MOLECULAR PROBES FOR DROUGHT STRESS IN TWO GEOGRAPHICALLY-SEPARATED POPULATIONS OF LOBLOLLY PINE

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Abstract.--Many native-plant species which grow in a variety of environments have evolved mechanisms for survival under chronic and acute water deficits. Some of these mechanisms have cellular and metabolic components. Osmotic adjustment, changes in metabolic pathways, appearance of new proteins, and production of polyamines have been proposed as components by which plants adapt to drought stress. Synthesis of molecular probes from successful species will facilitate our understanding of the basic cellular components which contribute to the adaptations of these species.

Messenger RNA was prepared from polysomes or total RNA. Polysomes were isolated from needles of loblolly pine *(Pinus taeda L.).* From the polysomes, mRNA (polyA$^+$ RNA) was separated by digestion with proteinase K and chromatography over oligo d(T) columns. Total RNA was isolated by LiCl precipitation, and mRNA isolated from Hybond-mAP. Total and mRNA's were translated *in vitro.* PolyA$^+$ RNA was used to synthesize cDNA and to construct cDNA libraries in lambda ZAP, which were subsequently converted to plasmids. Several clones were used in Southern analyses of restricted genomic DNA. The cDNA libraries from control and stressed seedlings from two geographically-separated populations will be screened for clones which are homologous to clones for drought-stressed genes from pea. Unique clones in stressed libraries will be selected by subtractive hybridization.

Keywords: *Pinus taeda* L., molecular probes, cDNA, drought-stress

INTRODUCTION

Native species have evolved mechanisms to withstand abiotic stresses; temperature (Newton and Goodin, 1989b), chronic and acute periods of water deficits (Newton and Goodin, 1989a), metals (Tomsett and Thurman, 1988), anoxia (McLeod, et al., 1986), etc. (Sach and Ho, 1986). Herbaceous plants also have evolved similar mechanisms and responses to drought stress (Jones et al., 1981). Some of these mechanisms have cellular and metabolic components. Plants adapt to drought stress through response mechanisms such as osmotic adjustment (Newton et al., 1986a), reduction in DNA content and enhanced tissue elasticity (Castro-Jimenez et al., 1989), changes in metabolic pathways with the accumulation of compounds such as proline, (Bhaskaran et al., 1985; Newton et al., 1986b, 1987), appearance of new proteins, (Hulbert et al., 1988; Valluri et al., 1988), synthesis of ABA (Guerrero and Mullet, 1986), and changes in transcription and translation (Guerrero and Mullet, 1988).

Synthesis of molecular cDNA probes from non-stressed (control) and drought-stressed plants can be used identify clones which represent polyA$^+$ RNA which are rapidly induced by drought stress. This report describes the synthesis of cDNA libraries from loblolly pine and the strategy for their use.

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METHODS

Plant Material

Stratified seeds of loblolly *pine,Pinus taeda,* L. (Texas Superior, MSSO 174; 1984M; Texas Forest Service) were germinated in moist vermiculite/peat mixture. Seedlings were grown under greenhouse conditions in flats. Ten-gram aliquots of needles were harvested and frozen in liquid nitrogen (LN2), ground in a mortar and pestle in liquid nitrogen, transferred to a coffee grinder with several pieces of dry-ice, ground further, and stored at -80° C until use.

Isolation of Polysomes

The ten-gram aliquots of ground tissue were extracted with grinding buffer (Wagner et al., 1987). After stirring with a spatula, the samples were squeezed through 8 layers of cheese cloth and centrifuged in 45-ml plastic tubes for 3 min at 27,000 rpm at 4° C. The supernatant fractions were transferred to a flask and 0.1 volume of 10% Triton X-100 were added. Aliquots were transferred to a Dounce homogenizer and homogenized (15 strokes). Pooled samples were centrifuged at 16,000 rpm for 7.5 min.

The supernatant fractions were layered over a step gradient (0.5 M sucrose with a 1.5 M sucrose in grinding buffer). After centrifugation at 27,000 rpm for 3 hours (SW 28 rotor), the supernatant fractions were aspirated, the walls of the tube washed and aspirated, and the cushion aspirated. The pellets which contained the polysomes were resuspended in 500 µL of polysome resuspension buffer and stored at -80° C.

Isolation of Total RNA

Nucleic acids from 10-gram aliquots of tissue were extracted as previously described (Wagner et al., 1987). Total RNA was precipitated with LiCl (Ausubel et al., 1987).

Isolation of PolyA$^{\pm}$ RNA

To 500-µl samples of polysomes, 500µl of 1 M NaCl and 10 Rl of 10 mg ml-^{1} proteinase K were added. After digestion at 25° C for 30 min, the samples were centrifuged for 2.5 min. The samples were heated to 65° C for 3 min and recentrifuged for 2 min and cooled to room temperature. The RNA was quantitated spectrophotometrically. Typical yield of RNA at this step was 2.5 mg. Oligo d(T) columns were prepared according to the manufacturers directions and samples loaded. The columns were washed with 0.5 M NaCl in TE (10 mM Tris, pH 8.5; 0.1 mM EDTA) until the A260 was less than 1.0 after which they were washed with 0.5 M KCl until the absorbance was less than 0.1. The polyA $^{+}$ RNA was eluted with four 400-µl aliquots of elution buffer. The RNA was ethanol precipitated, washed with ethanol, dried, resuspended in TE, and quantitated.

PolyA^{+} RNA was also isolated from the total RNA fraction with Hybond-mAP ® according to the manufacturers directions (Amersham).

Synthesis and Analysis of cDNA

Complementary DNA's (cDNA's) were constructed with several modifications of classical procedures. The first strand was synthesized with cloned MMLV reverse transcriptase. A

synthetic oligonucleotide was used instead of the classical oligo d(T) $_{12-18}$ primer, and it placed an *Xho* I site immediately downstream of the cDNA.

The second-strand synthesis was a modification (Gubler and Hoffman, 1983) of the RNAse H/DNA polymerase I/DNA ligase protocol (Okayama and Berg, 1982). The mRNA was removed by RNase H, and the second-strand synthesized with DNA polymerase I and DNA ligase. First- and second-strand synthesis were quantitated by determining the specific radioactivity of aliquots of each reaction. Reaction products were analyzed in alkaline-gels with ^{32}P end-labelled markers.

Preparation of cDNA Libraries

After synthesis of the second strand, the ragged ends of the cDNA were repaired with Klenow fragment of DNA polymerase. *Sac* I (isochizomer of *Sst* I) linkers were end-labelled with y-^{32}P ATP. The labelled linkers were ligated to the cDNA with T4 ligase. The linkered DNA was digested with *Sst* I and *Xho* I and dephosphorylated. Unligated linkers were removed by chromatography. The linkered-cDNA was eluted into digested lambda ZAP DNA. After precipitation and resuspension of the linkered cDNA and ZAP arms, the two were ligated with T4 ligase, and the ligated DNA was packaged. This procedure insures that the cDNA inserts are cloned into the vector in the same orientation, a requirement which is necessary for subtractive hybridization.

RESULTS AND DISCUSSION

Total and polyA$^+$ RNA supported *in vitro* translation (Figure 1). As others have observed, translation products for the "total" RNA samples is of higher molecular weight than that of the polyA$^+$ RNA samples. This may reflect protection from RNase attack of mRNA in the "total"

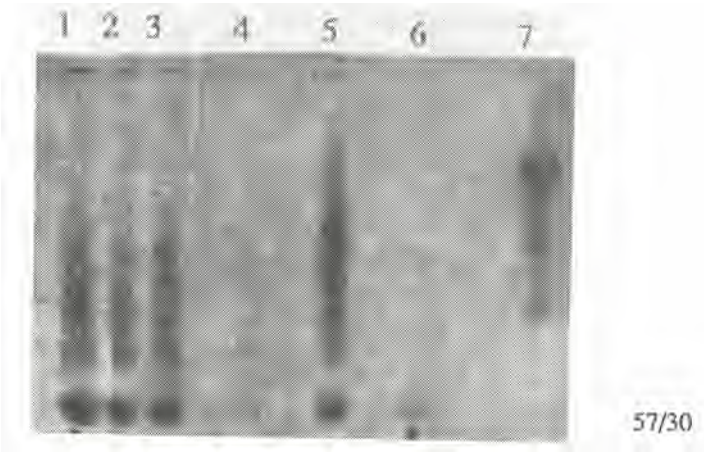

Figure 1. *In vitro* translation products of total and polyA$^+$ RNA from loblolly pine. Total and polyA$^+$ RNA was translated in a rabbit reticulocyte system according to the manufacturer's instructions (Progema) with ^{35}S methionine as the label. Translation productions were separated by SDS-PAGE and visualized by autofluorography. Poly $^+$ RNA, lanes 1-4; total RNA, lane 5; minus RNA control, lane 6; brome mosaic virus RNA control, lane 7.

Poly RNA from seedlings was used to construct cDNA libraries. Results of first and second strand synthesis of a typical experiment are shown in Figure 2. The bulk of the cDNA was between 500 and 2500 base-pairs in length.

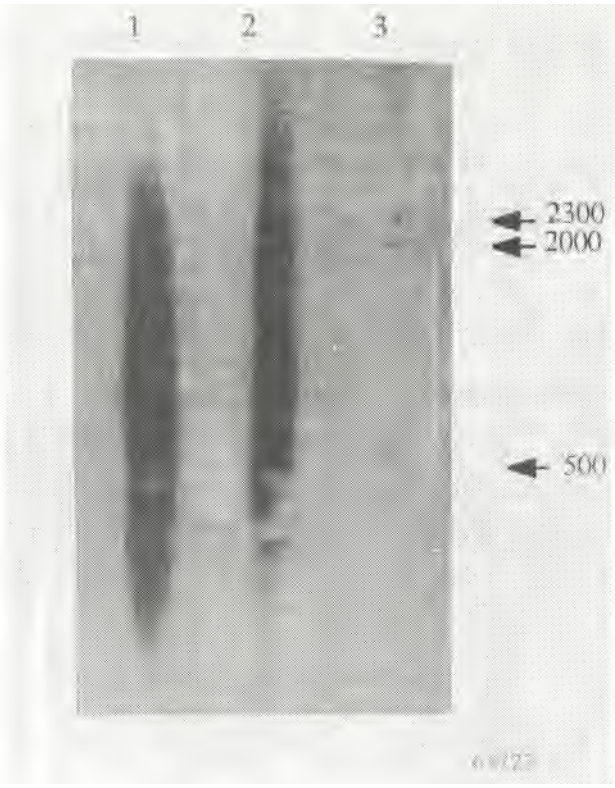

Figure 2. Synthesis of cDNA. Newly synthesized strands were end-labelled with ^{32}P and analyzed on alkaline agarose gel and visualized. Lane 1, first strand; Lane 2, second strand; Lane 3, standards. Arrows indicate the size of the standards in base pairs.

The directionally cloned cDNA was ligated to restricted lambda ZAP DNA and packaged. Table 1 summarizes the libraries constructed from seedling RNA. While the titer of these libraries is low, there is sufficient number of clones to begin screening. Several were selected as probes for Southern analyses.

Inserts from several clones from experiment 64/23 were labelled with ^{32}P by the random primer method according to the manufacturer's directions(Boehringer Mannheim). These probes were used in Southern blots of pine genomic DNA which was restricted with *Hind* III and *Eco R* I (Figure 3).

These data show the successful isolation of mRNA from loblolly pine and its use in the construction of cDNA libraries. Several clones from these libraries were used in southern analyses of restricted genomic DNA. This demonstrates their potential for RFLP mapping of loblolly pine.

Now that the method for routine construction of directionally cloned libraries has been established, libraries from mRNA isolated from stressed and control tissue will be constructed from seedlings of two geographically-separated populations of loblolly pine. By subtractive hybridization and differential screening, these libraries will be screened for clones which represent polyA$^+$ RNA's whose levels change in response to drought stress.

Table 1. Titer of cDNA libraries.

Experiment Number	total plaque forming units	percent transformants
64/17	7.7×10^4	2
64/23	1.1×10^3	99
64/32-59	0.3×10^3	64
64/32-64	0.2×10^3	87

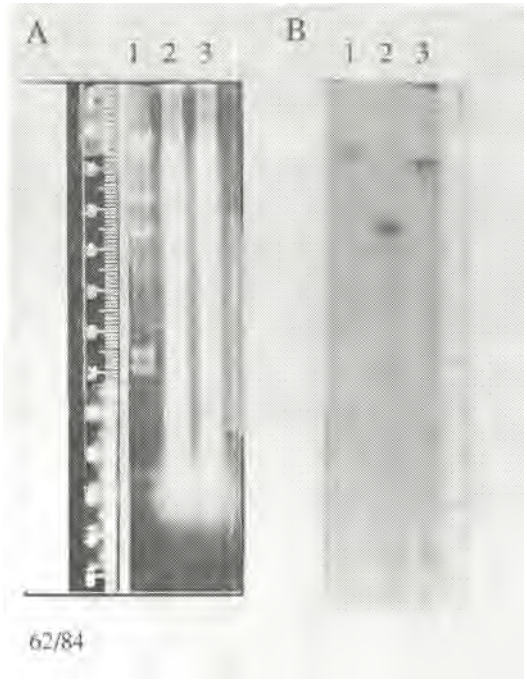

Figure 3. Southern blots of genomic DNA from loblolly pine. DNA was extracted from needles and restricted with ***Hind*** III (Lane 2) and ***Eco R*** I (Lane 3) and separated on an agarose gel along with standards (Lane 1). The DNA was transferred to nitrocellulose filters and probed with inserts from the library. A. Ethidium bromide stained gel. B. Nitrocellulose probed with clone from cDNA library.

LITERATURE CITED

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. Phenol/SDS method for plant RNA preparation. In Current Protocols in Molecular Biology. 4.3.2.

Bhaskaran, S., R. H. Smith, and R. J. Newton. 1985. Physiological changes in cultured sorghum cells in response to induced water stress. I. Free proline. Plant Physiol. 79:266-269.

Castro-Jamenez, Y., R. J. Newton, H. J. Price, and R. S. Halliwell. 1989. Responses to drought stress in Microseris species differing in nuclear DNA content. Amer. J. Bot. (In Press.

Gubler, W. and B. J. Hoffman. 1983. A simple and very efficient method for generating complementary DNA libraries. Gene. 25:263-270.

Guerrero, F. D., and J. E. Mullet. 1986. Increased abscisic acid biosynthesis during plant dehydration requires transcription. Plant Physiol. 80:588-591.

Guerrero, F. D., and J. E. Mullet. 1988. Reduction of turgor induces rapid changes in leaf translatable RNA. Plant Physiol. 88:401-408.

Hulbert, C., E. A. Funkhouser, E. J. Soltes, and R. J. Newton. 1988. Inhibition of protein synthesis of loblolly pine hypocotyls by mannitol-induced stress. Tree Physiol. 4:19-26.

Jones, M. M., N. C. Turner, and C. B. Osmond. 1981. Mechanisms of drought resistance. In The Physiology and Biochemistry of Drought Resistance in Plants. L. G. Paleg, D. Aspinall (ed.). Academic Press. N. Y. pp 15-37.

McLeod, K. W., L. A. Donovan, N. J. Stumpff, and K. C. Sherrod. Biomass, photosynthesis, and water-use efficiency of woody swamp species subjected to flooding and elevated water temperature. Tree Physiol. 2:341-346.

Newton, R. J., J. Puryear, S. Bhaskaran, and R. H. Smith. 1986a. Physiological changes in cultured sorghum cells in response to induced water stress. II. Soluble carbohydrates and organic acids. Plant Physiol. 81:626-629.

Newton, R. J., S. Sen, and J. D. Puryear. 1986b. Free proline in Pinus taeda L. callus in response to induced drought stress. Tree Physiol. 1:325-332.

Newton. R. J., J. D. Puryear, and S. Sen. 1987. Free proline in water-stressed pine callus. Tappi J. 70:141-144.

Newton, R, J, and J. R. Goodin. 1989a. Physiological Adaptation of Shrubs: Moisture Stress Adaptation. In Shrub Biology and Utilization. C. M. Mckell (ed.). Academic Press. N. Y. pp 365-383.

Newton, R, J, and J. R. Goodin. 1989b. Physiological Adaptation of Shrubs: Temperature Stress Adaptation. In Shrub Biology and Utilization. C. M. Mckell (ed.). Academic Press. N. Y. pp 385-402.

Sachs, M. M., and T.-H. D. Ho. 1986. Alteration of gene expression during environmental stress in plants. Annual Rev. Plant Physiol. 37:363-376.

Tomsett, A. B., and D. A. Thurman. 1988. Molecular biology of metal tolerances of plants. Plant Cell Enviorn. 11:383-394.

Valluri, J., W. J. Treat, R. J. Newton, and E. J/. Soltes. 1988. Water stress proteins induced in pine. Tree Physiol. 4:181-186.

Wagner, D. B., G. R. Furnier, M. A. Saghai-Maroof, S. W. Williams, D. B. Danick, and R. W. Allard. 1987. Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci. U.S.A. 84:2097-2100.