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An Aquaglyceroporin Is Abundantly Expressed Early in the Development
of the Suspensor and the Embryo Proper of Loblolly Pine (*Pinus taeda* L.)

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Title: An aquaglyceroporin is abundantly expressed early in the development of the suspensor and the embryo proper of loblolly pine (*Pinus taeda* L.)

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ABSTRACT

In contrast to angiosperms, pines and other gymnosperms form well-developed suspensors in somatic embryogenic cultures. This creates a useful system to study suspensor biology. In a study of gene expression during the early stages of conifer embryogenesis, we identified a transcript, *PtNIP1;1*, that is abundant in immature *Pinus taeda* zygotic and somatic embryos, but is undetectable in later-stage embryos, megagametophytes and roots, stems and needles from 1 year-old seedlings. Analysis of *PtNIP1;1* transcript in embryo proper and suspensor tissues by RT-PCR suggests preferential expression in the suspensor. Based on comparisons of derived amino acid sequences, PtNIP1;1 belongs to the NIP branch of the aquaporin (MIP) superfamily. Through heterologous expression in *Xenopus laevis* oocytes and the *Saccharomyces cerevisiae* *fpsI* mutant, PtNIP1;1 has been shown to be an active aquaglyceroporin.

INTRODUCTION

Embryogenesis is a critical period in the earliest stages of the sporophytic generation of plants. Following fusion of haploid male and female gametes to form the diploid zygote, embryo development and concomitant cellular differentiation commence. Subsequent early events in embryogenesis establish an embryo proper region and a separate suspensor region.

While angiosperm and gymnosperm embryo ontogeny share many features, gymnosperms have notable unique features. For example, in contrast to the double fertilization event and triploid endosperm characteristic of angiosperms, gymnosperm embryogenesis proceeds via a single fertilization of the female oocyte. Embryos, therefore, develop in a haploid female tissue, the megagametophyte. In angiosperms, the first zygotic division determines the basal cell, which gives rise to the suspensor, and a terminal cell, which gives rise to the embryo proper. Gymnosperms, however, undergo a free-nuclear phase where several nuclear divisions occur (three divisions in *Pinus* to yield 8 nuclei) prior to cell wall formation. Another round of division produces a four-tiered, sixteen-celled proembryo. With respect to the micropylar end of the seed, the four cells in the distal tier give rise to the embryo proper and the next tier will form the suspensor. Also common in gymnosperm embryogeny is a phenomenon called cleavage polyembryony whereby each embryo proper can cleave into four individual embryos, each with its own intact suspensor (Spurr, 1949). Ultimately, one of the embryos dominates and the others degenerate. Finally, while not unique to gymnosperms, their

embryos and suspensors tend to be large (Figure 1E), which is conducive to facile dissection, visualization, and molecular techniques.

The role of the suspensor in embryogenesis has been studied almost exclusively in angiosperms (reviewed in Schwartz et al., 1997). Suspensor elongation and development is rapid, usually preceding embryo development. The suspensor stimulates growth of the embryo by synthesizing growth factors such as gibberellins (Cionini, 1987) and by acting as a conduit for nutrients from the surrounding cells or medium to the growing embryo (Yeung, 1980). Later, the suspensor undergoes programmed cell death and is absent or shrunken in the mature seed. Support of embryo growth appears to be achieved in a variety of ways, as suspensors exhibit a wide variety of shapes (filamentous, columnar, spherical, or irregular), sizes (minute, unicellular to large, multicellular), ploidy, and metabolic activity. Smaller suspensors appear to promote growth via nutrient transport (e.g., suspensors of *Capsella* have structural modifications to facilitate nutrient transport (Schulz and Jensen, 1969). Larger suspensors may serve as a storage tissue and they appear to be more involved in macromolecular biosynthesis; thereby providing nutritional support for the embryo proper (Yeung and Meinke, 1993; Panitz et al., 1995; Cairney et al., 2000). From a genetic perspective, recently isolated developmental mutants are providing insights into suspensor function (reviewed in Schwartz et al., 1997; Yadegari and Goldberg, 1997).

Suspensors usually fail to develop when somatic embryos of angiosperms are produced in culture (Yeung and Meinke, 1993). However, when pines and other conifers undergo somatic embryogenesis in culture, embryos develop with an attached suspensor that can readily be isolated from somatic embryos (Figure 1C and D). This creates a

unique system to study suspensor molecular and cellular biology. This system has so far seen limited exploitation by molecular biologists (Cairney et al., 2000).

From an applied perspective, somatic embryogenesis is of particular interest to forest products industries as a method for mass-producing elite genotypes of commercially important coniferous species (Timmis, 1998; Grossnickle and Sutton, 1999). For loblolly pine (*Pinus taeda* L.), the predominating timber species of the southeastern United States (Schultz, 1999), the technology remains inefficient. The biochemical and metabolic reasons that underlie aberrant somatic embryo development are largely unknown. To better understand molecular events that are critical to proper embryogenesis, we have been exploring stage-specific gene expression during zygotic and somatic embryogenesis of loblolly pine, treating zygotic embryogenesis as the model against which somatic embryogenesis is judged (Cairney et al., 1999, 2000). For accurate comparison of somatic and zygotic embryos, we use a nine-stage system that is based on embryo morphology (Pullman and Webb, 1994) (Figure 1A and B).

We have used differential display RT-PCR (Liang and Pardee, 1992) to search for genes expressed in very young somatic and zygotic embryos, prior to the formation of cotyledons. Here we report cloning, expression analysis, and functional characterization of one early-expressed message, *PtNIP1;1*, that is very similar to members of the NIP branch of the MIP superfamily. The mRNA appears exclusive to early embryo development. Evidence from functional analyses suggests that *PtNIP1;1* forms an aquaporin channel upon expression in *Xenopus laevis* oocytes and, similar to *AtNLM1* (Weig and Jakob, 2000a), functions as a glycerol permease upon expression in *fps1* *Saccharomyces cerevisiae*. Taking advantage of large, easily dissectable loblolly pine

embryos and suspensors, we performed RT-PCRs on embryo proper and suspensor tissues. Results suggest *PtNIP1;1* expression is at least preferential for the suspensor. This result is consistent with previous reports of up-regulated MIP expression during cell elongation (Ludevid et al., 1992; Schünmann and Ougham, 1996; Smart et al., 1998; Weig and Eisenbarth, 2000) and may suggest a role for PtNIP1;1 in suspensor elongation. Alternatively, as a channel protein in suspensor cells, PtNIP1;1 may play a role in the transport of nutrients to the developing embryo proper.

RESULTS

***PtNIP1;1* mRNA is Detected Early in Embryogenesis and is Most Homologous to the NIP Branch of the MIP Superfamily**

To identify genes involved in early events of *Pinus taeda* embryogenesis, we performed a modified differential display procedure (Xu et al., 1997) on somatic embryos from all stages of development and cloned several cDNAs that appeared to be exclusive to early-stage embryos. To confirm early-stage somatic embryo expression, northern blots of liquid suspension culture and late-stage somatic embryo RNA were probed with radio-labelled differential display ESTs. One particular EST displayed very striking early-embryogenesis specificity, so using a biotin-streptavidin bead strategy (Ciavatta and Cairney, 2000), its full-length cDNA was obtained. Blastx searches with the cDNA sequence against the National Center for Biotechnology Information GenBank database revealed significant primary amino acid sequence homology to nodulin-like members of the MIP superfamily (NIPs) (Weig and Jakob, 2000b). To reflect recently proposed MIP nomenclature (Johanson et al., 2001), this full-length cDNA was subsequently named *PtNIP1;1*. The predicted *PtNIP1;1* amino acid sequence is very similar to other functionally characterized NIPs, sharing 41% identity + 21% similarity to *Arabidopsis thaliana* AtNLM1, 43% identity + 21% similarity to *Glycine max* GmNOD26, 43% identity + 21% similarity to *Lotus japonicus* LjLIMP2, and near identity to AtNLM1, GmNOD26 and LjLIMP2 at 5 key positions that are significant for aquaporins or glycerol permeases (Weig et al., 1997; Rivers et al., 1997; Weig and Jakob, 2000a; Dean

et al., 1997; Guenther and Roberts, 2000; Froger et al., 1998) (Figure 2). A cladogram of a multiple sequence alignment of PtNLM1;1 with selected MIPs demonstrates proper assignment of PtNIP1;1 to the NIP branch of the MIP superfamily (Figure 3).

To characterize expression further, we performed northern blots and slots blots with different embryo and vegetative tissues. Closer analysis of *PtNIP1;1* expression throughout somatic embryogenesis revealed a drastic drop in expression that coincides with embryo maturation (Figure 4A). Because our somatic embryo maturation protocol involves a switch from liquid maintenance medium to gelled, semi-solid maturation medium, we were interested to know if the drop in *PtNIP1;1* expression was triggered by this change from a submerged, aqueous environment to a more arid growth plate environment. To check if this environmental switch had an effect on *PtNIP1;1* expression, cells from liquid suspension culture were plated on maintenance medium that had been amended with a gelling agent. After 4 weeks on gelled, semi-solid maintenance medium, however, northern analysis of RNA from the plate-grown tissue revealed no change in expression of *PtNIP1;1* (not shown) (i.e., *PtNIP1;1* was still abundantly expressed in early-stage somatic embryos growing on plates). Since the environmental switch did not appear to influence expression of *PtNIP1;1*, we were interested to know if the *PtNIP1;1* expression profile during somatic embryogenesis (high early, sharp decline, undetectable late) would be conserved during zygotic embryogenesis. Upon analysis of zygotic embryos, *PtNIP1;1* mRNA was again detected in early-, but not late-stage embryos (Figure 4B). Finally, to broaden the scope of our expression analyses, more northern blots were done with non-embryo tissues. These northern blots indicated that *PtNIP1;1* expression was not detected in megagametophytes throughout embryogenesis (Figure

4C), nor was it detected in roots, stems, and needles of 1 year-old *P. taeda* seedlings (Figure 4D). Therefore, all expression analyses detected *PtNIP1;1* mRNA only in young precotyledonary somatic and zygotic embryos. To our knowledge, no aquaporin-like gene has been shown to be expressed this early in embryogenesis.

Aquaporin expression has been shown to be up-regulated in regions of cellular elongation (Ludevid et al., 1992; Schünmann and Ougham, 1996; Smart et al., 1998; Weig and Eisenbarth, 2000) and then down-regulated when cells became fully elongated (Weig and Eisenbarth, 2000). During early stages of conifer zygotic embryogenesis, suspensors undergo extensive elongation as the embryo proper advances into the corrosion cavity in the megagametophyte. Likewise, somatic embryo cultures are replete with elongating suspensor-like structures (Figure 1D). However, as the embryo proper reaches middle to later developmental stages, suspensor cells of zygotic (Jones and Dangl, 1996) and somatic embryos (Filonova et al., 2000) undergo programmed cell death. When RNA was isolated from embryo tissues in preparation for northern and slot blots (Figures 4A through D), no dissection was made to separate embryo proper from embryo suspensor. Instead, embryo proper with attached embryo suspensor were used for RNA isolations. To examine the site of *PtNIP1;1* expression, we dissected zygotic embryo suspensors from embryo propers of stage 3 embryos and performed RT-PCR with *PtNIP1;1*-specific primers on 1 ng of poly-A⁺ RNA isolated from each tissue. The results indicate greater *PtNIP1;1* mRNA abundance in suspensor cells than in embryo proper cells (Figure 5). This result needs to be verified via mRNA *in situ* hybridization on early-stage somatic and zygotic embryos.

PtNIP1;1 is an Aquaglyceroporin

In light of primary amino acid sequence similarity, we were interested to know if PtNIP1;1 would function similarly to AtNLM1 and GmNOD26. Since AtNLM1 and GmNOD26 were previously characterized as aquaporins (Weig et al., 1997; Rivers et al., 1997) and glyceroporins (Weig and Jakob, 2000a; Dean et al., 1997), we assessed the aquaporin and glyceroporin function of PtNIP1;1. To test aquaporin function, *PtNIP1;1* cRNA, *AtNLM1* cRNA (positive control) and water (negative control) were injected into *Xenopus laevis* oocytes and the average oocyte membrane osmotic water permeability, P_{os} , was determined for the three treatments. Results showed that P_{os} of PtNIP1;1-expressing oocytes was about 10 times greater than the P_{os} of the negative control (water-injected oocytes) and slightly greater than the P_{os} of the positive control (AtNLM1-expressing oocytes), suggesting PtNIP1;1 does function as an aquaporin (Figure 6).

To test glyceroporin function, we used a strategy that had been previously used by Weig and Jakob (2000a) to demonstrate glycerol permease ability for AtNLM1. For these experiments, PtNIP1;1 was expressed in a *Saccharomyces cerevisiae* strain that lacks the glycerol facilitator protein, Fps1. Because of this mutation, *fps1*⁻ yeast cannot rapidly modulate their internal glycerol concentration, and are consequently sensitive to hypo-osmotic shock (Tamás et al., 1999). Yeast transformants were tested for complementation of osmosensitivity and for increased ability to take up [³H]glycerol from surrounding medium. Results from the complementation experiments showed that PtNIP1;1 had a rescuing effect comparable to that of a proven aquaglyceroporin, AtNLM1 (Figure 7A). Similarly, glycerol uptake experiments indicated that *fps1*⁻ cells

expressing PtNIP1;1 accumulated glycerol faster than vector transformed controls, and about as well as cells expressing AtNLM1 (Figure 7B). Together, complementation and glycerol uptake experiments suggest that PtNIP1;1 can function as a glyceroporin.

DISCUSSION

The distinctive aspects of conifer embryogenesis render the system worthy of careful study for the light shed on gymnosperm embryogenesis and plant embryogenesis in general. Somatic embryogenesis in conifers is an established, tractable system which facilitates study; certain cell lines produce healthy embryos capable of germination, other cell lines show consistent patterns of aberrant development. The system also offers a unique opportunity to explore embryo suspensor development *in vitro* – a fact that, until recently (Cairney et al., 2000), has been largely unexploited. Here we report the identification and cDNA cloning of an mRNA expressed in the earliest stages of loblolly pine embryogenesis. The mRNA encodes a novel aquaglyceroporin whose expression pattern differs from previously identified members of the MIP superfamily. Evidence from RT-PCR experiments suggests that this mRNA may be located preferentially in the suspensor.

An Aquaglyceroporin that is Expressed Exclusively During Early Embryogenesis

Major intrinsic proteins (MIPs) are integral membrane proteins that facilitate transmembrane movement of small polar molecules. These proteins belong to a superfamily that is ubiquitous throughout bacteria, fungi, plants, and animals. In plants, the family is large with 35 members identified in *Arabidopsis thaliana* (Johanson et al., 2001) and 34 in *Zea mays* (Chaumont et al., 2001). The superfamily is divided into PIPs, TIPs, NIPs, and SIPs. Recently, crystallographers have been able to resolve the membrane conformation of AQP1 and GlpF (Murata et al., 2000; Fu et al., 2000). These

reports have shown which amino acid residues are important in forming the transmembrane channel pore and provide a basis for envisioning how water or solute molecules traverse the membrane.

Expression of MIPs has been shown to be an integral part of the embryogenesis program. Reports of embryogenesis-related MIPs, however, are limited to the late embryogenesis-expressed α -TIP, a seed- and embryo-specific aquaporin that has been described in several plants such as *Phaseolus vulgaris*, *A. thaliana*, and *Picea abies* (Johnson et al., 1989; Höfte et al., 1992; Oliviusson and Hakman, 1995). This protein is situated in protein storage vacuolar membranes and may therefore play an important role in stock-piling nutrients necessary for proper embryo maturation and germination. There is also a report of MIP expression in reproductive tissues of *Zea mays* where two NIPs, three SIPs, and four TIPs were shown to be exclusively or nearly exclusively identified in cDNA libraries from different developmental stages of reproductive tissues (Chaumont et al., 2001). However, it is not clear to what extent embryo tissue contributed to the reproductive tissues from which the cDNA libraries were derived.

In contrast to α -TIPs that are expressed in late-stage angiosperm and gymnosperm embryos and megagametophytes (Johnson et al., 1989; Höfte et al., 1992; Oliviusson and Hakman, 1995), *PtNIP1;1* transcript is detected only in the earliest, precotyledonary-stage *P. taeda* somatic and zygotic embryos. Interestingly, when an antiserum against the seed-specific α -TIP of *Phaseolus vulgaris* was used to probe western blots of protein preparations from *P. abies* whole ovules, a low molecular mass band (~26 kDa) was detected from about the time of fertilization to early embryo stages (Oliviusson and Hakman, 1995). As embryos matured, detection of the higher molecular mass α -TIP

(~27 kDa) became apparent while the earlier-expressed band vanished. The early expression profile of the lower molecular mass protein noted by Oliviusson coincides with the mRNA expression profile of *PtNIP1;1*, which raises the possibility that the α -TIP antiserum from *Phaseolus vulgaris* cross reacts with a *PtNIP1;1*-like protein in *P. abies* (*PtNIP1;1* shares 29% identities plus 16% similarities to the original *Phaseolus vulgaris* α -TIP from which the antiserum was raised and 29% identities plus 17% similarities to the *P. abies* MIPfg). If the α -TIP antiserum is cross-reacting to a *PtNIP1;1*-like protein in *P. abies*, this would imply that it is smaller than the *PtNIP1;1* predicted molecular mass of 28-29 kDa. Alternatively, the antiserum may simply be recognizing another early embryogenesis TIP isoform from embryos, megagametophytes or both.

Examples of tissue-specific expression, while not abundant, are not uncommon for plant MIPs (Johnson et al., 1989; Weig and Jakob, 2000b). More specifically, within the NIP subgroup (accessions listed at <http://mbclserver.rutgers.edu/CPGN/AquaporinWeb/Aquaporin.Table.html>), four analyzed *Zea mays* cDNAs show different expression patterns (*ZmNIP2-1* and *ZmNIP2-2* are exclusive to aerial vegetative tissues and *ZmNIP1-1* and *ZmNIP3-1* are nearly exclusive to reproductive tissues) (Chaumont et al., 2001), and similarly, *Arabidopsis thaliana* *AtNLM1*(*AtNIP1;1*) and *AtNIP4;1* seem to be exclusive to roots (Weig and Jakob, 2000b). The specific expression pattern of *PtNIP1;1* is consistent, therefore, with what has previously been reported for some MIPs.

In light of NIP diversity within plants (five NIPs identified in *Zea mays* (Chaumont et al., 2001), nine in *A. thaliana* (Johanson et al., 2001)), other NIPs would be

expected in *P. taeda*. A search of the Pine Gene Discovery Program EST database (<http://www.cbc.umn.edu/ResearchProjects/Pine/DOE.pine/index.html>) returned a 350 bp EST derived from loblolly pine normal xylem. Alignment and cladogram of the theoretical translation of the xylem EST with other MIPs revealed 85% nucleotide identity to *PtNIP1;1* spanning 292 bases, 77% amino acid identity spanning 114 amino acids, and clearly established the xylem EST as a NIP (not shown). Perhaps not coincidentally, this is another example of MIP expression in a region characterized by cell elongation. The 85% nucleotide identity of the xylem EST to *PtNIP1;1* over 292 bases, would suggest a reasonably high degree of 3' UTR similarity. It is curious, however, that we fail to detect a band in northern blots of 1 year-old stem tissue probed with the *PtNIP1;1* 3' UTR (Figure 4D). This might suggest a very low abundance of the xylem NIP in our 1 year-old seedling stem RNA samples and/or significant divergence in the 3' UTR regions of the *P. taeda* embryogenesis (*PtNIP1;1*) and xylem NIPs.

Opportunities for Deciphering the Biological Function of *PtNIP1;1*

Multiple members in a gene family and tissue specific expression such as that seen for *PtNIP1;1* argues for specialized function. Knowledge of such biological function for *PtNIP1;1* should prove useful to understanding embryogenesis in greater detail. More information is needed, however, to establish a biological role. Narrowing the expression profile to a few weeks early in embryogenesis begs finer localization on a cellular level (embryo suspensor versus embryo proper) and subcellular level (vacuolar,

plasma or other membrane) so the exact location of PtNIP1;1 channels can be determined.

Work by Panitz et al., (1995) in *Vicia faba*, showed transient accumulation of storage proteins and their mRNAs in suspensor and endosperm preceded synthesis in the embryo proper. We have observed differential accumulation of an array of transcripts in suspensor, megagametophyte and embryo proper of loblolly pine (Cairney et al., 2000 and unpublished data). During embryo dissections in preparation for our RT-PCRs, contamination of embryo proper tissue with suspensor tissue and vice versa was unavoidable. Despite problems of accurately separating embryo proper from embryo suspensor, present RT-PCR results (Figure 5) indicate greater *PtNIP1;1* expression in the suspensor. More qualitative expression analyses with earlier stages of development also show preferential *PtNIP1;1* expression in the suspensor (not shown). The profile and localization of *PtNIP1;1* expression is now being studied in greater detail to determine whether localization of expression varies over development, although present expression analyses (Figure 4A and B) would seem to preclude the type of suspensor-expression-early followed by embryo-proper-expression-late pattern described by Panitz et al., (1995).

Several possible roles can be envisioned for PtNIP1;1 channels in the suspensor. A role in suspensor elongation could be envisioned as aquaporins have been shown to be up-regulated in regions of cellular elongation (Ludevid et al., 1992; Schünmann and Ougham, 1996; Smart et al., 1998; Weig and Eisenbarth, 2000). That PtNIP1;1 fluxes glycerol in addition to water raises the possibility that it is a multifunctional solute channel as other plant (Rivers et al, 1997; Gerbeau et al., 1999) and mammalian

(Ishibashi et al., 1997; Tsukaguchi et al., 1998) aquaglyceroporins appear to be permeable to small, uncharged solutes when expressed in oocytes. As a solute channel, PtNIP1;1 may play a role in transporting nutrients to the developing embryo, or solute flux through PtNIP1;1 might be critical to maintaining turgor as suspensors elongate. In addition, permeation of NH₃ in peribacteroid membrane vesicles was shown to be partially mediated by proteinaceous channels (Niemietz and Tyerman, 2000), raising the question of GmNOD26 involvement in NH₃ flux. Assuming GmNOD26 and other NIPs like PtNIP1;1 flux NH₃, an immediate role could be envisioned for PtNIP1;1 since embryo maturation is marked by storage protein accumulation and suspensors likely synthesize storage proteins (Panitz et al., 1995; Cairney et al., 2000), both of which may require additional capacity for shuttling nitrogen.

Whatever the true biological function of PtNIP1;1 it is clear that more work is needed to establish its role in embryogenesis. Fortunately, established conifer embryo research programs are well positioned to decipher biological functions of embryogenesis genes by exploiting virtues of somatic embryogenesis. That conifer somatic embryogenic material is amenable to genetic transformation (reviewed in Ahuja, 2000), makes it a potentially workable system to study embryo- and suspensor-specific genes through transformation-dependent strategies (e.g., RNA interference, promoter-reporter fusions, etc.), and because large amounts of tissue can be rapidly generated, ample transgenic tissue is readily available for physiological and genome-wide expression studies (Cairney et al., 1999, 2000). In addition, the rather large size of conifer somatic embryos has proven useful for sectioning and mRNA *in situ* hybridization (Cantón et al., 1999; Sabala et al., 2000; Avila et al., 2001). Lastly, development of somatic embryos with attached

suspensors creates a useful system to study gene function during suspensor development (e.g. *PtNIP1;1*) and suspensor biology in general.

MATERIALS AND METHODS

Plant Tissue

Loblolly pine somatic embryo cultures were initiated as described by Becwar and Pullman (1995) with modifications. Somatic embryos were subsequently grown in liquid maintenance medium 16 and on gelled maturation medium 240 (Pullman and Webb, 1994). Weekly, aliquots of liquid suspension culture (LSC) were filtered with Miracloth (CALBIOCHEM[®], San Diego, CA, USA) to remove excess liquid medium, placed in 50 mL tubes, immediately frozen in liquid nitrogen, and stored at -70°C. For northern analysis to compare *PtNIP1;1* expression in early stage somatic embryos maintained in LSC versus gelled medium, 1 mL of LSC was plated on gelled maintenance medium (identical composition to LSC plus 2.5% Phytigel[™] (Sigma, St. Louis, MO, USA)). Weekly, the mass of early-stage embryos was transferred to fresh gelled maintenance medium. After 4 weeks, tissue was collected and frozen in liquid nitrogen in preparation for total RNA isolation. For later-stage embryos growing on gelled maturation medium, embryos were judged for stage of development under a dissecting microscope according to Pullman and Webb (1994), selected from plates, plunged into liquid nitrogen, and stored at -70°C. Zygotic embryo tissue was collected from cones of mother tree UC5-1036 (generously supplied by Union Camp Corporation). Cones were packed on ice, shipped overnight, and seeds were extracted upon receipt. Embryos were dissected from seeds, judged for stage of development according to Pullman and Webb (1994), frozen in liquid nitrogen and stored at -70°C until RNA extraction. No attempt was made to

separate embryo proper from embryo suspensor for any differential display or northern analysis.

Differential Display, *PtNIP1;1* Cloning and Sequence Analysis

Poly-A⁺ RNA was extracted from early stage somatic embryo tissue maintained in liquid suspension culture and more mature somatic embryos maintained on gelled maturation medium using oligo dT-coated beads (Dynal, Lake Success, NY, USA). Differential display was performed essentially as described previously (Xu et al., 1997). The full-length *PtNIP1;1* cDNA was captured from SMART[™] cDNA synthesized from somatic embryo liquid suspension culture RNA as described previously (Ciavatta and Cairney, 2000), cloned into pGEM T Easy (Promega, Madison, WI, USA) and sequenced by the dideoxy chain termination method. Sequence alignments were performed with CLUSTALW (Thompson et al., 1994). The shaded alignment was constructed with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html) The tree was assembled with TreeView(Win32) 1.6.5 (Page, 1996).

Northern Analyses

Total RNA for expression analysis was isolated by two methods. A modified CTAB (hexadecyltrimethylammonium bromide) procedure (Chang et al., 1993) was used for early stage somatic embryos from liquid suspension culture and vegetative tissues. For all zygotic and somatic embryo tissue other than liquid suspension culture, the

RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) was used with 1% PVP, 30K (Acros Organics, Fisher Scientific, Pittsburgh, PA) added to buffer RLT. For northern blots, total RNA was separate on a formaldehyde-containing agarose gel and transferred to Hybond N⁺ membrane (Amersham Pharmacia Biotech, Piscataway, NJ) according to the method in Ausubel et al. (1995). For slot blots, 2 µg of total RNA per slot were attached to Hybond N⁺ membrane (Amersham) with a PR 648 Slot Blot Filtration Manifold (Hoefer Scientific Instruments, San Francisco, CA, USA) according to the method in Ausubel et al. (1995). The RNAs were UV crosslinked to nylon membranes, prehybridized at 65°C for >3 h in several changes of hybridization buffer (0.5 M sodium phosphate buffer (pH 7.2), 5% SDS (w/v), 10 mM EDTA (pH 8.0), 1% BSA (w/v)) (Church and Gilbert, 1984). Probes were prepared from 50 to 100 ng DNA from the *PtNIP1;1* 3' UTR and 5 µL of α-³²P dATP (10 mCi/mL) (Amersham) with Ready-To-Go DNA Labelling Beads (Amersham) according to manufacturer's instructions. Prior to hybridization, probes were purified with Nick Columns (Amersham), heat denatured and placed on ice. All hybridizations were done overnight in the above hybridization buffer at 65°C. Blots were washed as follows: 2 times, 5 min each wash in 2X SSC, 0.1% SDS at room temperature; 2 times, 5 min each wash in 0.2X SSC, 0.1% SDS at room temperature; and 2 times, 15 min each wash in 0.2X SSC, 0.1% SDS at 65°C. Blots were exposed overnight to a phosphorimaging plate, images were read with a BAS1800 (software v1.0), and images were manipulated with ImageGauge (v2.54) (Fuji Photo Film Co., Ltd., Kanagawa, Japan).

Embryo Proper and Suspensor RT-PCR

Thirty stage 3 zygotic embryos were removed from megagametophytes, dissected into embryo proper and suspensor regions, and frozen in liquid nitrogen. Poly-A⁺ RNA was extracted from the two tissues with oligo dT-coated beads (Dynal, Lake Success, NY, USA). RNA concentration was measured with Ribogreen[®] RNA quantitation reagent (Molecular Probes, Eugene, OR, USA). In 20 µL reactions, 1 ng of each RNA was primed with Oligo dT₁₂₋₁₈ and reverse transcribed with Superscript II[™] and treated with RNase OUT[™] (Invitrogen[™] Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. For PCRs, 4 µL of reverse transcription products were PCR amplified in 100 µL reactions with either forward and reverse *PtNIP1;1* gene specific primers (0.2 µM each) or forward and reverse glyceraldehyde-3-phosphodehydrogenase (G3P) primers (0.2 µM each), dNTPs (0.2 mM each dNTP), 10X buffer (10 µL) and Advantage cDNA Polymerase Mix (2.0 µL) (Clontech, Palo Alto, CA, USA). Reaction conditions were: 94°C, 2 min; 35 X (94°C, 15 s; 67.3°C, 30 s; 72°C, 1 min); 72°C, 5 min for *PtNIP1;1* primers and 94°C, 2 min; 35 X (94°C, 15 s; 60.0°C, 30 s; 72°C, 30s); 72°C, 5 min for G3P primers. Aliquots (8 µL) were removed after every other cycle starting at cycle 17 and separated in 1.5% (*PtNIP1;1* products) or 2.0% (G3P products) agarose, stained with ethidium bromide (0.5 µg /mL) and photographed under UV illumination. Digitized signals were quantitated with ImageGauge (v2.54) (Fuji Photo Film, Co., Ltd.). All RT-PCRs were repeated three times to verify consistent results.

Complementation Test and Glycerol Uptake Assays

Complementation and uptake experiments were performed in *Saccharomyces cerevisiae* strain YSH6.114.-2A kindly donated by Dr. Alfons Weig. The *PtNIP1;1* ORF was excised from pGEM T Easy with *NotI* and subcloned into the *NotI* site of the yeast expression vector pDR195 (Rentsch et al.,1995). Proper sense orientation with respect to the PMA1 promoter was determined by restriction digestion and corroborated with dideoxy sequencing. Empty pDR195 (negative control), *PtNIP1;1*-containing vector, and *AtNLM1*-containing vector (positive control) were introduced into yeast cells via the SC EasyComp Transformation Kit (Invitrogen, Carlsbad, CA, USA) and transformants were selected with synthetic minimal medium lacking leucine and uracil (SC -Ura, -Leu) (required amino acids, 2% dextrose, 0.67% yeast nitrogen base w/o amino acids). Complementation tests and radioactive glycerol uptake experiments were conducted essentially as described by Weig et al. (2000) with the amendment that glycerol uptake is expressed on a per gram of dry yeast basis.

Expression of *PtNIP1;1* in *Xenopus oocytes*

To prepare the *PtNIP1;1* ORF for subcloning, the plasmid was linearized with *AvaI*, blunted with Klenow fragment, and the insert was released with *SpeI*. For directional cloning, the resulting *PtNIP1;1* ORF fragment was ligated into pAW2 (contains the 5' and 3' untranslated sequences of the *Xenopus* β -globin gene) that had been digested with *SpeI* and *EcoRV*. Resulting plasmid DNA was sequenced to verify construction and linearized with *NaeI*. Capped RNA was made with the T3 RNA

polymerase from the mMessage mMachine™ kit according to manufacturer's instructions (Ambion, Inc., Austin, Texas, USA).

Oocyte Osmotic Water Permeability Assay

Xenopus laevis oocytes (stages V and VI) were prepared as described previously (Zhang and Verkman, 1991) and incubated overnight at 16°C in ND96 buffer (96 mM NaCl, 5.0 mM HEPES, 1.0 mM MgCl₂, 2.0 mM KCl, 1.8 mM CaCl₂, 5.0 mM sodium pyruvate, and 0.1 mg·mL⁻¹ gentamicin) prior to injection. Oocytes were injected with 50 nL of 0.5 ng·nL⁻¹ *in vitro* synthesized transcripts (*PtNIP1;1* or *AtNLMI* (positive control) or nuclease-free water (negative control) and kept at 16°C. ND96 buffer was changed and dead oocytes were removed daily until swelling assays were conducted. Three days after injection, oocyte osmotic water permeability (P_{os}) was determined. At room temperature, individual oocytes experienced a five-fold drop in external osmolarity (200 mosmol to 40 mosmol) while oocyte images were captured every 5 s for 1.5 min with Scion Image software. Assuming oocytes were perfect spheres, P_{os} was calculated for each oocyte by: $P_{os} = V_o(d(V/V_o)/dt)S^{-1}V_w^{-1}(Osm_{in}-Osm_{out})^{-1}$ where V_o is the initial oocyte volume (determined for each oocyte), $d(V/V_o)/dt$ is the relative rate of volume change determined by the initial slope of V/V_o vs time, S is the initial oocyte surface area (determined for each oocyte), V_w is the molar volume of water (18 cm³·mol⁻¹), Osm_{in} is the osmolarity inside the oocyte (2.0 X 10⁻⁴ mol·cm⁻³) and Osm_{out} is the medium osmolarity (4.0 X 10⁻⁵ mol·cm⁻³) (Zhang and Verkman, 1991). Injections and subsequent

swelling assays were conducted on three separate replicates of oocyte preparations and five to seven oocytes were measured in each replicate.

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FIGURE LEGENDS

Figure 1. *Pinus taeda* (A) zygotic and (B) somatic embryos from the 9 developmental stages. The earliest 2 to 3 stages of somatic embryos typically are grown in (C) liquid suspension culture (LSC) maintenance medium; stages 3 through 9 are on gelled, semi-solid maturation medium. (D) Tissue in liquid cultures is characterized by dense embryo proper-like cell clusters (red arrows) surrounded by abundant, vacuolated suspensor-like cells (white arrows). (E) Stage seven zygotic embryo being dissected from megagametophyte (arrows: 1, embryo; 2 suspensor; 3, megagametophyte).

Figure 2. Alignment of PtNIP1;1, AtNLM1, and GmNOD26 to emphasize similarity of PtNIP1;1 to other aquaglyceroporins. According to a survey of more than 150 MIPs that identified 5 residues that are significant for either aquaporins or glycerol permeases (Froger et al., 1998), the NIPs are aquaporin-like at P₂₋₄ and glycerol permease-like at P₁ and P₅. The alignment was assembled with CLUSTALW (Thompson et al., 1994) and the shading was performed with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html)

Figure 3. Phylogenetic analysis of PtNIP1;1 with selected MIPs from the 4 classes: NIPs, PIPs, TIPS, and SIPs. The scale indicates nucleotide substitutions per amino acid position. Organism abbreviations are: At (*Arabidopsis thaliana*), Gm (*Glycine max*), Lj (*Lotus japonicus*), Os (*Oryza sativa*), Pa (*Picea abies*), Pt (*Pinus taeda*), Zm (*Zea mays*). Accession numbers are: AtNIP7;1 (AAF30303), ZmNIP3;1 (AF326486), OsNLM (BAA04257), ZmNIP1;1 (AF326483), AtNLM1 (CAA16760), LjLIMP2 (AAF82791), GmNOD26 (AAA02946), PaMIP-1 (CAB06080), PaMIP-2 (CAB07783), AtPIP0-0 (AAB65787), AtPIP2;8 (AAC64216), AtTIP5;1 (CAB51216), AtTIP0-0 γ (AAC62397), PaMIPr (CAA06335), PaMIPfg (CAB39758), AtSIP2;1 (CAB72165), AtSIP1;1 (AAF26804), AtSIP1;2 (BAB09487)

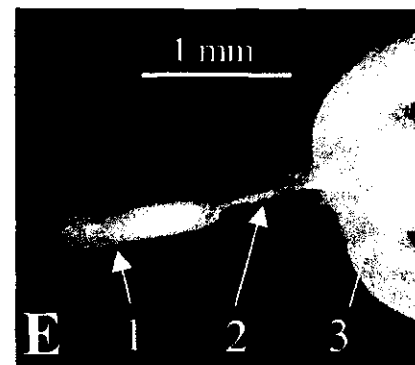
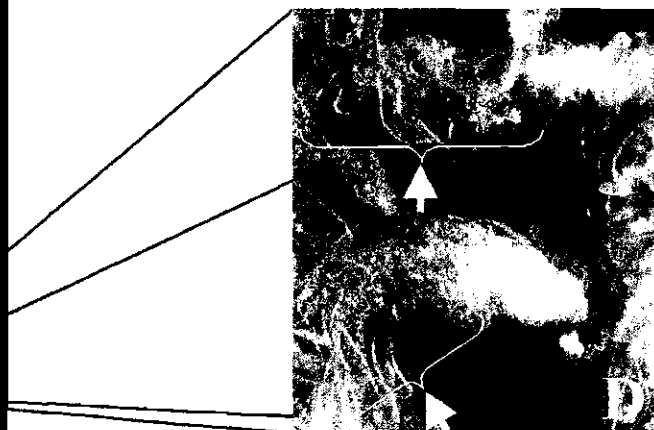
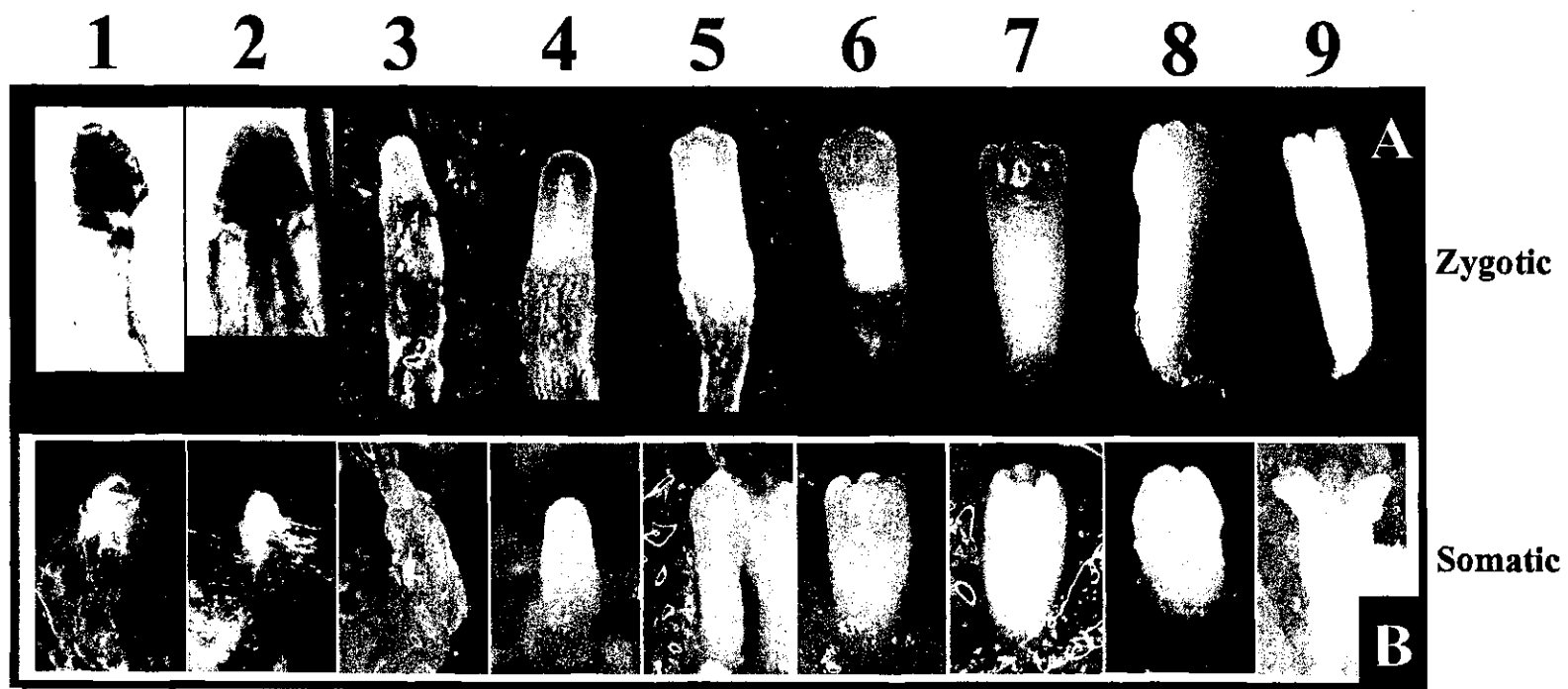
Figure 4. Expression analyses of *PtNIP1;1* in *P. taeda* tissues. All blots were hybridized with a ³²P-labelled 3' UTR fragment. (A) **Somatic embryo** northern analysis. Each lane contained five micrograms of somatic embryo total RNA. (B) **Zygotic embryo** slot blot. Each slot was loaded with two micrograms of zygotic embryo total RNA. (C) **Megagametophyte** northern analysis. Each lane contained ten micrograms of either somatic liquid suspension culture RNA (LSC) or megagametophyte total RNA. (D) **Vegetative** tissue northern analysis. Each lane contained 10 micrograms of either somatic embryo total RNA (LSC, late stage) or **vegetative** total RNA from 1 year-old seedlings (R, S, N). RNA designations: LSC, liquid suspension culture; L, late stage somatic embryo; R, root; S, stem, N, needle; numbers refer to embryo stages except for megagametophyte northern where numbers refer to stages of embryos removed prior to megagametophyte RNA isolation.

Figure 5. RT-PCR analysis of embryo proper and embryo suspensor total RNA. Aliquots for electrophoresis, ethidium bromide staining and subsequent quantification were removed after 29, 31 and 33 cycles (see Materials and Methods for PCR conditions). Numbers beneath PCR products (1.0, 2.9, 1.6, and 1.0) are normalized

signal intensities. Comparison of normalized signal intensities at equal numbers of PCR cycles during the linear range of the PCR reaction provides an estimate of *PtNIP1;1* mRNA relative abundance in the original embryo proper and suspensor RNA samples. (A) After background correction, measurement of ethidium bromide staining intensity indicates suspensor *PtNIP1;1* PCR product was nearly 3 fold greater than embryo proper products after 33 cycles. (B) As further support that approximately equal amounts of embryo proper and suspensor RNA were used in their respective RT reactions, embryo proper and suspensor RT products were PCR amplified with glyceraldehyde-3-phosphodehydrogenase (G3P) primers. Results indicate slightly higher expression of G3P in embryo proper compared to suspensor. Together, these results suggest a greater expression of *PtNIP1;1* in suspensor than in embryo proper.

Figure 6. Average osmotic water permeability, P_{os} , of oocytes injected with either cRNA (*PtNIP1;1* and *AtNLM1* (positive control)) or nuclease-free water (negative control). After measuring the rate of change of oocyte volume in response to a five-fold drop in external osmolarity, oocyte P_{os} was calculated according to the formula in Materials and Methods. Each treatment (injection of *PtNIP1;1*, *AtNLM1*, or nuclease-free water) was repeated three times with five to seven oocyte measurements per repeat. For each treatment, height of bars in the figure represents a mean of all oocyte measurements and error bars represent one standard deviation from the mean.

Figure 7. Demonstration of glyceroporin function for *PtNIP1;1*. Wild type yeast have a plasma membrane glycerol facilitator protein, *Fps1*, to modulate glycerol efflux and thereby maintain osmotic balance with the environment (Tamás et al., 1999). Lacking this protein, *fps1*⁻ yeast display sensitivity to hypo-osmotic shock (i.e., slowed growth, presumably due to rapid influx of water, after a sudden decrease in external osmolarity). (A) Complementation of *Saccharomyces cerevisiae fps1*⁻ osmosensitivity by *PtNIP1;1* and *AtNLM1*. *fps1*⁻ yeast transformed with *PtNIP1;1*, *AtNLM1* or vector-only (see Materials and Methods for plasmid construction) were grown to a common OD₆₀₀ in synthetic minimal medium amended with 1M glycerol. For each culture, ten-fold serial dilutions were made and 5 μL of each dilution were spotted on synthetic minimal medium plates with 1M glycerol (control) and without added glycerol (hypo-osmotic shock). Plates were incubated at 30°C for 12 hours and 36 hours prior to photography. Upon hypo-osmotic shock, yeast that expressed *PtNIP1;1* or *AtNLM1* grew significantly better than vector-transformed yeast (arrows). All transformants grew equally well on plates with 1M glycerol. (B) Radioactive glycerol uptake by *Saccharomyces cerevisiae fps1*⁻ yeast that express *PtNIP1;1* and *AtNLM1*. Yeast transformants were grown in synthetic minimal medium (no added glycerol) to OD₆₀₀ of 1.0. Fifty milliliters of each culture was washed twice and concentrated to 5 milliliters in 50 mM sodium phosphate buffer, pH 5.5. At 30°C and constant stirring, glycerol concentration was adjusted to 0.1 mM, 1% of which was ³H-labelled glycerol. At 5, 10, 15, and 20 minutes, 100 μL samples, three samples at each time point, were removed, filtered, and washed. Radioactivity of each sample was determined and converted to mass of glycerol. Data in 7B are from one experiment out of three that gave consistent results.



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PtNIP1;1 1 -----MAI[ ]L[ ]L[ ]G[ ]V[ ]A[ ]M[ ]P[ ]E[ ]G[ ]R[ ]R[ ]G[ ]R[ ]S[ ]C[ ]G[ ]S[ ]L[ ]L[ ]E[ ]S[ ]V[ ]R[ ]K[ ]W[ ]A[ ]E[ ]F[ ]L[ ]G[ ]T[ ]E[ ]F[ ]L[ ]I[ ]V[ ]C[ ]C[ ]G[ ]S[ ]V[ ]V[ ]
AtNLM1 1 [ ]V[ ]N[ ]L[ ]K[ ]D[ ]E[ ]V[ ]F[ ]H[ ]Q[ ]E[ ]N[ ]E[ ]D[ ]H[ ]N[ ]E[ ]R[ ]P[ ]L[ ]K[ ]D[ ]S[ ]L[ ]L[ ]S[ ]V[ ]S[ ]V[ ]P[ ]E[ ]L[ ]Q[ ]K[ ]I[ ]A[ ]E[ ]F[ ]L[ ]G[ ]T[ ]E[ ]F[ ]L[ ]I[ ]V[ ]C[ ]C[ ]A[ ]S[ ]V[ ]V[ ]
GmNOD26 1 [ ]M[ ]A[ ]D[ ]Y[ ]S[ ]A[ ]G[ ]T[ ]E[ ]S[ ]E[ ]V[ ]V[ ]N[ ]V[ ]T[ ]K[ ]N[ ]T[ ]S[ ]E[ ]T[ ]I[ ]C[ ]R[ ]E[ ]D[ ]S[ ]L[ ]S[ ]V[ ]S[ ]V[ ]P[ ]E[ ]L[ ]Q[ ]K[ ]I[ ]A[ ]E[ ]F[ ]L[ ]G[ ]T[ ]E[ ]F[ ]L[ ]I[ ]V[ ]C[ ]C[ ]A[ ]S[ ]V[ ]V[ ]

PtNIP1;1 52 [ ]D[ ]K[ ]I[ ]S[ ]N[ ]G[ ]S[ ]I[ ]H[ ]L[ ]D[ ]V[ ]S[ ]L[ ]V[ ]W[ ]G[ ]M[ ]A[ ]A[ ]I[ ]V[ ]Y[ ]S[ ]I[ ]G[ ]H[ ]I[ ]S[ ]G[ ]A[ ]E[ ]L[ ]N[ ]P[ ]A[ ]V[ ]T[ ]I[ ]A[ ]L[ ]A[ ]V[ ]K[ ]R[ ]F[ ]F[ ]W[ ]Q[ ]V[ ]P[ ]G[ ]Y[ ]I[ ]V[ ]
AtNLM1 61 [ ]M[ ]M[ ]N[ ]D[ ]V[ ]V[ ]T[ ]L[ ]P[ ]G[ ]I[ ]A[ ]I[ ]V[ ]W[ ]G[ ]L[ ]T[ ]E[ ]M[ ]V[ ]L[ ]Y[ ]S[ ]E[ ]G[ ]H[ ]I[ ]S[ ]G[ ]A[ ]E[ ]L[ ]N[ ]P[ ]A[ ]V[ ]T[ ]I[ ]A[ ]F[ ]A[ ]S[ ]C[ ]G[ ]R[ ]F[ ]P[ ]L[ ]K[ ]Q[ ]V[ ]P[ ]A[ ]Y[ ]V[ ]I[ ]V[ ]
GmNOD26 61 [ ]N[ ]E[ ]N[ ]Y[ ]Y[ ]N[ ]M[ ]I[ ]T[ ]F[ ]P[ ]G[ ]I[ ]A[ ]I[ ]V[ ]W[ ]G[ ]L[ ]T[ ]E[ ]M[ ]V[ ]L[ ]Y[ ]S[ ]E[ ]G[ ]H[ ]I[ ]S[ ]G[ ]G[ ]H[ ]E[ ]N[ ]P[ ]A[ ]V[ ]T[ ]I[ ]A[ ]F[ ]A[ ]S[ ]T[ ]R[ ]R[ ]F[ ]P[ ]L[ ]L[ ]Q[ ]V[ ]P[ ]A[ ]Y[ ]V[ ]I[ ]V[ ]

          P1
          F
PtNIP1;1 112 [ ]A[ ]Q[ ]V[ ]E[ ]S[ ]I[ ]S[ ]A[ ]G[ ]F[ ]L[ ]R[ ]E[ ]M[ ]F[ ]G[ ]-----[ ]E[ ]V[ ]A[ ]F[ ]M[ ]G[ ]I[ ]V[ ]E[ ]S[ ]G[ ]S[ ]E[ ]M[ ]O[ ]S[ ]E[ ]A[ ]E[ ]E[ ]I[ ]T[ ]I[ ]T[ ]S[ ]L[ ]I[ ]M[ ]F[ ]V[ ]M[ ]S[ ]A[ ]
AtNLM1 121 [ ]S[ ]Q[ ]V[ ]E[ ]G[ ]S[ ]T[ ]L[ ]A[ ]A[ ]A[ ]T[ ]L[ ]R[ ]L[ ]L[ ]E[ ]G[ ]L[ ]D[ ]H[ ]D[ ]V[ ]C[ ]S[ ]K[ ]H[ ]E[ ]V[ ]I[ ]S[ ]S[ ]E[ ]V[ ]G[ ]S[ ]D[ ]L[ ]O[ ]A[ ]F[ ]T[ ]M[ ]E[ ]F[ ]I[ ]M[ ]T[ ]E[ ]M[ ]P[ ]L[ ]I[ ]S[ ]G[ ]
GmNOD26 121 [ ]A[ ]Q[ ]L[ ]G[ ]S[ ]I[ ]L[ ]A[ ]S[ ]G[ ]T[ ]L[ ]R[ ]L[ ]L[ ]E[ ]-----[ ]M[ ]G[ ]N[ ]H[ ]E[ ]Q[ ]E[ ]S[ ]C[ ]T[ ]V[ ]E[ ]N[ ]G[ ]E[ ]N[ ]L[ ]O[ ]A[ ]F[ ]V[ ]E[ ]F[ ]E[ ]I[ ]M[ ]T[ ]E[ ]M[ ]F[ ]V[ ]I[ ]C[ ]G[ ]

          P2 P3 P4/5
          S A YL
PtNIP1;1 164 [ ]V[ ]A[ ]T[ ]D[ ]N[ ]R[ ]A[ ]V[ ]G[ ]E[ ]L[ ]E[ ]G[ ]L[ ]A[ ]I[ ]G[ ]A[ ]T[ ]I[ ]A[ ]N[ ]V[ ]A[ ]I[ ]S[ ]G[ ]P[ ]I[ ]S[ ]G[ ]A[ ]S[ ]M[ ]N[ ]F[ ]A[ ]R[ ]T[ ]I[ ]G[ ]S[ ]A[ ]V[ ]A[ ]G[ ]N[ ]K[ ]Y[ ]T[ ]S[ ]I[ ]W[ ]Y[ ]Y[ ]V[ ]E[ ]E[ ]P[ ]
AtNLM1 181 [ ]V[ ]A[ ]T[ ]D[ ]N[ ]R[ ]A[ ]V[ ]G[ ]E[ ]L[ ]E[ ]G[ ]L[ ]A[ ]I[ ]G[ ]S[ ]T[ ]Y[ ]L[ ]L[ ]N[ ]V[ ]L[ ]I[ ]A[ ]A[ ]F[ ]V[ ]G[ ]S[ ]A[ ]S[ ]M[ ]N[ ]I[ ]G[ ]R[ ]S[ ]L[ ]G[ ]P[ ]A[ ]L[ ]V[ ]Y[ ]C[ ]C[ ]K[ ]G[ ]I[ ]W[ ]I[ ]Y[ ]L[ ]V[ ]A[ ]P[ ]
GmNOD26 174 [ ]V[ ]A[ ]T[ ]D[ ]N[ ]R[ ]A[ ]V[ ]C[ ]E[ ]F[ ]A[ ]G[ ]I[ ]A[ ]I[ ]G[ ]S[ ]T[ ]E[ ]L[ ]L[ ]N[ ]V[ ]E[ ]I[ ]G[ ]G[ ]P[ ]V[ ]E[ ]G[ ]A[ ]S[ ]M[ ]N[ ]F[ ]V[ ]R[ ]S[ ]L[ ]G[ ]P[ ]A[ ]F[ ]V[ ]H[ ]G[ ]E[ ]Y[ ]E[ ]G[ ]I[ ]W[ ]I[ ]Y[ ]L[ ]H[ ]A[ ]P[ ]

PtNIP1;1 224 [ ]V[ ]I[ ]G[ ]A[ ]I[ ]M[ ]G[ ]A[ ]M[ ]S[ ]Y[ ]N[ ]M[ ]I[ ]R[ ]E[ ]T[ ]K[ ]M[ ]S[ ]E[ ]R[ ]E[ ]I[ ]M[ ]K[ ]S[ ]G[ ]S[ ]F[ ]M[ ]K[ ]D[ ]M[ ]G[ ]S[ ]S[ ]E[ ]S[ ]T[ ]A[ ]
AtNLM1 241 [ ]T[ ]I[ ]G[ ]A[ ]I[ ]G[ ]A[ ]W[ ]V[ ]Y[ ]N[ ]T[ ]V[ ]R[ ]Y[ ]T[ ]D[ ]K[ ]P[ ]L[ ]R[ ]E[ ]I[ ]T[ ]K[ ]S[ ]G[ ]S[ ]F[ ]L[ ]K[ ]T[ ]V[ ]R[ ]I[ ]G[ ]S[ ]T[ ]---
GmNOD26 234 [ ]V[ ]I[ ]G[ ]A[ ]I[ ]G[ ]A[ ]W[ ]V[ ]Y[ ]N[ ]T[ ]V[ ]R[ ]Y[ ]T[ ]D[ ]K[ ]P[ ]L[ ]S[ ]E[ ]T[ ]T[ ]K[ ]S[ ]A[ ]S[ ]F[ ]L[ ]K[ ]G[ ]R[ ]A[ ]S[ ]K[ ]---

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Figure 2.

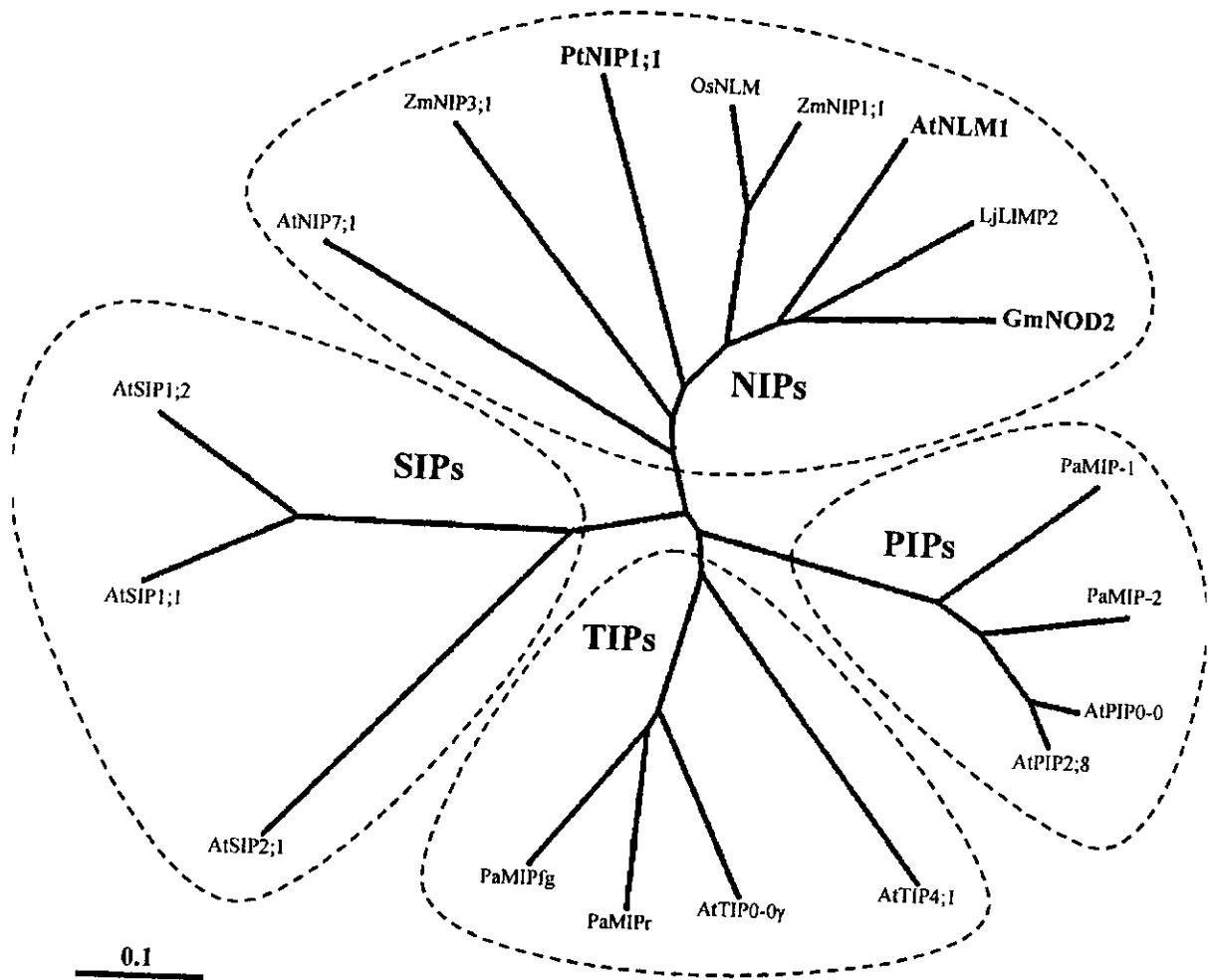


Figure 3.

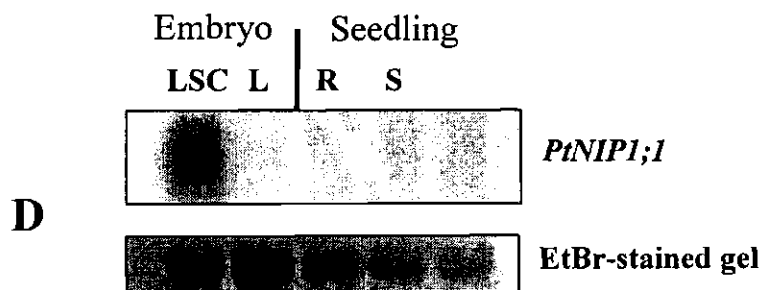
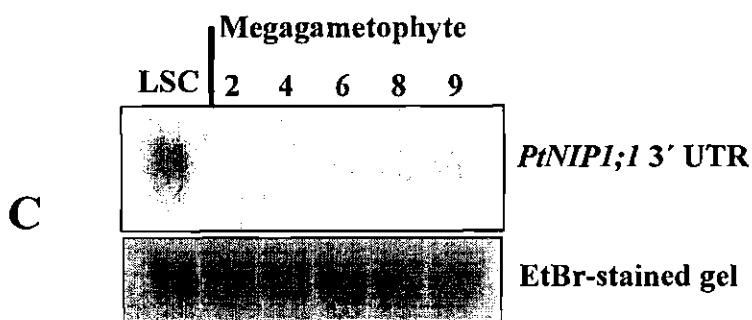
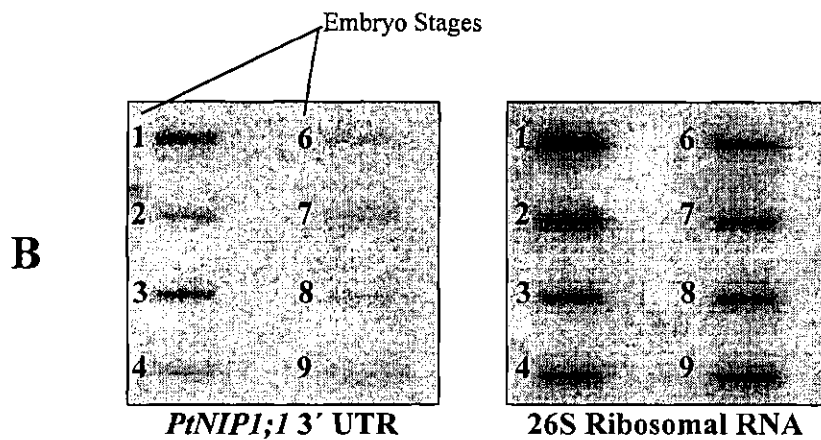
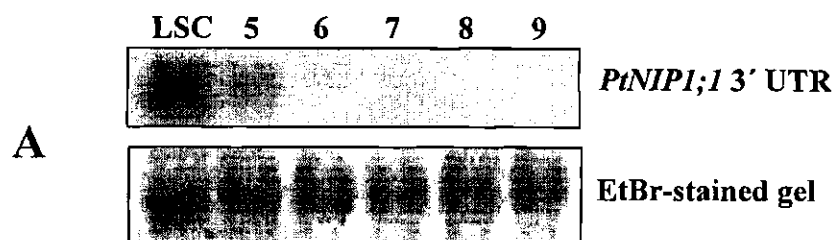


Figure 4.

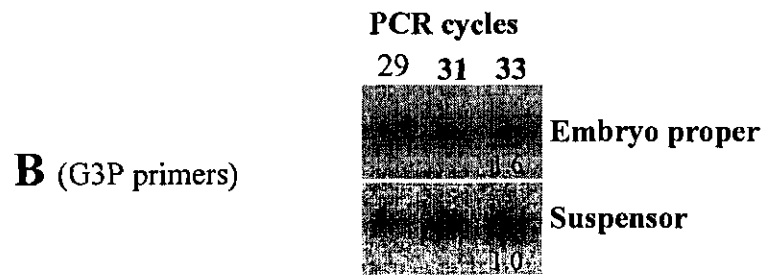
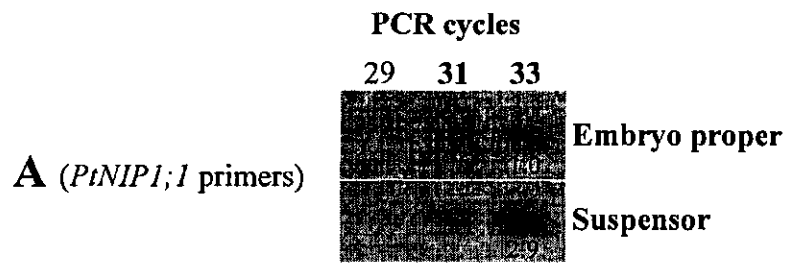
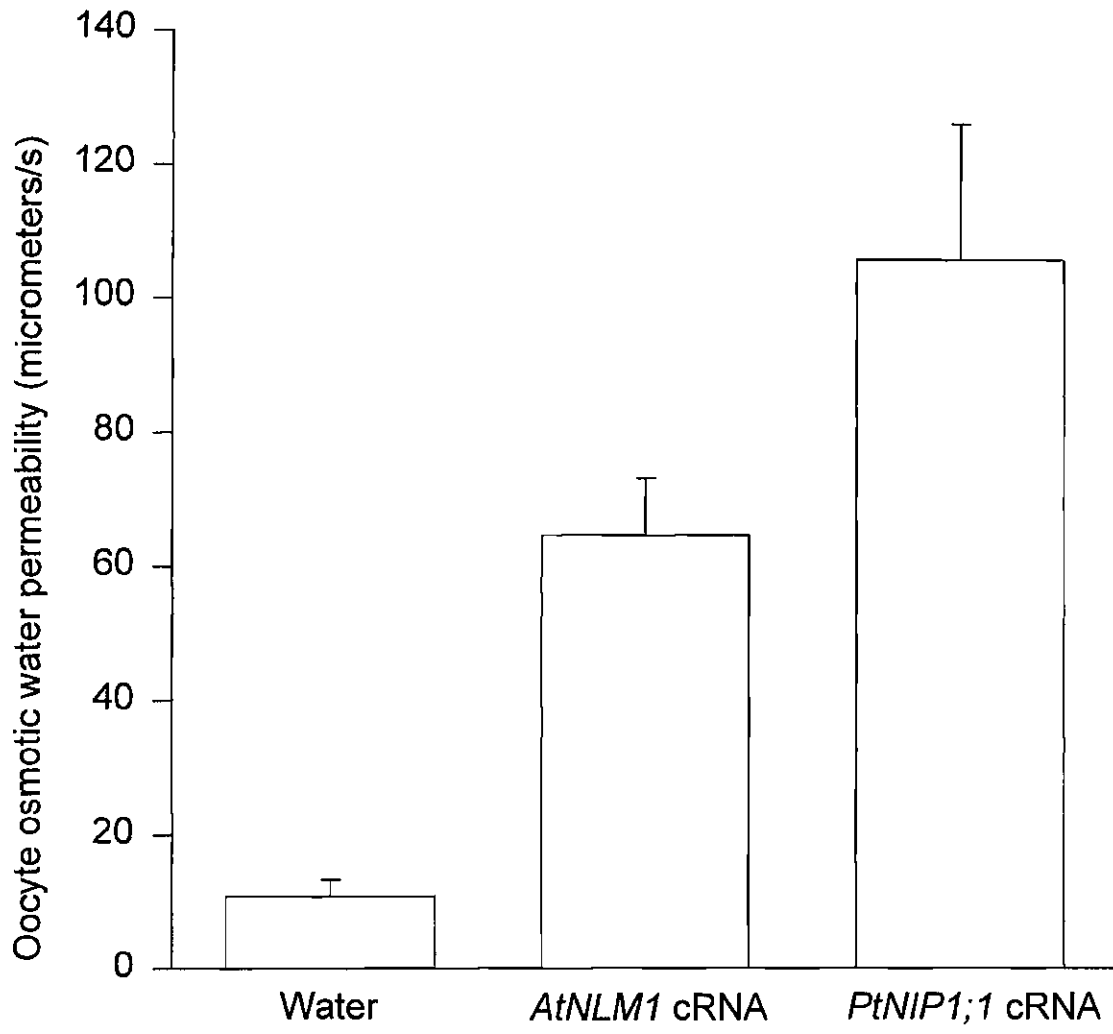


Figure 5.



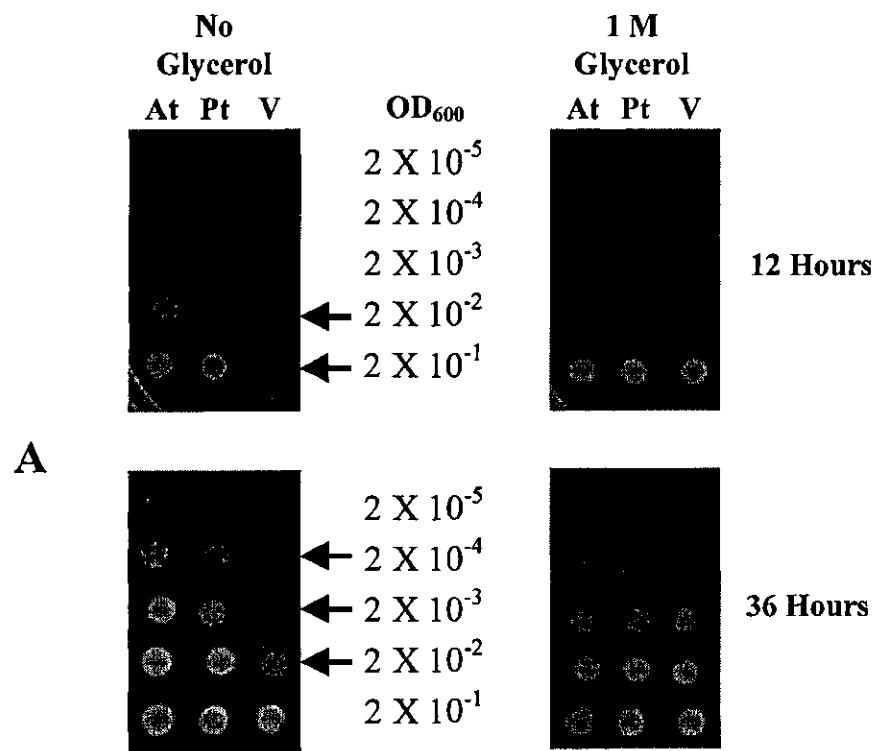


Figure 7A.

B

