 *A. thaliana* lines. The facility shipped these PCR reactions to us. Southern analysis was performed as described above using nick-translated PCR products of the various At-NLM genes that were amplified from genomic DNA of the Wassilewskija ecotype. PCR products from positive hits were cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen) and sequenced. Automated DNA sequencing was performed on a Perkin Elmer Applied Biosystems 373 DNA sequencer by the Molecular Biology Resource Facility at the University of Tennessee. Sequencing reactions were prepared with a Prism Dye Terminator Cycle sequencing kit (Perkin Elmer Applied Biosystems). A second round of PCR and Southern blot analysis was then performed with the same primer set to narrow the positive hit a specific pool of 225 lines. Seeds were then ordered from this pool and approximately 100 seeds from 225 lines were planted and grown in soil at 22°C under a 16h light/8h dark photoperiod for two weeks. Genomic DNA was isolated from the leaves of seedlings using the Promega Genomic Wizard Kit as described above. PCR reactions using the JL-202 and At-NLM3 reverse primer were then performed using Takara Ex Taq as described above to isolate two plants, each bearing the T-DNA insert of interest. These plants was then grown for 7 weeks to produce seed.

The seeds (T_2 generation) were germinated on MS agar containing 50 μg/ml kanamycin. Plants were transferred and grown for two weeks in soil and leaf tissue was harvested to isolate genomic DNA for PCR reactions. Reactions were done with 5' and 3' At-NLM3 primers (Table I) under the following conditions: 96° C for 5 min.,

followed by 36 cycles of 94° C, 15 sec.; 65° C, 30 sec.; 72° C, 2 min. and finally by one extension at 72° C for 4 min.

CHAPTER III

RESULTS

Identification of Nodulin 26-like Proteins (NLMs) in Arabidopsis

With the completion of the *Arabidopsis* genome (*Arabidopsis* Genome Initiative, 2000) the total member of family members in the MIP family in *Arabidopsis* is now clear (see www-biology.ucsd.edu/~ipaulsen/transport). All 39 MIP sequences were aligned and compared using the MEGALIGN program of DNASTAR. Clustering into the subfamilies described by Weig et al. (1997) was observed, including ten proteins that showed grouping into the nodulin 26 subfamily (Figure 2). Six of the proteins (At-NLM1 through 6) were previously described (Weig et al., 1997; Weig and Jakob, 2000; Johansson et al., 2000) and four additional members (At-NLM7 through 10) were found (Table II). All encode full length MIPs with all the structural hallmarks of the family, with the exception of At-NLM7 which encodes a 133 amino acid protein that has only the carboxyl terminal half of the prototypical MIP sequence. The proteins show varying levels of similarity to the family archetype, soybean nodulin 26, varying from 62.4% identity (At-NLM2) to 32.1% identity (At-NLM10) (Table II). This later protein clusters on one main branch by itself and shows limited similarity to the other family members (Figure 2). Interestingly, other family members are often found in homologous pairs underscoring proposal that many of the genes in the *Arabidopsis* genome have undergone

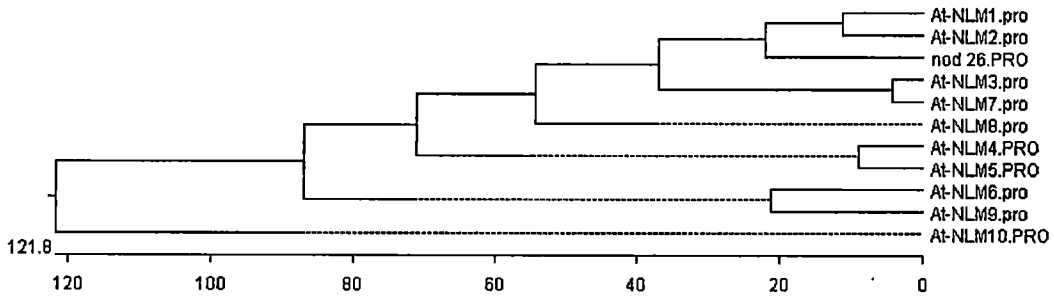


Figure 2: Phylogenetic alignment of ten NLMs of *A. thaliana* and soybean nodulin 26. Caldogram phylogenetic alignments were constructed using DNASTAR software. Branch distances correspond to sequence divergence. The units at the bottom of the tree indicate the number of substitution events.

Table II: Protein nomenclature, *Arabidopsis* gene number, % similarity of At-NLMs compared to nodulin 26, and accession numbers of at-NLMs.

Protein [a]	Arabidopsis gene # [b]	% identity to nodulin 26	Accession #
At-NLM1	At4g19030	61.6%	CAA16760;CAB78905
At-NLM2	At4g18910	62.4%	CAA16748;CAB78893
At-NLM3	At2g34390	49.4%	ACC27424;ACC26712
At-NLM4	At5g37810	52.0%	BAB10360
At-NLM5	At5g37820	53.1%	BAB10361
At-NLM6	At4g10380	40%	CAB78161
At-NLM7	At2g29870	45.3%	ACC3514
At-NLM8	At1g31880	44.6%	AAG50717
At-NLM9	At1g80760	39%	AAF14664
At-NLM10	At3g06100	32.1%	AAF30303

[a]AtNLM refers to "*Arabidopsis thaliana* nodulin 26-like MIP" AtNLM1-6 are proteins designed by Johansson et al. (2000). AtNLM7-10 are names used to designate other family members found since the completion of the *Arabidopsis* genome sequence (*Arabidopsis* Genome Initiative, 2000).

[b]The chromosome location and gene number based on the sequence of the *Arabidopsis* genome see www.biology.ucsd.edu/~ipaulsen/transport1.

duplication during evolution (*Arabidopsis* Genome Initiative, 2000). An examination of the phylogenetic tree (Figure 2) shows that At-NLM 1 and 2 (77.6% identity), At-NLM3 and 7 (87.8% identity), At-NLM4 and 5 (84.1% identity), and At-NLM6 and 9 (60.5% identity) form such pairs of highly similar sequences. However, At-NLM8 and At-NLM10, appear to be unique and show no homologous pairs (Figure 2).

Specific amino acid residues within the peptide sequence are characteristic of aquaporins versus aquaglyceroporins and can be used to predict which functional family that a particular MIP might fall within. The majority of plant PIPs and TIPs belong to the aquaporin group of MIPs based on sequence and functional analysis (reviewed in Weig and Jakob, 2000). However, the NLMs show amino acid signatures of both groups forming an 'intermediate' group between the classical 'aquaporins' (for example, plant PIPs and TIPs, animal AQP1) and glycerol facilitators/aquaglyceroporins (for example, GlpF, FPS1, AQP3 etc.) (Froger et al., 1998; Guenther and Roberts, 2000; Weig and Jakob, 2000). As shown in Figure 1 and Table III, sequences at five amino acid positions, P1-P5, have been shown to have characteristics either for aquaporins or glycerol permeases (Froger et al., 1998). P1 is found in loop C, P2 and P3 are found in loop D containing the second NPA motif, and P4 and P5 are found in the sixth transmembrane region. Support for this hypothesis came from studies in which substitutions at the P4 and P5 positions of an insect aquaporin with the residues found in GlpF abolished the water transport property of the protein, while rendering it capable of glycerol transport capabilities (Lagree et al., 1999). All of the At-NLMs, have residues at P2, P3, and P4 that are similar to residues in aquaporins such as AtTIP, AtPIP1b/2a, or the human aquaporin HsAQP1, while having residues at positions P1 and P5 that are

Table III: Conserved functional residues of nodulin 26 and At-NLMs [a].

protein	helix 2[b] *(selectivity)*	loop C[c] P_1	loop E *(selectivity)*	loop E *(selectivity)*	loop E P_2	loop E P_3	helix 6 P_4	helix 6 P_5
Nod 26	W*	F	A	R	S	A	Y	L
At-NLM1	W*	F	A	R	S	A	Y	M
NLM2	W*	F	A	R	S	A	Y	I
NLM3	W*	F	A	R	S	A	Y	L
NLM7	-	-	A	R	S	A	Y	L
NLM4	W*	F	A	R	S	A	Y	I
NLM5	W*	F	A	R	S	A	Y	I
NLM6	A*	F	A	R	S	A	Y	L
NLM9	A*	F	A	R	S	A	Y	L
NLM8	W*	F	A	R	S	A	Y	I
NLM10	W*	F	A	R	S	A	Y	L

[a] Residues in red indicate similarity to glycerol permeases, residues in blue indicate similarity to aquaporins. The invariant arginine residue of both glyceroporins and aquaporins is shown in black.

[b] The tryptophan, alanine, and arginine residues have been shown to be located within the selectivity filter of GlpF and to confer glycerol specificity (Fu et al., 2000). The tryptophan residue within helix 2 is an invariant residue within glycerol transporters.

[c] P1-P5 reisdues are discriminate residues (Froger et al., 1998) that are characteristic signature sequences for aquaporins or glyceroporins.

similar to bacterial and yeast glycerol permeases or the human aquaglyceroporin HsAQP3 (Weig and Jakob, 2000).

In addition to the five discriminate residues discussed above, there are three conserved residues that have been shown to be important for transport selectivity. These three residues have been shown to form the selectivity filter from the GlpF crystal structure (Fu et al., 2000). As shown in Figure 1, these include a tryptophan residue in helix 2, an alanine in loop E, and an arginine in loop E. The tryptophan residue is an invariant reside in glycerol transporters and is present in seven of the At-NLMs (At-NLM1-5, 8, and 10) (Table III). In contrast, At-NLM6 and At-NLM9 contain an alanine at this position, which is characteristic of aquaporins (Table III). Another interesting conserved feature within the nodulin 26 subfamily is the presence of putative phosphorylation sequences for calcium-dependent protein kinase (CDPK) similar to nodulin 26 (Table IV and Figure 1) (Weaver et al., 1991). At-NLM1-5, and 7 contain the CDPK phosphorylation motif, hydrophobic-X-basic-X-X-serine (X indicates any amino acid), in the carboxyl terminal domain suggesting they may be targets for regulation by CDPK (Table III) (Bachman et al., 1996).

Amplification of At-NLM Genomic Sequences

To attempt to identify and amplify At-NLM sequences PCR reactions were performed to amplify partial At-NLM1, At-NLM2, and At-NLM3 sequences from *A. thaliana* genomic DNA by using primers specific for these genes (Table I). Fragments of the expected size (At-NLM1/1288bp, At-NLM2/1761bp and At-NLM3/1045bp) were

Table IV: Putative calcium-dependent protein kinase (CDPK) phosphorylation sequences within At-NLMs

protein	Putative CDPK phosphorylation site[a]
Nod 26	S-E-*I*-T-*K*-S-A-*S*-F-L-K
At-NLM1	R-E-*I*-T-*K*-S-G-*S*-F-L-K
NLM2	R-E-*I*-T-*K*-S-G-*S*-F-L-K
NLM3	P-E-*F*-S-*K*-T-G-*S*-S-M-K
NLM7	P-K-*F*-T-*K*-T-G-*S*-S-H-K
NLM4	R-E-*L*-T-*K*-S-A-*S*-F-L-R
NLM5	R-E-*L*-T-*K*-S-A-*S*-F-L-R

[a] Six of the At-NLMs sequences contain the features of CDPK phosphorylation sequence motifs (hydrophobic-X-basic-X-X-Ser) shown in italics (Bachman et al., 1996).

amplified using touchdown PCR (Figure 3). The At-NLM3 fragment was cloned into the pCR2.1 vector by using the Invitrogen TA Cloning Kit. Restriction enzyme digest analysis was performed to verify the cloned sequence was the correct molecular weight (Figure 4).

Tissue distribution of NLMs

To help elucidate the specific function of NLMs in A. thaliana, their tissue distribution patterns were evaluated. Northern blot analysis with isolated total RNA produced no detectable signals presumably due to very low levels of transcripts, and thus reverse transcriptase PCR was performed to achieve greater sensitivity in detection. Total RNA from various Arabidopsis tissues was used to produce a complimentary DNA transcript using a retroviral reverse transcriptase and an oligo dT primer. This was followed by amplification of the cDNA using PCR . The PCR reaction is a co-amplification reaction that contains the gene specific primers s well as actin 2 primers. The actin 2 primers serve as an internal control to help monitor amplification efficiency and as a loading control (Table I).

The results of RT-PCR show that At-NLM1 is specifically expressed in two organs, the flowers and the roots (Figure 5). Based on comparison of At-NLM1 levels with actin 2 expression from internal control primers a standardized expression level for At-NLM1 was obtained. The results show levels of At-NLM1 expression in the roots was slightly higher than that in the flower (Figure 5). In contrast, At-NLM3 was found to be expressed in all organs tested, but the expression levels between tissues were different

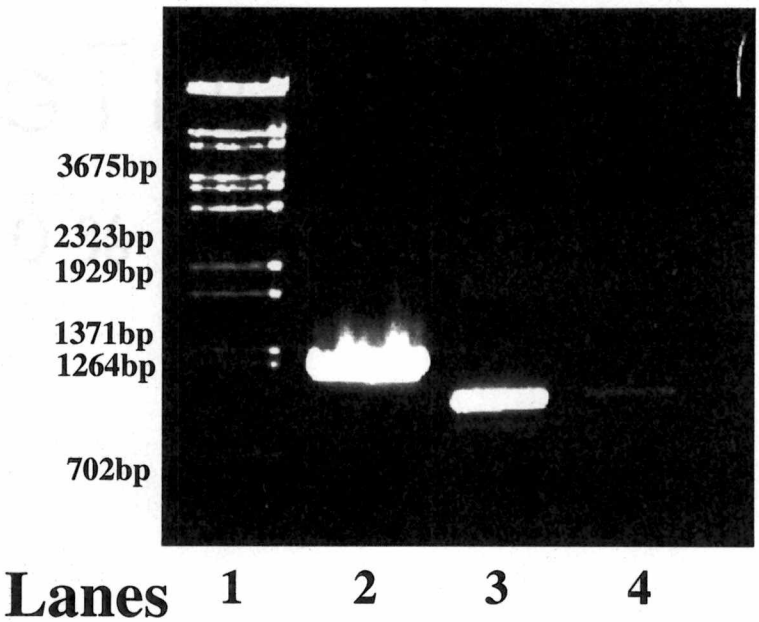


Figure 3: PCR amplification of At-NLMs from genomic DNA. 1% (w/v) agarose gel stained with ethidium bromide. **Lane 1**, Lambda/BstEII DNA ladder. **Lane 2**, At-NLM1. **Lane 3**, At-NLM2. **Lane 4**, At-NLM3.

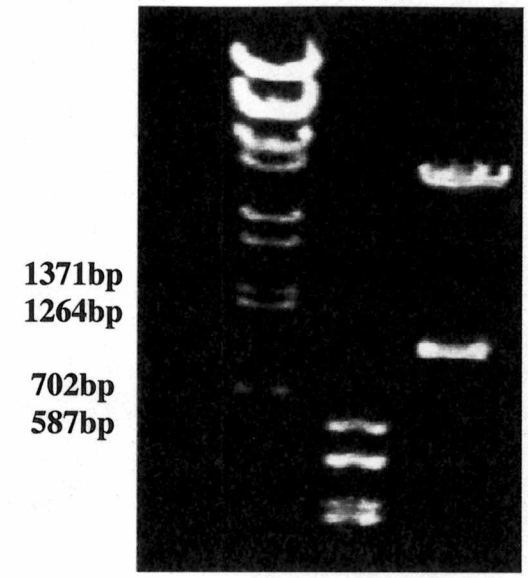


Figure 4: *EcoR*I restriction enzyme digest analysis of the pCR2.1 vector containing the At-NLM3 fragment. Lane 1- Lambda/*BstE*II DNA ladder. Lane 2- pUC18/HindIII DNA ladder. Lane 3- Positive result showing vector and At-NLM3 fragment released by *EcoR*I digestion.

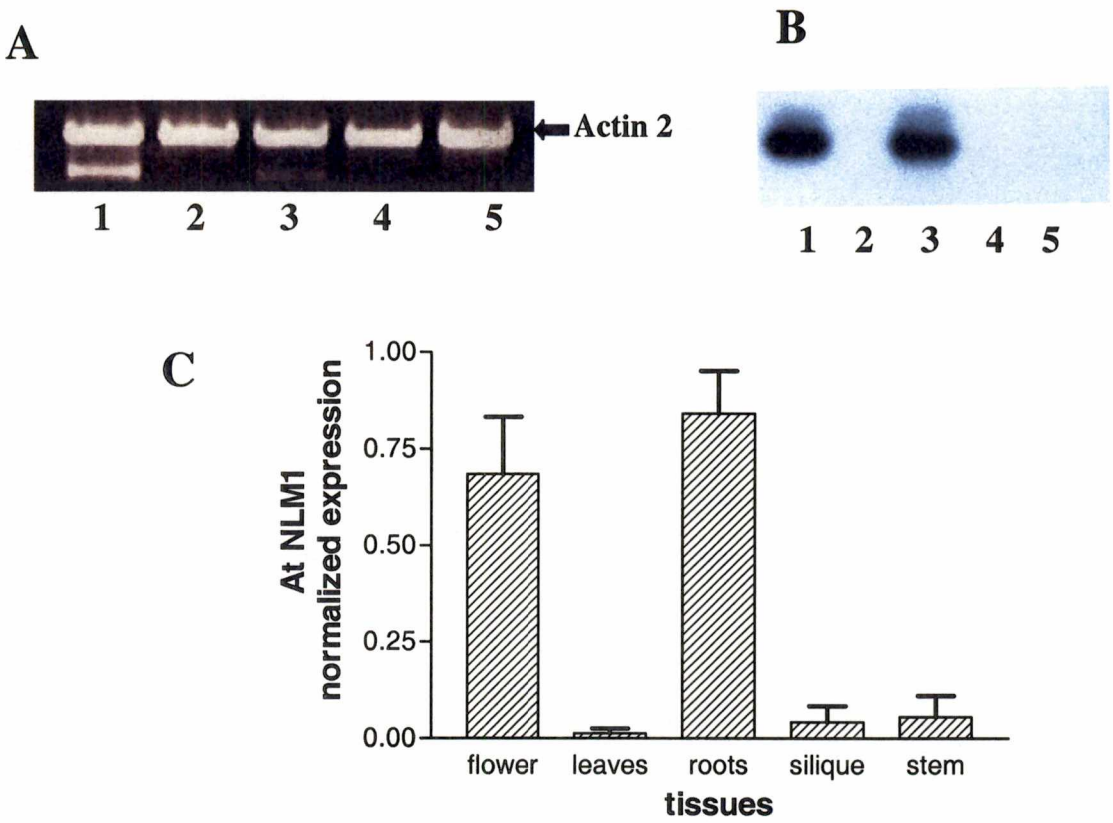


Figure 5: Tissue expression pattern of At-NLM1. Total RNA was isolated from tissues of 6-week old *A. thaliana* **Lane 1**, flower; **Lane 2**, leaf; **Lane 3**, root; **Lane 4**, silique; **Lane 5**, stem tissue. RNA was subjected to RT-PCR with At-NLM1 primers with actin 2 primers included as an internal control to monitor amplification efficiency and loading. Products were resolved by electrophoresis on 1% (w/v) agarose gels. **Panel A**, ethidium bromide stain showing actin 2 PCR products. **Panel B**, Southern analysis of RT-PCR reactionswith an At-NLM1 probe. **Panel C**, Graph representing relative expression of At-NLM1 in root and flower tissue. The At-NLM1 signal from Southern analysis was standardized to the actin signal and was normalized relative to the expression in the roots. Error bars show standard error of mean (n=5).

(Figure 6). At-NLM3 was expressed at the highest levels in roots and the stems and was expressed at lower levels in the flowers and leaves, and was nearly undetectable in the siliques (Figure 6). RT-PCR results of At-NLM2 show that it appears to be more highly expressed than At-NLM1 and 3 since a visible ethidium bromide-stained band at the expected molecular weight was observed (Figure 7). In contrast, the other At-NLMs, particularly At-NLM3, required Southern blot analysis after RT-PCR for detection. Southern blot analysis of the products with an At-NLM2 probe verified that the lower band is At-NLM2 (data not shown). Unlike At-NLM1 and 3, At-NLM2 was expressed nearly evenly in all organs tested (Figure 7).

Effects of Environmental Factors on NLM Expression

Adaptation to stress by gene regulation is a common theme in plant stress biology (reviewed in Johansson et al., 2000). Previous studies have shown MIP mRNAs are sensitive to turgor/osmotic signals resulting either in up regulation or down regulation of transcript levels (Weig et al., 1997; Yamada et al., 1995). This finding suggests that plants alter the permeabilities of their membranes to help adapt to stresses. To determine the potential involvement of At-NLMs in stress biology the analysis of At-NLM1 and At-NLM3 transcript levels upon challenge with various environmental signals was performed. The following osmotic conditions were examined: drought as a desiccating stress, NaCl as an ionic osmotic stress, and mannitol as a nonionic osmotic stress. The effects of the exogenous application of the naturally-occurring stress hormone absisic acid (ABA), which is involved in stress adaptation was also examined. The effects of

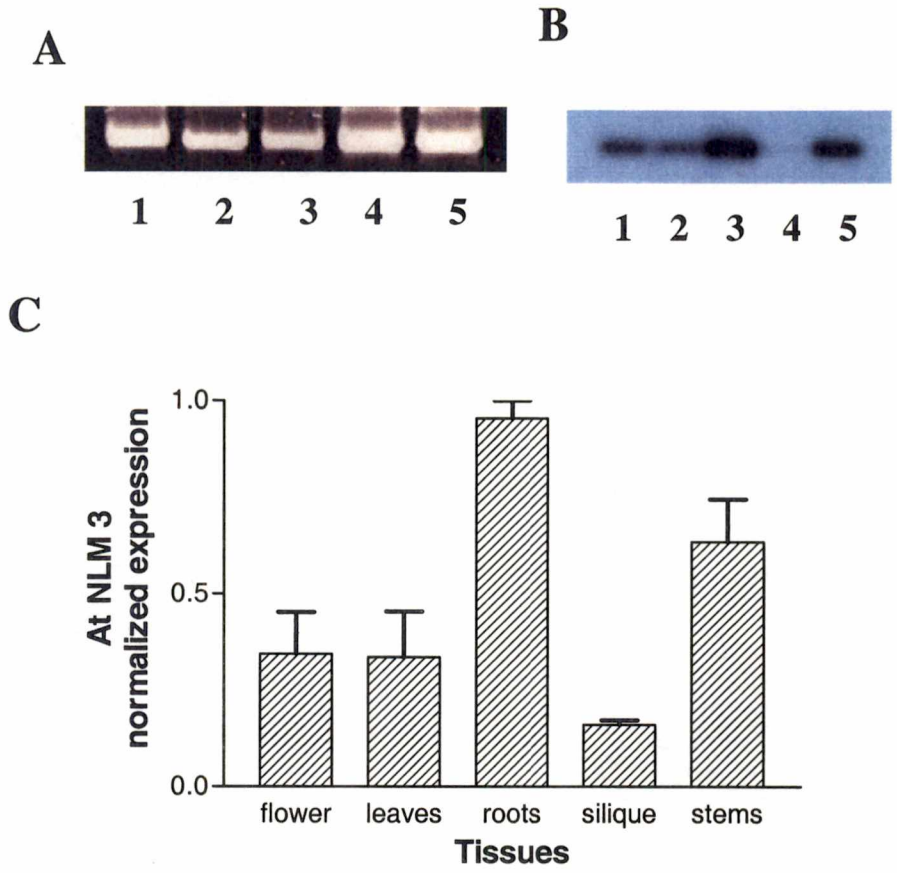


Figure 6: Tissue expression pattern of At-NLM3. Total RNA was isolated from tissues of 6-week old *A. thaliana.* **Lane1**,flower; **Lane 2**, leaf; **Lane 3**, root; **Lane 4**, silique; **Lane 5**, stem tissue. RNA was subjected to RT-PCR with At-NLM3 specific primers with actin 2 primers included as an internal control to monitor amplification efficiency and loading. Products were resolved by electrophoresis on 1% (w/v) agarose gels. **Panel A**, ethidium bromide stain showing actin 2 PCR products; **Panel B**, Southern analysis of RT-PCR reactions with an At-NLM3 probe. **Panel C**, Graph representing relative expression of At-NLM3 in all tissues. The At-NLM3 signal from Southern analysis was standardized to the actin signal and was normalized relative to the expression in the roots. Error bars show standard error of mean (n=5).

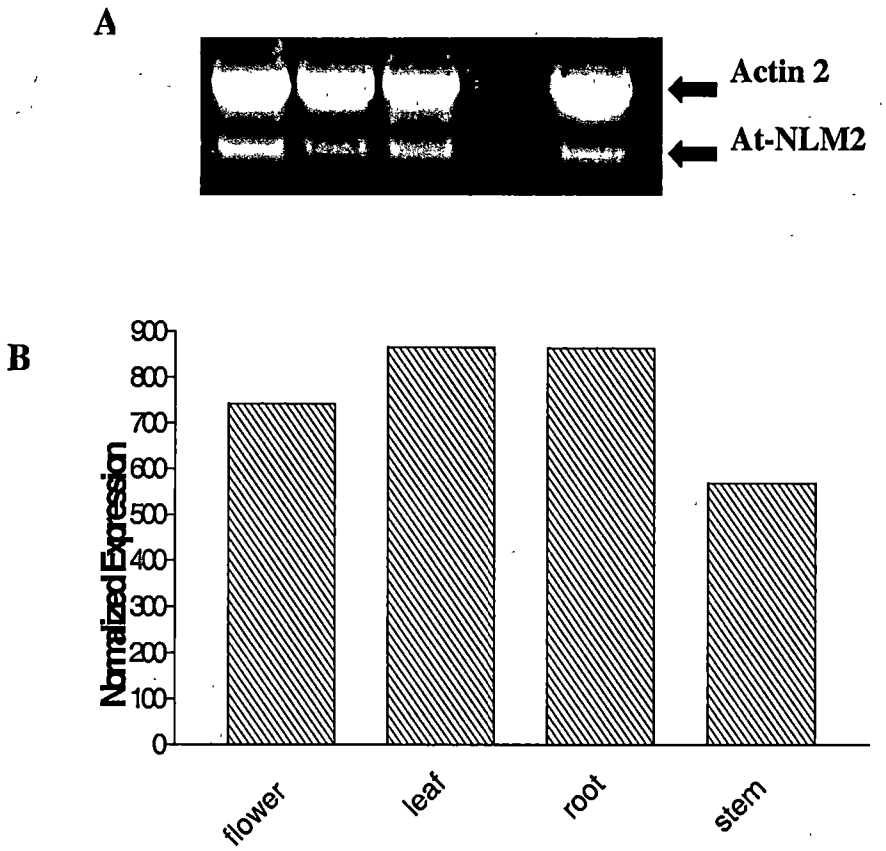


Figure 7: Tissue expression pattern of At-NLM2. Total RNA was isolated from tissues of 6-week old *A. thaliana* **Lane 1**, flower; **Lane 2**, leaf; **Lane 3**, root; **Lane 4**, stem tissue. RNA was subjected to RT-PCR with At-NLM2 primers with actin 2 primers included as an internal control to monitor amplification efficiency and loading. Products were resolved by electrophoresis on 1% (w/v) agarose gels. **Panel A**, ethidium bromide stain showing actin 2 PCR products. **Panel B**, Histogram representing relative expression of At-NLM2 in all tissue examined. The At-NLM2 signal from Southern blot analysis was standardized to the actin signal.

light on At-NLM expression was also examined with two different treatments. Etiolation, which is growth in the absence of light, was examined. In addition, the diurnal cycling of At-NLMs in green *Arabidopsis* was also examined. Lastly, the effects of decreased temperature on At-NLM expression was also investigated.

Effect of Osmotic and Cold Stress on At-NLM Expression

To determine whether At-NLM1 or 3 showed responsiveness to the various treatments, *Arabidopsis* plants were germinated on MS agar and were grown for 19 days on filter paper as described by Weig et al. (1997). At this point seedlings were exposed to three separate osmotic stresses: 1. drought (by removal of the filter paper discs and drying by exposure to air for 2.5 hrs); 2. salinity (0.3 M NaCl) and 3. high concentrations of an uncharged solute (0.3 M mannitol).

At-NLM3 levels showed a differential response to the applied stresses (Figure 8). At-NLM3 levels showed little sensitivity to mannitol, and showed a slight decrease with cold temperature and NaCl (Figure 8). However, At-NLM3 showed a higher sensitivity to drought, with a 2.5 fold reduction in At-NLM3 levels observed over 2.5 hours (Figure 8). In addition, the transcript level of At-NLM3 showed a 2.5 fold decrease with the application of 0.1 mM of the stress hormone abscissic acid (ABA) (Figure 10). Similar to At-NLM3, the application of 0.1 mM ABA decreased the amount of At-NLM1 mRNA by four fold (Figure 10). However, in contrast to At-NLM3, transcript levels of At-NLM1 exhibited a higher sensitivity to NaCl and decreased temperature, showing a 2-fold reduction for both treatments (Figure 9). As seen in Figure 11, the

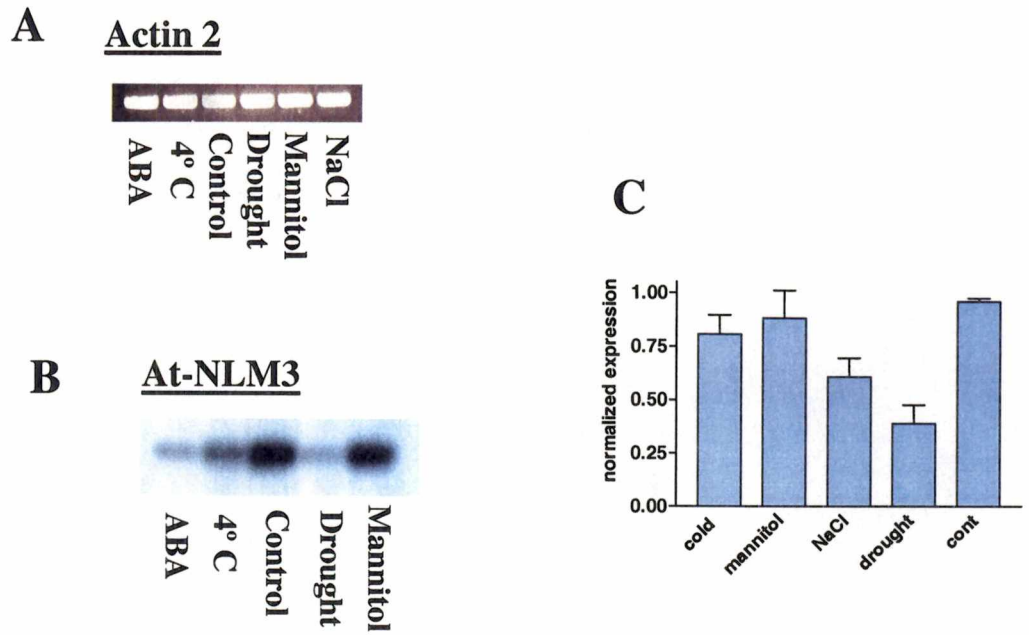


Figure 8: Effects of Stress/Environmental Stimuli on At-NLM3. Seedlings were grown on 1X MS agar for 17 days and then transferred to MS agar plates containing the various treatments indicated above. NaCl (0.3 M), mannitol (0.3 M), or 0.1 mM abscissic acid were adminstered for 24-hr. Cold (4°C) and drought (seedlings exposed to air on dry filter paper) treatments were done for 2.5 hr. RT-PCR analysis was done as shown in panel A using At-NLM3 and actin 2 primers. **Panel A** shows a 1% (w/v) agarose gel of representative results from RT-PCR of actin 2 used as a amplification and loading control. **Panel B** shows Southern blot of At-NLM3. **Panel C** shows graph of RT-PCR result showing expression levels standardized to actin signal and were normalized t untreated controls. Error bars show standard error of mean (n=4).

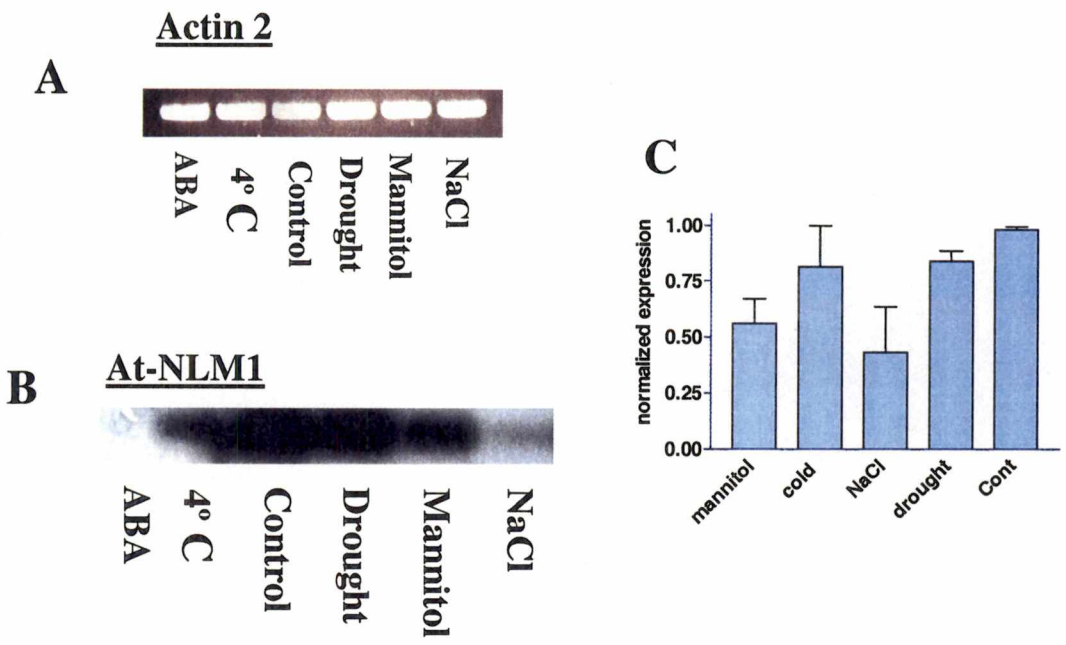


Figure 9: Effects of Stress/Environmental Stimuli on At-NLM1. Seedlings were grown on 1X MS agar for 17 days and then transferred to MS agar plates containing the various treatments indicated above. NaCl (0.3 M), mannitol (0.3 M), or 0.1 mM abscissic acid were adminstered for 24-hr. Cold (4°C) and drought (seedlings exposed to air on dry filter paper) treatments were done for 2.5 hr. RT-PCR analysis was done as shown in panel A using At-NLM1 and actin 2 primers. **Panel A** shows a 1% (w/v) agarose gel of representative results from RT-PCR of actin 2 used as a amplification and loading control. **Panel B** shows southern blot of At-NLM1. **Panel C** shows graph of RT-PCR result showing expression levels standardized to actin signal and were normalized t untreated controls. Error bars show standard error of mean (n=4).

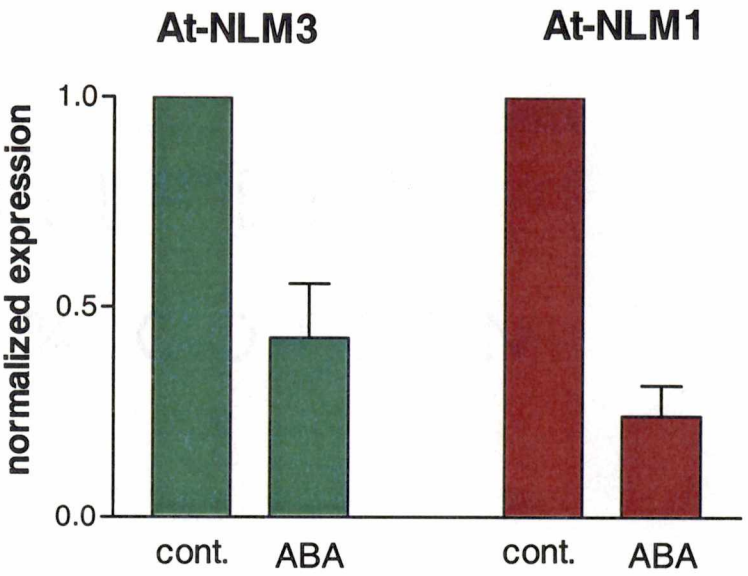


Figure 10: Effects of abscisic acid on At-NLM1 and At-NLM3. Seedlings were grown on 1X MS agar for 17 days and then transferred to MS agar plates containing 0.1 mM abscisic acid for 24-hr. RT-PCR analysis was done using At-NLM1 or At-NLM3 and actin 2 primers. Graph represents RT-PCR results showing expression levels standardized to actin signal and were normalized to untreated controls. Error bars show standard error of mean (n=4).

A

hours after NaCl: 0 0.5 2.3 7 27 34

B

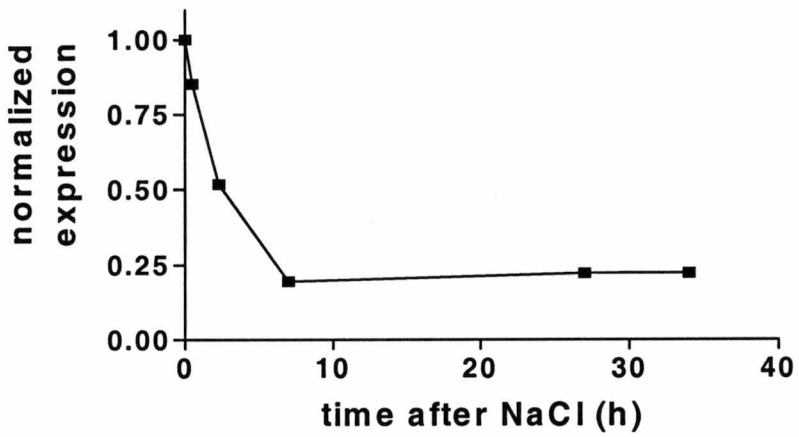


Figure 11: Effects of NaCl on expression levels of At-NLM1. Total RNA was isolated from 17 day old *Arabidopsis thaliana* seedling exposed to 0.3 M NaCl for 0, 30 minutes, 2h/17 minutes, 7h, 27h, and 34 h. **Panel A**, shows Southern blot analysis using At-NLM1 probe. **Panel B**, Graph representing relative expression of At-NLM1 in seedlings standardized to actin 2..

transcript levels of At-NLM1 respond rapidly to NaCl treatment, showing a decrease by 30 minutes. The transcript levels began to decrease within a 30 minutes and eventually reach a four-fold reduction at the 34 hour time point. The levels of At-NLM1 mRNA did not seem to be greatly affected by drought or mannitol (Figure 9).

Effect of Light/Diurnal Rhythms on At-NLMs

The flow of water and water relations in plants changes during dark/light cycling. In the light, there is a steady transpiration stream that begins with water being absorbed through the root system and ends with water release through stomatal pores in the leaves. Thus, the rate and direction of water flow changes depending on light/dark conditions and it is possible that the accompanying changes in water permeability and hydraulic conductivity of the membranes of various cells and tissues involves changes in the levels and/or regulation of MIPs and aquaporins. To investigate the changes in At-NLMs during this process the At-NLM levels were analyzed using seedlings entrained for a 16h light/8 h dark light cycle.

Whereas At-NLM3 showed no clear pattern of change in response to this treatment (data not shown), At-NLM1 expression showed diurnal variation in expression (Figure 12). The transcript levels were lowest at the end of the 8-hr dark period. Transcript levels begin to increase upon entering the light period, reaching a peak approximately 8-hr into the photoperiod. This diurnal expression pattern of At-NLM1 seemed to follow an internal circadian rhythm rather than responding to light (Figure 12). This was determined by entraining plants to a 16h light/8 h dark cycle and then allowing

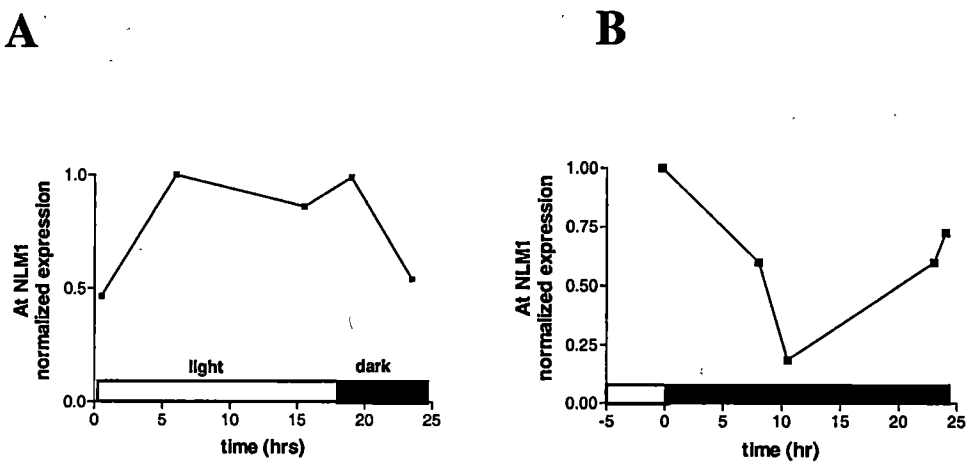


Figure 12: Diurnal rhythm of At-NLM1 expression in *Arabidopsis thaliana*. Total RNA was isolated for RT-PCR analysis from seedlings (17 d) grown under a 16-h light/8-h dark photoperiod. **Panel A**, results of RT-PCR representing expression pattern of At-NLM1 at various points (x-axis) throughout the photocycle. At-NLM1 expression was standardized to that of actin 2 and was normalized. The bar shows the duration of the light and dark periods. **Panel B**, 17 d old A. thaliana plants were entrained to a 16h light/ 8 hr dark photocycle and then the lights were turned off (time=0). Plants were collected at various time points, and total RNA was isolated and analyzed by RT-PCR as in Panel A.

the plants to cycle in complete darkness. As shown in Figure 12, during the first 9 hours of the dark period, the levels of At-NLM1 decrease 4-fold. After 9 hours, the levels begin to increase, as if anticipating the onset of the light period. The results show that the expression of At-NLM1, a root MIP, shows a higher expression in light, a time at which the transpiration stream and radial transcellular flow of water in roots is highest. Further, this fluctuation in expression appears to be regulated according to an internal circadian clock since after the entrainment a light stimulus was no longer required.

Developmental changes in response to light stimuli were also evaluated by growing *Arabidopsis* under four different light regimes (complete darkness for 19 days, light for 19 days, dark for 14 days then transferred to light for 5 days, or in low light for 19 days) and using the RT-PCR assay to analyze expression. As shown in Figure 13, actin 2 shows no variation in response to these treatments whereas At-NLM3 shows a substantial up regulation of 7-fold upon growth in darkness compared to growth in continuous light or in tissues greened for five days after etiolated growth. As shown in Figure 13 the At-NLM3 transcript is hardly detectable in the light grown plants due to the decrease in exposure time of the Southern blots in response to the significant up regulation of At-NLM3 in etiolated plants. In comparison, At-NLM1 showed more modest effects (Figure 14) with levels actually showing a decrease upon growth in darkness. The results show that At-NLM3 expression appears to be regulated by a photomorphogenic light stimulus and appears to be enhanced in the etiolated state.

Isolation of a Transferred (T-DNA) Knockout in At-NLM3

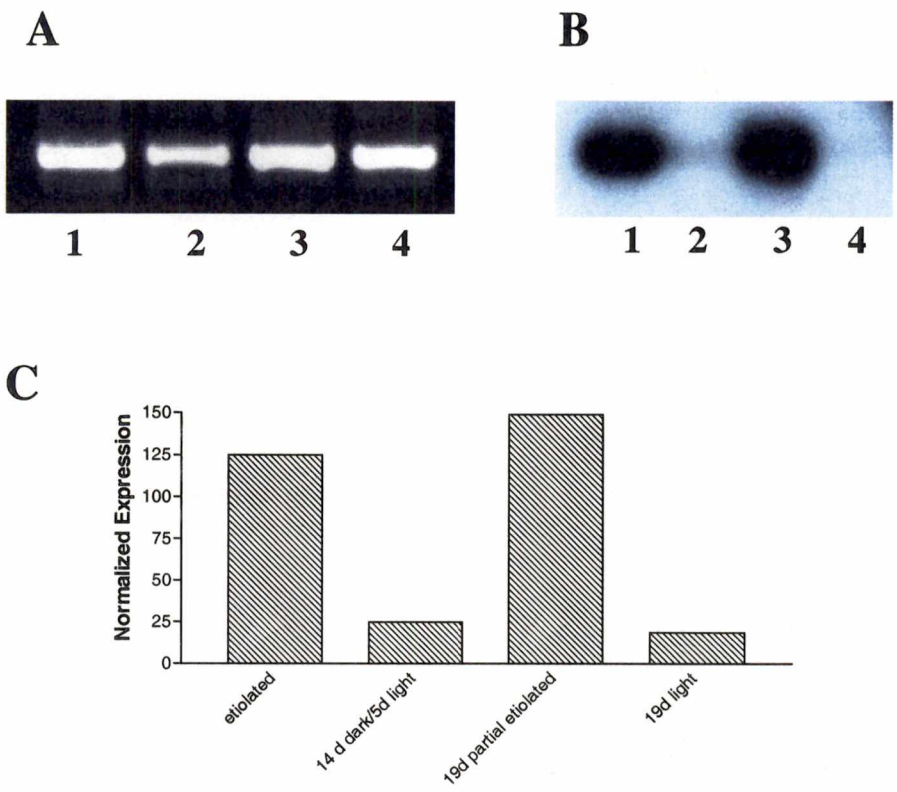


Figure 13: Effects of etiolation on expression pattern of At-NLM3. Total RNA was isolated from 19 day old *Arabidopsis thaliana* seedlings that either received no light for 19 days **(Lane 1),** 14 days dark and 5 days light **(Lane 2),** low light for 19 days **(Lane 3),** or light for 19 days **(Lane 4).** RNA was subjected to RT-PCR with At-NLM3 specific primers with actin 2 primers included as an internal control to monitor amplification efficiency and loading. **Panel A**: 1 % (w/v) agarose gel stained with ethidium bromide showing the actin 2 PCR product. **Panel B**: Southern blot of RT-PCR products using an At-NLM3 probe. Bands in the greened and light grown plants showed up upon a longer exposure time. **Panel C**: Histogram representing relative expression of At-NLM3 in seedlings.

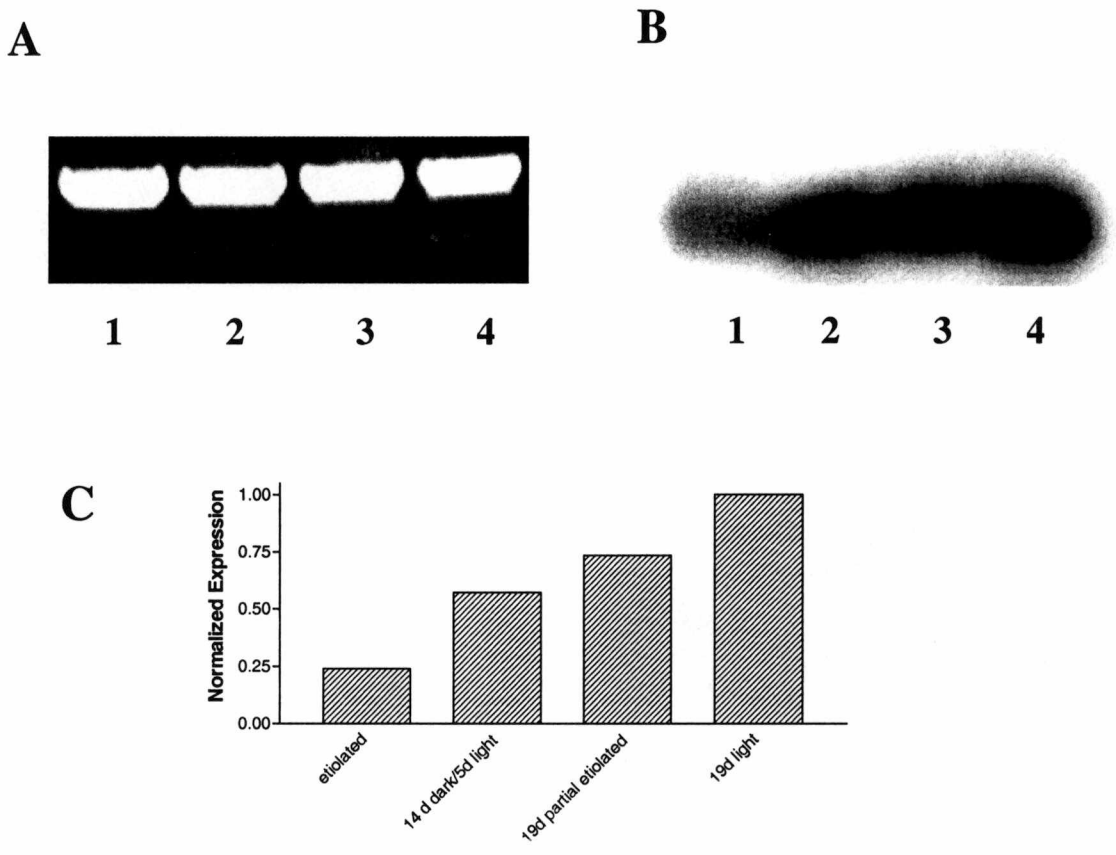


Figure 14: Effects of etiolation on expression pattern of At-NLM1. Total RNA was isolated from 19 day old *Arabidopsis thaliana* seedlings that either received no light for 19 days **(Lane 1),** 14 days dark and 5 days light **(Lane 2),** low light for 19 days **(Lane 3),** or light for 19 days **(Lane 4).** RNA was subjected to RT-PCR with At-NLM1 specific primers with actin 2 primers included as an internal control to monitor amplification efficiency and loading. **Panel A**: 1 % (w/v) agarose gel stained with ethidium bromide showing actin 2 band and lower band representing At-NLM1. **Panel B**: Southern blot analysis of RT-PCR products using an At-NLM1 probe. **Panel C**: Histogram representing relative expression of At-NLM1 in seedlings.

The expression patterns of AtNLM1 and At-NLM3 suggest they play a role in adaptation to changing osmotic conditions and also are developmentally controlled by light stimuli. However, the role of these putative channel proteins in stress biology and membrane function remains unclear. To begin to explore the functions of NLMs *in vivo*, a reverse genetic technique is being employed to try to obtain *Arabidopsis thaliana* lines in which these genes are silenced. To gain insight into its role in stress physiology, a T-DNA knockout mutant of At-NLM3 has been generated.

To begin this process an extremely large collection of T-DNA-transformed *A. thaliana* lines is necessary. These lines were generated via a transformation process using *Agrobacterium tumefaciens* (*A. tumefaciens*). During infection this bacterium transfers a DNA segment, T-DNA, which becomes randomly inserted in the plant host genome (Zambryski, 1988). Screening large populations of T-DNA lines to detect the insertion of the T-DNA within the gene of interest can be accomplished through a PCR technique using two primers (Krysan et al., 1999): one primer directed against the T-DNA and the second primer against the gene of interest. A positive PCR product indicates the insertion of the T-DNA close to the site of the gene of interest. Plants containing these gene disruptions can then be studied to understand the normal function of the gene (Krysan et al, 1996). T-DNA insertional mutagenesis has recently become an established method for studying genes in *A. thaliana*. Knockouts of many diverse genes have been isolated and studied including genes involved in signal transduction and ion transport, members from the actin gene family and genes essential for seed germination (McKinney et al, 1995; Dubreucq et al., 1996; Castle et al., 1993).

We began this process by purchasing the Jacks and Feldman T-DNA libraries from the *Arabidopsis* Biological Resource Center (ABRC), two commercially available *A. thaliana*/T-DNA knockout libraries consisting of 6,000 lines each (McKinney et al., 1995). Primers (Table I) were designed following the guidelines of the knockout facility and were generated with sequences at the extreme 5' and 3' end of the gene. Primers were 29 bp in length with melting temperatures of 65°C. A series of PCR and Southern blots were performed to screen both libraries for T-DNA insertions within the At-NLM1 and At-NLM3 loci. Positive results (i.e., low molecular weight PCR products stained with ethidium bromide) were initially observed. However, Southern blot analysis with At-NLM1 or At-NLM3 probes were negative, suggesting that these amplified products were not derived from these loci.

After several attempts with these lines, we decided to take advantage of the University of Wisconsin T-DNA knockout facility, which aids investigators in the design of primers and early aspects of screening. In addition, a collection of 60,480 *A. thaliana* lines are offered that have been transformed with the T-DNA vector pD991 (McKinney et al., 1995). Thus, many more lines are available raising the likelihood that an insert within the gene of interest can be identified. As outlined in Figure 15, the screening process involves two rounds of PCR. The first round was done on the entire population (30 super pools) for T-DNA inserts with the At-NLM3 primers.

The ethidium bromide stained agarose gel of the primary PCR screen of thirty pools, each containing 2025 lines, is shown in Figure 16. Southern blot analysis with an At-NLM3 probe showed three hybridizing "positive hits" corresponding to PCR products within the desired size range, between 1100bp and 1500bp (Figure 17). These PCR

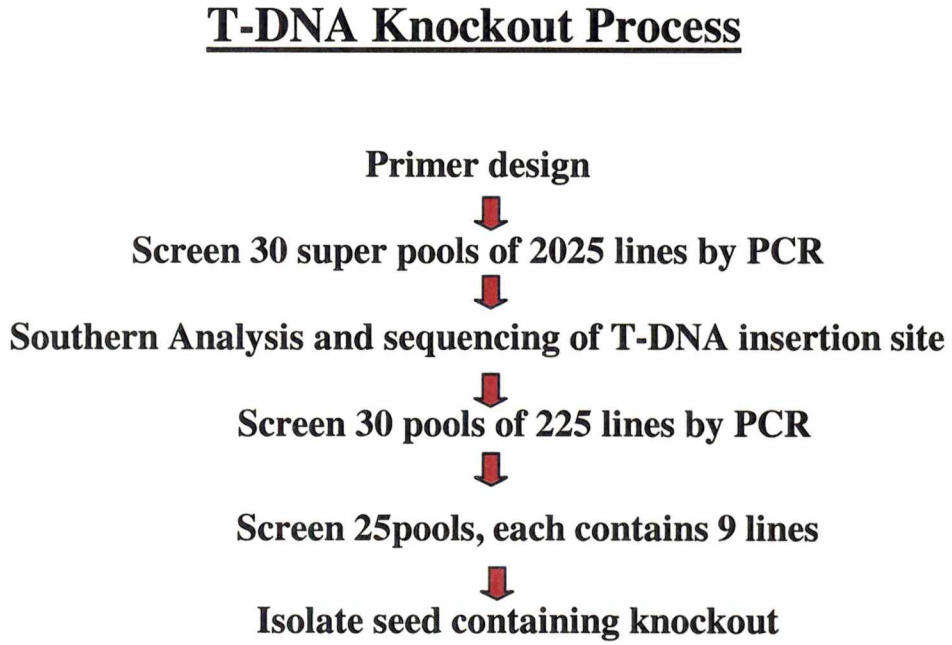


Figure 15: Organization and screening of 60,480 T-DNA-transformed *Arabidopsis* lines. PCR primers refers to both 5' and 3' At-NLM3 primers and the T-DNA left border primer as shown in Figure 19 and Table 1.

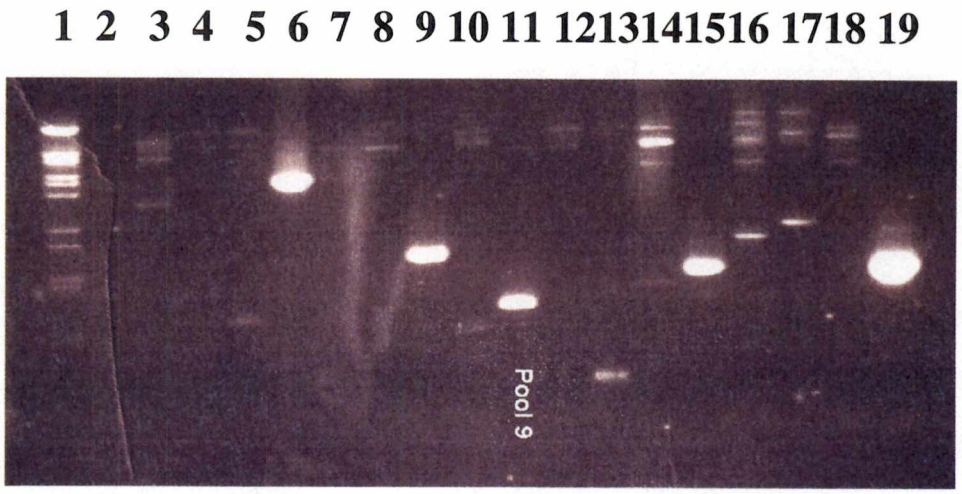


Figure 16: Results of primary PCR screen for a T-DNA knockout within the At-NLM3 gene. Primers designed to the extreme 5' and 3' ends of the first and fourth exons were used by the *Arabidospsis* Knockout Facility at the University of Wisconsin to conduct a primary screen of thirty super pools of 2025 lines each from their T-DNA tagged library. Reactions were resolved on a 1% (w/v) agarose gel and were stained with ethidium bromide. **Lane 1**, Lambda/*BstE*II DNA ladder. **Lanes 2-18,** representative reaction products of pools 1-17. **Lanes 9, 11, and 14** contain the three positive hits detected by Southern analysis (Figure 17). **Lane 19**, positive control (At-NLM3 pcr product). The location of the positive band in pool 9 is indicated.

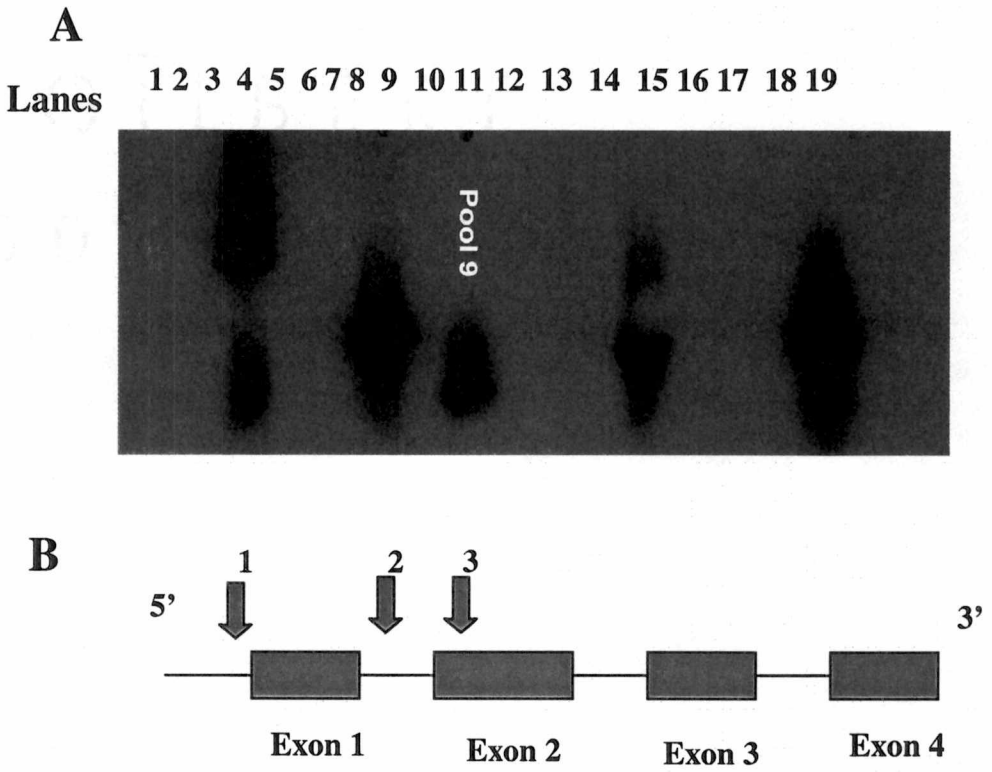


Figure 17: Location of T-DNA inserts in At-NLM3 gene. Panel A, Autoradiogram of a Southern blot of PCR reactions 1-17 from Figure 20 probed with At-NLM3 probe. Primers were designed to the extreme 5' and 3' coding regions of At-NLM3 and the left border of the T-DNA (Table I). **Panel B,** Location of T-DNA inserts from positive hits in lane 9, (pool 7), lane 11, (pool 9) and lane 15, (pool 13) based on DNA sequence analysis.

products (pool 7, 9, 13) were cloned into the pCR2.1 vector using the TA cloning kit and sequenced. The location of the T-DNA inserts based on sequence analysis is shown in Figure 17. The first hit (pool 13) was 170bp downstream of the transcription start site within the first intron. The second hit (pool 7) was 180bp upstream from the transcription start site within the putative promoter region. Pool 9, on the other hand, has a T-DNA insert located 531bp downstream from the transcription start site, within the second exon. This insertion would most likely completely diminish the gene's ability to correctly function (Figure 17). The sequence surrounding the site of insertion is shown in Figure 18.

A similar approach was used to attempt to isolate an insertion in the At-NLM1 gene. Multiple potential hits were detected by Southern blot of PCR reactions in the primary screen (not shown). However, sequence analysis of these showed that the T-DNA was inserted within the second intron and therefore are less likely to provide a gene disruption. At-NLM1 knockout mutagenesis was therefore not pursued further as a part of this study.

The knockout mutant of At-NLM3 detected in pool 9 was taken to a secondary screen of 30 pools each containing 225 lines. By PCR/Southern blot analysis, a subpool containing the desired T-DNA insert was identified. Next, seed was ordered in which this subpool was derived into 25 pools each containing 9 separate T-DNA tagged lines. Seeds were planted, grown for two weeks and genomic DNA was then isolated from leaf tissue. The At-NLM3 knockout plants was then identified using a PCR based strategy using the reverse At-NLM3 primer and the left border T-DNA primer. The results of the PCR screen identified two positive plants (from pool 4 and 8) as shown in Figure 19.

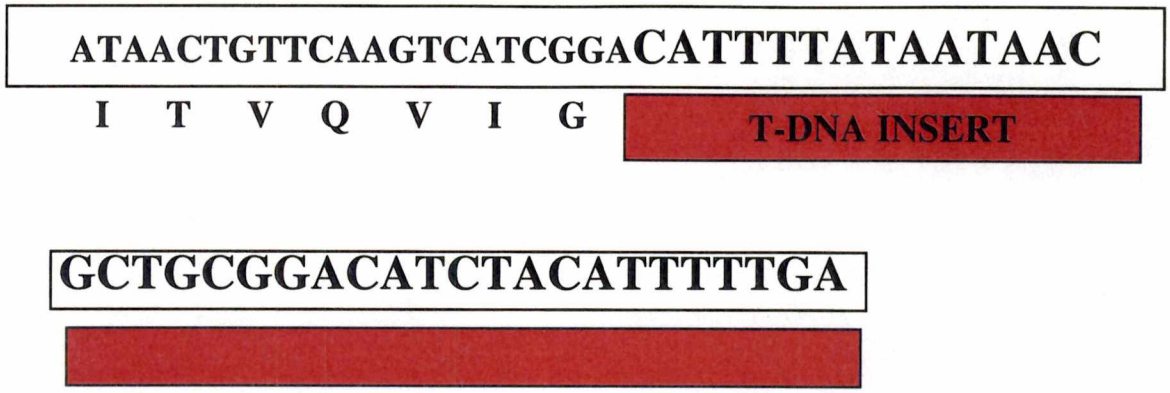


Figure 18: Sequence analysis data showing location of DNA insert within the second exon of At-NLM3. Boxed nucleic acids represent the At-NLM3 gene and the T-DNA insert from sequence analysis data. Amino acids flanking the insertion site are seen below nucleic acids, beginning with the Glycine 134. Part of the sequence of the T-DNA insert from sequence data (bold) is shown. The location of the T-DNA insert within the second exon is shown in red.

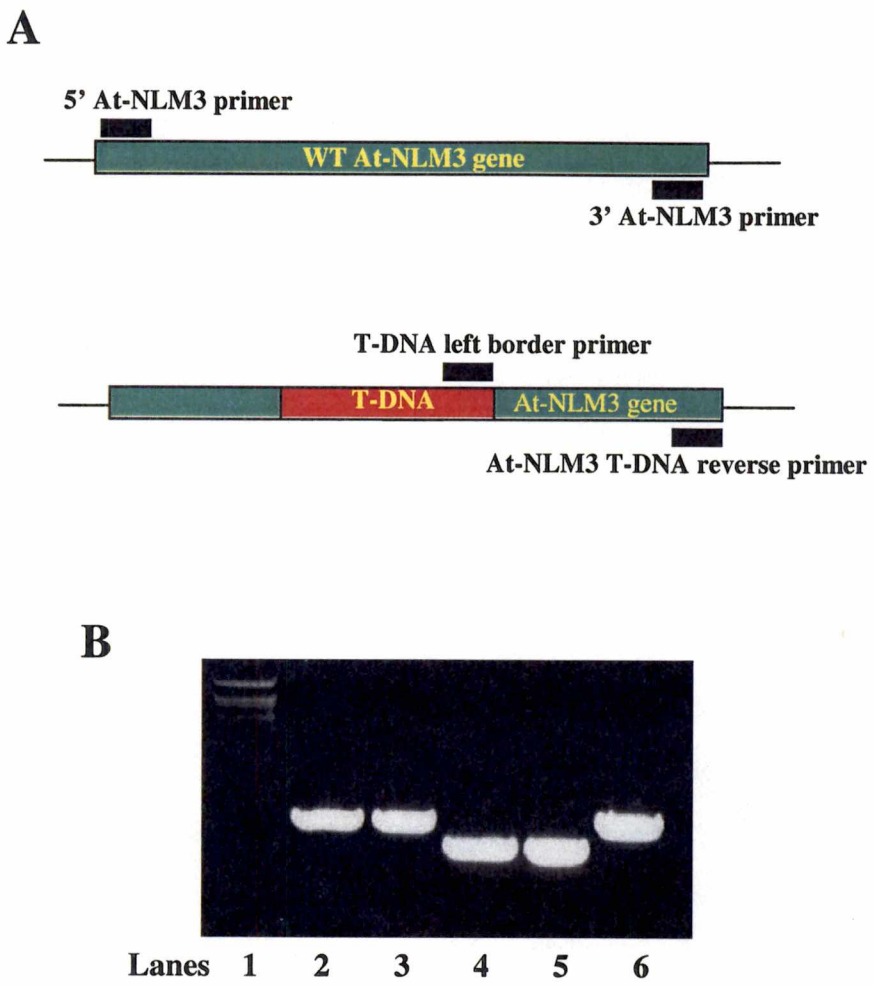


Figure 19: Isolation of At-NLM3 knockout plants. Panel A, Diagram illusrating PCR stategy used to isolate At-NLM3 knockout plants. PCR reaction were performed with both WT and putative At-NLM3 knockout plants genomic DNA with gene specific and knockout combination of primers. **Panel B,** PCR reactions were resolved on 1% (w/v) ethidium bromide stained gel. Lane 1, Lambda/BstEII DNA marker. Lane 2 and 3, PCR reactions with genomic DNA from two putative knockout plants using the 5' and 3' At-NLM3 primers. Lane 3 and 4, PCR reactions using genomic DNA from two knockout plants using the 3' At-NLM3 primer and the T-DNA left border primer. Lane 5, PCR reaction with wild type genomic DNA using the 5' and 3' At-NLM3 primers.

Genomic DNA from these plants gave the positive 1264 bp product with the At-NLM3 gene specific / left border T-DNA primer pair. Seed from this plant were collected and stored.

The isolated seed from the plant could have one of three genotypes. It could be: 1. Homozygous containing two At-NLM3 gene disruptions; 2. Heterozygous containing one At-NLM3 disruption and a normal At-NLM3 allele; or 3. Homozygous containing two normal At-NLM3 alleles. The first genotype above was the one desired to obtain a complete At-NLM3 knockout. To obtain this, seed were germinated with kanamycin selection. Under these conditions only plants having a T-DNA insert will grow. Next, the homozygous plant was identified by PCR using the At-NLM3 gene specific primers. As shown in Figure 20, heterozygous plants containing one wild type copy of the At-NLM3 gene will yield a product corresponding to the normal size of the gene. In contrast, a homozygous plant with insertions within both copies of the At-NLM3 gene will yield no products with the normal PCR conditions due to the large T-DNA insert (Figure 20). As shown in Figure 20, a plant homozygous for the At-NLM3 disruption was identified. Seed was collected from this plant and was used for the preliminary phenotype analysis described below.

Growth and Development of At-NLM3 Knockout Plants

Preliminary phenotype analyses were done on *A. thaliana* plants homozygous for the At-NLM3 knockout showed two reproducible growth defects, one with green plants (Figure 21) and one observed with etiolated plants (Figure 22). Prior to flowering

A

Isolation of homozygous plant

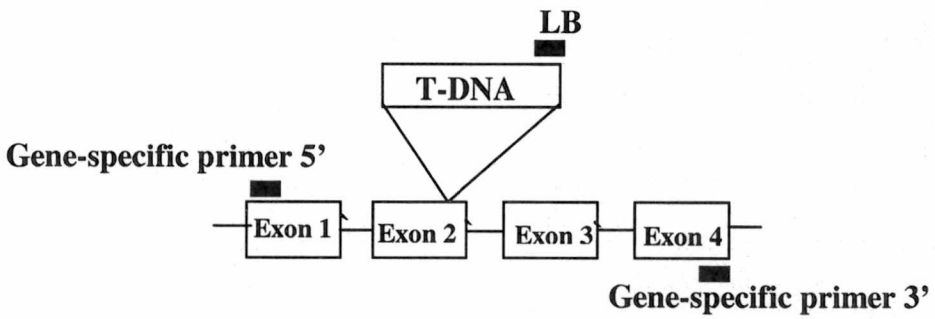
From T_2-seed

↓

20 plants

↓

PCR screen for T-DNA insert and for At-NLM3 gene



B

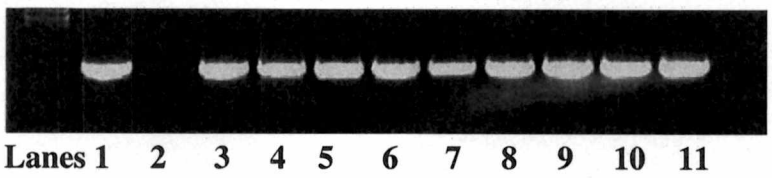


Figure 20: PCR strategy used to isolate the homozygous At-NLM3 knockout plant. Panel A, Flow chart describing process used to isolate the At-NLM3 homozygous knockout plant. Panel B, genomic DNA was isolated and used as a template for PCR from plants that were selected by growing on MS agar plants containing kanamycin. PCR reactions were performed using the 5' and 3' At-NLM3 primers to isolate the homozygous plant. Lane 1, Lambda/BstEII DNA marker. Lane 2, and 4-11, PCR reactions from heterozygous plants showing products of wild type At-NLM3 gene A. Lane 3, PCR reaction from homozygous plant yielding no PCR product due to large T-DNA insert.

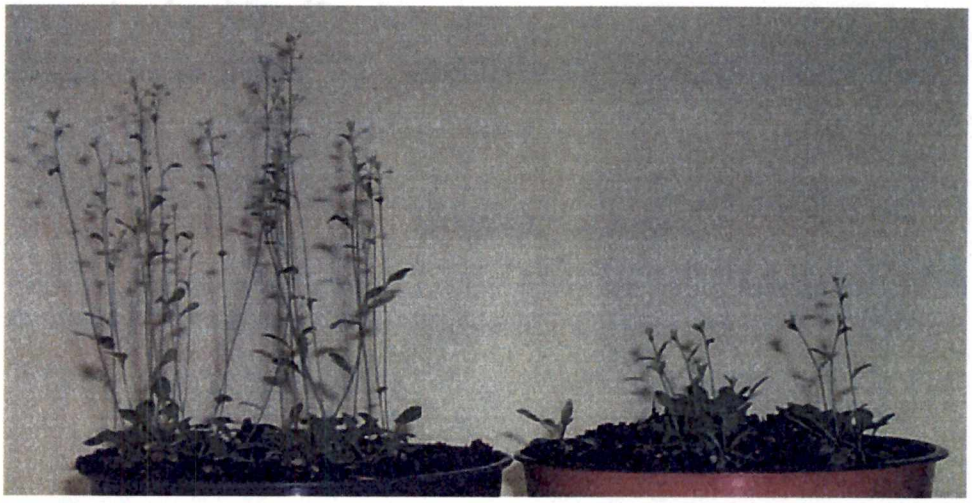
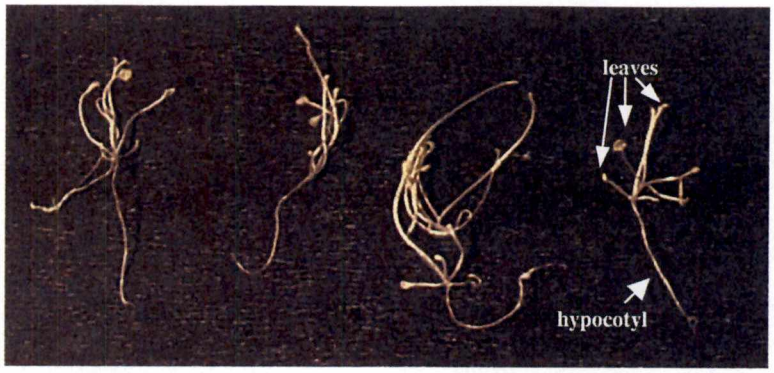


Figure 21: Comparison of stem growth of 4 week old *Arabidopsis thaliana* plants. Comparison of stem growth At-NLM3 knockout plants (plants in left pot) and wild type plants (right pot) after four weeks of growth (16h light/8 h dark photocycle).

A



B

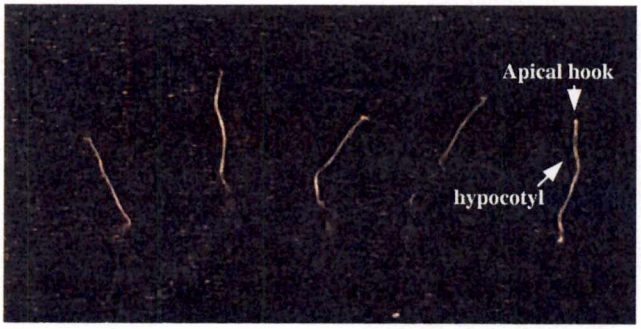


Figure 22: 3 week old *Arabidopsis thaliana* etiolated seedlings. *Arabidopsis thaliana* seeds were planted on 1X MS agar and grown at 22°C in complete darkness for 3 weeks. **Panel A**, At-NLM3 knockout seedlings. **Panel B**, wild type seedlings. Shown are the hypocotyls of each and the unfolded rosette leaves of the mutant compared to the folded apical hook of the wild type plants.

Arabidopsis plants undergo a rapid stem growth phase called bolting. During the growth of At-NLM3 seedlings in light they showed premature bolting with increased stem growth whereas wild type plants showed a delay of the onset of stem bolting. Specifically, At-NLM3 knockout plants bolting was observed 28 days after germination under the growth conditions used. In contrast, bolting in wild type plants was delayed and occurred approximately 10 days later. Additionally, the stems of At-NLM3 knockout plants appeared less rigid and the plants showed difficulty remaining upright and tended to fall over at more advanced growth stages.

A more pronounced phenotype was observed upon extended growth of At-NLM3 seedlings in the dark (Figure 22). Upon germination in darkness, wild type and At-NLM3 knockout plants initially show the same growth pattern. However, upon extended etiolated growth wild type *Arabidopsis* plants show the typical single hypocotyl with an apical hook and folded leaves. In contrast, the At-NLM3 knockout plants develop multiple extended leaf structures.

Since At-NLM3 expression is disrupted by the introduction of the T-DNA insert, the possibility that other At-NLM expression levels might be altered to compensate was tested. As shown in Figure 23, At-NLM1 was up regulated in At-NLM3 knockout plants by 2-fold.

A

WT At-NLM3-

B

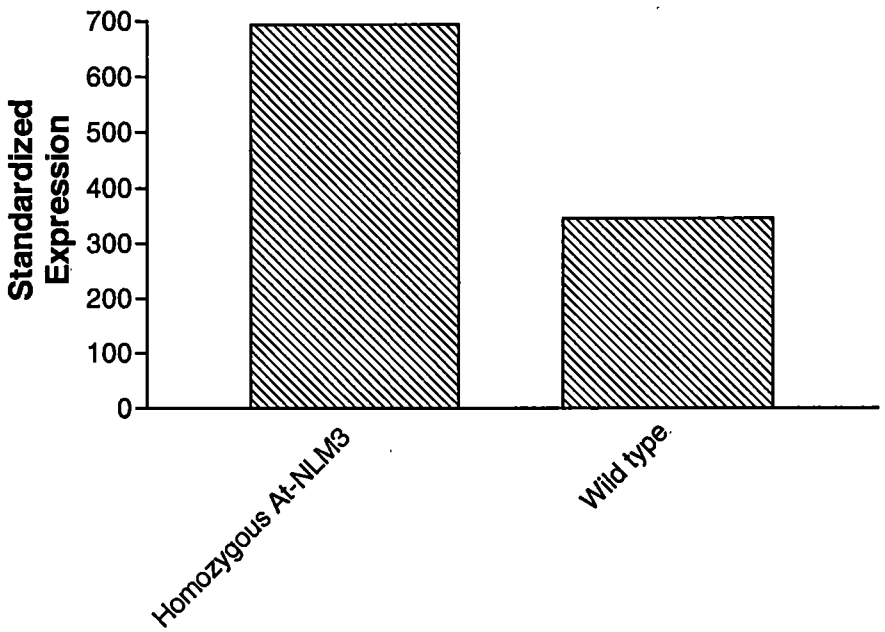


Figure 23: Expression levels of At-NLM1 in *Arabidopsis thaliana* At-NLM3 knockout seedlings and wild type seedlings. Arabidopsis thaliana seeds were planted on 1 X MS agar and grown at 22°C for 3 weeks. Total RNA was isolated from seedlings and subjected RT-PCR with At-NLM1 specific primers. **Panel A**, Southern blot analysis using At-NLM1 as a probe. **Panel B**, Histogram representing relative expression of At-NLM1 standardized to actin 2.

CHAPTER IV

DISCUSSION

Plants were among the earliest organisms in the fossil record, but they did not colonize land until about 450 million years ago, or in the last 10% of the age of the earth (Boyer, 1985). Apparently, the complexities of obtaining water in a dry environment had something to do with this delay. Roots and an advanced vascular system were necessary to gather water while an epidermis and stomata were necessary to conserve it (Boyer, 1985). Water must transverse many different cell types as it moves along its transport path. Some tissues such as the xylem were modified for water movement whereas others such as parenchyma were not (Boyer, 1985). Together with other difficulties in measurement techniques initially caused researchers to approach the subject of water transport in plants blindly by measuring forces and determining water flows without knowing the precise nature of the flow path.

In plants, water transport across tissues plays an important role during many fundamental processes. Results consistent with the presence of proteins facilitating transmembrane water flow in plant cells (Wayne and Tazawa, 1990), and the identification of several major plant membrane proteins with high sequence homology to AQP1 and MIP (also known as AQP0) (Fortin et al., 1987; Sandal and Marcker, 1988; Johnson et al., 1989; Yamamoto et al., 1991; Höfte et al., 1992; Yamaguchi-Shinozaki et al., 1992) led to the identification of a water channel protein in plant membranes (Maurel

et al., 1993). Most plant aquaporins investigated are located in either the tonoplast or the plasma membrane. The abundance and diversity of aquaporins in plants is greater than their mammalian and microbial counterparts. For example, after the completion of the sequencing of the *Arabidopsis* genome, we now know that there are 39 members of the MIP family in *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000). According to amino acid sequence similarities, *Arabidopsis* MIPs either belong to the MIPs of the tonoplast (tonoplast intrinsic proteins, TIPs), to the MIPs of the plasma membrane (the plasma membrane intrinsic proteins, PIPs) or to the NLMs (nodulin 26-like MIPs). The PIPs, TIPs, and NLMs form three distinct phylogenetic groups (Weig et al., 1997; Kjellbom et al., 1999). The PIPs can be further divided into the PIP1 and the PIP2 subfamilies, whereas the TIPs can be divided in to three subfamilies: αTIPs, δTIPs and the γTIPs (Schäffner, 1998). In the present study, I have focused on the less studied NLM subfamily, of which soybean nodulin 26 is the archetype.

Phylogeny of Nodulin 26 Proteins

Nodulin 26 is expressed solely in nitrogen fixing root nodules where it is targeted to the symbiosome membrane and constitutes 10-15% of the total protein of the symbiosome membrane (Weaver et al., 1991; Rivers et al., 1997). In addition, nodulin 26 from soybean nodules was the first aquaglyceroporin to be discovered in plants and has been shown to transport both water and small uncharged solutes, such as glycerol and formamide (Dean et al., 1999; Rivers et al., 1997) and to be phosphorylated by a calcium dependent protein kinase (CDPK) (Weaver et al., 1991; Weaver et al., 1992).

The finding that nodulin 26 is a major component of the symbiosome membrane, has unique transport properties, and is a target for calcium dependent phosphorylation has generated considerable interest in its biological function. The finding of Weig et al. (1997), and elaborated by the work shown here, illustrates that the nodulin 26 subfamily is also well represented in non-nodulating plant species such as *Arabidopsis*. To date NLMs are published from six plant species: GmNOD26 from *G. max* (Fortin et al., 1987), LIMP2 from *Lotus japonicus* (Guenther and Roberts, 2000), OsrMIP from *Oryza sativa* (Liu et al., 1994), NaPIP28 from *Nicotiana alata* (Genbank accession number AJ243308) and the At-NLMs from *A. thaliana* (Weig et al., 1997; Weig and Jakob, 2000). While, detailed biochemical information is only known for GmNOD26, several observations suggest that other family members have conserved functional properties, for example, recently an orthologous protein (LIMP2) was described from another legume, *L. japonicus* (Guenther and Roberts, 2000). LIMP2, similar to nodulin 26, is expressed solely in the nodules and transports both water and glycerol (Guenther and Roberts, 2000). In addition, both At-NLM1 and At-NLM2 from *A. thaliana* were recently shown to transport both water and glycerol when expressed in *S. cerevisiae* (Weig and Jakob, 2000).

The identification of this unique subgroup in *A. thaliana*, a non-nodulating plant, suggests these proteins are playing other roles than in nitrogen fixation and symbiosis. From the phylogenetic analysis presented here, we propose that there are ten members of this subgroup in *A. thaliana*. The proteins are referred to as At-NLM1-10 and range from 32.1% to 62.4% identity to nodulin 26. Sequence alignment analysis shows that the At-NLMs cluster into pairs with as high as 87.8% identity, underscoring the fact that ~60%

of the *Arabidopsis* genome has been duplicated (*Arabidopsis* Genome Initiative, 2000). Interestingly, seven of the At-NLMs (At-NLM1-5, 8 and 10) have conserved structural/functional features, suggesting that they transport both water and glycerol. At-NLM1-5, 8 and 10, similar to nodulin 26 have a hybrid sequence signature with characteristics of both aquaporins and aquaglyceroporins. Possibly the most interesting sequence conservation is the tryptophan residue in helix 2. As shown in the high resolution crystal structure of the *E.coli* GlpF (Fu et al., 2000), this residue resides in the narrowest constriction of the channel and helps provide selectivity for glycerol by helping to form an amphipathic channel. The finding that this residue is conserved in these At-NLMs strongly suggests that they will form multifunctional aquaglyceroporin activities similar to nodulin 26. The exception to this is At-NLM6 and At-NLM9, which have an aquaporin-like substitution at this location. It will be of interest to analyze their functional properties by expression in *Xenopus* oocytes (Rivers et al., 1997; Dean et al., 1999; Guenther and Roberts, 2000) to test these predictions. In addition, six of the At-NLMs (At-NLM1-5, 7) contain conserved sequence motifs suggesting they may be targets for phosphorylation by calcium-dependent protein kinase. In particular, it has been found that members of the CDPK family tend to recognize the hydrophobic-X-basic-X-X Ser motif (Bachman et al., 1996; Harmon et al., 2000). The conservation of this motif in these NLMs within their hydrophilic carboxyl terminal domains argues that they also will be targets for calcium-dependent regulation by CDPKs.

To understand the multiple roles that aquaporins play in plants, we will have to catalog them and study their expression patterns. While PIPs and TIPs have been characterized to some extent in several species, not very much is known about the NLMs.

The identification of this unique subgroup in *A. thaliana* prompted us to try to clarify two questions. Firstly, we want to understand the roles/physiological functions of these proteins in *Arabidopsis* and secondly, by understanding these roles/functions the At-NLMs we might gain insight into the role of nodulin 26 in the symbiosome. In the present study we have investigated NLMs in *Arabidopsis thaliana* from three different angles: 1) First, we wanted to determine the tissue specific expression patterns of NLMs to gain insight and clues on the specific role each protein plays in the plant; and 2) Secondly, to determine how the levels and patterns of NLM expression are affected by various environmental stimuli; and 3) Lastly, to deduce the functions of the NLMs in vivo through reverse genetics using transferred (T-DNA) mutagenesis technology.

Tissue Specific Expression of Aquaporins

There is an astonishingly high number of plant genes encoding (putative) aquaporins, indicating both a fundamental role and a complex and/or redundant regulation (Schäffner, 1998). Aquaporins are expressed in organ-, tissue-, and cell type-specific manners. Analyzing the expression patterns of different aqauporins is essential for many reasons. It will provide clues to the functions of aquaporins at the whole plant level. Details of the specific roles and involvement in physiological processes are still lacking. Secondly, different aqauporins are differentially expressed. It is not known whether members of different subgroups are present in the same cell. In addition, aquaporins of different subfamilies may differ in activity, specificity and regulation.

Determining characteristics and expression patterns is the first step in the clarification of the distinct roles of individual aquaporins and how they collaborate.

We used reverse transcriptase PCR (RT-PCR) to determine the tissue specific expression patterns. Reverse Transcriptase PCR is the most sensitive method available for gene expression. Irrespective of the relative quantity of the specific mRNA, it is possible to detect the RNA transcript of any gene. An RNA template is copied onto a complimentary DNA transcript using a retroviral reverse transcriptase, followed by amplification of the cDNA using PCR.

We found At-NLM1 to be expressed predominantly in the flowers and the roots of six week old *Arabidopsis* plants. Finding At-NLM1 in only two of the tissues tested perhaps suggests a more specialized role of water transport. Apparently, the root and the flowers are sites where water transport is critical. Flower formation, the transition from the vegetative to the reproductive phase, is a cardinal point in the development of the plant. The apical meristem that produced leaves in the vegetative phase switches to producing flowers in the reproductive phase (Bowman, 1994). When the plant becomes florally induced, the apical meristem switches to producing flowers. Subsequent to the production of the first few flower primordia, the plant bolts due to increased internode elongation between the uppermost leaves and between flowers (Bowman, 1994). Controlled and rapid transcellular water flow is essential during the enormous amount of growth and physiological changes that take place in the different stages of flower formation . Obviously, a large amount of water is transported through root tissue. Although small amounts of water can be absorbed by the above ground parts of plants,

the root system effectively constitutes the region of entry for virtually all water and minerals absorbed in higher plants.

In contrast to At-NLM1, both At-NLM3 and At-NLM2 were found to be expressed in all types of tissue analyzed. While At-NLM3 was shown to be expressed in all tissue analyzed the highest expression was in the roots and the stems. Again, the roots are an obvious region of the plant where water transport is high and must be regulated. The pathway of radial water movement from the root surface is a zone of rapid absorption involving transport across the epidermis, the root cortex and the endodermis (Slatyer, 1967). The stem structure is also a site where there is rapid water flow and the cells must keep a tight control of water balance to maintain turgor. The expression of At-NLM2 was higher and was relatively even in all tissue examined, suggesting a more general role in water transport in more cells than At-NLM1 and 3. It will be interesting to catalog the expression patterns of all the NLMs, determine subcellular expression and the coordination between the different proteins. In particular, the investigation of At-NLM expression using higher resolution techniques such as in situ hybridization or reporter gene/promoter constructs will help identify particular cellular locations of expression which may provide additional information on their potential role in water relations in *A. thaliana.*

The tissue-selective expression of MIPs has been investigated for other members of the MIP family. For example, for α-TIP was initially identified as a seed specific protein (Johnson et al., 1989). In tobacco, TobRB7, was found to be a root specific TIP (Conkling et al., 1990; Yamamoto et al., 1991). Most other PIPs and TIPs, although

usually restricted to one cell type within an organ are usually found in more than one organ. Additional studies investigating expresion pattern of MIPs are discussed below.

Three MIPs, MipA, MipB and MipC, from the ice plant (*Mesembryanthemum crystallinum*) were initially detected in salt-stresses plants. The tissue-specific expression patterns were then characterized showing MipB in the roots, whereas MipA and MipC were detected both the roots and the leaves (Yamada et al., 1995). So far, many aquaporins seem to be excluded from meristematic tissue, an exception being TobRB7 in tobacco roots (Yamamoto et al., 1991). Yamamoto et al. (1991) characterized the expression pattern of the tobacco root-specific gene TobRB7. By performing in situ hybridizations and using the beta-glucuronidase (GUS) reporter system they found TobRB7 in root meristem and immature central cylinder regions. Since meristems are sinks actively taking up and exchanging solutes this observation may point to a more general role of aquaporins in transport physiology. Aquaporin-enhanced water permeability may be beneficial or even necessary to achieve quick osmotic balancing during highly active transport of solutes and solvent (Schäffner , 1998).

The TIPs and PIPs have also been located in zones of elongation and cell growth that are undergoing vacuolization. For example, this expression pattern has been reproted for γ-TIP of *Arabidopsis* (Ludevid et al., 1992), and maize (Chaumont et al., 1998; Barrieu et al., 1998), and δ-TIP of spinach (Karlsson et al., 2000). Cell expansion is believed to be accomplished by loosening of the pectin network of the primary cell wall in combination with the vacuole driving a sustained water influx because of its high osmotic potential. In order for the vacuolar and cytosolic cell compartments to play this role in cell enlargement, and in order for mature cells to maintain turgor, a tight

osmoregulation of the cytosol must be exerted at all times (reviewed in Johansson et al., 2000). The high capacity to take up water rapidly may require a high water permeability of the tonoplast membranes which is conferred by high levels of TIP proteins.

A related function in controlling cell volume is suggested by the expression of *Arabidopsis* AthH2/PIP1b in guard cells (Ludevid et al., 1992; Kaldenhoff et al., 1995). Kaldenhoff et al. (1995) used biochemical analyses and electron microscopic immunocytochemistry to elucidate the subcellular localization of the AthH2 protein. The results clearly demonstrated that it is an exclusive constituent of the plasmalemma. In addition, by using the beta-glucouronidase reporter system they found that the specific promoter is temporally activated by light in expanding and/or differentiating cells comprising newly formed tissues and organs: root elongation zone, guard cells of stomata, vascular bundle sheaths, filaments of stamen and young siliques (Kaldenhoff et al., 1995).

Several aquaporins have been associated with the germination and fruit-ripening processes: in these cases osmotically active compounds are generated and distributed or huge amounts of precursors are transported into cells and tissues (reviewed in Schäffner, 1998). A prominent example is the expression of the seed specific α-TIP during germination in protein storage vesicles. After the mobilization and conversion of large storage molecules into smaller ones, α-TIP is successively substituted by the vegetative γ-TIP isoform (Culianez-Macia and Martin, 1993; Fray et al., 1994; Maurel, 1997). Also, the high density of aquaporins in plasma-membrane invaginations in *Arabidopsis* mesophyll has been interpreted as assisting high rates of local transport (Robinson et al., 1996).

Many aquaporins of both the tonoplast and plasma membrane are expressed around vascular bundles throughout plants, e.g. the *Arabidopsis* δ-TIP (Daniels et al., 1994), the maize γ-TIP ortholog ZmTIP1 (Karlsson et al., 2000), the *Arabidopsis* PIP1b (Kaldenhoff et al., 1995), and the ice plant PIP1 ortholog MipA (Yamada et al., 1995). Inhibition studies using mercury chloride, known to inhibit aquaporins by binding to cysteine residues near the aqueous pore, suggest that aquaporins of the xylem parenchyma cells are involved in maintaining the transpiration stream by refilling gas filled (embolized) xylem vessels thereby increasing the hydraulic conductivity of the tissue (Holbrook and Zwieniecki, 1999; Tyree et al., 1999).

Regulation of At-NLM by Light and Environmental Stresses

Plants are exposed to different environmental stresses that have in common an effect on plant water status. Although plants vary in their sensitivity and response to water deficit, all plants appear to have encoded genetic information for stress perception, signaling; response and adaptation (Barrieu et al., 1999). Plants respond to conditions of drought or water deficit by a number of physiological and developmental changes, which include stomatal closure, reduction in the rate of photosynthesis, reduced growth of leaves, stems and hairy roots, and biosynthesis of the plant hormone abscisic acid (reviewed in Mansfield, 1987; Quatrano, 1987). In addition, many genes have been described that are regulated or induced by various stress treatments .

Investigating aquaporin gene regulation in plants exposed to water stress is particularly important if we are to understand water-channel involvement in regulating

plant water balance under stress conditions. Abscisic acid is produced under such environmental stresses, and it is important in the tolerance of plants to drought, high salinity, and cold (Davies and Jones, 1991). Many genes that respond to stresses such as drought and cold stress are also induced by the exogenous application of abscisic acid (Shriver and Mundy, 1990; Bray, 1993; Daives and Jones, 1991; Chandler and Robertson, 1994). It appears that stress conditions trigger the production of abscisic acid, which, in turn induces various genes. We have investigated the effects of various environmental stimuli on the expression patterns of the NLMs in *Arabidopsis.*

We have investigated the effects of three different osmotic stresses (1) drought, a desiccating condition, (2) NaCl, an ionic stress and (3) mannitol, a nonionic stress. We also wanted to look at the effects of the exogenous application of the stress hormone abscissic acid, which has been shown to be involved in stress adaptation. We have also investigated the effects of both the absence of light and the cycling of light throughout the photocycle and lastly, the effects of decreased temperature

Both transcripts were down regulated by osmotic stresses, but showed different response patterns. At-NLM1 showed a greater sensitivity to NaCl treatment (4-fold down regulation) as compared to At-NLM3. In contrast, At-NLM3 exhibited a greater sensitivity to drought stress (2.5-fold down regulation) whereas the levels of At-NLM1 were not similarly affected. Both transcripts, however, were not greatly affected by the application of mannitol, suggesting that ionic factors may play a role in the sensitivity. Both At-NLM1 and At-NLM3 are strongly down regulated by the application of abscisic acid (4-fold and 2.5-fold, respectively). These results provide further support that At-NLM1 and At-NLM3 are stress regulated genes, and that decreasing their abundance may

help the plants to adapt to stressful conditions. The regulation of AtNLM1 and At-NLM3 by stress/environmental stimuli suggest they play a role in adaptation to changing osmotic conditions. In addition, not only are they differentially expressed but they are also responded differently to various stresses.

Other MIPs in plants have been shown to be subject to stress regulation although the patterns are complex and both up and down regulation can be observed depending on the MIP transcript in question and the nature of the stress. Some environmental factors have been shown to enhance the expression of aquaporins. For example, At-RD28 was first identified in a cDNA library of water-stressed *Arabidopsis* plants (Yamaguchi-Shinozaki et al., 1992). A related protein, RD29 was also shown to be induced by desiccation, cold, high salt, and ABA (Yamaguchi-Shinozaki and Shinozaki, 1993). RD29 exhibited a two-step induction in response to desiccation. Early induction occurred within 20 minutes and secondary induction occurred three hours after the start of desiccation (Yamaguchi-Shinozaki and Shinozaki, 1993). The tonoplast aquaporin BobTIP26-1 was also shown to be up regulated in response to desiccation and osmotic stress (Barrieu et al., 1999). Osmotic stress caused a rapid and substantial increase in BobTIP26 mRNA and exposure to desiccation showed a slower but equally large rise in BobTIP26 mRNA followed by a rapid decline upon rehydration (Barrieu et al., 1999). Their results indicate that under water-deficit conditions, expression of the tonoplast aquaporin gene is subject to precise regulation that can be correlated with important cytological changes in the cells.

The expression levels of three MIPs from the common ice plant (*Mesembryanthemum crystallinum*) were shown to be altered by salt-stress conditions

(Yamada et al., 1995). They found that seawater-strength salt stress initially results in wilting of the plant, but that full tugor is restored within two days. To attempt to explain this behavior they analyzed changes in gene expression in root coincident to the onset of stress. They found that the transcript levels of three MIPs, MipA, MipB and MipC, declined initially during the salt stress (Yamada et al., 1995). The most dramatic decline was seen with MipA and MipC. In addition, the timing of the mRNA fluctuations coincided with turgor changes in the leaves.

Recently, Kirch et al. (2000) also studied MIPs in *Mesembryanthemum crystallinum* and found them to be sensitive to salt stress. After stressing the plants for 2 weeks with 200 mM NaCl they found that the amount of MIP-F protein in the leaves was decreased and that the amount of MIP-C protein in the roots increased (Kirch et al., 2000). Their findings may help to explain the plant's tolerance to osmotic stress. They suggest that the down regulation of the tonoplast MIP-F in the leaves is a mechanism for restricting the loss of water from the vacuoles. In addition, they also suggest that the up regulation of MIP-C in the PM fraction of the roots might be controlled by endosome trafficking to increase the cellular up-take of water in plants (Kirch et al., 2000).

Dehydration tolerance is a complex phenomenon and involves many changes on both biochemical and physiological levels (Ingram and Bartels, 1996). Recently Mariaux et al. (1998) isolated and characterized several MIPs that are involved in the dehydration response in the resurrection plant *Craterostigma plantagineum.* They grouped the 11 cDNA clones in to four groups, Cp-PIPa, Cp-PIPb, Cp-PIPc, and Cp-TIP based on sequence homologies. Cp-PIPa, Cp-PIPc and Cp-TIP transcript accumulation was regulated by dehydration and abscisic acid. Within the Cp-PIPa group transcripts were

up regulated either by drought only or by drought and abscisic acid, indicating that ABA-dependent and –independent signal transduction pathways lead to Cp-PIPa expression (Mariaux et al., 1998). In addition, the Cp-PIPa genes displayed a biphasic expression pattern, suggesting a role for these genes in an early and a late response to dehydration. They suggest the late response may be in preparation for the rehydration. The differential regulation by dehydration of these transcripts encoding putative aquaporins from both the plasma and the vacuolar membranes suggests a concerted action of both protein classes in the regulation of plant cell turgor and/or transcellular water transport (Mariaux et al., 1998).

Differentially regulated PIPs that respond to drought- and salt-stresses were also identified in *Nicotiana excelsior* (Yamada et al., 1997). Their results show that mRNA of all three genes, Ne-Mip1, Ne-Mip2 and Ne-Mip3, increased under salt- and drought-stresses. However, they showed different accumulation patterns. Under salt-stress, mRNA for NeMip1 and Ne-Mip2 steadily increased, but the increase in Ne-Mip1 was greater. In contrast, the mRNA level of Ne-Mip3 rapidly increased after the initiation of salt-stress and then reached a plateau. The time course profiles of mRNA levels of the three genes after drought-stress were quite different from those after salt stress. mRNA for Ne-Mip3 greatly increased after drought-stress and then decreased gradually, whereas mRNA for Ne-Mip2 increased steadily. The mRNA for Ne-Mip1 moderately increased in the beginning and then did not change thereafter (Yamada et al., 1997). The differential regulation in gene expression and multiplicity of PIP genes in *N. excelsior* suggests that individual PIP isogenes must be precisely regulated under stressed conditions and probably under unstressed conditions as well (Yamada et al., 1997). By

having multiple genes that can be expressed differently and thus respond to environmental changes differently, plants may maintain their sound water status (Yamada et al.,1997).

Regulation by Light

MIP genes have also been shown to be activated by hormones, blue light, and pathogen attack. Gibberellins increase the expression of the gene corresponding to γ-TIP in an *Arabidopsis* mutant with low levels of endogenous gibberellins (Phillips and Huttly, 1994). Addition of gibberellins to this mutant induces stem elongation by stimulation of cell division and cell elongation. The activation of γ-TIP gene in this system may reflect its importance in rapidly elongating cells, a theory supported by the enhanced expression of γ-TIP of *Arabidopsis* (Ludevid et al., 1992).

Kaldenhoff and Richter (1996) isolated a gene that is expressed during blue light-induced initiation of flowering. The *Arabidopsis* PIP1b gene was shown not only to be activated by blue light, but also by two phytohormones, ABA and gibberellins (Kaldenhoff and Richter, 1996). The expression of the root-specific aquaporin, TobRB7, was induced by infection by root-knot nematodes (Oppermann et al., 1994). These parasites induce the formation of a feeding site within the plant root and alter the expression of plant genes at this site. The TobRB7 promoter sequence responsible for nematode infection-induced expression was determined and found to be different from the sequence responsible for root-specific expression (Oppermann et al., 1994).

Light is one environmental stimuli to which plants must respond and adapt to on many different levels in order to survive. Interestingly, our results indicate that both At-NLM1 and At-NLM3 are affected by light. Firstly, At-NLM1 transcript levels from seedlings entrained in a 16h light/8h dark photoperiod were shown to fluctuate diurnally. The transcript levels were lowest at the end of the 8 hr dark period, beginning to rise upon entering the light period and reaching a peak approximately 8 hr into the photoperiod. In addition, this expression pattern appears to follow an internal rhythm since mRNA levels showed the same pattern even in the absence of light. Thus, it appears that At-NLM1, which is expressed highly in the roots is up-regulated during the light hours when there is an active transpiration stream and radial transcellular flow of water in roots is high. As discussed below a similar regulation of PIPs has been observation *Lotus japonicus*.

Two studies have been performed that correlate diurnal root hydraulic conductance (L_{pr}) with the expression of aquaporins in *Lotus japonicus*. Henzler et al. (1999) found that the hydraulic conductance of excised roots of the legume *Lotus japonicus* to vary over a 5-fold range during a day/night cycle. They took mRNA from roots and probed it with cDNA from the *Arabidopsis* aquaporin AthPIP1a gene and found an abundant transcript that varied in abundance diurnally (Henzler et al., 1999). The pattern of fluctuations resembles closely the diurnal pattern of variation in root L_{pr}. Another study performed by Clarkson et al. (2000) also correlated changes in L_{pr} from nutrient deprived plants to expression levels of PIP1 and PIP2 aquaporins in *Lotus japonicus*. They concluded that the roots are capable of monitoring the nutrient content of the solution in the root apoplasm and initiating responses that anticipate any metabolic

disturbances caused by nutrient deficiencies (Clarkson et al. 2000). This in turn, results in changes in water channel activity close to the initiation of responses. Our finding that At-NLM1 is similarly regulated, suggests that it too may contribute to diurnal changes in the water permeability of the root . More detailed analysis of the subcellular localization of this protein may yield additional insight into the specific role that At-NLM1 plays in control of water movement in roots.

The stems of plants raised in the dark elongate much more rapidly than normal, a phenomenon called etiolation. It is a mechanism that increases the probability of the germinating plant in soil reaching the light. Rapid transcellular water flow is the basis for the rapid expansion growth involved in etiolation, which is a turgor-driven cell expansion growth. As discussed above, MIP proteins have been shown to be localized to areas of turgor-driven stem and root elongation, which is the basis of the hypocotyl growth in etiolated plants, as well as stem elongation in green plants. We have investigated the At-NLMs in etiolated *A. thaliana* at the transcription level to determine if they are involved in the rapid water transport during etiloation. Both At-NLM1 and At-NLM2 transcripts were not greatly affected by etiolation. In contrast, At-NLM3 was significantly up regulated (7-fold) in etiolated plants. Therefore, it appears At-NLM3 which, is expressed at high levels in the roots and stems, is up regulated in response to etiolation and may help in the rapid transport of water during turgor-driven stem expansion growth. Again, subcellular localization of these proteins will aid in determining which cells in the elongating stem contain At-NLM3 and may provide additional insight into its cellular role.

Transferred (T-DNA) Knockouts

Forward genetics approaches involve the initial identification of a mutant phenotype and eventually results in the elucidation of the genotype and the gene sequence causing the mutant phenotype. Reverse genetics on the other hand involves the rational manipulation of known gene sequence and the study of the resulting phenotype to elucidate gene/protein function. The relatively new approach of reverse genetics for studying the function of genes and has been exploding with the recent completion of genome sequencing projects. Gene knockouts, or null mutants, are important because they provide a direct route to determining the function of a gene product *in situ* (Krysan et al., 1999). There are many different ways to mutate or knockout a specific gene. To analyze the functions of Nodulin 26-like MIPs in *Arabidopsis* we used insertional mutagenesis with transferred (T-DNA) DNA.

The ultimate goal of genome research on the model flowering plant *Arabidopsis thaliana* is the identification of all of the genes and understanding their functions. The completion of the sequencing of the *Arabidopsis* genome was a major step towards this goal. However, functional studies of newly discovered genes have not yet kept up to this pace. Gene knockouts, or null mutations, are important because they provide a direct route to determining the function of a gene product in situ (Krysan et al., 1999). Currently, the most effective method for disrupting gene function is insertional mutagenesis (Parinov and Venkatesan, 2000). In *Arabidopsis*, this involves the use of either transposable elements or T-DNA. An alternative approach that that has been effective in inactivating specific genes is by anti-sense or co-suppression approaches.

To gain insight into its role in stress physiology, a T-DNA knockout mutant of At-NLM3 has been generated. Preliminary analysis of the knockout mutant suggests some developmental defects including reduced stem and leaf growth, reduced stem thickness and stability, increase in the timing of bolting and developemental defects in etiolated plants. Presently, the reason why a At-NLM3 knockout results in enhanced stem growth in both dark and light grown plants is puzzling. The absence of At-NLM3 could affect the homeostasis of water flux resulting in altered growth and developmental phenotypes in stems and hypocotyls. The phenotype is made even more complex by the observation that At-NLM1 is up regulated in the At-NLM3 knockout plants. The up regulation of other NLMs could perhaps be a way the plant is trying to compensate for the loss of one NLM. A more detailed analysis of the phenotype of the knockout plant, and the analysis of the expression of other AtNLMs needs to be performed to draw more concrete conclusions.

Antisense technology was used in *Arabidopsis* to demonstrate the importance of PIPs in water transport (Kaldenhoff et al., 1998). *Arabidopsis* plants were transformed with a pip1b antisense construct, resulting in reduced levels of PIP1b and PIP1a in both roots and leaves. Analysis of water permeabilites of protoplasts prepared from leaves showed a 3-fold reduction of P_f in protoplasts from transformed plants as compared tot hose from control plants (Kaldenhoff et al., 1998). The phenotype of the transformed plants was similar to that of control plants except for the root system, which was five times larger in the transformed plants (Kaldenhoff et al., 1998). The rate of water uptake by the roots was similar in transformed plants and control plants, indicating that the

plants compensate its reduced level of aquaporins by increasing the size of the root system. Their results demonstrate the importance of aquaporins in water uptake in vivo.

Although we have addressed the expression and biological function of two of the At-NLMs, the methods are in place to investigate the functional properties of the other nine family members. Moreover by using negative backgrounds (knockouts) we may be able to use mutants of the genes (i.e., that lack a phosphorylation site) to investigate protein function and regulation. Finally, by using knockout approaches, multiple At-NLM genes can be silenced, and the functional consequences of this can be evaluated. Overall, these approaches should provide considerable insight into the function of NLM proteins in plant development and stress biology.

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VITA

Rana Ferrebee was born in Parkerburg, West Virginia on January 26, 1976. She moved to Morgantown, West Virginia in the early 1980's and graduated from Morgantown High School, in 1994. In the fall of 1994 she enrolled as an undergraduate student at West Virginia University (WVU) in Morgantown, West Virginia. In May of 1998 she graduated summa cum laude with a Bachelors of Art degree in biology. While obtaining her degree at WVU, she engaged in two semesters of undergraduate research in a Reproductive Endocrinology laboratory under the tutorship of Jorge Flores. In August 1998 she transferred her studies to the University of Tennessee in Knoxville and entered the graduate program in Biochemistry, Cellular, and Molecular Biology. She joined the laboratory of Dr. Daniel M. Roberts and received the Masters degree in Biochemistry, Cellular, and Molecular Biology in May of 2001.