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An auxin-inducible gene from loblolly pine (*Pinus taeda* L.) is differentially expressed in mature and juvenile-phase shoots and encodes a putative transmembrane protein

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Abstract We have isolated a gene from loblolly pine, *5NG4*, that is highly and specifically induced by auxin in juvenile loblolly pine shoots prior to adventitious root formation, but substantially down-regulated in physiologically mature shoots that are adventitious rooting incompetent. *5NG4* was highly auxin-induced in roots, stems and hypocotyls, organs that can form either lateral or adventitious roots following an auxin treatment, but was not induced to the same level in needles and cotyledons, organs that do not form roots. The deduced amino acid sequence shows homology to the *MtN21* nodulin gene from *Medicago truncatula*. The expression pattern of *5NG4* and its homology to a protein from *Medicago* involved in a root-related process suggest a possible role for this gene in adventitious root formation. Homology searches also identified similar proteins in *Arabidopsis thaliana* and *Oryza sativa*. High conservation across these evolutionarily distant species

suggests essential functions in plant growth and development. A 38-member family of genes homologous to *5NG4* was identified in the *A. thaliana* genome. The physiological significance of this redundancy is most likely associated with functional divergence and/or expression specificity of the different family members. The exact biochemical function of the gene is still unknown, but sequence and structure predictions and *5NG4*:GFP fusion protein localizations indicate it is a transmembrane protein with a possible transport function.

Keywords Adventitious root formation · Auxin · Gene expression (*5NG4*) · Maturation · Nodulin · *Pinus*

Abbreviations *ABA* Abscisic acid · *BA* Benzylaminopurine · *EST* Expressed sequence tag · *NAA* 1-Naphthaleneacetic acid · *GFP* Green fluorescent protein · *ORF* Open reading frame

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Introduction

During development from seed to mature plant, trees undergo dramatic phenotypic changes, known as phase change or maturation (Poethig 1990; Haffner et al. 1991). Although maturation has widespread effects on a number of phenotypic characteristics (Poethig 1990; Greenwood and Hutchison 1993), the focus of our efforts has been to study the relationship between maturation and adventitious root formation in loblolly pine (*Pinus taeda* L.). As trees grow older, cuttings obtained from them gradually lose the competence to form adventitious roots (Hackett 1988). It is likely that maturation causes changes in the expression of genes involved in adventitious rooting (Greenwood and Hutchison 1993). Knowledge of changes in gene expression during maturation in woody perennials is incomplete. Hutchison et al. (1990) showed that a light-harvesting chlorophyll *a/b*-binding protein (CAB) gene

is expressed at a 30% higher level in juvenile than in mature larch needles. Similarly, a CAB gene from English ivy was expressed at higher levels in juvenile than in mature petioles (Woo et al. 1994). The dihydroflavonol reductase (DFR) gene in English ivy was completely repressed in mature petioles (Murray and Hackett 1991). In contrast, a proline-rich protein (PRP) from English ivy was expressed at higher levels in mature petioles (Woo et al. 1994). Little is known about changes in gene expression during maturation in loblolly pine.

Auxin is the primary signal for adventitious root formation (Blakesley 1994). Besides adventitious rooting, auxins also regulate stem elongation, lateral root formation, tropic responses, apical dominance and other developmental processes in plants (Davies 1995). Recent evidence has also linked auxin to the symbiotic process of nodulation (Mathesius et al. 1998; de Billy et al. 2001; Mathesius 2001). *Rhizobium* inoculation causes inhibition of auxin polar transport in root cortical cells (Mathesius et al. 1998). It is believed that the flavonoids synthesized by the plant in response to bacterial lipochitinoligosaccharides that activate the bacterial NOD genes act as endogenous auxin-transport inhibitors (Mathesius et al. 1998). This transport inhibition most likely creates temporary, localized elevated auxin concentrations (Mathesius et al. 1998). Plants respond to increased auxin concentrations by transcriptional activation of a number of auxin-induced genes (reviewed in Abel and Theologis 1996). The elevation of auxin concentration and the early steps in nodulation occur within a short time-frame in the same tissues. Hence, genes that have been identified as "nodulation-induced" may, in fact, be induced by auxin independently of nodulation. In support of such a scenario, a highly auxin-inducible *GH3* promoter fused to the β -glucuronidase (*GUS*) reporter gene was found to be up-regulated at the site of nodule initiation in transgenic white clover (Mathesius et al. 1998). In a screen of nodulins, 4 out of 22 were induced by auxin (Jimenez-Zurdo et al. 2000). Thus, by manipulating the concentration of host signaling molecule, in this case auxin, *Rhizobium* may trigger a nonsymbiotic developmental process that can be modified for the purposes of the symbiotic interaction. One candidate for such a developmental process is lateral root formation, and recent experimental data suggest a link between lateral root formation and nodulation. The hypernodulating mutant *har1* from *Lotus japonicus* shows a phenotype of extreme lateral root proliferation (Woperies et al. 2000). *Pisum sativum* plants, transformed with the promoter of a nodulin gene (*ENOD12A*) fused to the *GUS* reporter gene, expressed the reporter gene in the region of lateral root emergence without NOD factor treatment (Schneider et al. 1999). A subtilisin protease that is induced during nodule formation in *Alnus* (Ribeiro et al. 1995) and *Casuarina* (Laplaze et al. 2000) is similar to the *Arabidopsis* protein AIR3 that is expressed at higher levels during lateral root formation (Neuteboom et al.

1999). Moreover, a loblolly pine gene with substantial similarity to AIR3 is induced by auxin in stem cuttings (Busov 2001). Thus, some nodulins, particularly those that are regulated by auxin, may have a function in lateral root formation.

In this study, we report a nodulin-like gene from loblolly pine that is highly induced by auxin in the base of loblolly pine shoots, prior to adventitious root formation, a process with similarities to lateral root formation (Cheng et al. 1995; Smith and Federoff 1995). The possible involvement of this gene in adventitious rooting is suggested by a correlation between loss of adventitious root competence and down-regulation of the gene in mature shoots. The phylogenetic relationships, putative biochemical functions and expression patterns of this gene are discussed.

Materials and methods

Plant material and treatments

Scions from a 23-year-old, mother tree (mature) of loblolly pine (*Pinus taeda* L.) and from 1-year-old, open-pollinated, seedlings (juvenile) from the same mother tree were grafted onto 1-year-old rootstock from the same family. The grafted stock plants were fertilized, watered and sheared in the same manner for three growing seasons to provide a consistent supply of cuttings similar to those used in applied rooting experiments (e.g. Murthy and Goldfarb 2001). Shoots from these stock plants were used to compare gene expression in the mature and juvenile phases. All shoots were approximately 8 cm in length and the basal 3 cm of each was submerged in 10% ethanol with or without 1.6 mM NAA for 10 min. This auxin treatment was chosen because it results in extensive adventitious root formation in cuttings of loblolly pine hypocotyls (Diaz-Sala et al. 1996). After treatment, the shoots were inserted in moist sand and placed in 16 h/8 h (light/dark) photoperiod at 25°C. Shoots were then removed from the sand and rinsed with distilled water. The bottom portions of the shoots were stripped of needles and the basal 2.5 cm was severed and immediately frozen in liquid nitrogen and stored at -70°C until further processing.

To study *5NG4* expression in different organs, 2-week-old seedlings, open-pollinated progeny from the same mother tree, were submerged for 10 min in 10% ethanol (v/v) with or without 1.6 mM NAA. Following treatments, seedlings were placed in moist sand and incubated in a growth chamber for 24 h under a 16 h/8 h photoperiod at 25°C. Hypocotyls, cotyledons, primary needles and roots from 50 seedlings were cut and pooled separately and frozen in liquid nitrogen. The RNA was immediately extracted.

In order to evaluate *5NG4* expression in response to different growth regulators, shoots were collected from 4-year-old, continuously pruned stock plants (see above). Fifty shoots were cut to approximately 8 cm long and the bases (3 cm) were submerged for 10 min in the following treatment solutions: 1.6 mM 1-naphthaleneacetic acid (NAA), 1.6 mM gibberellic acid, 1.6 mM benzylaminopurine (BA), 3.2 mM abscisic acid (ABA), 0.04 N NaOH (control for BA and ABA treatments), and 10% ethanol (v/v; control for gibberellic acid and NAA treatments). Following treatment, shoots were incubated and processed as described above.

For heat-shock treatment, 40, two-year-old seedlings were grown in a growth chamber at 27°C constant temperature and a 16 h/8 h photoperiod. Water was applied to each pot until the potting medium was saturated. After 14 days of acclimation to the chamber, seedlings were separated into 2 groups of 20 seedlings each, 1 group for the stress treatment and 1 for a control. Heat stress was applied to the treatment group by raising the

temperature to 42°C for 2 h, while the control group was maintained at 27°C. The developing xylem was harvested as described by Allona et al. (1998).

Nucleic acid isolation and gel-blot analysis

For northern analyses, total RNA was isolated as described by Chang et al. (1993). Ten- to twenty- μ g aliquots were fractionated on 1% agarose, 2.2 M formaldehyde gels and transferred to uncharged nylon membrane (Micron, Boston, MA, USA) as described by Ausubel et al. (1995). Transferred RNA was UV cross-linked to the membranes using a Stratalink (Stratagene, La Jolla, CA, USA). Probes were ³²P-labeled cDNAs generated by random prime labeling using the Prime-A-Gene Kit (Promega, Madison, WI, USA) and with subsequent removal of unincorporated nucleotides using the JetNick Kit (Genomed, Bad Oeynhausen, Germany). The membranes were prehybridized and hybridized following standard formamide hybridization procedures (Ausubel et al. 1995). To remove non-specifically bound probe, membranes were washed twice at room temperature for 5 min each with 2 \times sodium chloride–sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) and 0.2 \times SSC, 0.1% SDS, and twice for 15 min with 0.2 \times SSC, 0.1% SDS at 42°C. Membranes were exposed to Kodak BioMax film (Kodak, Rochester, NY, USA) for 1–5 days at –70°C. Membranes were stripped (Ausubel et al. 1995) and subsequently hybridized with a larch 18S ribosomal DNA probe (Hutchison et al. 1990) as a loading control. Transcript size was estimated relative to RNA size standards that were included on the gels used for northern blotting. All autoradiographs were scanned and hybridization signals quantified using LabWorks software (UVP, Upland, CA, USA).

For Southern analysis, nuclear-enriched DNA was isolated from needles of the same loblolly pine mother tree, using the procedures described in Goldfarb et al. (2003). Genomic DNA was restricted with *Eco*RI and *Hind*III and blotted, and high-stringency hybridization and washing were carried out as previously described (Goldfarb et al. 2003).

Sequence analysis of 5NG4

5NG4 was identified as being differentially induced by auxin treatment in loblolly pine shoots in a microarray screen of (partially sequenced) ESTs (Busov 2001). The original cDNA from the EST library was then sequenced in its entirety by automated sequencing at the DNA sequencing facility of Iowa State University at Ames and yielded a putative complete open reading frame (ORF) described below. Sequence homology searches and sequence analyses were performed using the NCBI BLAST server (<http://www.ncbi.nlm.nih.gov/>) and the University of Wisconsin Genetics Computer Group (GCG) software package (Devereux et al. 1984). Protein predictions were performed using the CBS server (<http://www.cbs.dtu.dk/>) and the hidden Markov model (Erik et al. 1998). Sequence alignments were carried out using CLUSTAL W (Thompson et al. 1994) and the EMBL server (<http://www.ebi.ac.uk/clustalw/>). Phylogenetic analyses were performed using the MEGA 2.1 software (Kumar et al. 2001).

Preparation of the 5NG4::GFP construct

We amplified 5NG4 cDNA from EST clone 5NG4 (Allona et al. 1998) using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and the following primers: forward (5'-GGGGGATCCTCCCTGTGCA GGAAGCACT-3') and reverse (5'-GGGGGATCCTGGCTGT GGCTCGTCTGA-3'). The resulting PCR product was cut with *Bam*HI and cloned into the *Bam*HI site of the *pBINmGFP4* vector (Haseloff et al. 1997). This insertion site was immediately downstream of the *CaMV* 35S promoter and the 5NG4 sequence was fused in frame with the (*GFP4*) mutant version of the *Aequorea victoria* green fluorescent protein (GFP). The sequences of several clones

harboring the construct were analyzed to select only the insertions in the sense orientation.

Plant material and *Agrobacterium tumefaciens*-mediated transient expression

Nicotiana tabacum L. SR1 (cv. Petit Havana) seeds were disinfected with 10% (v/v) commercial bleach and plated on Murashige and Skoog (MS) basal salt medium (Sigma, St. Louis, MO, USA), containing 0.7% (w/v) agar and 3% (w/v) sucrose. Individual seedlings were serially propagated under sterile conditions on the same medium. The two newest fully expanded leaves from these plants were used to obtain 15 mm \times 15 mm leaf square sections for the transient-expression studies. *Agrobacterium tumefaciens* strain LBA4404 was transformed with the plasmid containing the 5NG4::GFP fusion construct by electroporation. A single transformed colony was used to inoculate 10 ml of YEB medium (1 g l⁻¹ yeast extract, 5 g l⁻¹ beef extract, 5 g l⁻¹ tryptone, 5 g l⁻¹ sucrose and 0.5 g l⁻¹ MgSO₄·7H₂O), supplemented with 100 mg l⁻¹ kanamycin sulfate (Sigma). Bacterial cultures were incubated at 28–30°C with constant agitation until reaching stationary phase (OD₆₀₀ = 1.0). Five milliliters of culture were pelleted by centrifugation at 3,000 g for 10 min at room temperature. The pellet was resuspended in infiltration buffer (Batoko et al. 2000) to adjust the concentration to OD₆₀₀ = 1.0. Leaf disks were co-cultivated with 150 μ l of bacterial suspension plus 3 ml of infiltration buffer at 25°C for 2 days. After co-cultivation, leaf discs were washed at least three times with infiltration buffer and transferred to MS basal salt medium (Sigma) containing 0.7% (w/v) agar, 3% (w/v) sucrose, 400 mg l⁻¹ (w/v) Timentin (SmithKline Beecham, Philadelphia, PA, USA) and 50 mg l⁻¹ kanamycin sulfate (Sigma). GFP fluorescence was monitored using an SMZ1000 zoom stereomicroscope (Nikon, Melville, NY, USA), equipped with an epi-fluorescence attachment P-FLA (Nikon). We observed maximum fluorescence from all constructs between 3 and 5 days after transfer.

Confocal microscopy

Leaf pieces that expressed GFP were embedded in a thin layer of 1.3% (w/v) low-melting-point agarose VII (Sigma) in the MS buffer described above on a wetted slide. Confocal fluorescence image stacks were acquired in optical sections of about 1 μ m in thickness with a Leica TCS SP confocal system using a Leica DM IRBE microscope and a 20 \times N.A. 0.6 dry objective or 40 \times N.A. 1.2 oil immersion objective (Leica, Wetzlar, Germany). Samples were excited with an argon laser at 488 nm and fluorescence emission was collected from 515 to 545 nm. Images were processed with Leica TCS SP confocal software. Volume rendering of confocal stacks was performed using a maximum projection algorithm.

Results

Phenotypic characteristics and adventitious rooting of mature and juvenile shoots

Grafted stock plants originating from the juvenile and mature scion material (see Materials and methods for details), produced shoots that were quite different in a number of characteristics. Mature shoots were longer, had larger diameters and produced longer fascicular needles (Table 1). When stock plants were placed in a horizontal position, mature shoots exhibited more-rapid tropic responses than juvenile shoots (Table 1). These two types of shoots also differed markedly in the production of adventitious roots. When treated with 1.6 mM NAA for

Table 1 Phenotypic and developmental characteristics of mature and juvenile *Pinus taeda* shoots. All measurements and treatments were performed on shoots from grafted hedges originating from scions of 1- (juvenile) or 23-year-old (mature) donor trees. The numbers in the table indicate the mean (standard error)

Phenotypic trait	Mature	Juvenile
Fascicle needle length (mm)	73.0 (2.0)	50.0 (2.0)
Shoot diameter (mm)	3.8 (0.1)	1.6 (0.1)
Shoot length (mm)	83.0 (2.0)	63.0 (2.0)
Gravitropic bending ($^{\circ}$) ^a	75.0 (2.0)	26.0 (2.0)
Rooting (%) ^b	0.0 (0.0)	69.0 (22.0)

^aGravitropic bending was determined by placing two mature and two juvenile hedges at a horizontal orientation and measuring the angle from horizontal of 40 shoots after 24 h

^bRooting percentage was determined by treating 8-cm shoots with 1.6 mM NAA for 10 min and placing them in 60% perlite and 40% peat in a greenhouse under intermittent mist for 12 weeks. Shoots with one or more emerged roots were scored as rooted

10 min, 69% of juvenile shoots produced adventitious roots (Table 1), but no roots were observed in the mature shoots. Based on these observations, we concluded that shoots from these stock plants would provide suitable material for the investigation of differences in gene expression between mature and juvenile shoots during auxin response and adventitious root formation.

Identification of differentially expressed genes

Northern blot analyses were conducted to determine which, if any, of 14 loblolly pine auxin-responsive genes were differentially expressed between the juvenile and mature phases. Five auxin-induced genes from loblolly pine (*PTIAA1*–*PTIAA5*; Goldfarb et al. 2003), with sequence homology to members of the plant *Aux/IAA* gene family (Abel and Theologis 1996) and nine genes

Table 2 Expression of 14 loblolly pine genes in mature and juvenile shoots. Shoots from hedges, grafted with scions originating from juvenile (1-year-old) and mature (23-year-old) trees were treated in 10% ethanol (–NAA) or 10% ethanol plus 1.6 mM NAA (+NAA) for 10 min. Treated shoots were harvested 24 h following the treatments. The experiment was performed twice (in

Clone	Homologous gene	Juvenile –NAA	Mature –NAA	Juvenile +NAA	Mature +NAA
5NG4	MtN21 nodulin	0.22 (0.02)	0.15 (0.01)	0.90 (0.02)**	0.32 (0.03)
2cd6	Caffeoyl-CoA-methyltransferase	0.45 (0.26)	0.51 (0.25)	0.97 (0.52)	0.62 (0.39)
2cf5	Serine hydroxy-methyltransferase	1.30 (0.70)	1.13 (0.50)	2.69 (1.34)	1.96 (0.51)
2naa12	K ⁺ channel	0.65 (0.34)	0.75 (0.41)	1.65 (0.82)	1.36 (0.23)
5ca7	Light-inducible protein	0.34 (0.21)	0.34 (0.13)	0.72 (0.45)	0.55 (0.36)
5ng3	Methionine synthase	0.37 (0.14)	0.45 (0.01)	1.04 (0.59)	0.69 (0.24)
6ca1	Subtilisin protease	0.72 (0.30)	0.66 (0.43)	1.33 (0.30)	0.90 (0.16)
7cg8	Proline-rich protein	0.32 (0.02)	0.34 (0.08)	0.66 (0.06)	0.55 (0.01)
9228	DNA-binding protein	0.91 (0.57)	0.95 (0.50)	2.15 (1.40)	1.60 (0.96)
PTIAA1	Aux/IAA	0.15 (0.02)	0.17 (0.00)	0.56 (0.12)	0.56 (0.04)
PTIAA2	Aux/IAA	0.28 (0.00)	0.27 (0.11)	1.03 (0.26)	1.64 (0.58)
PTIAA3	Aux/IAA	0.45 (0.38)	0.39 (0.33)	2.93 (2.40)	2.51 (1.93)
PTIAA4	Aux/IAA	0.26 (0.03)	0.20 (0.03)	0.55 (0.06)	0.56 (0.09)
PTIAA5	Aux/IAA	0.17 (0.05)	0.21 (0.00)	0.35 (0.01)	0.31(0.00)

**Expression value for *5NG4* in NAA-treated juvenile shoots was significantly greater ($P=0.0036$) than in NAA-treated mature shoots. No other juvenile–mature comparisons were significantly different ($P<0.05$) using LSD tests

identified by microarray screening (Busov 2001) were tested. cDNAs of the 14 auxin-induced clones were used as probes to measure the RNA abundance in mature and juvenile shoots 24 h after treatment, with or without 1.6 mM NAA. The experiment was performed twice.

We found that auxin induction of *5NG4* was significantly greater in juvenile than mature shoots (Table 2). Elevated *5NG4* mRNA levels in auxin-treated juvenile shoots could still be detected 5 days after treatment, while reversion to basal levels occurred as early as 3 days after treatment in mature shoots (Fig. 1). Some induc-

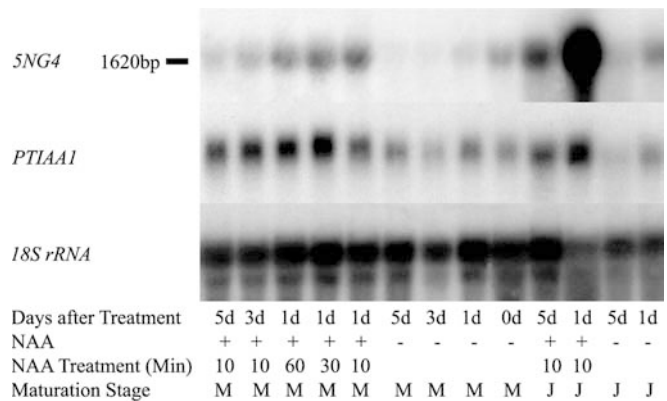


Fig. 1 Expression of *5NG4* in mature and juvenile shoots. Scions from 23-year-old, mature (M) and 1-year-old, juvenile (J) loblolly pine (*Pinus taeda*) trees were grafted onto 1-year-old rootstocks and continuously pruned under the same conditions for three growing seasons. Shoots from these stock plants were treated for the times indicated with (+) or without (–) 1.6 mM NAA and sampled at the time intervals indicated for RNA isolation and gel blot analysis. The blot was hybridized with ³²P-labeled full-length cDNAs of *5NG4* and pine clone *PTIAA1* (homologous to the *Aux/IAA* gene family), as well as an 18S larch rDNA for a loading control

February and October). Total RNA was extracted and 15 μg subjected to electrophoresis followed by northern blot analysis. Each blot was also hybridized with an 18S rDNA probe for the loading control. Numbers in the table show the relative expression after the correction for loading differences and the means of the two experiments. Standard errors are in parentheses

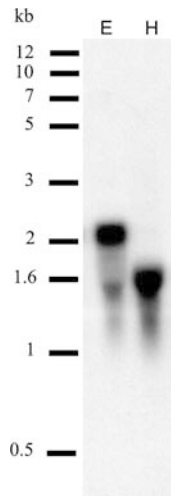
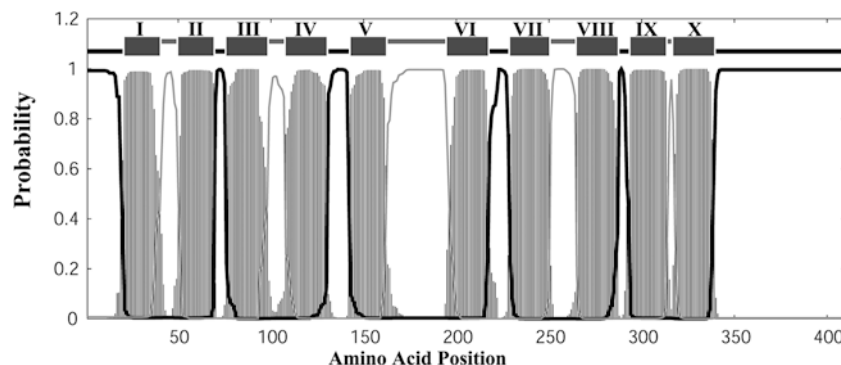


Fig. 2 Genomic Southern analysis showing the hybridization pattern of loblolly pine *5NG4*. Approximately 25 μ g of nuclear-enriched genomic DNA was digested with *Eco*RI (*E*) and *Hind*III (*H*) and hybridized with the 32 P-labeled full-length *5NG4* cDNA probe. Hybridization and washing at high stringency shows the probe hybridizing primarily to a single band

tion was also seen in the control juvenile shoots where, at 1 day after treatment, *5NG4* was expressed at a higher level than in mature shoots. This expression was reduced to basal levels by 5 days. None of the other 13 genes tested exhibited greater expression in juvenile than in mature shoots.

In a second experiment, we tested whether treating mature shoots with longer auxin exposures would result in *5NG4* transcript levels similar to auxin-treated juve-

Fig. 3 Prediction of transmembrane helices in the loblolly pine *5NG4* amino acid sequence. The full amino acid sequence of *5NG4* was analyzed using the THMMH (v.2.0) server (<http://www.cbs.dtu.dk/services/TMHMM/>) and the hidden Markov model. The *x*-axis indicates the position in the amino acid sequence starting at the N-terminal end and the *y*-axis indicates the probability of residing inside of, outside of, or in the membrane. Different positions relative to the membrane are coded as follows: *thick line* inside the membrane, *thin line* outside the membrane, and *solid bars* transmembrane helix. A schematic diagram of the protein is presented at the top of the graph. *Boxes* indicate transmembrane domains and are numbered consecutively by Roman numerals starting from the protein N-terminus



nile shoots. Mature shoots were treated for 10, 30 and 60 min with 1.6 mM NAA, and mRNA abundance was compared with that of juvenile shoots following a 10-min auxin pulse. The *5NG4* transcript levels in the longer auxin exposures did not exceed those in the 10-min treatment (Fig. 1). Moreover, the *5NG4* mRNA abundance in the juvenile shoots receiving the 10-min auxin treatment exceeded that of all the mature treatments. In contrast, the abundance of mRNA of *PTIAA1* increased following the 30-min auxin treatment in mature shoots (Fig. 1).

To determine whether the *5NG4* cDNA probe used in northern analyses was hybridizing to mRNA of the same locus, we conducted Southern blot analyses under the same stringency conditions and with the same probe as the northern blots. The single bands detected suggest that the expression patterns reported are those of *5NG4* (Fig. 2).

Predicted protein has 10 putative transmembrane helices

The cDNA of *5NG4* contains 1,629 nucleotides [excluding the poly(A) tail], corresponding to the length of the transcript detected in northern blotting (approx. 1,620 bp; Fig. 1), and encodes a predicted polypeptide of 410 amino acids with a calculated molecular mass of 44.98 kDa and an isoelectric point of 9.33. Topology analysis of the predicted protein structure indicates the presence of 10 putative transmembrane helices (Fig. 3). The helices are arranged in 2 groups of 5, separated by a 31-residue region that lies outside the membrane. The N- and C-terminal ends were predicted to reside inside the cytoplasm. Analysis of subcellular localization using the TargetP server of CBS (<http://www.cbs.dtu.dk/TargetP>) indicated that the protein might be targeted to the secretory pathway. Primary sequence comparisons to other membrane proteins and auxin influx and efflux carriers (e.g. AUX1, PIN1; Bennett et al. 1996; Galweiler et al. 1998; Luschnig et al. 1998; Chen et al. 1998) from *Arabidopsis thaliana* showed no significant homology. In addition, no significant homology was found to a recently identified loblolly pine gene encoding a nodulin-like transporter, and showing developmentally specific transcriptional regulation during embryogenesis (Ciavatta et al. 2001).

High degree of sequence conservation with proteins from other species

Similarity searches identified homologous proteins from *A. thaliana*, *Oryza sativa* and *Medicago truncatula*. The gene from *Medicago* was first identified as being induced during nodulation and so was classified as a nodulin (Gamas et al. 1996). The genes from *Arabidopsis* and *Oryza* were identified during genome sequencing projects and the corresponding putative proteins are of unknown function. Sequence alignments displayed a high degree of conservation among the homologous proteins (Fig. 4). Conservation was highest in the region of the transmembrane helices and lowest in the spacer region separating the first five helices from the second five and at the N- and C-terminal ends (Fig. 4).

A large gene family in *Arabidopsis thaliana*

We found 38 sequences encoding putative polypeptides similar (below the e^{-20} level of probability) to 5NG4 in the *A. thaliana* genome (Table 3). All of these appear to be transmembrane proteins, with the majority having 10 similar transmembrane domain structures to the pine clone (Table 3). Eleven of these genes are scattered across the 5 chromosomes as small clusters of 2–3 genes positioned next to each other and are probably the result of local duplications.

All of these proteins, except 4g19180 (ALO21687) are of unknown function (Table 3). 4g19180 shows homology to a class of enzymes known as apyrases (EC 3.6.1.5.) that hydrolyze both di- and tri-phosphate nucleotides to nucleotide monophosphates (Handa and Guidotti 1996), although the apyrase activity of the 4g19180 protein has yet to be demonstrated. The similarity of 4g19180 to other apyrase proteins is restricted to the C-terminal end, while it is the N-terminal portion of the protein that shows homology to the other 37 members of the family and 5NG4.

We performed phylogenetic analysis using all of the *Arabidopsis* family members, as well as *Oryza*, *Medicago* and 5NG4 predicted protein sequences (Fig. 5). The phylogenetic tree indicates four major lineages. The first one includes only one member, 3g45870. The second group consists of two *Arabidopsis* proteins, 5g45370 and 4g19180, the protein with the apyrase domain that was discussed above. The third distinct cluster, separated with very high bootstrap support includes three *Arabidopsis* proteins, the rice and pine proteins. The rest of the *Arabidopsis* proteins and the MtN21 (*Medicago*) protein are part of a remaining large group that contained several sub-groups. In several cases, *Arabidopsis* genes that are positioned next to each other on chromosomes were grouped together by the cluster analysis, supporting the hypothesis that these genes have indeed occurred through local duplications. For example, 1g11450 and 1g11460 are positioned next to each other on chromosome 1 and are clustered in one group and 5g40230 and

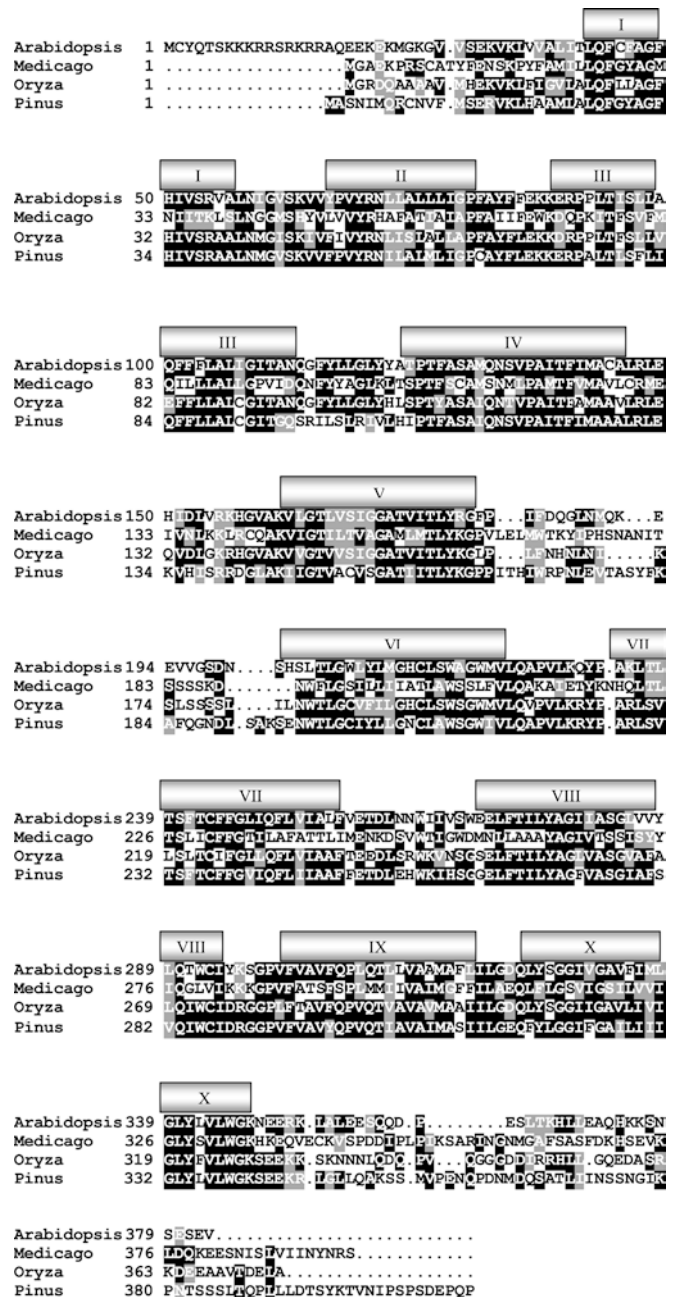


Fig. 4 Alignment of the deduced loblolly pine 5NG4 amino acid sequence with three angiosperm sequences. The deduced amino acid sequences for 5NG4, *Arabidopsis thaliana* (accession no. ABO20749), *Oryza sativa* (accession no. BAB17350) and *Medicago truncatula* (accession no. BAB02033) were aligned using the Pileup program from the GCG Package (Genetic Computer Group, Madison, WI). Output was produced using the program BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Positions where at least 50% of the residues are identical are printed in *white type*. Amino acids similar to the consensus are in *gray boxes*. Gaps introduced to improve alignment are indicated by *dots*. *Boxes above the alignment* indicate the position of the predicted transmembrane helix in 5NG4 and are numbered in Roman numerals starting from the N-terminal end

5g40240, positioned next to each other on chromosome 5, are grouped together by the cluster analysis (Fig. 5).

Table 3 Predicted transmembrane domains of 38 *Arabidopsis thaliana* proteins and sequence similarity to loblolly pine 5NG4. The full amino acid sequence of each *A. thaliana* protein, with a probability of sequence similarity of $P < e^{-20}$ was analyzed for the presence of transmembrane helices using the THMMH/CBS server (<http://www.cbs.dtu.dk>) and the Hidden Markov Model

Accession number	AGIcode ^a	Probability ^b	Transmembrane helices
ACOO6434	At1g75500	e-124	9
AB020749	At3g18200	e-102	10
AL132958	At3g53210	5e-075	8
AB010697	At5g07050	1e-052	10
AC004218	At2g39510	2e-052	10
AL080252	At4g08290	3e-052	10
AF160182	At4g30420	6e-052	9
AC013482	At1g21890	1e-050	10
AC002409	At2g40900	6e-050	10
AC003970	At1g09380	9e-049	10
AL162459	At3g45870	4e-048	8
ACO20576	At1g44880	9e-047	10
AL163972	At3g56620	9e-047	10
AT5g64700	At5g64700	9e-047	10
AL080252	At4g08300	1e-046	10
AB006704	At5g13670	6e-046	10
AF069300	At4g13670	2e-045	9
AC005896	At2g37460	6e-044	10
AC005896	At2g37450	6e-043	10
AT5g45370	At5g45370	6e-043	7
AC002376	At1g11450	2e-041	9
AL035524	At4g28040	4e-041	10
AP001314	At3g30340	1e-040	9
AC007323	At1g11070	1e-036	10
AC002376	At1g11460	2e-035	10
AF096370	At4g01450	3e-035	10
AF069300	At4g10430	5e-035	10
AL021687	At4g19180	8e-033	6
AT5g40230	At5g40230	2e-031	10
AB028616	At3g28050	8e-031	10
AC016447	At1g68170	2e-029	8
AC005966	At1g60050	1e-028	9
AT5g40240	At5g40240	3e-028	9
AC079374	At1g25270	6e-028	10
AT5g47470	At5g47470	2e-026	9
AC009526	At1g43650	2e-025	9
Z97335	At4g16620	5e-024	11
AC002062	At1g70260	4e-022	6

^a*Arabidopsis* Genomic Initiative (AGI) code: the first number represents the chromosome position (**bold**), followed by g (gene) and five-digit code, numbered from top to bottom of chromosome

^bThe probability that the similarity between 5NG4 and the corresponding protein can occur by chance

Induction is auxin-specific and localized to stems, hypocotyls and roots

Using northern blots, we characterized the expression pattern of 5NG4 in 2-week-old seedlings. Low levels of transcript were detected in all organs of the untreated seedlings (Fig. 6). Following auxin treatment, 5NG4 mRNA abundance increased markedly in hypocotyls and roots, but only slightly in cotyledons, and remained unchanged in needles. The overall expression levels following the auxin pulse were slightly lower in roots than in hypocotyls. To further characterize the regulation of 5NG4, we measured the expression response in shoots from pruned stock plants that were treated with NAA,

gibberellic acid, BA, ABA or heat shock. The 5NG4 mRNA abundance substantially increased only in the auxin treatment (Fig. 7).

Transient expression of a 5NG4::GFP fusion protein in tobacco leaves

To characterize the subcellular localization of 5NG4 we fused the full 5NG4 ORF to *gfp4* and transiently expressed the construct in tobacco leaves. For controls, we used GFP4, expressed in the cytoplasm and nucleus, and GFP5ER, which is targeted to the endoplasmic reticulum (Haseloff et al. 1997). All three coding regions were under the control of the *CaMV 35S* promoter.

GFP5ER fluorescence was observed in a cytoplasmic network and at the periphery of the nucleus, consistent with the distribution of the endoplasmic reticulum (Fig. 8a). As expected, GFP4 showed bright fluorescence in the nucleus and cytoplasm (Fig. 8b). The 5NG4::GFP4 fusion protein fluorescence was detected in the periphery of cells and in a series of small punctate structures (Fig. 8h,i; arrows) scattered throughout the cytoplasm (Fig. 8c-i; video sequence 1 in Electronic Supplementary Material).

Discussion

We have identified a loblolly pine auxin-inducible gene, 5NG4, that is differentially regulated in mature and juvenile-phase shoots. Although not completely repressed, 5NG4 was down-regulated in mature shoots. It is unlikely that this could have been the result of different rates of auxin uptake, because it was the only one of the 14 auxin-induced genes tested that showed reduced expression in mature shoots. Moreover, exposing mature shoots to auxin for longer time periods did not increase the transcript abundance of 5NG4, but did result in increased levels of another auxin-inducible gene, *PTIAA1*. Thus, it appears that 5NG4 is differentially regulated in ontogenetically mature and juvenile shoots. The nature of this regulation is not yet clear, but could be the result of a difference in availability of transcription factors, progressive methylation of the gene, or expression of the gene only in a particular tissue or cell type that is absent from mature shoots.

In this study, we did not find genes that were completely active or inactive in either the mature or juvenile phases. Our data and other studies (Hutchison et al. 1990; Woo et al. 1994) suggest that differential gene expression of many genes between the two phases has a quantitative character. Similar quantitative expression differences were reported for genes important for phase changes in annual plants. An *emf* (*embryonic flower*) mutant in *A. thaliana* causes flowering at the embryonic stage (Chen et al. 1997). Analysis of the activity of the corresponding gene (a putative transcription regulator) in wild-type plants indicates that it is expressed at a

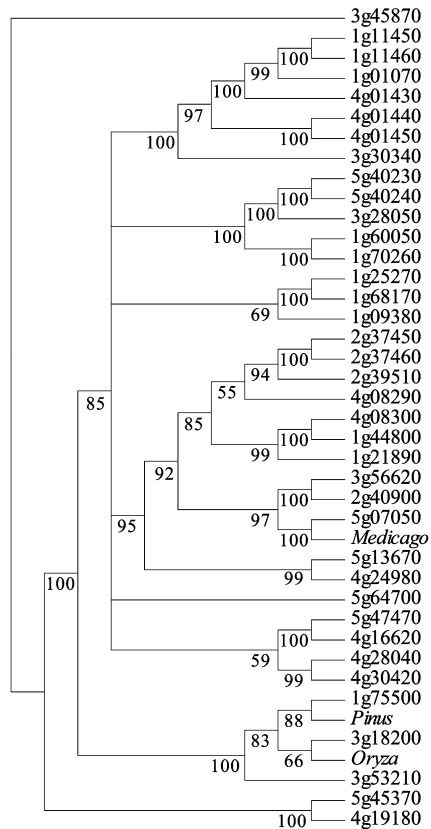


Fig. 5 Phylogenetic tree of loblolly pine 5NG4-related sequences in *Arabidopsis*, *Oryza* and *Medicago*. Phylogenetic analysis was performed using MEGA2 software (<http://www.megasoftware.net>). Sequences were aligned using Clustal W (<http://www.ebi.ac.uk/clustalw/>). Pairwise deletions were used to deal with gaps and the distance between sequences was estimated after Poisson correction. The unrooted tree was constructed using the neighbor-joining method. The bootstraps indicated at each joint point were created from 1,000 samplings. Nodes with less than 50% bootstrap confidence were collapsed. The *Arabidopsis* sequences are numbered according to the new *Arabidopsis* Genome Initiative (AGI) codes. The At abbreviation in front of each was omitted. The first number represents the chromosome position, followed by g (gene) and the five-digit code, numbered from top/north to bottom/south of the chromosome. *Pinus* (5NG4), *Oryza* (accession no. BAB17350) and *Medicago* (MtN21 accession no. BABO2033) were also used in the tree construction

20–30% higher level in mature, flowering parts than in other tissues (Aubert et al. 2001). A loss-of-function mutation *squint* (*sqn*) in *Arabidopsis* results in accelerated transition to adult vegetative phase (Berardini et al. 2001). The corresponding gene encodes a protein similar to cyclophilin 40 and was expressed in both mature and juvenile organs, but in different abundances. *LEAFY* is a gene important for transition to flowering in *Arabidopsis* (Blazquez et al. 1997). The expression of *LEAFY* in wild-type plants shows a gradual increase during plant transition from vegetative to reproductive development (Blazquez et al. 1997). Experiments with different numbers of *LEAFY* genes introduced into transgenic plants demonstrated that a certain expression threshold was necessary for flower initiation. However, the study also demonstrated that the threshold was not absolute, but

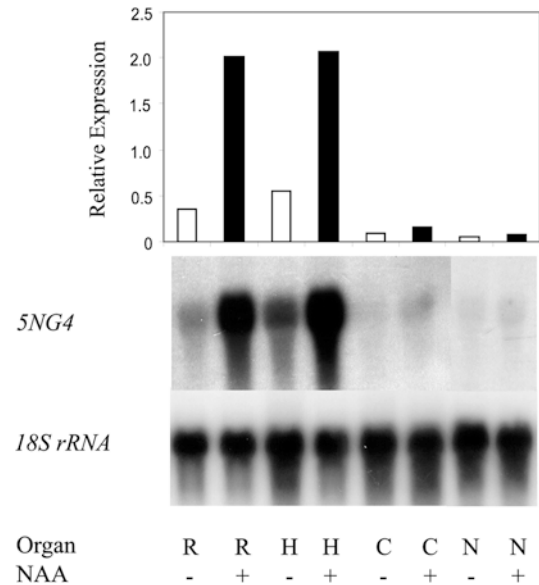


Fig. 6 Expression of 5NG4 in different organs of young loblolly pine seedlings. Total RNA was isolated from roots (R), hypocotyls (H), cotyledons (C) and needles (N) of 2-week-old seedlings treated for 10 min in 10% ethanol with (+) or without (-) 1.6 mM NAA. Total RNA (15 μ g) was separated by denaturing electrophoresis, blotted on a nylon membrane, and hybridized with 32 P-labeled full-length 5NG4 cDNA. Results are shown graphically as volume measurements normalized to an 18S ribosomal probe from larch. Filled bars represent auxin-treated samples and open bars controls

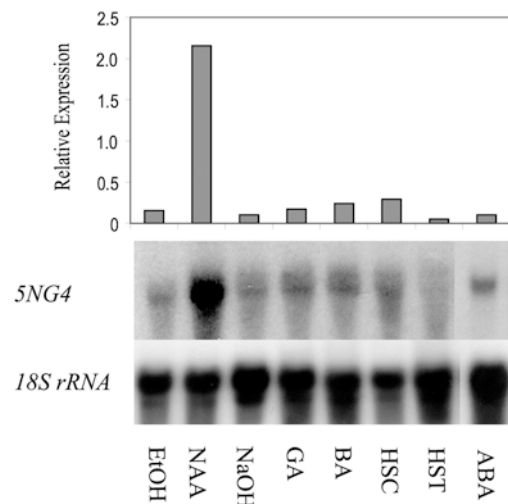


Fig. 7 Expression of 5NG4 in response to different growth regulators and heat shock. Shoots from 4-year-old continuously pruned loblolly pine stock plants were immersed for 10 min in the following solutions: 1.6 mM NAA, 1.6 mM gibberellic acid (GA), 1.6 mM BA, 3.2 mM ABA, 10% ethanol (EtOH; solvent for NAA and gibberellic acid) or 0.04 N NaOH (solvent for ABA and BA). Seedlings were also subjected to heat-shock treatment (HST) and control heat-shock treatment (HSC), and sampled 24 h after treatment (see text for details). Total RNA (15 μ g), isolated 24 h after the treatments, was subjected to northern blot analysis using 32 P-labeled full-length 5NG4 as a probe. Volume measurements are as described in Fig. 6

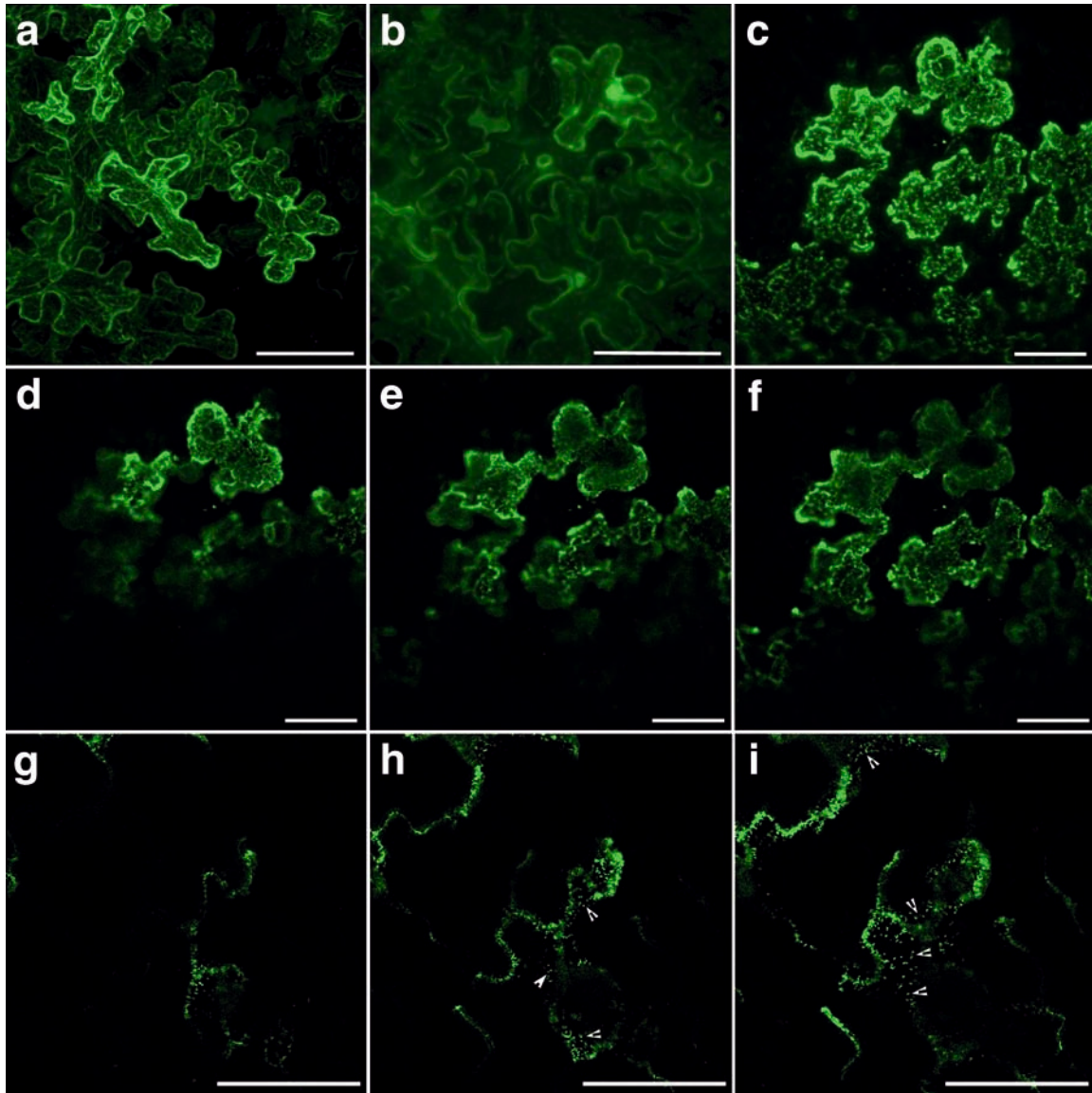


Fig. 8a-i Cellular location of transiently expressed GFP5ER, GFP4 and 5NG4 in tobacco (*Nicotiana tabacum*) epidermal cells visualized with confocal laser scanning microscopy. **a-c** Z-stack projections composed of 34 (**a,b**) or 53 (**c**) sections scanned in z-steps of 1 μm . **a** GFP5ER targeted to the endoplasmic reticulum (Haseloff et al. 1997); **b** GFP4, expressed in the cytoplasm and nucleus; **c** 5NG4::GFP4 fusion expressed in a punctate pattern around the cell periphery and throughout the cytoplasm. **d-f** 5NG4::GFP4 fusion, individual z-sections, numbers 10 (**d**), 22 (**e**) and 33 (**f**); full-stack projection shown in **c**. **g-h** 5NG4::GFP4 fusion, individual z-sections 5 μm apart, imaged at higher resolution (40 \times , N.A. oil immersion objective); *arrowheads* point at small punctate structures in the cytoplasm. Bars = 100 μm

relative to the abundance level of other yet unknown factors. These studies in annual and woody plants, including this report, suggest that the expression of many genes that change during phase transition vary quantitatively and that the relative amount of a particular gene product to other interacting factors can be critical for the initiation or inhibition of a particular developmental process.

The cDNA sequence of *5NG4* encodes a putative transmembrane protein. The protein is predicted to have 10 transmembrane helices and a central pore region. This protein structure is reminiscent of plant molecular transporters (Delrot et al. 2000). Comparisons with known auxin influx and efflux carriers and other known transporters from *Arabidopsis* showed no primary sequence similarity. This suggests that *5NG4* is most likely involved in the transport of another molecule that functions downstream of the auxin response. The transient expression of the *5NG4::GFP4* fusion in tobacco cells indicates that the protein accumulates in the periphery of the cell and in a series of punctate structures. The peripheral distribution supports the sequence predictions for plasma membrane localization. Although the identity of the punctate structures remains to be established, it is possible that they represent elements of the secretory pathway (for example Golgi cisternae). Our protein sequence

analysis also suggests that the protein may be targeted to the secretory pathway.

We found several homologous proteins from *Medicago truncatula*, *Arabidopsis thaliana* and *Oryza sativa* with high sequence conservation. The genes from *Arabidopsis* and *Oryza* were discovered during genome sequencing projects and are of unknown function. The protein from *Medicago* is induced during nodule development (Gamas et al. 1996). The high sequence conservation in evolutionarily distant species, including gymnosperms, monocots and dicots, suggests an essential function of this protein in plant growth and development. Because *Medicago* is the only species of the four that develops nodules, it is unlikely that the function is necessary only for nodulation. Other genes previously thought to be expressed specifically during nodulation are also expressed in nonsymbiotic organs of plants (Jorgensen et al. 1999). This suggests that nodule formation is a modification of a process or processes common to higher plants (Gualtieri and Bisseling 2000).

A large number of sequences (38) for homologous, inferred proteins were found in *Arabidopsis*. High levels of redundancy have been reported for other genes in *Arabidopsis* (Blanc et al. 2000) and are most likely the result of inter- and intra-chromosomal duplications (*Arabidopsis* Genome Initiative 2000). About 75% of the inferred gene products in the *Arabidopsis* genome share significant similarity with at least one other such protein from *Arabidopsis* (Bancroft 2000). We also found 6 *Oryza*, 20 *Medicago* and 6 *Populus* partial EST sequences that share significant similarity ($< e^{-20}$ level of probability) to 5NG4 (<http://www.tigrblast.tigr.com>, <http://www.biochem.kth.se/PopulusDB/>). As these sequencing projects were not yet complete at the time of this writing, the number of the recognized family members in these species may increase. This suggests that the multigene structure found in *Arabidopsis* may be a common organizational structure for this gene family in plants. The functional significance of multigene families may be 3-fold: (i) transcription and translation from numerous genes may result in a larger quantity of the protein; (ii) different proteins may have diverged in function; and (iii) different family members may provide a means for expression specificity through differential regulation (Durbin et al. 2000). Our data provide support for the latter two possibilities. Although most of the *Arabidopsis* proteins are of unknown function, one family member, 4g19180, has a domain with homology to apyrase enzymes that is not present in the other family members. In addition, phylogenetic analysis clustered 4g19180 with another family member in an isolated lineage and separated three other groups of proteins, suggesting possible functional divergence within the gene family. Many membrane proteins function in the context of multi-subunit structures (Isacoff et al. 1990; Baizabal-Aguirre et al. 1999). Interactions between different family members may impart different properties to the protein complex. These could be important for diverse functions in the wide variety of auxin responses. Alternatively,

there may be differential temporal and spatial regulation of the family members. Many plant gene families are regulated in tissue-, organ- or time-specific manners (Durbin et al. 2000; Reidy et al. 2001). We found that 5NG4 is highly induced by auxin in stems, hypocotyls and roots but not in needles and cotyledons. It would be informative to study members of the *Arabidopsis* family and to clone and test additional pine family members for tissue and organ specificity.

Several lines of evidence suggest that this gene may be involved in adventitious root formation. First, the down-regulation of the gene in mature shoots correlates with the loss of competence to form adventitious roots. Second, the gene was induced by auxin only in organs such as roots, hypocotyls and juvenile shoots that are competent to form either lateral or adventitious roots following an auxin pulse. In contrast, we did not observe the same level of induction in cotyledons and needles, which do not form roots. Finally, the derived protein sequence shows high homology to a nodulin from *Medicago*. Recent evidence suggests a direct connection between lateral root formation and nodulation (Woperies et al. 2000) and the former is a process with similarities to adventitious root formation (Cheng et al. 1995; Smith and Federoff 1995). Taken together, the expression pattern and sequence homology to a gene involved in a root-related process suggest a possible role of 5NG4 in adventitious root formation. However, we cannot rule out the possibility that 5NG4 functions in other auxin-mediated processes.

We report here the characterization and analysis of a gene sequence for an auxin-regulated nodulin-like protein from loblolly pine that is down regulated in mature-phase shoots and is predicted to encode a transmembrane protein. It shares sequence similarity with genes of a large gene family in *A. thaliana* and other plant species that all contain predicted transmembrane helices. Although the first gene in this family to be identified was isolated during nodulation in *M. truncatula*, the presence of the pine gene in a non-nodule-forming species, its specific induction by auxin, and its decreased expression in mature shoots suggest that it may have a more general developmental function, possibly related to lateral or adventitious root formation.

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