2002

Localization of Expansin Expression During Adventitious and Lateral Rooting in Response to Auxin in Loblolly Pine

Fuyu Xu

LOCALIZATION OF EXPANSIN EXPRESSION DURING ADVENTITIOUS AND LATERAL ROOTING IN RESPONSE TO AUXIN IN LOBLOLLY PINE

By

Fuyu Xu

B.S. Fujian Agriculture and Forestry University, 1984

M.S. Institute of Applied Ecology, Chinese Academy of Sciences, 1990

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Forestry)

The Graduate School

The University of Maine

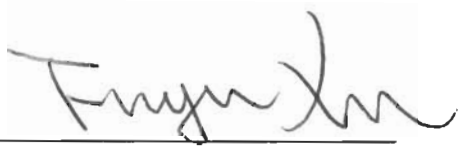
August, 2002

Advisory Committee:

Michael S. Greenwood, Ruth Hutchins Professor of Forest Tree Physiology and Professor of Forest Resources, Advisor
Katherine K. Carter, Associate Professor of Forest Resources
Keith W. Hutchison, Professor of Biochemistry
Mike Vayda, Professor of Biochemistry

LIBRARY RIGHTS STATEMENT

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at The University of Maine, I agree that the Library shall make it freely available for inspection. I further agree that permission for "fair use" copying of this thesis for scholarly purposes may be granted by the Librarian. It is understood that any copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Signature: 

Date: 8/23/2002

LOCALIZATION OF EXPANSIN EXPRESSION DURING ADVENTITIOUS AND LATERAL ROOTING IN RESPONSE TO AUXIN IN LOBLOLLY PINE

By Fuyu Xu

Thesis Advisor: Dr. Michael S. Greenwood

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
Degree of Master of Science
(in Forestry)
August, 2002

Loblolly Pine is the most important and widely cultivated timber species in the southern United States. Due to its fast growth, it is extensively planted for lumber and pulpwood. Vegetative propagation will enhance gains from genetic improvement of tree species. Rooted-cutting is at present the most reliable non-somatic embryogenesis method for cloning specific genotypes. However, an abrupt decline of adventitious rooting capacity has hindered the application of vegetative propagation in loblolly pine. Unraveling the rooting mechanism may facilitate a way to overcome this barrier.

Regulation of lateral and adventitious root formation by auxin has been demonstrated through the application of exogenous auxin to roots. Natural lateral root initiation may depend on the localization and redistribution of IAA at the root tip. We studied spatial and temporal responses of lateral root formation to exogenous NAA (1-naphthaleneacetic acid) on the primary roots of loblolly pine seedlings. A significant increase in the frequency and the growth of lateral root primordium (LRP) in response to NAA pulses could be detected at 24 and 48 h. The region 0.5~2.0 cm behind from root tip responds to auxin treatments, but the most responsive region is 0.5~1.5 cm with the

largest number of LRPs at both 24 and 48 h. Four poles of primary xylem are often seen in loblolly pine roots. Positioning of successive LRP formation in a given vascular pole is not random. The probability that the next LRP will develop in the same file is nearly 0. Treatment with an exogenous pulse of NAA increases the probability that the next primordium will form in the same file, which suggests that LRP formation may deplete the local auxin concentration in the vascular pole, lowering the probability of successive LRP forming close by.

In loblolly pine, there exists a stepwise decrease in auxin-induced rooting response from root, hypocotyl, to epicotyl in young seedlings. Competence to organize root meristems is normally confined to cells in pericycle or vascular parenchyma located centrifugal to primary xylem poles. Auxin can induce cellular reorganization and cell division in all parts of the seedling, but does not always promote the organization of root meristems in epicotyls. Expansins were found to be auxin-inducible while searching for auxin-induced genes specific to adventitious rooting. To investigate the localization and time course of expansin expression during adventitious and lateral root formation, 25-day-old hypocotyls, 50-day-old hypocotyls and epicotyls, and 10-day-old primary roots were treated with auxin at different concentrations. Non-radioactive *in situ* localization of expansin mRNA using digoxigenin-labeled probes was used to compare expansin expression at the cellular level in different parts of the seedling. Expansin expression was observed in the auxin-treated hypocotyl and epicotyl tissue, but no or a very weak signal was observed in the untreated tissue. In addition, the auxin-induced increase of expansin mRNA in 25-day-old hypocotyls and primary roots was highly localized to the region of the parenchyma from which adventitious roots will form. In the lateral rooting zone of

primary root, strong expression, without auxin treatment, occurred in the pericycle cells prior to lateral root primordium organization. Different patterns of expansin gene expression in response to auxin were found between hypocotyl and epicotyl cuttings. Relatively strong and localized expression in vascular parenchyma of hypocotyls contrasts with relatively weak and diffuse expression in cortex cells in epicotyl cuttings. A preliminary Western blot, detected the expansin protein in hypocotyls at both 24 and 48 h after auxin treatment but not in epicotyls. Collective results suggest auxin-induced expansin expression may play a role in both lateral and adventitious root formation in loblolly pine seedlings.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Michael Greenwood, my thesis advisor, for all his academic guidance, encouragement, and support in all of my time here at the University of Maine. Acknowledgement is also given to my other thesis committee members, Dr. Keith Hutchison, Dr. Michael Vayda and Dr. Catherine Carter for their constructive comments and suggestions over the course of this study.

Thanks also to Charlene Kozerow for her assistance with the cloning and other lab work.

I appreciate all the help from the faculty and staff in the Department of Forest Ecosystem Science and the Department of Biochemistry, Microbiology and Molecular Biology.

Finally, I would like to thank my wife and son for their patience, support and spiritual encouragement during my thesis research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter One:

SPATIAL AND TEMPORAL RESPONSES OF LATERAL ROOT FORMATION TO EXOGENOUS AUXIN IN LOBLOLLY PINE (*PINUS TAEDA* L.)

ABSTRACT

The regulation of the frequency and radial positioning of lateral roots on the primary roots is an unsolved basic problem in plant developmental biology. We studied spatial and temporal responses of lateral root formation to exogenous NAA (1-naphthaleneacetic acid) on the primary roots of loblolly pine seedlings. A significant increase in the frequency and the growth of lateral root primordium (LRP) in response to NAA pulses could be detected at 24 and 48 h. The region 0.5~2.0 cm behind from root tip responds to auxin treatments, but the most responsive region is 0.5~1.5 cm with the largest number of LRPs at both 24 and 48 h. Four poles of primary xylem are often seen in loblolly pine roots. Positioning of successive LRP formation in a given vascular pole is not random. The probability that the next LRP will develop in the same file is nearly 0. Treatment with an exogenous pulse of NAA increases the probability that the next primordium will form in the same file, which suggests that LRP formation may deplete the local auxin concentration in the vascular pole, lowering the probability of successive LRP forming close by.

INTRODUCTION

Lateral root formation is the primary way in which plants expand their root system and requires the coordination of many developmental processes such as cell division, cell expansion and differentiation. The frequency and placement of lateral roots are highly responsive to nutritional cues but auxin localization is a key factor in the regulation of LRP formation (Malamy and Ryan 2001). Lateral roots are initiated in pericycle parenchyma, the outermost layer of the vascular cylinder. Only some of pericycle cells, called founder cells, are competent to initiate lateral roots (Dubrovsky et al. 2000). These cells are predictably located with respect to the protoxylem poles, shorter and more mitotically active than other pericycle cells. In *Arabidopsis*, the pericycle cells that are competent to form lateral roots are immediately adjacent to the two protoxylem poles (Laskowski et al. 1995, Malamy and Benfey 1997a).

An auxin requirement for lateral root formation has been demonstrated by many studies (see Malamy and Benfey 1997b for review). Application of exogenous auxin to roots stimulates an increase of lateral root initiation (Blakely et al. 1988). In *Arabidopsis*, the *sur* ('superroot') and *rty* ('rooty') mutants, with significantly increased internal concentrations of indole-3-acetic acid (IAA), form many more lateral roots than wild-type plants (King et al. 1995). In addition, many auxin resistant mutants either lack lateral roots or have less than the normal number (Hobbie and Estelle 1995, Mirza et al. 1984). The normal process of lateral root formation is disrupted in the *Arabidopsis* mutant *alf4* such that lateral roots are not initiated even in the presence of exogenous auxin (Celenza et al. 1995). In the *alf3-1* mutant, lateral roots are initiated but die early in development, but the natural phenotype is fully restarted by exogenous IAA or other auxins. The auxin-

resistant mutants *axr1*, *axr4* and *aux1* exhibit drastically reduced numbers of lateral roots, which further demonstrates the critical role of auxin in lateral root formation (Hobbie and Estelle 1995; Timpte 1995). Furthermore, application of auxin transport inhibitors inhibits lateral root formation (Muday and Haworth 1994). The *tir3* ('transport inhibitor response') mutants have altered responses to polar auxin transport inhibitors and exhibit reduced numbers of lateral roots due to changes of IAA distribution (Ruegger et al. 1997), suggesting mutations that affect the polar transport mechanism also affect lateral root initiation.

The initiation and development of lateral root primordia are affected by many factors, some originating from the root tip and others from the shoot (Wightman and Thimann 1980). It is possible that these factors are transported through the vascular system of the primary root. Therefore there should be some relationship between the vascular system of the primary root and LRP positioning (Barlow 1984). LRP initiation sites have been studied in several species. Riopel's studies (1966, 1969) in banana and five other monocotyledons showed that the distribution of LRPs is non-random. Mallory et al. (1970) reported that the arrangement of LRPs along the primary root of the fern *Ceratopteris thalictroides* is highly ordered, arising alternately opposite one protoxylem pole or the other. However, *Victoria trickery* and *Eichhornia crassipes* have a greater number of vascular poles associated with a less ordered sequence of LRP formation. After working with several species whose roots contain a large number of vascular poles, Charlton (1975, 1982, 1983) suggested that the major feature of the distribution pattern is a rather regular spacing of LRPs along protoxylem-based ranks (or files). Within a given

file, LRPs are initiated in an orderly fashion due to mutual influences between neighbor LRPs.

Loblolly pine is a species of major economic importance and is the most widely planted conifer in the southern United States. Consequently, considerable effort has been put into genetic improvement of this species (Wright 1976). Rooted cuttings represent one of the most reliable and cost-effective vegetative propagation methods in tree improvement. However, adventitious rooting capacity by stem cutting declines rapidly in loblolly pine due to maturation (Greenwood and Nussbaum 1981; Greenwood and Hutchinson 1993; Diaz-Sala et al. 1996; Hamann 1998). The maturation-related decline in rooting could be due to loss of competence at the cellular level to respond to auxin. Rooting-competent cells are in some way more sensitive to auxin than incompetent cells and show a strong response in cell division after auxin treatment (Diaz-Sala et al. 1996). Lateral root initiation continues spontaneously in the root axis possibly in response to endogenous auxin, which provides a good system to study rooting mechanism.

Loblolly pine seedling primary roots have 3 to 5 poles of primary xylem, most commonly 4 (see Figure 1.1), each with centrifugal vascular parenchyma where lateral primordia usually form. The object of this study was to investigate the response of different regions of primary roots to exogenous auxin, as well as the lateral root distribution pattern in loblolly pine in relation to the vascular system of the primary root. The role of auxin in the placement of LRP is also discussed.

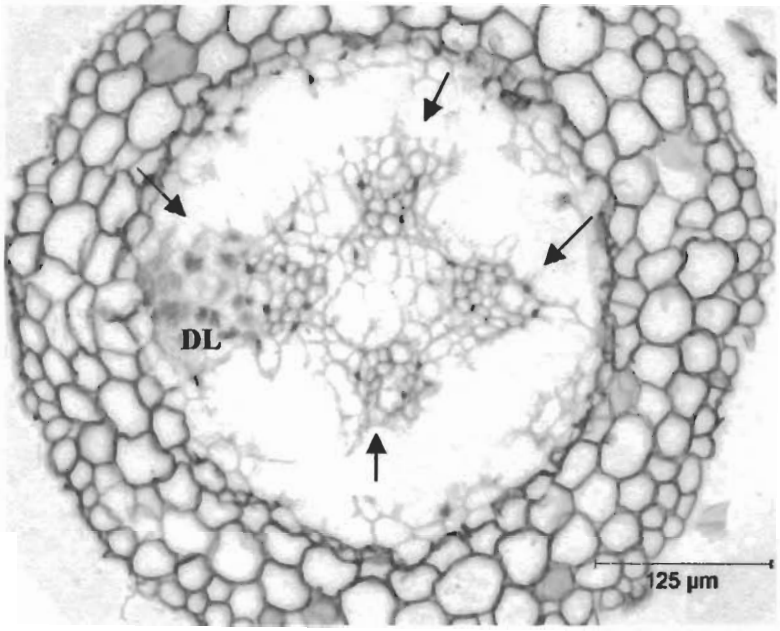


Figure 1.1 Cross section of primary root of loblolly pine showing four poles of vascular tissue. Roots of 10-d seedlings were fixed in FAA, embedded in paraffin, and sectioned at 12 µm thick. Arrows point to four poles of vascular tissue; DL stands for developing lateral root primordium.

MATERIALS AND METHODS

All experiments were performed using the loblolly pine half-sib family 8-05 (kindly provided by the Weyerhaeuser Co.). The seeds were stratified at 4°C for about 4~6 weeks, sterilized in 1% hydrogen peroxide, then sown in vermiculite in plastic flats in a growth chamber with a 16 h (28^0C)/8 h (18^0C) light/dark cycle. Seven-day-old seedlings of similar size were selected for all treatments. Primary roots of intact seedlings were pulsed for 5 minutes in 1, 5, 10, 25, and 50 mg/l NAA respectively, then put into 30 ml vials with distilled water in the growth chamber set as described above. 20 seedlings were used for each treatment.

Roots were detached after either 24 or 48 h at the root-hypocotyl junction and submerged in 5% chromium trioxide for 24 h for fixing and clearing. The roots were then rinsed in several changes of distilled water for 2-3 hours to permit visualization of LRP under a stereo dissecting microscope (Wild Heerbrugg). The number of lateral root primordia and their relative position were recorded. The lengths of the primary roots and distances between LRP were determined with transparent ruler under the petri dish. Each primary root was oriented with respect to the position the first LRP. The position of each subsequent LRP was recorded as occurring in the left file, right file or opposite file. The process was repeated acropetally to the root base. Both χ^2 ($\sum \frac{(Observed - Expected)^2}{Expected}$ (Steel et al. 1997)) and analysis of variance (using SAS) were used to evaluate the effect of auxin on the successive placement and frequency of LRP.

RESULTS

The response of LRP formation to different concentrations of exogenous auxin is shown in Figure 1.2. LRP frequency is increased 2 fold 24 h after treatment with 5 mg/l NAA pulse, and about 3 fold at 48 h after treatment with 25 mg/l NAA pulse. The ANOVA results showed that the increase in number of LRP between treatments is significantly different at both 24 and 48 h (24 h: P-value = 1.484×10^{-12}, F= $23.787>>Fcrit = 2.386$; 48 h: P-value = 9.998×10^{-18}, F= $43.393>>Fcrit = 2.386$). The maximum response of the LRP formation was reached at 24 h after 5 mg/l NAA 5 min pulse, and at 48 h after 25 mg/l treatment, respectively. It can be observed that the exogenous auxin treatment not only increases the frequency of LRP but also greatly increases the size of the LRP (see Figure 1.3).

Different regions of the primary root vary in their response to exogenous auxin treatment. In 7-d-old loblolly pine seedlings, the total average length of primary roots is 2.8 cm, and the region within 2.0 cm behind from the root tip responds to exogenous auxin, with the most responsive region located 0.5-1.5 cm at both 24 and 48 h (Figure 1.4 and 1.5). Beyond 2.0 cm there is little difference in LRP formation between the control and auxin treatments. The region 0.5-2.0 behind the tip contributes most of the LRP that form after NAA treatment. The region >2.0 cm only responded to the highest auxin concentration, especially at 48 h after auxin treatment (Figure 1.4 and 1.5).

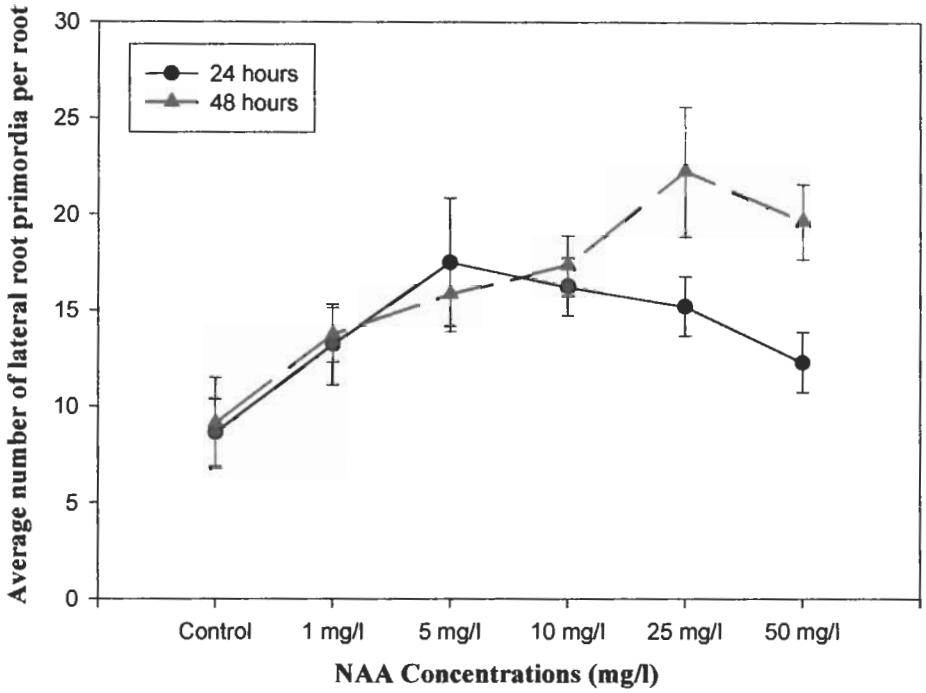


Figure 1.2 Effect of NAA concentration on the number of lateral root primordia formed on roots of 7 d loblolly pine seedlings. Measurements were made at 24 and 48 h after a treatment 5 min NAA pulse. Error bars represent standard errors of the mean (SE), n = 20.

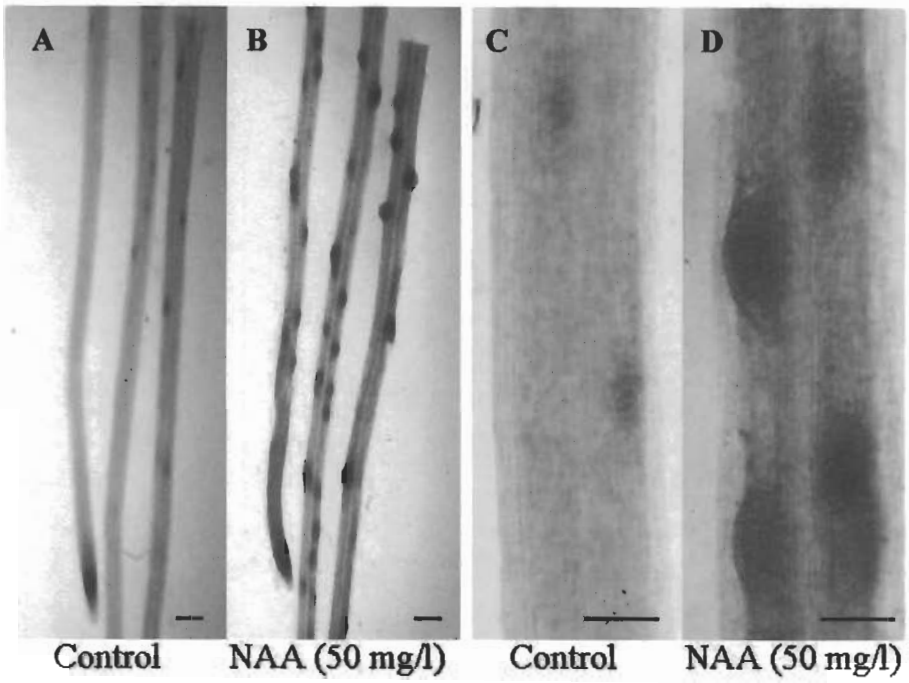


Figure 1.3 LRP formation in response to exogenous auxin at 48 h. Roots of 7 d loblolly pine seedlings were fixed and cleared in 5% chromium trioxide for 24 h. A: non-auxin treated (control), scale bar, 1 mm; B: 50 mg/l NAA 5 min pulse, scale bar, 1 mm; C: non-auxin treated (control), scale bar, 400 μm; D: 50 mg/l NAA 5 min pulse, scale bar, 400 μm.

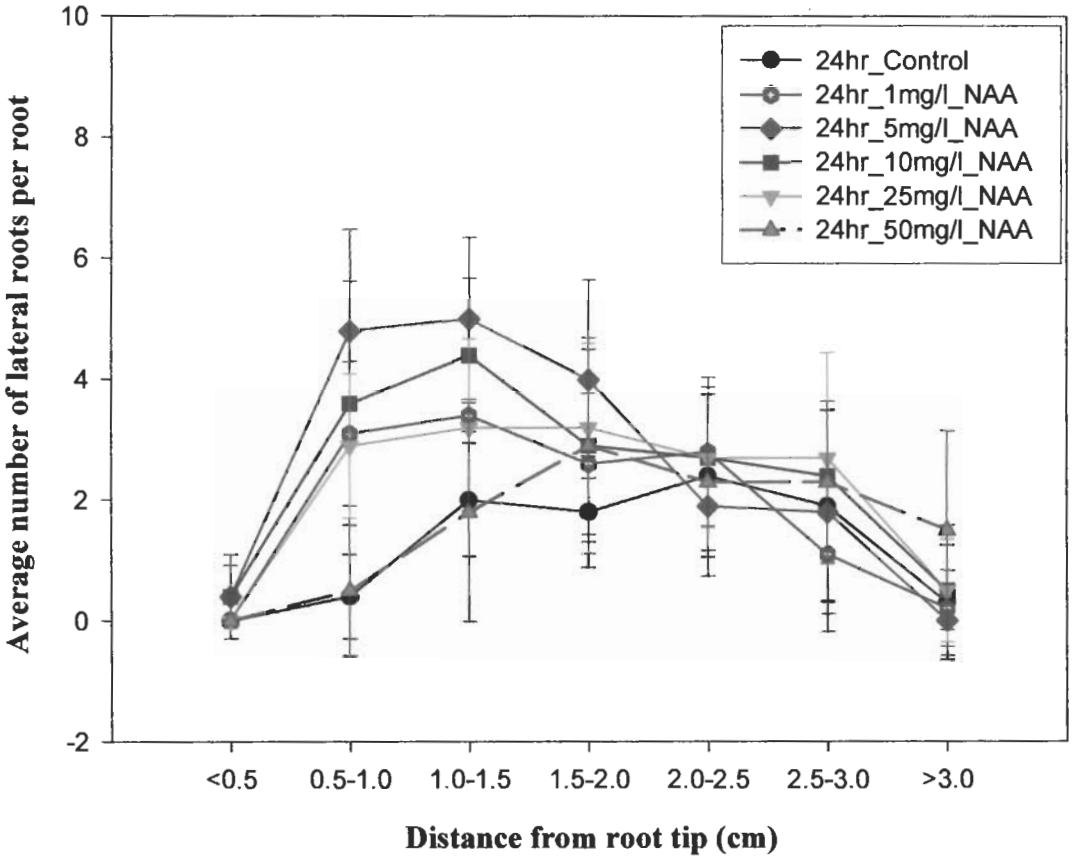


Figure 1.4 Variation of rooting responses to NAA pulses in different regions of the primary root at 24 h after treatment. Error bars represent standard errors of the mean (SE), n = 20.

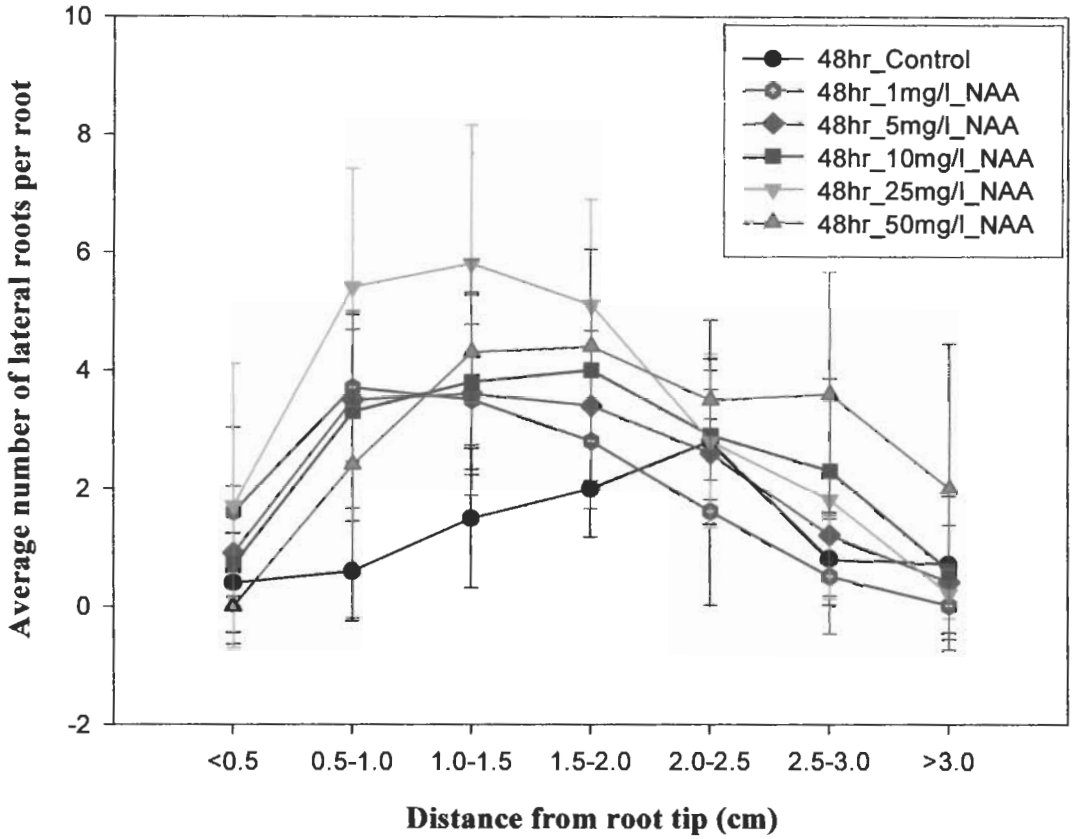


Figure 1.5 Variation of rooting responses to NAA pulses in different regions of primary root at 48 h after treatment. Error bars represent standard errors of the mean (SE), n = 20.

The results of the LRP placement frequency and χ^2 test results for 4 files in all treatments at both 24 and 48 h are listed in Table 1.1. The probability of next LRP placement in the same file is extremely low (χ^2 greatly exceeds the critical value of 7.81). However, placement among the other three files is random with an equal probability of the next LRP occurring in the left, right or opposite file (Table 1.2), in both non-auxin treated and auxin treated materials.

The effect of exogenous auxin treatment on distribution of the successive LRPs was also reflected with percentages of LRP placement in different files for all treatments (Table 1.3). The chance of successive LRP placement in the same file increases about 4 and 6 fold at the highest NAA concentrations after 24 and 48 h, respectively. ANOVA showed that the increase was statistically significant at $P<0.000$ (Table 1.3). ANOVA also shows that auxin significantly increases the probability of LRP occurrence in the 3 files ($P<0.01$). Clearly, exogenous auxin treatment alters the chance of next LRP occurring in all files.

Table 1.1 LRP placement and χ^2 test in 4 files

Treatments	Same	Left	Right	Opposite	χ^2	Probability
Control 24	1	30	29	18	27.949	3.7×10^{-6}
NAA 1-24	1	49	37	33	42.000	4.0×10^{-9}
NAA 5-24	5	65	48	50	47.571	2.627×10^{-10}
NAA 10-24	7	45	65	46	43.503	1.924×10^{-9}
NAA 25-24	8	54	50	38	34.640	1.451×10^{-7}
NAA 50-24	3	42	38	24	34.794	1.3×10^{-7}
Control 48	1	24	35	21	29.765	1.5×10^{-6}
NAA 1-48	2	51	42	33	42.563	3.0×10^{-9}
NAA 5-48	2	42	58	47	48.074	2.1×10^{-10}
NAA 10-48	7	60	57	43	42.509	3.1×10^{-9}
NAA 25-48	16	70	78	54	41.743	4.5×10^{-9}
NAA 50-48	11	56	66	46	38.408	2.3×10^{-8}

* If $\chi^2 \geq \chi^2(0.05, 3) = 7.815$, then frequencies are significantly different at $p< 0.05$

Table 1.2 χ^2 test LRP placement in other 3 files

Treatments	Left	Right	Opposite	χ^2	Probability
Control 24	30	29	18	3.455	0.1778
NAA 1-24	49	37	33	3.496	0.1741
NAA 5-24	65	48	50	3.178	0.2041
NAA 10-24	45	65	46	4.885	0.0870
NAA 25-24	54	50	38	3.648	0.1614
NAA 50-24	42	38	24	3.308	0.1913
Control 48	24	35	21	4.075	0.1304
NAA 1-48	51	42	33	3.857	0.1454
NAA 5-48	42	58	47	2.735	0.2548
NAA 10-48	60	57	43	3.088	0.2136
NAA 25-48	70	78	54	4.436	0.1088
NAA 50-48	56	66	46	3.571	0.1677

* If $\chi^2 \geq \chi^2(0.05, 2) = 5.991$, then frequencies are significantly different at $p< 0.05$

Table 1.3 Dose effect of auxin on lateral root positioning; percentages of the LRP placement frequency in all four files; the difference between treatments is significant when P-value is less than 0.05.

Treatments	Same file	Left file	Right file	Opposite file
Control 24	1.3%	38.5%	37.2%	23.1%
NAA 1-24	1.7%	40.0%	30.8%	27.5%
NAA 5-24	3.0%	37.2%	29.3%	30.5%
NAA 10-24	4.3%	27.6%	39.9%	28.2%
NAA 25-24	5.3%	36.0%	33.3%	25.3%
NAA 50-24	2.8%	39.3%	35.5%	22.4%
P-value*	0.0227	0.0427	0.0024	0.0002
Control 48	1.2%	29.6%	43.2%	25.9%
NAA 1-48	1.6%	39.4%	33.1%	26.0%
NAA 5-48	2.0%	28.2%	38.3%	31.5%
NAA 10-48	4.2%	35.9%	34.1%	25.7%
NAA 25-48	7.3%	32.1%	35.8%	24.8%
NAA 50-48	6.1%	31.3%	36.9%	25.7%
P-value*	1.8×10^{-6}	0.0122	0.0041	0.0113

* P-value, probabilities from ANOVA.

DISCUSSION

Response of LRP to exogenous auxin treatment is rapid

The response of LRP formation in loblolly pine to NAA pulses occurs at relatively low concentrations of NAA and can be detected at 24 and 48 h. These results are very similar to those from *Arabidopsis*, radish, tomato, and lettuce. Laskowski et al. (1995) reported that most of developing primordia of 2-4 cell layers were generated 24 hours after IAA application, and that there are more primordia from the auxin application than prior to the auxin treatment. In lettuce roots continually treated with NAA (10^{-5} *M*), 2-4 pericycle cells at the protoxylem poles divided periclinally at 20 hours, and periclinal and anticlinal divisions could be observed at 24 hours and large and distinct LRP were clearly visible at 72 hours (MacIsaac et al. 1989).

In loblolly pine, the lateral rooting response to auxin is much faster than that for adventitious rooting on hypocotyls from seedlings. Lateral rooting proceeds without exogenous auxin, but a low concentration of exogenous auxin can highly increase the frequency of LRP. In auxin-induced adventitious rooting by loblolly pine hypocotyls, rapid cell division can be observed only during 6 to 8 days after auxin treatment and organization of a root meristem takes 10 to 12 days in 20-day-old hypocotyls treated with a 500 mg/l NAA 5 min pulse (Diaz-Sala etl al. 1996). No roots can form without exogenous auxin treatment on hypocotyls.

Spatial distribution characteristics of LRP along primary root

The results of this study clearly indicate that LRP formation in loblolly pine does not randomly occur in pericycle cells adjacent to the 4 primary xylem poles. The position of successive LRP formation is highly related to the position of the previous formed LRP. There have been few studies on positioning of LRP formation since Riopel (1966) reported that in banana successive LRPs tended to occur more than 30° apart around the primary root, and within 30° were longitudinally further apart. He also found similar patterns in 5 other monocotyledons (Riopel 1969), i.e., lateral roots are formed in a nonrandom dispersed distribution in all cases, and pointed out that establishment of a root primordium in some way influences the position of subsequent LRP origin. Similarly in our study, the probability of successive LRP in the same file is dramatically reduced, but occurrences of the LRP are almost equally distributed among the other three files. Riopel (1969) explained that any change in the availability of those substances required for lateral development could substantially influence the positions where later laterals will arise. Therefore, the distribution of laterals in this way may be the result of a depletion of certain metabolites utilized by the previous LRP.

Distribution of LRP along primary root may be associated with the localized concentration of auxin. Our hypothesis is that lateral root initiation requires a localized threshold concentration of auxin, and its formation consumes most of this auxin, which drastically reduces the auxin amount at the next potential rooting position. In loblolly pine, the next LRP placement is much less on the same file than on other three, and application of exogenous auxin greatly increases the probability of an LRP forming in the same file. In addition, auxin increases the frequency of, and decreases the distance

between, LRP in all files. Auxin transport has been implicated in LRP formation, and failure of the transport mechanism reduces LRP (Casimiro et al. 2001), which implies that auxin is transported along each vascular pole, although the direction of the auxin transport, basipetal or acropetal, is still undetermined. Early investigators agree that the main root apex produces an inhibitor of lateral root formation, which leads to the formation of LRPs some distance behind the root tip in many species (McCully 1975). However, Casimiro et al. (2001) demonstrated that both root basipetal and leaf acropetal auxin transport activities are required during lateral root initiation. Nevertheless, the amount of auxin at a specific site is a function of previous consumption and the accumulation from continuing polar transport of auxin. That exogenous treatment of auxin increases the probability of placement of successive LRP on the same file supports this explanation.

There is considerable evidence that auxin provides positional cues for root patterning (Doerner 2000). Through studying the behavior of *Arabidopsis thaliana* mutants *axr1-3, axr1-12, pin1-1, eir1-1*, and *aux1-7*, effects of polar auxin transport inhibition, and laser ablation experiments, Sabatini et al. (1999) demonstrated that auxin is likely to be involved in providing positional cues required for distal meristem patterning, and also emphasized the role of auxin flux in plant patterning. Berleth and Sachs (2001), and Kuhlemeier and Reinhardt (2001) also reviewed the role of auxin in phyllotaxis and plant developmental pattering processes and pointed out that the auxins act as intercellular messengers in patterning processes in meristems and vascular development. We can speculate that the non-random distribution of LRP along the primary root is mainly caused by the consumption of the auxin at one position which

affects the accumulation or localization at the adjacent region on the same file. However, more studies at molecular and cellular levels are needed to elucidate the exact mechanism for the role of auxin in regulation of LRP positioning.

Chapter Two:

LOCALIZATION OF EXPANSIN EXPRESSION DURING ADVENTITIOUS AND LATERLAL ROOTING IN LOBLOLLY PINE (*PINUS TAEDA* L.)

ABSTRACT

During early seedling development, an abrupt decline of adventitious rooting capacity has hindered the application of vegetative propagation in loblolly pine. Unraveling the rooting mechanism may facilitate a way to overcome this barrier. In loblolly pine, there exists a stepwise decrease in auxin-induced rooting response from root, hypocotyl, to epicotyl in young seedlings. Competence to organize root meristems is normally confined to cells in specific locations, such as pericycle cells in roots or vascular parenchyma in hypocotyls located centrifugal to primary xylem poles. Auxin can induce cellular reorganization and cell division in all parts of the seedling, but does not always promote the organization of root meristems in epicotyls. While searching for auxin-induced genes specific to adventitious rooting, we found that auxins induce expansins, a family of extracellular proteins that can increase primary cell wall extensibility and function as wall-loosening enzymes for plant cell growth. To investigate the localization and time course of expansin expression during adventitious and lateral root formation, 25-day-old hypocotyls, 50-day-old hypocotyls and epicotyls, and 10-day-old primary roots were treated with auxin at different concentrations. Non-radioactive *in situ* localization of expansin mRNA using digoxigenin-labeled probes was used to compare expansin expression at the cellular level in different parts of the seedling. Expansin expression was observed in the auxin-treated hypocotyl and epicotyl tissue, but no or a very weak signal

was observed in the untreated tissue. In addition, the auxin-induced increase of expansin mRNA in 25-day-old hypocotyls and primary roots was highly localized to the region of the parenchyma from which adventitious roots will form. In the lateral rooting zone of primary root, strong expression, without auxin treatment, occurred in the pericycle cells prior to lateral root primordium organization, but decreased rapidly as the meristem organized. Different patterns of expansin gene expression in response to auxin were found between hypocotyl and epicotyl cuttings. Relatively strong and localized expression in vascular parenchyma of hypocotyls contrasts with relatively weak and diffuse expression in cortex cells in epicotyl cuttings. A preliminary Western blot, detected the expansin protein in hypocotyls at both 24 and 48 h after auxin treatment but not in epicotyls. Collective results suggest auxin-induced expansin expression may play a role in both lateral and adventitious root formation in loblolly pine seedlings.

INTRODUCTION

Loblolly Pine is the most important and widely cultivated timber species in the southern United States. Due to its fast growth, it is extensively planted for lumber and pulpwood. Therefore, considerable effort has been put into genetic improvement of this species (Wright 1976). Vegetative propagation will enhance gains from genetic improvement of tree species. Rooted-cutting technology is at present the most reliable non-somatic embryogenesis method for cloning specific genotypes. Adventitious rooting ability of stem cuttings allows the mass production of elite families for industrial plantations. However, adventitious rooting competence declines very quickly with maturation in loblolly pine (Diaz-Sala et al. 1996), even in the seedling stage. This has hindered the large-scale use of genetically improved families or clones.

Regulation of lateral and adventitious root formation by auxin has been demonstrated through the application of exogenous auxin to roots (Blakely et al. 1988), and by the fact that an *Arabidopsis* mutant with elevated internal concentrations of indole-3-acetic acid (IAA, the major auxin in higher plants) has a higher density of lateral roots than wild type plants (Boerjan et al. 1995). Natural lateral root initiation may depend on the localization and redistribution of IAA at the root tip (Bhalerao et al. 2002). In addition, many auxin resistant mutants either lack lateral roots or have less than the normal number (Hobbie and Estelle 1995, Mirza et al. 1984).

There are many genes involved in adventitious and lateral root formation. Two major stages of lateral and adventitious root formation have been observed and identified (Laskowski et al. 1995; Malamy and Benfey 1997a; Diaz-Sala et al. 1996), including dedifferentiation and proliferation of pericycle cells to form a primordium, and formation

of a root meristem. Expression of different genes during dedifferentiation to organization of root meristem is needed. Many genes associated with little after two stages of lateral and adventitious root formation have recently been reported. Keller and Lamb (1989) isolated the *HRGPnt3* gene encoding a novel cell wall hydroxyproline-rich glycoprotein. Localization of expression of this gene using GUS suggests it is involved in the initiation of lateral roots. Smith and Fedoroff (1995) described a gene, called *LRP1*, which is expressed in lateral and adventitious root primordia of *Arabidopsis*, and the expression of this gene is only limited at the early period of root primordium development and is shut down just before lateral roots emerge. An auxin-induced gene, *RSI-1*, was expressed in both lateral and adventitious root initiation in tomato, and its expression increased tenfold by 72h compared to that at 4 h after auxin treatment (Taylor and Scheuring 1994). Transcripts for one of the *PI-II* (proteinase inhibitor II) family members increased ten-fold at 72 h after auxin treatment, which occurred in tomato roots and hypocotyls but not in epicotyls (Taylor et al. 1993). Based on the results of differential screening, Neuteboom et al. (1999) pointed out that many genes encoding for extracellular proteins could be activated during auxin-induced lateral root formation. Chen et al. (1996) isolated two mRNA, designated *MII*-3 and *MII*-4, which are present in IBA-treated mungbean hypocotyl cuttings, but not present in control hypocotyls. Thus there are a number of examples of auxin-induced gene expression, whose products might be involved in the auxin-regulated adventitious root formation.

While searching for auxin-induced genes specific to adventitious root formation in loblolly pine using differential display-RT-PCR, Hutchison et al. (1999) found that auxins induce expansins in hypocotyl cuttings. Results of northern blots showed that

expansin mRNA levels increase during the early stages of induction of adventitious root formation in hypocotyls in response to the application of exogenous auxin and peak expression occurs at 24 h. Exogenous auxin increases expansin mRNA levels by 50 to 100 fold. Expansins are a highly conserved class of cell wall proteins that induce loosening of the primary cell wall (Cosgrove 2000). They enable wall loosening without any degradation of the polymers or an overall weakening of wall structure during expansion by disrupting the hydrogen bonds that exist between cellulose microfibrils and hemicelluloses allowing them to slide apart (McQueen-Mason et al. 1995, Cosgrove 1996).

Given that auxin induces both root primordium initiation and expansin expression, we compared expansin expression patterns at the cellular level in tissues with different rooting competence. One hypothesis would be that expansin expression occurs with or without auxin in roots, where laterals form without exogenous auxin, but depends on auxin in hypocotyls. Our objective is to correlate the expansin expression with rooting ability via contrasting the expression patterns in hypocotyls and epicotyls, and preliminarily defining the possible role of expansin expression in both lateral and adventitious root formation in loblolly pine seedlings.

MATERIALS AND METHODS

Plant materials

All experiments were performed using the loblolly pine family 8-05 (kindly provided by the Weyerhaeuser Co.). The seeds were stratified in refrigerator at 4°C for about 4~6 weeks, sterilized in 1% hydrogen peroxide, then sown in vermiculite in plastic flats. The growth chamber was set up with 16 hr (28^0C)/8 hr (18^0C) light/dark cycle. 10, 20, 25, 50-day-old seedlings were used for different treatments and studies, respectively. For each age, seedlings with similar development level were chosen for all treatments. Whole primary roots of intact 10-day-old seedlings were pulsed for 5 minutes in 5 and 50 mg/l NAA respectively, then put into 30 ml vials with distilled water. All vials were placed in the growth chamber set as described above, and left for 24 or 48 h. Hypocotyl and epicotyl cuttings from 20 or 50-day-old seedlings were treated with either 10 μM IBA or a 5 minute pulse of 500 mg/l (2.7 mM) α-napthalene acetic acid (NAA). One cm segments from the bases of the cuttings were cut at 24 and 48 hours after auxin treatment for thin sectioning for *in situ* hybridization. An identical set of control cuttings, treated with distilled water, was also prepared.

Preparation of Dig-labeled RNA probe

A digoxigenin (DIG) (Genius 4) RNA Labeling Kit (cat. No. 1175 025) was used to prepare DIG labeled RNA probes according to the manufacture's instructions (Boehringer Mannheim 2000). The clone DD21.4-1 was obtained using a gel-purified differential display-RT-PCR and its GenBank accession number is U64889 (Hutchison et al. 1999). This clone contains conserved expansin sequence (325 base pairs) in a

pBluescript vector, which allows for *in vitro* transcription using T3 and T7 RNA-polymerase. The template DNA was linearized with Hind III for generating an antisense probe while using T3 RNA-polymerase, and with Xba I for synthesizing a sense probe while using T7 RNA-polymerase.

In situ hybridization

In situ hybridization experiments were performed mainly according to the *Nonradioactive In Situ Hybridization Application Manual* obtained from Boehringer Mannheim and the protocol described by Fleming et al. (1993), with minor modifications. Briefly, plant tissues (segments of primary root, hypocotyl and epicotyl cuttings) were fixed in 50% FAA (Formalin : Acetic Acid : Ethanol) at room temperature for 16 hr, dehydrated in a graded series of ethanol and xylene, and then embedded in Paraplast Plus. Cross sections and longitudinal sections 12μM thick were cut with a rotary microtone and attached to Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) coated with poly-L-lysine. The sections were deparaffinized with xylene, and rehydrated through a graded ethanol series (100%, 95%, 85%, 70%, 50%, 30%, 15%). Then they were digested with 1 μg ml^{-1} protease K in 100 mM Tris-HCl, pH 7.5 and 50 mM EDTA at 37°C for 30 min, and dehydrated in graded ethanol and dried under vacuum for 2 hours. Sections were hybridized with sense or antisense probes in 50%(v/v) formamide, 300 mM NaCl, 10 mMTris-HCl, pH 7.5, 1 mM EDTA, 5% (w/v) dextran sulfate, 1% (w/v) Blocking Reagent (from Boehringer Mannheim), and 150 μg ml^{-1} tRNA in humid chamber at 42~50°C for 16 hours. After washing in 2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate) and 0.2× SSC for 20 min each at room

temperature, sections were treated with 2 μg ml^{-1} RNAse A in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 500 mM NaCl at 37°C for 30 min, and washed tree times in 0.2× SSC for 15 min each at room temperature. For immunological detection, the sections were subsequently treated with 1% (w/v) Blocking Reagent in 10 mM maleic acid-NaOH, pH 7.5, 150 mM NaCl, and 1% (w/v) BSA (Sigma, catalog #A7030) in TNT (100mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.3% (v/v) Triton-X 100) for 45 min at room temperature. Following the blocking reaction, sections were incubated with ani-digoxygenin-alkaline phosphatase conjugate (Boehinger Mannheim, 1:500 dilution) in 1% BSA solution at room temperature for 2 hours, washed three times in TNT for 45 min, and rinsed with alkaline phosphatase buffer (100 mM Tris-HCl, pH9.5, 100 mM NaCl, and 50 mM $MgCl_2$). The color reaction was performed in alkaline phosphatase buffer containing nitroblue tetrazolium/5-bromo-4chloro-3indolyl phosphate and stopped by placing the slides into 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. For making permanent slides, sections were dehydrated in graded ethanol, dipped in xylene, and mounted with Permount (Fisher Scientific). Sections were checked with a Zeiss microscope, and photographed with a digital microscope system SPOT 2e (Diagnostic Instruments, Inc.).

Western blot

Hypocotyl and epicotyl cuttings were treated with or without 10 μM IBA, and protein extracts were prepared 24 and 48 hours after auxin treatment. Proteins were extracted from cell walls according to the protocol of Li et al. (1993). After tissue homogenization, the cell walls were collected by filtration (Spectra/Mesh nylon mesh with 70 μM holes)

and expansin extracted with 1M NaCl with 20mM Hepes buffer, 2mM EDTA and 3mM sodium metabisulfite. The wall extracts were precipitated with 0.4g/ml of ammonium sulfate, and the resulting preciptate redissolved in extraction buffer and desalted with 30 KD Cetricon prior to SDS-polyacrylamide gel electrophoresis. 25 μg of protein was loaded on all acrylamide gels. Blotting onto nitrocellulose must be done with care since expansin can readily pass through the membrane. Next, horse serum blocking buffer was applied prior to the antiserum. Antiserum conjugated with alkaline phosphatase was used. Protein extract from 3-day-old expanding cucumber shoots was used as a positive control. The antisera prepared against cucumber expansin was a gift from Dr. Daniel Cosgrove in Penn State University.

RESULTS

Rooting responses to auxin by plant tissues with different rooting competence

Figure 2.1 and Table 2.1 clearly show a pronounced stepwise decrease in the auxin-induced rooting response from the root to the epicotyl. With auxin treatment, cuttings from hypocotyls of 20-50-day-loblolly pine seedlings root well within 30 days, while cuttings from epicotyls don't root at all in this period and auxin does not promote rooting. Hypocotyl cuttings do not root without exogenous auxin treatment. Lateral roots can occur from primary roots of seedlings without exogenous auxin treatment, and exogenous auxin significantly promotes both the lateral root initiation and growth, but the optimum response at a 10 fold lower auxin concentration (see Figure 2.1, Table 2.1). However, too high concentration of the auxin (3000 μM) hurt the delicate root materials and hindered the lateral root initiation, even caused rotten after 4-5 days (data not shown).

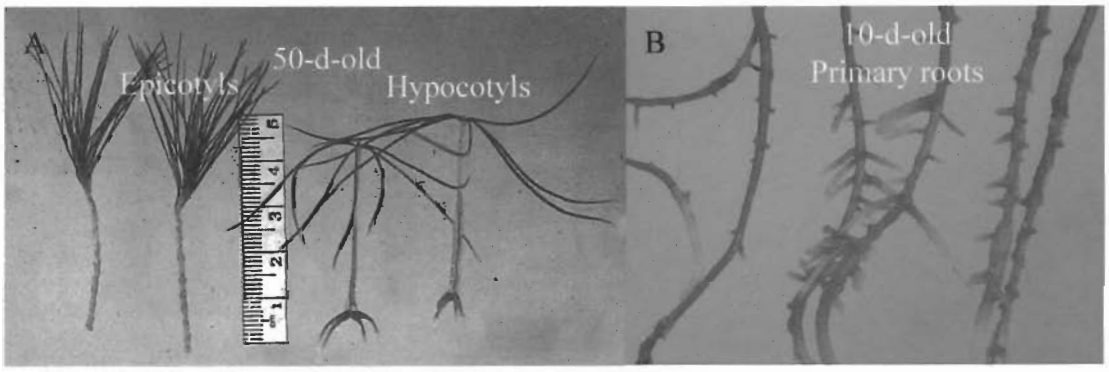


Figure 2.1 Auxins promote adventitious and lateral rooting in juvenile tissues but not in mature epicotyls. A: 50-day-old hypocotyl and epicotyl cuttings with NAA pulse; B: primary roots of 10-day-old seedlings with NAA 5 min pulses.

Table 2.1 Rooting responses to serial NAA pulses in tissues with different rooting competence[1]

NAA concentration[2] (μM)	Lateral rooting[3]	25-day-old Hypocoty rooting[4]	50-day-old Epicotyl rooting [4]
0	0	0	0
30	9	1	0
300	19	4	0
3000	0	18	0

Note: 1: rooting response is referred to average root number per cutting or primary root.

2: 5 min pulse in NAA solution, then placed to vials with distilled water.

3: result at day 6 after NAA treatment.

4: result at day 20 after NAA treatment.

Comparison of expansin expression between hypocotyl and epicotyl cuttings with auxin treatment

A comparison of expansin expression between hypocotyl and epicotyl cuttings after *in situ* mRNA hybridization, both in cross and longitudinal sections, is shown in Figures 2.2, 2.3, 2.4. The antisense probe for expansin mRNA showed strong hybridization signal (Figure 2.2A, 2.2C, 2.3A, 2.3C, 2.4), and the sense probe did not show any detectable signal in the corresponding areas (Figure 2.2B, 2.2D, 2.3B, 2.3D). In hypocotyls, localized expansin gene expression is clearly observed in the vascular parenchyma centrifugal to the resin canals (Figure 2.2A, 2.3A). However, in epicotyls the expansin expression is diffuse, evenly distributed over the cortical tissues (Figure 2.2A, 2.3A). The Western blot showed that the expansin protein was detected in hypocotyls but

not in epicotyls (Figure 2.4), but these results were not repeated and must be considered preliminary.

Localized expansin gene expression in lateral root formation sites

The distribution of expansin mRNA in the primary roots during lateral root formation is similar to that in hypocotyl cuttings, in that expansin expression was observed along the pericycle cells from which lateral roots will originate. A major difference is that expansin expression evident without auxin treatment (Figure 2.5, 2.6). In addition, the longitudinal distribution of expansin mRNA is consistent with the localization in cross sections. Furthermore, in both auxin treated and untreated roots expansin expression is localized in these lateral root formation sites (Figure 2.5A).

Most expansin expression can be observed in vascular parenchyma opposite four protoxylem poles. However, once lateral root primordia start to organize, the expansin expression declines (Figure 2.7). There is no longer much expansin expression after the primordium organizes (Figure 2.7, 2.8), and hybridization signal to antisense probe is not seen.

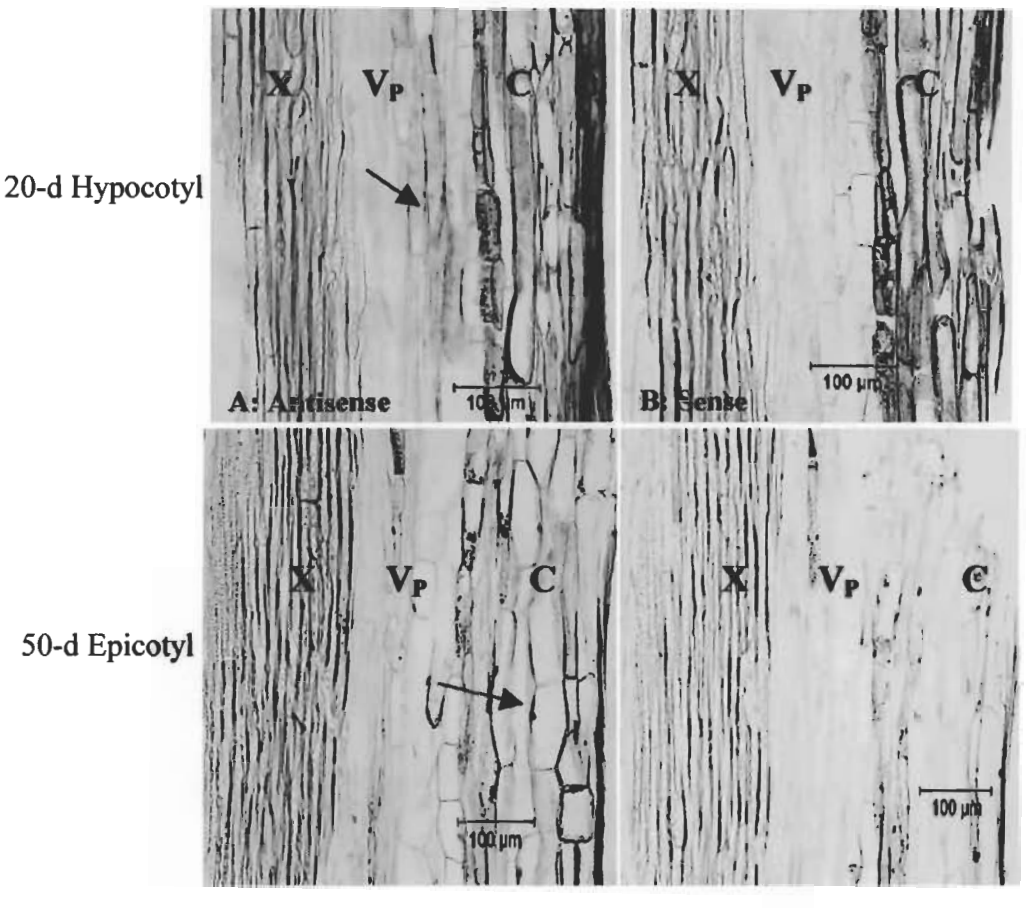


Figure 2.2 Comparison of expansin expressions in response to NAA between 20-day-old hypocotyls and 50-day-old epicotyls, 12 µm longitudinal sections. A: Hybridized with antisense probe, localized expansin expression in vascular parenchyma in 20-day-old hypocotyl cuttings; C: Localization of relatively weak diffusive expression of expansin in cortex in 50-day-old epicotyl cuttings; B, D: no hybridization signal when hybridized with sense probe. X=Xylem, V_P=Vascular parenchyma, C=Cortex. Arrows point to the hybridization signal (blue staining).

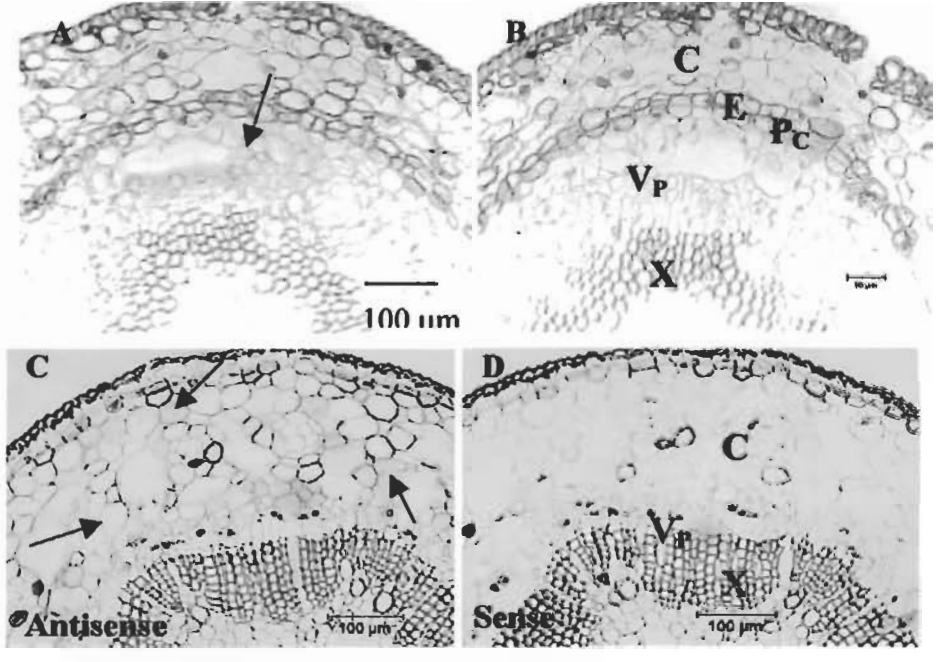


Figure 2.3 Comparison of expansin expressions in cross sections between hypocotyl and epicotyl **cuttings with auxin treatment.** A: Localized expansin expression **in vascular parenchyma in 20-day –** old hypocotyl cuttings; C: Expansin **expression is diffused over** in cortex tissue in 50-day –old **epicotyl cuttings; B and D are controls** hybridized with sense probe **for A and C, respectively. X=Xylem,** V_P=vascular parenchyma, P_C=**Pericycle, E=Endodermis, C=Cortex.** Arrows point to the hybridization **signal (blue staining).**

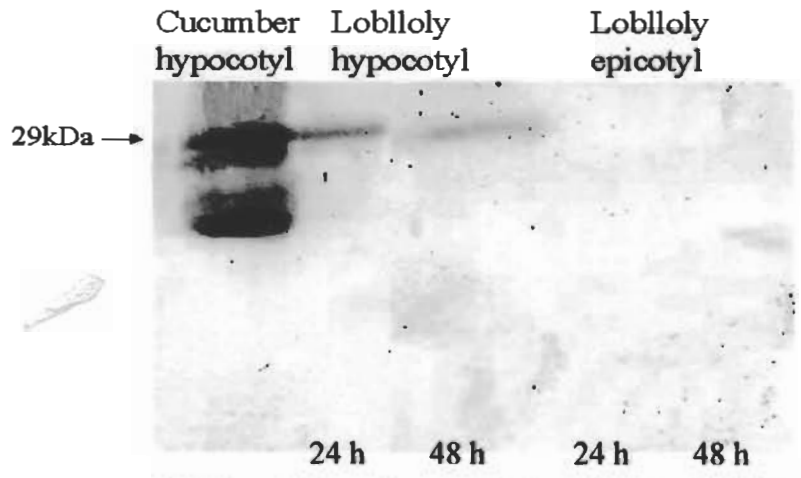


Figure 2.4 Western blots using cucumber antisera. A western blot was prepared using protein extracts from elongating cucumber hypocotyls, or fully elongated loblolly pine hypocotyls in the presence of 10□μM IBA, or fully elongated loblolly pine epicotyls in the presence of 10□μM IBA. The blot was probed with antisera prepared against cucumber expansin (gift from *from D. Cosgrove, Penn State*). Only one band with 29kDa could be detected in hypocotyls, but not in epicotyls.

Figure 2.5 Expansin expression in cross sections of lateral rooting zone without auxin treatment before LRP formation. In situ hybridization of expansin probe DD21.4 to cells in serial cross sections of 10-day-old primary roots without auxin treatment. A: hybridized with antisense probe; B: with sense probe. Arrows point to the hybridization signal (blue staining) in vascular parenchyma or differentiation primary xylem.

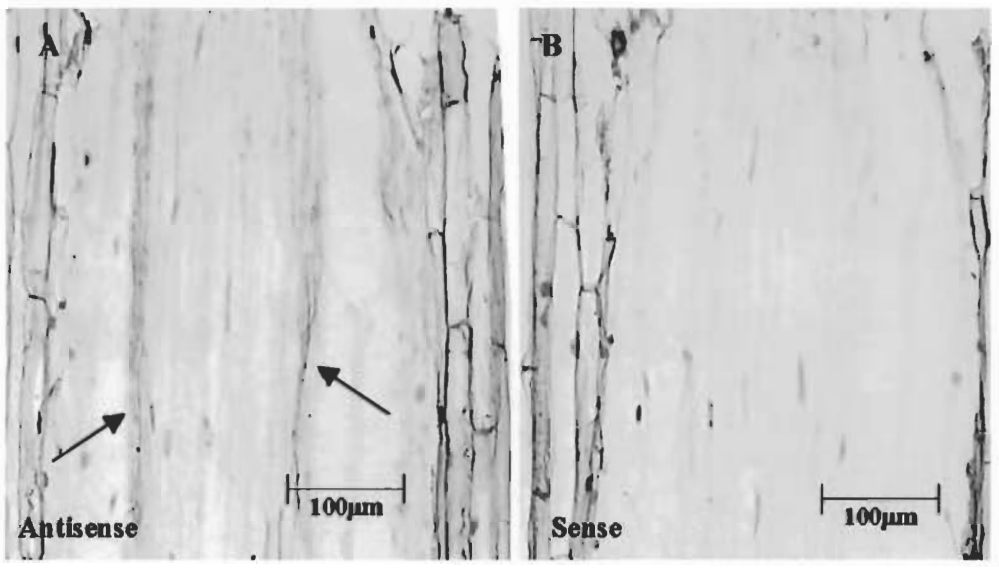


Figure 2.6 Expansin expression in response to 5mg/l NAA 5min pulse in longitudinal sections of lateral rooting zone at 48 hr after treatment. Expansin gene expression could be found along the pericycle cells when treated with antisense probe (A), but not with sense probe (B). Arrows point to the hybridization signal (blue staining) in vascular parenchyma.

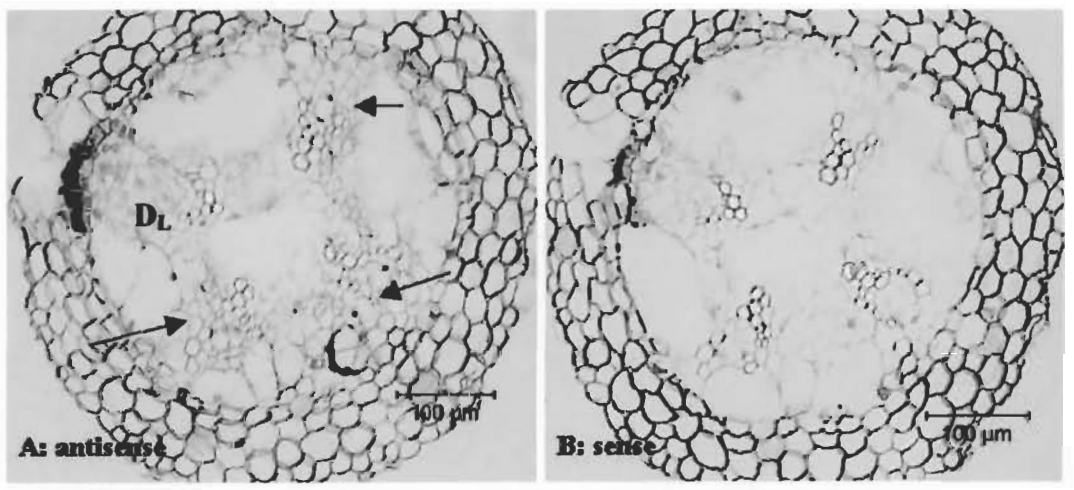


Figure 2.7 Expansin gene expression in cross sections of lateral rooting zones of primary roots without auxin treatment during lateral root initiation. More **expansin expression can be observed around four poles. A and B are** hybridized **with antisense and sense probe, respectively. DL=Developing LRP.** Arrows **point to the hybridization signal (blue staining) in vascular parenchyma.**

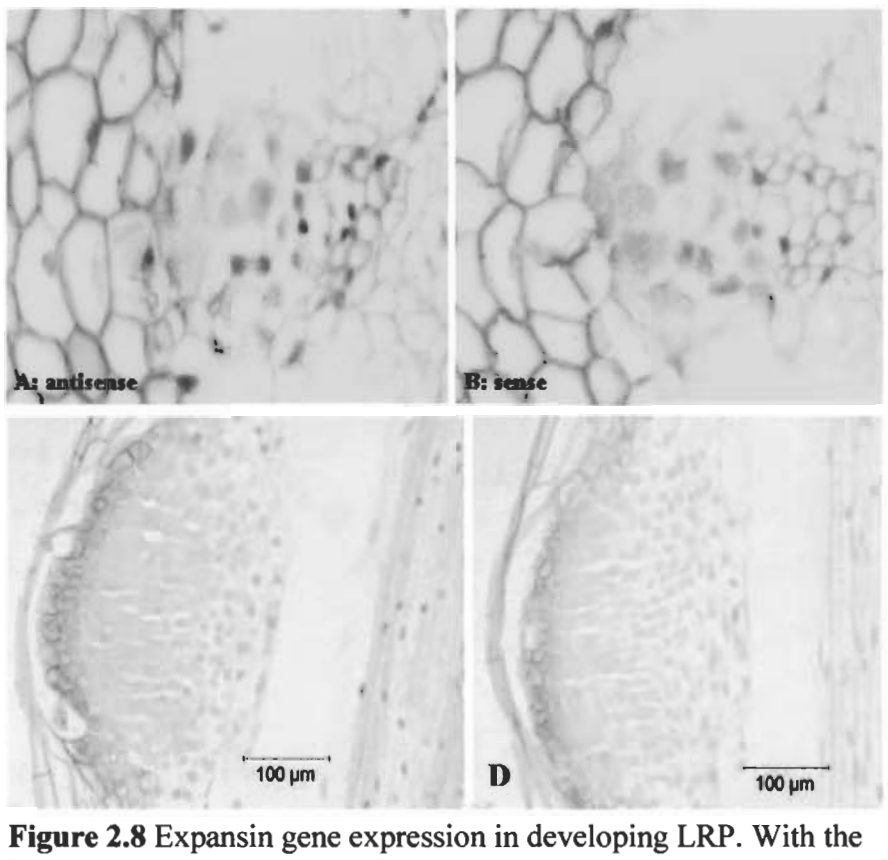


Figure 2.8 Expansin gene expression in developing LRP. With the lateral root primordium development, the expansin gene expression is decreasing rapidly. There is no longer much expansin expression during LRP development. A and C are hybridized with antisense probe; B and D are hybridized with sense probe.

DISCUSSION

Auxin can induce cellular reorganization and cell division, but does not always promote the organization of root meristems (Greenwood et al. 2001). In loblolly pine competence to organize root meristems is normally confined to pericycle cells of roots or vascular parenchyma centrifugal to 3-5 primary xylem poles in hypocotyls (Diaz-Sala et al. 1996). A stepwise decrease in rooting response to exogenous auxin from root to shoot in loblolly pine was demonstrated in this study. Quick loss in rooting competence by epicotyls is very common in conifers, unlike bean and other herbaceous plants where epicotyls root well (Friedman et al. 1979). Clearly, primary roots of loblolly pine seedling are more sensitive to exogenously applied auxin in terms of rooting response in that they respond to much lower concentrations. Variation of rooting responses to exogenous auxin by spatially different plant tissues reflects the declining of rooting capacity with maturation in woody plants that occurs in young seedlings. There is a considerable evidence that auxin regulates development by affecting gene expression through a signal transduction pathway (Guilfoyle et al. 1998, Macdonald 1997).

Expansins, which cause cell wall-loosening, cause cell enlargement and elongation of primary tissues (Cosgrove 2000, Fleming et al. 1997). Localized application of expansin in resin beads will induce leaf primordia on the apical meristem of tomato at the site of application (Fleming et al. 1997, 1999, Reinhardt et al. 1998), implying that the expansin may play a key role in the early stages of leaf initiation. Similarly, cell wall loosening and tissue expansion are also very critical to root formation, which results from cell dedifferentiation and subsequent root primordium formation, which requires changes in cell shape and cell division.

Different patterned expansin expression in hypocotyl and epicotyl cuttings may account for the reduced rooting competence in epicotyls. Since expansin is auxin inducible (Hutchison et al. 1999) and promotes loosening of the primary wall, it may play a key role in the early stages of root meristem formation. Thus localized expansin expression in vascular parenchyma or pericycle cells may be critical for adventitious rooting process. However, in epicotyl cuttings, diffuse expression of expansin mRNA is detected only in cortex cells which do not form roots.

Combined analysis of *in situ* hybridization results and the preliminary Western blot might indicate that expansin expression at the mRNA level does not necessarily result in active protein in all tissues. If these results can be repeated it may indicate that expression of expansin protein is under translational control. This may be the one of reasons why hypocotyl cuttings root readily and epicotyl cuttings don't root at all with auxin treatment.

Localized expansin expression was observed in pericycle cells or vascular parenchyma in lateral rooting zones of primary roots even without exogenous auxin treatment, similar to the auxin induced expression pattern in hypocotyls. This further demonstrates the significance of localized expansin expression in rooting sites for the lateral root initiation. Expansin expression in non-auxin-treated roots occurs in same tissues where the natural lateral rooting occurs, which may result from endogenous auxin, because the primary root is much more sensitive than hypocotyl or epicotyl cuttings.

Expansin expression decreased rapidly as the lateral root meristem organizes. Expansin affects the plasticity of the primary wall, and an increase in plasticity may precede cellular dedifferentiation that occurs prior to meristem organization. This may

mean that early expansin in some vascular parenchyma is required for lateral root initiation.

In summary, this study further demonstrated that the expansin expression is auxin inducible and mainly occurs in vascular parenchyma or pericycle cells centrifugal to the primary xylem poles, from which adventitious or lateral roots will form. In addition, auxin-induced expansin expression may be related to the tissue sensitivity in rooting competence, i.e., different expression patterns in epicotyl, hypocotyls and lateral root are consistent with their rooting capacity in response to auxin. Finally, stronger expansin expression before or during lateral root initiation might suggest the function of expansins in some way at early stages during adventitious and lateral root formation. Collective results show the role of expansin in the rooting process.

REFERENCES

Barlow, P. W. (1984) Positional controls in root development. *In* Positional controls in plant development (P. W. Barlow and D. J. Carr, Eds.). Cambridge University Press, Cambridge, UK, pp. 281-318.

Berleth, T. and Sachs, T. (2001) Plant morphogenesis: long-distance coordination and local patterning. Current Opinion in Plant Biology 4: 57-62.

Bhalerao, R.P., Eklof, J., Ljung, K., Marchant, A., Bennett, M. and Sandberg, G. (2002) Shoot-derived auxin is essential for early lateral root emergence in *Arabidopsis* seedlings. Plant Journal 29: 325-332.

Blakely, L. M., Blakely, R. M., Colowit, P. M. and Elliott, D. S. (1988) Experimental studies on lateral root formation in radish seedling roots. II. Analysis of the dose-response to exogenous auxin. Plant Physiology 87: 414-419.

Blakely, L. M., Blakely, R., Colowit, P. and Elliott, D. (1988) Experimental studies on lateral root formation in radish seedling roots. II. Analysis of the dose response to auxin. Plant Physiology 87: 414-419.

Boehringer Mannheim (2000) Nonradioactive In Situ Hybridization Application Manual. http://www.roche-applied-science.com/prod_inf/manuals/InSitu/InSi_toc.htm

Boerjan, W., Cervera, M. T., Delarue, M., Beekman, T., DeWitte, W., Bellini, C., Caboche, M., Van Onckelen, H., Van Montagu, M. and Inzi, D. (1995) *SUPER ROOT*, a recessive mutation in *Arabidopsis*, confers auxin over production. Plant Cell 7: 1405-1419

Brummell, D.A., Harpster, M.H. and Dunsmuir, P. (1999) Differential expression of expansin gene family members during growth and ripening of tomato fruit. Plant Molecular Biology 39: 161-169.

Casimiro, I., Marchant, A., Bhalerao, R. P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inze, D., Sandberg, G. Casero, P.J. and Bennett, M. (2001) Auxin transport promotes *Arabidopsis* lateral root initiation. Plant Cell 13: 843-852.

Celenza, J.L., Grisafi, P.L. and Fink, G.R. (1995) A pathway for lateral root formation in *Arabidopsis thaliana.* Genes & Development 9: 2131-2142.

Charlton, W.A. (1975) Distribution of lateral roots and sequence of lateral initiation in *Pontederia cordata* L. Botanical Gazette 136: 225-235.

Charlton, W.A. (1982) Distribution of lateral root primordia in root tips of *Musa acuminata* Colla. Annals of Botany 49: 509-520.

Charlton, W.A. (1983) Patterns of distribution of lateral root primordia. Annals of Botany 51: 417-427.

Chen, J., Wu, D., Witham, F.H., Heuser, C.W. and Arteca R.N. (1996) Molecular cloning and characterization of auxin-regulated genes from mungbean hypocotyls during adventitious root formation. Journal of the American Society for Horticultural Science 121: 393-398.

Cho, H.T. and Kende, H. (1997a) Expansins in deepwater rice internodes. Plant Physiology 113: 1137-1143.

Cho, H.T. and Kende, H. (1997b) Expansins and internodal growth of deepwater rice. Plant Physiol. 113: 1145-1151.

Cho, H.T. and Kende, H. (1997c) Expression of expansin genes in rice is correlated with growth. Plant Physiol. 114: 810-815.

Cho HT, Kende H (1998) Tissue localization of expansins in deepwater rice. Plant Journal. 1998. 15: 6, 805-812

Clowes, F. A. L. (1961) Duration of the mitotic cycle in a meristem. Journal of Experimental Botany 12: 283-293.

Clowes, F.A.L. (1981) The difference between open and closed meristems. Annals of Botany 48: 761-767.

Cosgrove, D.J. (1996) Plant cell enlargement and the action of expansins. BioEssays 18(7): 533-540.

Cosgrove, D.J., Bedinger, P.A. and Durachko, D.M. (1997a) Group I allergens of grass pollen as cell wall loosening agents. Proceedings of the National Academy of Sciences 94: 6559-6564.

Cosgrove, D.J. (1997b) Creeping walls, softening fruit, and penetrating pollen tubes: The growing roles of expansins. Proceedings of the National Academy of Sciences 94: 5504-5505.

Cosgrove, D.J. (1997c) Relaxation in a high-stress environment: The molecular bases of extensible cell walls and cell enlargement. Plant Cell 9: 1031-1041.

Cosgrove, D.J. and Li, Z.-C. (1993) Role of expansin in developmental and light control of growth and wall extension in oat coleoptiles. Plant Physiology 103: 1321-1328.

Diaz-Sala, C., Hutchison, K.W., Boldfarb, B. and Greenwood, M.S. (1996) Maturation-related loss in rooting competence by loblolly pine stem cuttings: The role of

auxin transport, metabolism and tissue sensitivity. Physiologia Plantarum 97: 481-490.

Doerner, P. (2000) Root patterning: Does auxin provide positional cues? Current Biology 10:R201-R203.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B. (1993). Cellular organization of the *Arabidopsis thaliana* root. Development 119: 71-84.

Dubrovsky, J.G., Doerner, P.W., Colon-Carmona, A. and Rost, T.L. (2000) Pericycle cell proliferation and lateral root initiation in *Arabidopsis*. Plant Physiology 124: 1648-1657.

Fleming, A.J., Mandel, T., Roth, I. and Kuhlemeier, C. (1993) The patterns of gene expression in the tomato shoot apical meristem. Plant Cell 5: 297-309.

Fleming, A.J., McQueen-Mason, s., Mandel, T., Kuhlemeier, C. (1997) Induction of leaf primordia by the cell wall protein expansin. Science 276: 1415-1418.

Friedman, R, Altman, A and Zamski, E (1979) Adventitious root formation in bean hypocotyl cuttings in relation to IAA translocation and hypocotyl anatomy. Journal of Experimental Botany 30: 769-777.

Greenwood, M.S., Cui, X. and Xu, F. (2001) Response to auxin changes during maturation-related loss of adventitious rooting competence in loblolly pine (*Pinus taeda* L.) stem cuttings. Physiolgia Plantarum 111: 373-380.

Greenwood, M.S. and Nussbaum, E.S. (1981) Rooting ability and vegetative growth performance of stem cuttings from one- and five-year-old ortets of loblolly pine. Proceedings of the 16th southern tree improvement conference, May 27-28, Blacksburg.

Greenwood, M.S. and Hutchinson, K.W. (1993) Maturation as a developmental process. *In* Clonal forestry. I. Genetics and biotechnology (M.R. Ahuja M.R and W.J. Libby, Eds.). Springer, Berlin Heidelberg New York, pp 14-33.

Guilfoyle, T., Hagen, G., Ulmasov, T. and Murfett, J. (1998) How does auxin turn on genes? Plant Physiology 118: 341-347.

Hamann, A. (1998) Adventitious root formation in cuttings of loblolly pine (*Pinus taeda* L.): developmental sequence and effects of maturation. Tree 12: 175-180.

Hobbie, L. and Estelle, M. (1995) The *axr4* auxin-resistant mutants of *Arabidopsis* define a gene important for root gravitropism and lateral root initiation. Plant Journal 7: 211-220.

Huang, J., Takano, T. and Akita, S. (2000) Expression of alpha-expansin genes in young seedlings of rice (*Oryza sativa* L.). Planta 211: 467-473

Hutchison, K.W., Singer, P.B., McInnis, S., Diaz-Salaz, C. and Greenwood, M.S. (1999) Expansins are conserved in conifers and expressed in hypocotyls in response to exogenous auxin. Plant Physiology, 120: 827-831.

Keller, B. and Lamb, C.J. (1989) Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation. Genes & Development 3: 1639-1646.

Keller, E. and Cosgrove, D.J. (1995) Expansins in growing tomato leaves. Plant Journal 8(6): 795-802.

King, J.J., Stimart, D.P., Fisher, R.H. and Bleecker, A.B. (1995) A mutation altering auxin homeostasis and plant morphology in *Arabidopsis*. Plant Cell 7: 2023-2037.

Kuhlemeier, C. and Reinhardt, D. (2001) Auxin and phyllotaxis. Trends in Plant Science 6: 187-189.

Laskowski, M.J., Williams, M.E., Nusbaum, H.C. and Sussex, I.M. (1995) Formation of lateral root meristems is a two-stage process. Development 121: 3303-3310.

Link, B.M. and Cosgrove, D.J. (1998) Acid-growth response and α-expansins in suspension cultures of bright yellow 2 tobacco. Plant Physiology 118: 907-919.

Macdonald, H. (1997) Auxin perception and signal transduction. Physiologia Plantarum 100(3): 423-430.

MacLeod, R. D. and Thompson, A. (1979) Development of lateral root primordia in *Vicia faba*, *Pisum sativum*, *Zea mays* and *Phaseolus vulgaris*: rates of primordium formation and cell doubling times. Annals of Botany 44: 435-449.

MacIsaac, S.A., Sawhney, V.K. and Pohorecky, Y. (1989) Regulation of lateral root formation in lettuce (*Lactuca sativa*) seedling roots: Interacting effects of α–naphthaleneacetic acid and kinetin. Physiologia Plantarum 77: 287-293.

Malamy, J.E. and Benfey, P.N. (1997a) Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. Development 124: 33-44.

Malamy, J.E. and Benfey, P.N. (1997b) Down and out in *Arabidopsis*: the formation of lateral roots. Trends in Plant Science 2: 390-396.

Malamy, J.E. and Ryan, K. S. (2001) Environmental regulation of lateral root initiation in *Arabidopsis*. Plant Physiology 127: 899-909.

Mallory, T.E., Chiang, S.H., Cutter, E.G. and Gifford, Jr. E.M. (1970) Sequence and pattern of lateral root formation in five selected species. American Journal of Botany 57: 800-809.

McQueen-Mason, S.J. and Cosgrove, D.J. (1995) Expansin mode of action on cell walls: analysis of wall hydrolysis, stress relaxation, and binding. Plant Physiology 107: 87-100.

McQueen-Mason, S., Durachko, D.M. and Cosgrove, D.J. (1992) Two endogenous proteins that induce cell wall extension in Plants. Plant Cell 4: 1425-1433.

McQueen-Mason, S.J. and Rochange, F. (1999) Expansions in plant growth and development: an update on an emerging topic. Plant Biology 1: 19-25.

McQueen-Mason, S.J. (1995) Expansins and cell wall expansion. Journal of Experimental Botany 292: 1639-1650.

MucCully, M.E. (1975) The development of lateral roots. *In* The development and function of roots (Torrey, E.G. and Clarkson, D.T., Eds). Pp. 105-124. Academic Press, New York, London.

Muday, G.K. and Haworth, P. (1994) Tomato root growth, gravitropism, and lateral development: Correlations with auxin transport. Plant Physiology and Biochemistry 32: 193-203.

Neuteboom, L.W., Ng, J.M., Kuyper, M., Clijdesdale, O.R., Hooykaas, P.J. and van der Zaal, B.J. (1999) Isolation and characterization of cDNA clones corresponding with mRNAs that accumulate during auxin-induced lateral root formation. Plant Molecular Biology 39: 273-287.

Reinhardt, D., Wittwer, F., Mandel, T. and Kuhlemeier, C. (1998) Localized upregulation of a new expansin gene predicts the site of leaf formation in the tomato meristem. Plant Cell 10: 1427-1437.

Riopel, J.L. (1966) Regulation of lateral root positions. Botanical Gazette 130: 80-83.

Riopel, J.L. (1969) The distribution of lateral roots in Musa acuminata 'Gros Michel'. American Journal of Botany 53: 403-407.

Rose, J.K.C., Lee, H.H. and Bennett, A.B. (1997) Expression of a divergent expansin gene is fruit-specific and ripening-regulated. Proceedings of the National Academy of Sciences 94: 5955-5960.

Ruegger, M., Dewey, E., Hobbie, L., Brown, D., Bernasconi, P., Turner, J., Muday, G. and Estelle, E. (1997) Reduced naphthylphthalamic acid binding in the *tir3*

mutant of *Arabidopsis* is associated with a reduction in polar auxin transport and diverse morphological defects. Plant Cell 9: 745-757.

Sabatini, S. Beis, D., Wolkenfelt, H., Murfett, J. Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P. Scheres, B. (1999) An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. Cell 99: 463-472.

Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C. and Weisbeek, P. (1994). Embryonic origin of the *Arabidopsis* primary root and root meristem initials. Development 120: 2475-2487.

Shcherban, T.Y., Shi, J., Durachko, K.M., Guiltinan, M.J., McQueen-Mason, S.J., Sheh, M. and Cosgrove, D.J. (1995) Molecular cloning and sequence anylysis of expansins - a highly conserved, multigene family of proteins that mediate cell wall extension in plants. Proceedings of the National Academy of Sciences 92: 9245-9249.

Smith, D.L. and Fedoroff, N.V. (1995) LRP1, a gene expressed in lateral and adventitious root primordia of *Arabidopsis*. Plant Cell 7: 735-745.

Steel, R.G.O., Torrie, J.H. and Dickey, D.A. (1997) *Principles and procedures of statistics: a biometrical approach*. –3rd Ed. McGraw-Hill, Inc. New York.

Taylor, B.H., Young, R.J. and Scheuring, C.F. (1993) Induction of a proteinase inhibitor II-class gene by auxin in tomato roots. Plant Molecular Biology 23: 1005-1014.

Taylor, B.H. and Scheuring, C.F. (1994) A molecular marker for lateral root initiation: the RSI-1 gene of tomato (*Lycopersicon esculentum* Mill) is activated in early lateral root primordia. Molecular & General Genetics 243: 148-157.

Timpte, C., Lincoln, C., Pickett, F.B., Turner, J. and Estelle, E. (1995) The *axr1* and *aux1* genes of *Arabidopsis* function in separate auxin-response pathways. Plant Journal 8: 561-569.

Wightman, F. and Thimann, K.V. (1980) Hormonal factors controlling the initiation and development of lateral roots. I. Sources of primordia-inducing substances in the primary root of pea seedlings. Physiologia Plantarum 49: 13-20.

Wright, J.W. (1976) *Introduction to forest genetics*. Academic Press. New York, 463 pg.

Wu, Y., Sharp, R.E., Durachko, D.M. and Cosgrove, D.J. (1996) Growth maintenance of the maize primary root at low water potentials involves increases in cell-wall extension properties, expansin activity, and wall susceptibility to expansins. Plant Physiology 111: 765-772.

APPENDICES

Appendix A: Protocol for RNA probe preparation

A) The probes:

Generally two probes are synthesized. The first probe hybridizes with the RNA of interest, and is generally called antisense, anti-mRNA, or (+) strand probes. You must also synthesize a control probe that will not hybridize with the RNA of interest. The most convenient thing to do is to synthesize an RNA probe in the opposite orientation as the anti-RNA probe. Probes of this type are called sense, mRNA, or (-) strand probes.

A typical experiment consists of 10 slides; 8 of these are hybridized with the anti-RNA probe and 2 are hybridized with the control probe.

B) Solutions:

1. 200 mM EDTA (DEPC-treated and autoclaved)

2. 4 M LiCl (DEPC-treated and autoclaved)

3. 200 mM Na_2CO_3 (pH = 11.4)--prepare just prior to use.

4. 200 mM $NaHCO_3$ (pH = 8.2)--prepare just prior to use.

5. 1 M NaOAc pH 4.7 (DEPC-treated and autoclaved)

pH must be adjusted with acetic acid

6. TE (DEPC-treated and autoclaved)

C) Materials:

1. Boehringer Mannheim RNA Labeling Kit (#1175 025)

2. 20 mg/ml RNase-free Glycogen (Boehringer Mannheim #901 393):

D) Template preparation:

- Digest with the appropriate restriction enzyme to linearize the template so that a "run-off" transcript can be generated. Avoid the use of enzymes that generate 3' overhangs--they can act as promoters for the polymerases. Set up reaction system as below:

	For Antisense Probe	For Sense Probe
10X buffer	2 µl (High)	2 µl (Medium)
DNA	? µg	? µg

DdH_2O	? µl (depends on the volume of DNA)	?
Enzyme	1 or 2 µl (XbaI)	1 µl (Hind III)
Total	20 or 50 µl	20 or 50 µl

- Centrifuge briefly, mix well
- Incubate in water block for 1.5—2 hours at 37°C
- Add 0.5M EDTA to a final concentration of 10 mM to stop the reaction
- Add 1/10 volume 3M NaAc and 2.5 volume EtoH (100%)
- Precipitate at –20°C at least 2 hr or overnight
- Centrifuge 10-15 min at 4°C
- Wash with RNAse free 70% EtoH (1/2 tube volume)
- Centrifuge 5 min at 4°C. Dry pellet at room temperature or 37°C in incubator
- Resuspend in EDPC-water or TE at about 1µg/µl

Measure linearized DNA concentration with spectrophotometer and check on the agarose gel

E) Probe synthesis:

1. Transcription reaction:

The 20 µl reaction described here should yield ~10 µg of DIG-labelled RNA. If more DIG-labelled RNA is required, scale up the reaction proportionally.

(a) Mix:

H_2O 12 µl (or depends on the volume of template DNA)

10x Transcription Buffer (vial 8) 2 µl

NTP Labeling Mix (vial 7) 2 µl

Template DNA 1 µl (1 µg)

RNase Inhibitor (vial 10) 1 µl (20 units)

(b) Add 2 µl of RNA polymerase (SP6, T3, or T7).

(c) Incubate at 37°C for greater than or equal to 2 hours.

2. DNase treatment:

(a) Add 2 µl of RNase-free DNase (vial 9).

(b) Incubate at 37°C for 15 min.

3. Add 2 µl of 200 mM EDTA to stop the reaction.

4. Precipitate by adding 2.5 µl of 4 M LiCl and 75 µl of Ethanol. Mix well and incubate at -20°C overnight. Do not add tRNA carrier because its presence will interfere with monitoring the hydrolysis reaction in step D.3.

5. Centrifuge, wash pellet 2X in 70% ethanol, and dry pellet. Resuspend in 100 µl of DEPC-treated H_2O. Let resuspend for 1-2 hours on ice.

6. Store frozen at -70°C or -20°C until ready for the hydrolysis step.

Note: Boehringer-Mannheim recommends a modified version of the transcription reaction if you are synthesizing probes of <500 bp. The modifications for a typical 20 µl reaction include: (1) using 500 ng gel isolated insert DNA as a template; (2) increasing the reaction time from 2 to 4-6 hrs.; (3) increasing the spermidine concentration by 1-5 mM; and (4) adding 40 U RNase inhibitor (Boehringer-Mannheim #799 017).

F) Probe Hydrolysis:

We reduce the size of our probes to allow better penetration into the tissue. Small probes (50-100 nt) give higher signals than larger probes (>150 nt). We chemically degrade our probes to a mean length of ~100 nt. The RNA probes are chemically degraded by incubation in a pH 10.2 carbonate buffer at 60°C.

1. Before doing the hydrolysis reaction, test the hydrolysis buffers by mixing 5 ml of H_2O with 2 ml of 200 mM NaHCO3 and 3 ml of 200 mM Na2CO3. The pH should be ~10.2.

2. Calculate incubation time:

Incubation time (in minutes) = (Lo - Lf) / (K)(Lo)(Lf)

t = hydrolysis in minutes

Lo = starting length in kb

Lf = final length in kb = 0.100 kb

K = rate constant for hydrolysis = .11 kb-1 min-1

Sample calculation, 1 kb transcript to a mean size of 100 nt:

[(1) - (0.100)]/[(0.11)(1.0)(0.100)] = 82 minutes.

3. Hydrolyze half of your probe:

(a) Take off a 2 µl sample as an unhydrolysed control.

(b) Take off 50 µl for hydrolysis.

(c) To the 50 µl of RNA, add:

20 µl 200 mM NaHCO3 (add first)
30 µl 200 mM Na2CO3

(d) Mix and incubate at 60°C for the calculated time in the paraffin oven.

(e) Stop the reaction by adding 10 µl of 1 M NaOAc pH 4.7.

4. Precipitate the probes by adding the following:

1 µl 20 mg/ml Glycogen (optional)
10 µl 4 M LiCl2
300 µl Ethanol

Incubate at -20°C overnight.

G) Prepare Probe for Hybridization:

1. Spin down the probe, wash 2X with 70% ethanol, and resuspend in 50 µl of TE.

2. There is no good way to quantify a DIG-labeled probe. We estimate the concentration of the probe two ways:

(a) Remove greater than or equal to 5 µl and run on a gel to check the size and to estimate probe concentration.

(b) [RNA] can be estimated by scanning a 1:50 dilution using 100 µl cuvette in the spectrophotometer. Expect to obtain between 100-200 µg/ml.

3. To determine the best [probe] to use, we do a pilot hybridization experiment using 200, 400, and 800 ng/ml per kb of probe complexity.

4. For long-term storage (months), it is essential that the probe be stored in hybridization solution. We usually dilute the probe to 10 µg/ml in hybridization solution for long-term storage. RNase inhibitor (1-2 µl) can also be added to inhibit possible contaminating RNases.

Appendix B: Modified Protocol for *in situ* hybridization with non-radioactive probe

FIXATION AND EMBEDDING

A) Required Materials:

Bulk 95% ethanol--use this for everything except the 100% ethanol steps
Bulk 100% ethanol --use only for the 100% ethanol steps
Glacial acetic acid
37% formaldehyde
20 ml glass scintillation vials (e.g. S/P Baxter #R2550-14)
Xylenes
Eosin Y; Eosin Yellow (Manufacturing Chemists #B286)
Paraplast Plus Chips (contains <1% DMSO)
Paraplast Chips
Index cards (3x5)
Superfrost Plus Treated Microscope Slides (Fisher # 12-550-15)
Dissecting needle

B) Required Equipment:

Oven set to 60°C
Eberbach Micro Slide Warming Table (product #2750; phone, 800-422-2558)
TissuePrep Flotation Bath (e.g. Lab-Line # 26103/Baxter # M7655) set to 42°C.
Slide warmer (e.g. Fisher # 12-594) set to 45-50°C.
Microtome

C) Fixation:

We have tried a variety of fixation procedures. For Arabidopsis floral tissue, the best signals come from tissue fixed in FAA. Floral tissue fixed in glutaraldehyde gives no signal. There are numerous reports that paraformaldehyde-fixed tissue gives the best signals. We have not compared FAA-fixed and paraformaldehyde-fixed tissue.

1. Fixative Mix:

	% in Mix	Amount Add
Ethanol	50.0%	50 ml
Acetic Acid	5.0%	5 ml
37% Formaldehyde	3.7%	10 ml
Water	41.3%	35 ml
		100 ml

2. Place 10-15 ml of fixative into 20ml scintillation vials.

3. Cut a cluster of flowers at the apex of the floral stem that includes stages 1-14, and immediately immerse in fixative. Cut so that 1-2 mm of floral stem is present--this helps to orient the tissue when sectioning. Cut off older flowers if you are not interested in these stages. If possible, cut tissue while immersed. Place as many pieces of tissue that will easily cover the bottom of a vial as a single layer of tissues.

4. The tissue will float in the fixative. If desired, the tissue can be forced down with a Nytex screen cut to the size of the scintillation vials.

5. Place tissue/fixative in a vacuum (>20 atm) for 15 minutes. Pull the vacuum very slowly (this should take at least 10 minutes). This step pulls the air out of the tissue, allowing the fixative to penetrate better. Usually, air bubbles will come out of the solution and get trapped in the trichomes, causing the tissue to float.

6. After 15 minutes, release the vacuum very slowly. Again this should take at least 10 minutes.

7. Gently swirl contents of vial to remove trapped air bubbles. At this point the tissue will usually sink when the second vacuum pulse is released. If the tissue does not sink, repeat steps 5 and 6 until the tissue does sink.

8. Incubate at room temperature until the tissue has been exposed to the fixative for a total of 16 hours. NO LONGER! Overfixation can result in the loss of signal.

9. Rinse out fixative--remove fixative and add 50% ethanol. Incubate at room temperature for 30 minutes. Repeat this step.

D) Dehydration:

The tissue must be completely dehydrated before exposure to xylenes; otherwise, the water and xylenes will form a white emulsion. We also stain the tissue with Eosin Y during these steps to help orient the tissue when sectioning. This is an optional step.

1. Remove 50% ethanol and replace with 60% ethanol. Incubate for 30 minutes.

2. Repeat for the following ethanol solutions: 70%, 85%, 95%.

3. Leave overnight in 95% ethanol with 0.1% Eosin Y.

4. Next day, remove as much as possible of the Eosin Y/95% ethanol and replace with 100% ethanol. Incubate for 1 hour. The tissue destains slowly in 100% ethanol, so do not incubate too long (e.g. overnight).

5. Remove as much as possible of the 100% ethanol, replace with fresh 100% ethanol, and incubate for 30 minutes. Repeat this step if it is impossible to remove virtually all of the solution in steps 4 and 5.

E) Clearing:

The tissue must be permeated with xylenes because paraffin is not miscible in ethanol. The tissue does not destain (Eosin) in xylenes.

1. Remove 100% ethanol and replace with 25% xylenes:75% ethanol. Incubate at room temperature for 30 minutes.

2. Repeat for the following xylene:ethanol solutions:

50% xylenes: 50% ethanol
75% xylenes: 25% ethanol

3. Remove the 75% xylenes and replace with 90% xylenes:10% chloroform. Incubate at room temperature for 1 hour.

4. Repeat step 3 two times. Fill the vial half full with 90% xylenes:10% chloroform the final time.

F) Infiltration:

Xylenes is typically used as an organic solvent for infiltration of paraffin. In the presence of 100% xylenes paraffin will sink to the bottom of the vial, resting on the tissue. By contrast, in the presence of 90% xylenes:10% chloroform, paraffin floats. For this reason, we use 90% xylenes:10% chloroform as an organic solvent to allow for a more gradual infiltration of paraffin into the tissue.

Preliminary step. Place a beaker full of Paraplast Plus chips in an oven at 60°C well in advance; it will take >5 hours to melt.

1. Add 20 chips of Paraplast Plus to each vial. Incubate overnight at room temperature. If in a hurry, go to step 2 without the overnight incubation.

2. Next morning, the paraplast will be only partially into solution. Place vials in a 42°C incubator. After about 30 minutes, the paraplast chips will be in solution.

3. Add 20 more Paraplast Plus chips, and incubate at 42°C until chips are in solution--about 30 minutes. Swirl occasionally.

4. Repeat step 3 until the vial is full (4-5 times; total of about 100 chips).

5. Pour off xylene/paraplast solution. Add molten Paraplast Plus. Swirl to mix. Incubate at 57-62°C for at least 10 hours.

6. Change the molten paraplast twice a day (morning and night; minimum 10 hrs. between changes) for 2 days. If you are able to remove almost all of the molten paraplast each time, 4 changes should be adequate. If your tissue floats and you are not able to remove most of the wax, change the molten paraplast a few extra times.

G) Pouring Boats:

1. Fold up an index card so that it has raised edges of about 1 cm. Tape the edges with Scotch tape inside and outside.

2. Place the boat on the hottest part of the Eberbach hot plate.

3. Pour the infiltrated tissue into the boat.

4. Top off the boat with molten Paraplast (not Paraplast Plus).

5. Arrange the tissue into a regular array using a dissecting needle. Orient tissue pieces with the stems either straight up (for transverse sections) or lying on their side (for longitudinal sections). Tissue pieces must be at least 5 mm apart.

6. Slowly move the boat to the cooler part of the hot plate. You probably will need to arrange the tissue again.

7. Label each boat by inserting a flagged string.

8. To harden, float the boat for greater than or equal to 1 hour in water.

H) Sectioning:

1. Cut out blocks of embedded tissue and mount onto microtome blocks.

2. Section tissue at 8-10 μm.

3. Cut ribbons into ~1.5 cm pieces. Float ribbon pieces on 42°C water for >1 minute. This step takes the compression out of the tissue.

4. Put slide in water just under the floating ribbon.

5. Bring slide up so as to catch the ribbon. Use a teflon-coated spatula to position the ribbon. Minimize the amount of water trapped between section and slide. Place slide on slide warmer for at least 30 minutes before adding more ribbon pieces. (Note: a convection or gravity air incubator set at 45-50°C can also be used to affix ribbons to slides).

6. Repeat for as many different ribbon pieces as you want to place on a given slide.

7. Incubate slide on a slide warmer greater than or equal to 24 hours (the longer the better) to "bake" the ribbon piece onto the slide. This is a very important step. This must be done immediately, and the slide warmer must be set to 45-50°C or the sections may fall off during the hybridization and wash steps. Use the dustcover.

HYBRIDIZATION

A) Solutions:

1. Set up 10 staining dishes containing the following ethanol solutions: 100%, 100%, 95%, 85%, 70%, 50%, 30%, 15%, H_2O, H_2O. Will need 250 ml of each solution if hybridizing less than or equal to 10 slides (small staining dishes) or 500 ml if hybridizing 11-20 slides (large staining dishes).

2. 20X SSPE:
Composition: 0.2 M NaH2PO4
20 mM EDTA
3 M NaCl

Amount Required: 100 ml per experiment
Formula for 1 L: 27.6 g NaH2PO4-H_2O
175.3 g NaCl
7.4 g EDTA

Adjust pH to 7.4, increase volume to 1 L

Autoclave

3. Proteinase K Solution:
Composition: 100 mM Tris (7.5)
50 mM EDTA

Amount Required: 250 ml for less than or equal to 10 slides
500 ml for 11-20 slides

Formula for 500 ml: 50 ml 1 M Tris (7.5) or 6.1 g Tris base
50 ml 0.5 M EDTA or 9.3 g EDTA

Adjust pH to 7.5, increase vol to 500 ml

4. 50% Dextran Sulfate

We use Dextran Sulfate from Pharmacia (# 17-0340-01)

Make up 25-50 ml and store in the refrigerator

NOTE: 50% dextran sulfate is difficult to pipet, especially when cold. Warm it up by placing in the microwave for a short time (~15 seconds)

5. 10 mg/ml RNase-free yeast tRNA

We use tRNA from BRL (# 5401)
Stored in the -20 freezer

6. 10X Hybridization salts:
Composition: 100 mM Tris (7.5)
10 mM EDTA
3 M NaCl

Amount Required: 1 ml per experiment
Formula for 100 ml: 10 ml 1 M Tris (7.5) or 1.2 g Tris base
2 ml 0.5 M EDTA or 372 mg EDTA
17.5 g NaCl

7. 10X Blocking Reagent (10X BR);

See Immunological Detection section for composition and formula
Need <1 ml per experiment

8. Hybridization Solution:
Composition: 50 % Formamide
300 mM NaCl
10 mM Tris (7.5)
1 mM EDTA
5 % Dextran sulfate
1 % Blocking reagent
150 µg/ml tRNA
500 µg/ml Poly A (only if hybridizing with cDNA clone sequences)

Amount Required: Use ~150 µl per slide for a surface area of 24 x 50 mm. For convenience, make up 10 ml at a time and store frozen.
Formula for 10 ml: 1.85 ml H_2O
5.00 ml Formamide
1.00 ml 10X Hybridization salts
1.00 ml 50% Dextran sulfate
1.00 ml 10X Blocking reagent
0.15 ml 10 mg/ml tRNA

9. Other Reagents/Materials:

Distilled Formamide (We use BRL # 5515)
Tupperware box for hybridization (22 x 33 x 6 cm). We bought our box from Life Science Products, Inc. (1-800-245-5774), product #T-541
Plastic test tube rack that fits in the Tupperware box. A 13 mm Nalgene Unwire rack (Baxter #S9248-13) works well, but it must cut in half to shorten its height
Coverslips
Sigmacote (Sigma #SL-2)

B) Affix sections to slides:

1. Before hybridizing, place the slides in a 45°C incubator overnight or longer to bake the sections onto the slides. Should be done immediately after sectioning--baking later does not help.

C) Set up for experiment:

1. Prewarm proteinase K solution to 37°C in a staining dish.

2. Set up staining dishes with alcohol solutions for dehydration step.

3. Remove Hybridization solution from freezer and warm to room temperature.

4. Treat coverslips with Sigmacote:

(a) Dip coverslips in Sigmacote and air dry.

(b) Dip coverslips in 100% alcohol and air dry.

D) Dewaxing:

1. Put slides in a staining dish slide holder.

2. Fill a staining dish with xylenes, and place a small stir bar at the bottom. Place slides into this staining dish.

3. Stir for 10 minutes. Stir very slowly--just fast enough to mix the solution a bit.

4. Repeat with fresh xylenes.

5. If you are processing many racks of slides, you can use the same two xylenes solutions.

E) Hydration:

1. Remove slides from xylenes and place into 100% ethanol (95% ethanol will not work). Dip up and down about 15 times or until the "streaks" go away. Incubate for 5 minutes to remove Eosin Y.

2. Repeat step 1.

3. Process the slides through the following ethanol solutions: 95%, 85%, 70%, 50%, 30%, 15%, H_2O, H_2O. Once again, dip up and down 15 times or until the "streaks" go away. Begin with the 95% ethanol and end with the H_2O, so as to hydrate the tissue gradually. If the sections are still orange in the 85% solution, leave in 85% for no longer than 5 minutes.
4. Change the last water for each rack of slides.

F) Proteinase K digestion:

Proteinase K is used to partially digest the tissue to allow better probe penetration. This step increases the signal. For Arabidopsis floral tissue, the best incubation time is 30-45 minutes. Less time gives a weaker signal, and greater time tends to destroy the morphology of the tissue. The timing was calibrated with a Proteinase K solution that was prepared just before the incubation.

1. Prewarm the Proteinase K Solution to 37°C in a staining dish.

2. Add Proteinase K to the prewarmed solution to a final concentration of 1 µg/ml. We make up a fresh 5 mg/ml solution and add 50 µl of this to 250 ml of solution (100 µl to 500 ml if using large staining dishes).

3. Incubate at 37°C for 30 minutes.

4. After the incubation, remove the slides from the Proteinase K solution and place into a staining dish filled with H_2O. Dip up and down a few times to rinse off the Proteinase K. Repeat.

G) Dehydration

1. Process the slides through the following ethanol solutions: H_2O, 15%, 30%, 50%, 70%, 85%, 95%, 100%. You can use the same solutions used in step E except for the 100% solution.

2. Air dry sections in a desiccator under vacuum for about 1 hour.

H) Hybridization

1. Look at the slides and rank them according to quality. Plan to hybridize ~8 slides with the anti-mRNA probe and ~2 slides with the control probe. Use the best 2-3 slides for

hybridizing with the anti-mRNA probe. Be sure to save some reasonably good slides for hybridizing with the control probe.

2. Apply Hybridization solution to slides:

(a) Add probe to the hybridization solution. The amount added should be optimized for each probe preparation (see Probe Synthesis section, step II.G)

(b) Pre-warm the hybridization solution to 45°C. This makes it easier to spread.

(c) Elevate one side of the slide about 1 cm and apply the hybridization solution in a pool to the elevated side of the slide. Spread evenly with a yellow tip. This step is made easier by using a lot (200 µl) of hybridization solution. The amount required depends upon the number of sections on the slide.

(d) Cover slide with a Sigmacote-treated coverslip. Avoid bubbles--if you get a lot of bubbles, start over.

3. Place slides in a humidified box and incubate overnight at 42°C. Place several layers of wet paper towels at the bottom of the tupperware box. Elevate the slides using plastic test tube racks cut to fit the box. If the plastic racks are not available, use a pair of 10 ml plastic pipets.

WASH

A) Solutions:

1. 20X SSPE (see above)

2. RNase Buffer:
Composition: 10 mM Tris (7.5)
1 mM EDTA
500 mM NaCl

Amount Required: 250 ml for 6-10 slides

Formula for 500 ml: 5 ml 1 M Tris (7.5) or 0.6 g Tris base
1 ml 0.5 M EDTA or 0.2 g EDTA
14.6 g NaCl

Adjust pH to 7.5, increase vol to 500 ml

3. 10 mg/ml RNase Stock Solution:

Add RNase Buffer directly to the bottle to achieve a concentration of 10 mg/ml. Store frozen at -20°C. This is a 2000X stock. This RNase stock will be good for >1 year. We use Ribonuclease A from Sigma (catalog # R-5503).

4. 10X PBS:
Composition: 1.3 M NaCl
30 mM NaH_2PO4
70 mM Na_2HPO4

Amount Required: 50 ml

Formula for 1 L: 76.0 g NaCl
3.6 g NaH_2PO4
9.9 g Na2HPO4

Autoclave

B) Other Materials:

Tupperware boxes for washes
Slide mailers (Baxter # M6271)

C) Set up for wash:

1. Prewarm 100 ml of the RNase buffer to 37°C.

2. Thaw the RNase stock solution.

3. Prewarm 3L of 0.2X SSPE to 50°C (2L for use before RNase step, 1L after)

D) Remove coverslips and hybridization solution:

1. Fill two 150 - 250 ml beakers with 2X SSPE. Dip each slide up and down in the first beaker until its coverslip comes off. Dip each slide a few times in the second beaker of 2X SSPE.

2. Place slides into a staining dish containing 2X SSPE. After all of the slides have been processed, proceed to the next step.

E) First High Stringency Wash:

1. Wash slides in 1 L of 0.2X SSPE at 55°C for 1 hour with gentle agitation (can do in tupperware).

2. Transfer slides to a slide mailer containing RNase buffer preequilibrated to 37°C. Rinse slides 2 X in RNase buffer, 37°C.

F) RNase Treatment:

This step significantly reduces background. However, DIG-labeled RNA is apparently more sensitive to RNase than 35S-labeled probes. We did an experiment using 0, 5 µg/ml, and 25 µg/ml RNase and found that 0 or 5 µg/ml RNase gave about equal levels of signal and background, but that there was very little signal at 25 µg/ml RNase. We suggest first using 5 µg/ml RNase. Increase the RNase concentration if you have background problems; decrease the concentration if you have problems with low signals.

1. Prewarm 70 ml of the RNase Buffer to 37°C (35 ml per disposable plastic tube).

2. Thaw the 10 mg/ml RNase stock, dilute to 5 µg/ml by adding 17.5 µl to the 35 ml of prewarmed RNase Buffer, and mix by swirling gently.

3. Using a disposable plastic pipet, dispense 16 ml (16 ml for 5 slides--more solution if fewer slides or you can fill the empty spots with blank slides) of 5 µg/ml RNase to each of 2 slide mailers.

4. Place slide mailers in a 37°C water bath and incubate for 30 minutes.

5. After the 30 minute incubation, pour 5 µg/ml RNase directly into sink drain and rinse slides in slide mailers two times with the remaining RNase buffer (pre-warmed to 37°C also.)

6. Toss the slide mailers into the trash and treat all glassware with DEPC.

G) Second High Stringency Wash:

1. Place slides into slide rack and wash in 1 L of 0.2X SSPE at 55°C for 1 hr with gentle agitation.

2. Wash slides for 5 minutes in 0.2X SSPE at 37°C in a large staining dish. Repeat.

H) Final Rinse:

1. Rinse in 1X PBS in staining dish.

2. Proceed immediately to the immunological detection. If you prefer to do the immunological detection the following day, you can store the slides in 1X PBS overnight.

IMMUNOLOGICAL DETECTION

A) Materials:

Boehringer Mannheim DIG Nucleic Acid Detection Kit (#1175 041)

B) Buffers:

1. Buffer 1:
Composition: 100 mM Maleic Acid (7.5)
150 mM NaCl

Amount Required: 500 ml

Formula for 500 ml: 5.8 g Maleic Acid (MW = 116)
4.4 g NaCl
3.5 g NaOH (solid)

Adjust pH to 7.5, increase vol to 500 ml

Autoclave

2. 10X Blocking Reagent (10X BR):

Composition: 10% (w/v) Blocking Reagent in Buffer 1
Amount Required: 100 ml
Formula for 100 ml: 10 g of blocking reagent (vial 6)
Buffer 1 to a final vol of 100 ml

Will need to heat to ~60°C to get into solution

Autoclave, store in the refrigerator

3. 1X Blocking Reagent (1XBR):

Composition: 1% Blocking Reagent in Buffer 1
Amount Required: 100 ml
Formula for 100 ml: 10 ml 10X Blocking Reagent
90 ml Buffer 1

4. 1 M MgCl2

5. BSA Wash solution:

NOTE: The BSA you use is very critical. You must use BSA that is free of endogenous phosphatases or you will have a lot of background. We use Sigma #A-7030.

Composition: 1% BSA
0.3% Triton-X-100
100 mM Tris-Cl, pH 7.5

150 mM NaCl

Amount Required: ~20 ml for 4 slides per step if using tip box lids (~400 ml for 16 slides)
Formula for 500 ml: 5 g BSA
1.5 ml Triton X-100
50 ml 1 M Tris-Cl, pH 7.5
15 ml 5 M NaCl

Bring volume up to 500 ml.

6. TNM Buffers:

Use TNM-5 if using polyvinyl alcohol in the color reaction

Use TMN-50 if not using polyvinyl alcohol in the color reaction

TNM-5 Buffer:

Composition: 100 mM Tris (9.0)
100 mM NaCl
5 mM MgCl2

Amount Required: ~ 100 ml
Formula for 500 ml: 50 ml 1 M Tris or 6.1 g Tris base
10 ml 5 M NaCl or 2.9 g NaCl
2.5 ml 1 M $MgCl_2$
Adjust pH to 9.0; increase vol to 500 ml

TNM-50 Buffer:
Composition: 100 mM Tris (9.5)
100 mM NaCl
50 mM $MgCl_2$
Amount Required: ~ 100 ml
Formula for 500 ml: 50 ml 1 M Tris or 6.1 g Tris base
10 ml 5 M NaCl or 2.9 g NaCl
25 ml 1 M $MgCl_2$
Adjust pH to 9.5; increase vol to 500 ml

7. TNP Buffer:

This buffer is needed only if you are using polyvinyl alcohol in the color reaction
Composition: 100 mM Tris (9.0)
100 mM NaCl
10% Polyvinyl alcohol of 70-100kDa (Sigma # P-1763)

Amount Required: ~20 ml per set of 5 slides
Formula for 100 ml: 10 ml 1 M Tris
2 ml 5 M NaCl
10 g PVA

Add Tris and NaCl; adjust pH to 9.0
Dissolve PVA at 90°C; increase vol to 100 ml

Cool to room temperature before making PVA Color-Substrate Solution.

8. T10E1 (8.0):
Composition: 10 mM Tris (8.0)
1 mM EDTA
Amount Required: 500 ml
Formula for 500 ml: 5 ml of 1 M Tris
1 ml of 0.5 M EDTA
Adjust pH to 8.0, increase vol to 500 ml
Autoclave

B) Blocking:

1. Place slides in a tupperware box of the correct size (or top from a yellow pipette tip box) and cover with 1X Blocking Reagent. Incubate at room temperature with gentle agitation for 45 min.

2. Pour out blocking solution and replace with BSA Wash Solution. Incubate at room temperature with gentle agitation for 45 min.

C) Antibody reaction:

The optimal antibody dilution will probably be different with different probes. Try a 1:600 dilution first. If your signal is too weak, try a lower dilution. If your signal is very strong, try a higher dilution to save antibody.

1. Dilute the anti-DIG-AP conjugate (vial 3) in BSA Wash Solution. If using slide mailers, make up 15-16 ml for each set of 5 slides.

2. Place slides in slide mailer and add antibody solution. Incubate at room temperature for 2 hours with slow rotation.

D) Antibody wash:

1. Remove slides from the antibody solution and place on the bottom of a Tupperware box. Add enough BSA Wash Solution to cover the slides. Work fast so that the antibody does not dry up on the slides.

2. Incubate for 30 minutes at room temperature with gentle agitation.

3. Repeat step 1 twice for a total of 3 washes in BSA Wash Solution.

E.1) Color reaction without polyvinyl alcohol:

The color reaction can be done in one of two ways--either with or without polyvinyl alcohol. If you wish to do the color reaction with polyvinyl alcohol, go to section E.2.

1. Wash slides in TNM-50 Buffer for 5 minutes at room temperature with gentle agitation.

2. Repeat step 1.

3. Make up Color-Substrate Solution just before use. Wrap in foil to keep dark until used. If you do the color reaction in slide mailers, you will need 15-16 ml for each set of 5 slides. For 32 ml, mix the following:

32 ml TNM-50 Buffer

112 µl X-phosphate-solution (vial 5)

144 µl NBT (vial 4)

4. Place slides in slide mailers and add the Color-Substrate Solution. Incubate in the dark at room temperature without shaking.

E.2) Color reaction with polyvinyl alcohol

It has been reported that PVA decreases the incubation time and increases the cellular localization of the color reaction by slowing the diffusion of reaction intermediates (see De Block and Debrouwer, 1993). In our experience, PVA does not significantly decrease the incubation time. However, we have observed that PVA does improve cellular resolution. PVA can be a hassle to deal with (thick and gummy); thus, for your first experiment, it might be best to eliminate the PVA.

1. Wash slides in TNM-5 Buffer for 5 minutes at room temperature with gentle agitation.

2. Repeat step 1.

3. Make up PVA Color-Substrate Solution just before use. Wrap in foil to keep dark until used. If you do the color reaction in slide mailers, you will need 15-16 ml for each set of 5 slides. For 32 ml, mix the following:

32 ml TNP Buffer

160 µl 1 M MgCl2

112 µl X-phosphate-solution (vial 5)

144 µl NBT (vial 4)

4. Place slides in slide mailers and add PVA Color-Substrate Solution. Incubate in the dark at room temperature without shaking.

F) Monitor the color reaction:

1. For a typical probe, monitor the reaction progress beginning at about 6 hours. If you expect the signal to be very strong, begin monitoring at 1 hour.

2. To monitor the reaction progress, choose a test slide, rinse with T10E1, wipe off the back of the slide, and observe using an old microscope. Never observe unmounted slides using a good microscope!!! Put the test slide back into the color solution if a longer incubation is required.

3. When the color reaction is complete, wash the slides for 5 minutes with T10E1. Repeat twice.

SLIDE MOUNTING

A) Materials/Solutions:

1. Ethanol series in coplin jars: 25%, 50%, 75%, 100%, 100%

2. Xylenes in a coplin jar

3. Coverslips

4. Cytoseal or Permount

B) Procedure

1. Do this procedure in the hood. Process 1 slide at a time because the color fades in the xylene.

2. Dehydrate through the ethanol series. Dip slides in the ethanols until streaks go away (3-4 times).

3. Dip in the xylenes until the streaks go away. Let sit for another 10-20 seconds.

4. Remove the slide from the xylenes and place on a clean paper towel. Quickly (before the xylenes evaporate) add 1-2 drops of Cytoseal to the slide and quickly (before the xylenes evaporate) place a coverslip on the slide.

5. Squeeze out air bubbles and excess Cytoseal by pressing down gently on the coverslip with a kimwipe. This will usually cause Cytoseal streaks to form on top of the coverslip. The Cytoseal streaks can be removed by rubbing gently with a xylenes-moistened kimwipe.

6. If you wish to observe the slides at this point, use the old microscope. Never use the good microscope until step 7 has been completed.

7. Place slides in a 42°C incubator overnight to harden the Cytoseal. Before observing the slides, dip in xylenes a few times to remove the excess Cytoseal, and then air dry (in the hood) for greater than or equal to 5 minutes. It is now OK to use the good microscope.

BIOGRAPHY

Fuyu Xu was bourn in Shaowu, Fujian Province, PR China on February 4, 1965. He moved to Zhejiang province with his family when he was four years old. He received his early education in Wenchen, Zhejiang Province and graduated from First Wenchen High School in 1980. He subsequently entered the Fujian Forestry College (now merged as Fujian Agriculture and Forestry University) and graduated in 1984 with a Bachelor's in Forestry. Then taught courses here for three years. After that, in 1987, Fuyu Xu joined the graduate program in Ecological Genetics and Tree Improvement at the Institute of Applied Ecology, Chinese Academy of Sciences. He finished his course work in Graduate School of Chinese Academy of Science in Beijing in 1988. Fuyu Xu Graduated in 1990 with Master of Science in Ecology, with a concentration in Ecological Genetics and Tree Improvement. He worked as a research associate at the Institute of Applied Ecology, Chinese Academy of Sciences, for six years after graduation. He came to the University of Maine as a visiting scholar in September, 1996. In 1998, he joined the graduate program in Forest Ecosystem Science at the University of Maine, where he worked as a graduate research assistant. Fuyu Xu is a candidate for the Master of Science degree in Forestry from The University of Maine in August, 2002.