REGULATION OF DEFENSE/REPAIR GENE EXPRESSION IN WOODY PLANTS IN RESPONSE TO WATER DEFICIT

J. Cairney [1], D.K. Villalon', S. Chang, M.A.D. Dias[2] and R.J. Newton-3

Abstract:--Four cDNA clones and two genomic clones of different members of a PI gene family have been isolated from *Atriplex canescens*. The sequence of the coding regions are very similar however promoter sequences and AU-rich sequences within the transcribed regions suggest different levels of regulation. Gene specific oligonucleotides reveal some of these differences and will facilitate gene regulation studies and, as part of a broader program, the assignment of physiological roles to individual family members. An Loblolly Pine mRNA of unusual structure encodes a protein similar to a number of chitinases. The 5' UTR of this mRNA accounts for almost half the molecule and possesses numerous inverted repeat and uORF, features found in many translationally regulated mRNAs. RT-PCR results which suggest additional transcripts possess some of these sequences. Cloning and sequencing of these gene fragments along with in vivo studies of the reported genes will illuminate the types of gene regulation which operate in pines.

Keywords: Gene regulation, Reverse Transcription-PCR, sequence specific oligonucleotides, upstream open reading frames, secondary structure.

INTRODUCTION

The study of plant gene expression in response to environmental stress has resulted in the elucidation of a number of biochemical defense and repair mechanisms (Skriver and Mundy 1990. Keen 1992). The application of that knowledge, through the isolation of genes, their modification and subsequent transfer into plant cells, has brought spectacular successes (Chrispeels and Sadava 1994). As recognition of the efficacy of biotechnologies has spread, the desire among researchers and corporate enterprises to avail themselves of new opportunities has broadened the field. An increasing numbers of researchers now conduct molecular experiments with plants and with their arrival, the less highly publicized technical problems of gene transfer and expression have begun to surface (Matzke and Matzke 1995). The recognition of the complexities of gene regulation has led researchers to seek more fundamental understandings of gene expression. In this paper we discuss the regulation of two gene families whose members display variable responses to environmental and hormonal cues. The sequence and structural features of the genes and

Assistant Professor, Graduate Student and Research Associate, Institute of Paper Science and Technology, 500 10th Street, Atlanta, GA 30318
[2] Graduate Student, Department of Forest Science, Texas A&M University, College Station, TX 77843
[3] Professor, Department of Forest Science, Texas A&M University, College Station, TX 77843

mRNAs suggest tiers of regulation which would allow rapid modulation of protein synthesis in response to stress.

METHODS

Plant material and water deficit stress treatments. Saltbush *(Atiplex canescens* (Pursh Nutt.): Whole plants were desiccated after growth in germination medium (GM) for 4-6 weeks. Plants were left to desiccate on Whatman 3MM paper at 22°C and 60% humidity under dim light following published protocols (Yamaguchi-Shinozaki and Shinozaki, 1993 a and b). For ABA treatment, plants were removed from GM and grown hydroponically in half strength either MS media or MS media supplemented with 10μM ABA for 14 hours.
Loblolly pine *(Pinus taeda* L.):Seedlings were full-sibling, resulting from the cross of S6PT2 and S6PT3 sources from east Texas. The seeds were sown in 5-liter cylindrical containers filled with a fitted clay medium adapted for pine seedlings (Meier et al. 1992). The seedlings were randomly separated into 7 groups, and water was then withheld from the seedling groups in staggered fashion. Seedlings were harvested pre-dawn on the same day at which point the water potentials were taken. (Chang et al 1995).
Molecular methods and cDNA library construction. For Atriplex a cDNA library was made from the leaves of seedlings with water potential - 0.94 MPa (Adair et al 1992). The pine library was made from the entire root tissue of an 8-month-old seedling with a water potential of -1.1 MPa (Cairney et al 1993). In both cases the vector was the Xgt10-derived vector, Lambda-ZAP (Stratagene, La Jolla, CA).
Reverse Transcription-Polymerase Chain Reaction. One microgram of total RNA was used for Reverse transcription using MMLV Reverse Transcriptase (Promega, Madison, WI). One tenth of the reaction products were then used for PCR. Conditions were, 94°C, 15 s(econds), 50-55°C, 15s, 72°C 30s, 35 cycles. Reaction products were transferred to membranes, probed with a labelled cDNA and the membrane scanned and signals quantitated.
DNA sequencing and homology comparison. DNA sequencing was performed by the dideoxy chain termination method (Sanger et al. 1977). Standard molecular methods were used in other cases (Sambrook et al. 1989). For comparing the putative proteins encoded by these pine cDNA clones with similar proteins from other organisms, sequences were aligned using the LaserGene program (DNASTAR, Madison, WI).

RESULTS AND DISCUSSION

Halophytes which survive long periods with little moisture, possess both physiological and biochemical adaptations which permit them to prosper under conditions where most plant would perish. A study of halophyte physiology may shed light on novel molecular mechanisms of stress tolerance or may reveal a source of new genes. In parallel projects we have chosen to study gene expression under water deficit in the woody desert shrub, Saltbush *(Atriplex canescens)* and the commercially important conifer, Loblolly Pine *(Pinus taeda L.).* In both cases cDNA libraries

were constructed from water stressed plants and differentially screened to isolate clones of genes whose steady-state mRNA levels fluctuate under water deficit. Details of clone isolation have been reported elsewhere (Adair et al 1992, Cairney et al 1993. Chang et al 1995).

An Atriplex clone, p23-3, isolated from a cDNA library by differential screening was shown to encode a protein with strong similarity to Proteinase Inhibitor-I (PI-I) from soybean and potato Figure 1). The clone proved distinctive in hybridizing to a broad mRNA band in Northern analysis. This signal appeared be divided between two principal bands, of about 0.5 and 0.7kb, suggesting closely related transcripts. By rescreening the library four additional cDNA clones which hybridized to p23-3 were isolated. DNA sequencing showed them to be 95% identical at the nucleotide level. They appeared to part of a multigene family. Most of these clones were around 0.4 kb however clone p8-3 was 0.65 kb and differed from the other clones, principally, in possessing a longer 3'UTR which contained a AU-rich region.

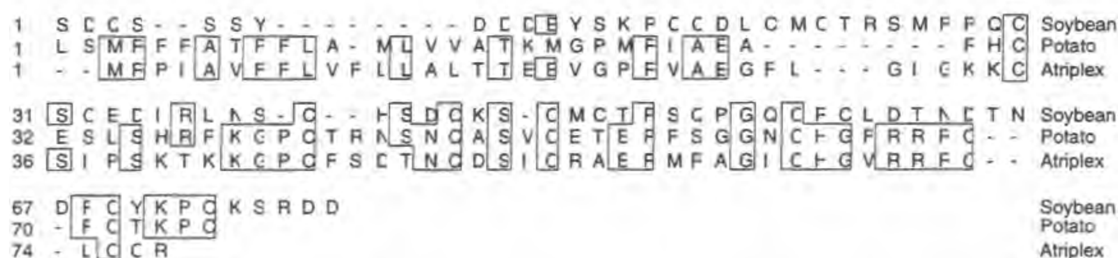

Figure 1. Comparison of amino acid sequences of proteinase inhibitor CII(Bowman Birk) of soybean, potato (p322, Steikema et al., 1988), and predicted polypeptide of Atriplex clone 23-3.

Since the translation product of these mRNA molecules was essentially the same, the presence of several transcript sizes within a given organ begs questions about their role. Several explanations are possible;

1. The PIs may differ in subtle but significant ways, fulfilling different roles in the cell, their cognate genes responding to different cues.
2. These transcripts may be produced in different cell types within an organ, each with a appropriate stability and translational efficiency.

The altered cytoplasmic conditions of water deficit may favor one transcript over another. A number of AU-rich sequences have been identified in the 3'UTRs of transcripts. These sequences have been assigned roles in mRNA instability (Green 1993), translational enhancement (Gallie 1993) regulated/cytoplasmic polyadenylation and deadenylation (Bachvarova 1992).

Attempts to purify the protein and determine its function are underway, this paper discusses advance made in the study of PI-I gene regulation.

Genomic Southern blots gave a complex pattern indicating many copies of the PI-I genes. To

shed light on the transcriptional regulation of these genes genomic clones were isolated. Two clones, pG 12-95 and pG 18-1 were sequenced. The coding regions and introns of both genomic clones were determined on the basis of nucleotide sequence comparison with the cDNA clones. Neither genomic clones matches a cDNA exactly; their putative exonic sequences show 95% identity to the cDNAs. Both pG12-95 and pG18-1 contain one intron of 931 and 422 bp in length, respectively. The first exon of both clones are identical in length. coding for 17 amino acids. Therefore, the introns of both genes vary at the 3' end in length.

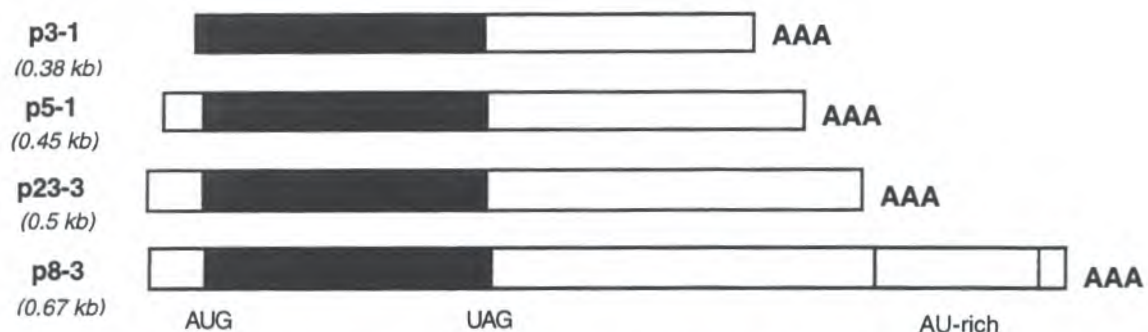

Figure 2. Schematic showing alignment and placement of open reading frames and additional sequence features of Atriplex PI-1-like cDNA clones (p3-1, p5-1, p23-3, p8-3).
Key: Gray area; open reading frame, Stippled area; AU-rich region, AAA: polyA tail.

Preliminary analysis of the promoters reveals a number of motifs which have been identified in other plants as transcription factor binding sites. These include ABA-Responsive Elements (ABREs).A number of unusual repeat structure are conspicuous, their function is being investigated. There is no AU-rich region in the second exon of pG 12-95 however pG 18-1, while differing in sequence from the other clones, contains a 3'AU-rich sequence identical to that of cDNA clone p8-3.

Multigene families contain many pseudogenes and sequence analysis alone cannot distinguish these from transcriptionally active form. Transgenic studies would indicate the inducibility of these genes however with large families of genes such experimentation is laborious and time consuming. We have sought a swift,sensitive and specific assay of gene expression through the use of specific oligonucleotides and Reverse-Transcription-PCR (RT-PCR). Oligonucleotides specific to each transcript and suitable for PCR were designed. Each should amplify a product of a characteristic size, facilitating identification. Preliminary assay using the cDNA and genomic clones showed that these primers displayed fidelity either with single templates or a mixture of templates.

RT-PCR experiments were carried out with RNA from Atriplex seedlings water stressed for varying periods of time. Each transcript showed some level of induction however the magnitude of the response and its' triggering point were greatly different. The same individual patterns were observed during ABA induction. These results suggest that our technique is sufficiently sensitive

to discriminate between individual members of a multigene family and at present these experiments are being repeated to establish levels of confidence with the assay. Such a technique will be of great use in determining which members are induced under different circumstances. Such assessment, in combination with other biochemical and physiological data, would allow workers to chose the most appropriate protein for their purposes from among a family of similar polypeptides. In gene regulation studies pseudogenes or genes whose level of induction is low, specific to a tissue or environmental signal, could be identified.

As part of a parallel project, examining gene expression in response to water deficit in Loblolly Pine, a cDNA library was constructed from the roots of five month old pine seedling which had been deprived of water for 11 days. The library was differentially screened using polyA+ RNA from control seedling (water potential, -0.4MPa) and stressed seedlings (-1.3MPa) and examples of inducible and repressible clones were selected (Cairney et al 1993). One clone, pLP6, is strongly expressed in the roots and stems of well-watered plants but mRNA levels decline rapidly as plants dehydrate. The same pattern is seen in needles although the absolute level of gene expression is much lower. A single transcript of 1.5kb is dectected, the same size as the cDNA suggesting that almost all the information in the mRNA is present in the cDNA clone.

The nulceotide sequence of pLP6 consists of 1488 nulceotides, concluding with a polyA tail. The longest open reading frame commencing with an ATG could encode a polypeptide of 216 amino acids, which has a predicted molecular weight of 24.2 kD and PI of 5.05. However this open reading frame does not start until nucleotide 721 almost halfway through the mRNA (Figure 3).

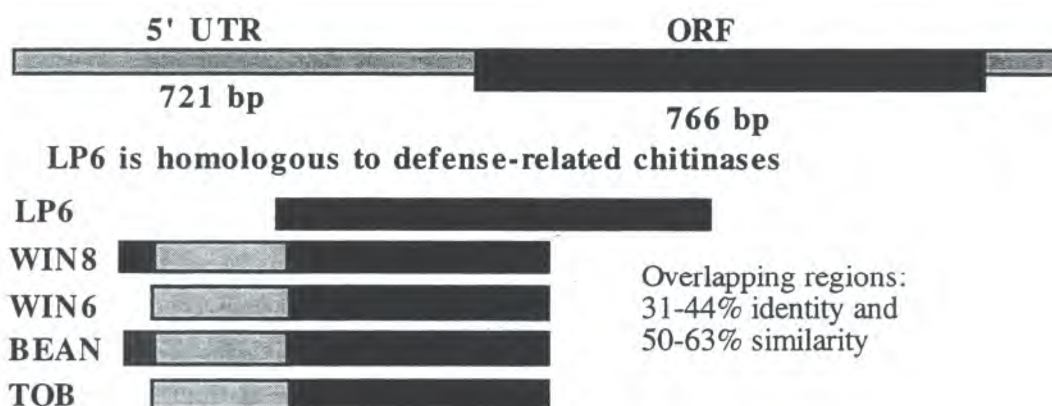

Figure 3. Schematic showing structure of pLP6 5'UTR and major ORF (above) and the alignment of the putative LP6 polypeptide and several Class I chitinases (below).

To demonstrate that pLP6 is not a "double clone" fortuitously hybridizing to an mRNA of similar size we repeated hybridization using a 500 bp 5' probe generated using a convenient EcoRI site and found a similar hybridization pattern . To eliminate any possibility of an abundant mRNA maskin^{g}, the expression of a rare transcript, we performed RT-PCR using primers near the 5' terminal of the cDNA and from within the open reading frame. A fragment of

predicted size was amplified and cloned. The sequence of approximately 150 nucleotides from either end of this molecule was determined, this is identical to the corresponding region of pLP6 (data not shown). These results confirm that pLP6 is derived from a single mRNA molecule.

The polypeptide encoded by pLP6 shows strong homology to a number of Class I Chitinases from bean, tobacco and poplar (Broglie et al 1986, Shinshi et al 1987, Parsons et al 1989) however the simililarity is only with the carboxy half of these proteins. The signal peptide, cysteine-rich chitin binding domain and Glycine/Proline rich "hinge" region, all located in the amino terminal portion of the Class I chitinases (Collinge et al 1993, Raikhel et a 1993) are absent from LP6. Neither the putative catalytic site nor the carboxy terminal sequence involved in translocation to the vacuole is present. In addition the pLP6 protein has a carboxy extension of 69 amino acids not present in any of the chitinases though no homolgies with any sequence in the gene bank could be detected. .

Since chitinases are wound-inducible we wounded 12 seedlings with pliers and chose seedlings from this group for assay lh, 2h, and 6h after wounding. The water potentials of these plants were assayed and gene expression was compared to unwounded control plants harvested at the same time. Steady state RNA levels of pLP6 are greatly reduced 6h after wounding. Similar results have now been obtained for roots and needles (data not shown).

At present we have no information on the function of the putative LP6 protein. The physiological function of chitinases has long proved elusive however recently, with the elucidation of the biochemical nature of Nod factors (Denarie and Cullimore 1993) and the demonstration that a protein capable of rescuing a developmentally blocked carrot embryo mutant was a chitinase (De Jong et al 1992), a role for chitinases in development is being suggested. It is possible that the LP6 protein fulfills some role in develpment and to investigate this possibility expression in early embryos is being investigated. The unusual structure of pLP6 may be explained if the protein is a `proto-chitinase', such gene variants have been suggeted (Shinshi et al 1990).

The sequence of the 5'UTR of pLP6 revealed several inverted repeats which could form stem loop structures of moderate to high stabiliy (-11kcal/mol to -17kcal/mol). In addition eight upstream open reading frames (uORFs) were identified. Both these feature are found often in genes exhibiting post transcriptional regulation (Gallie 1993). The 600 nucleotide 5'UTR from Cauliflower Mosaic Virus posseses extensive secondary sturcture and several uORFs and recently a novel translation mechanism, a "ribosome shunt", was proposed to control expression of the downstream cistron (Fiitterer et al 1993). At present we are cloning copies of the LP6 5'UTR into plant expression vectors to determine whether reporter gene expression can be influenced by this sequence or truncated and mutated variants.

CONCLUSIONS

Sequence anaysis and preliminary regualtory studies show stress genes are regulated at several levels. In vivo analysis and additional environmental and hormonal treatments will illuminate the relative contribution of each step and provide information for efficient biotechnological applications in forest species.

ACKNOWLEDGEMENTS

Acknowledgement. The authors thank Dr. Larry Harris-Haller of DNA Technologies Laboratories at Texas A&M University. This work was supported by the Temple Inland Foundation, Diboll, Texas, the Texas Agricultural Experiment Station and USDA-BARD grant US-2212-92.

LITERATURE C11 LD

Adair, L. S., D.L.Andrews, J. Cairney, E.A. Funkhouser, R.J. Newton, and E.A. Aldon. 1992. Characterizing gene responses to drought stress in fourwing saltbush *[Atriplex canescens* (Pursh.) Nutt.]. J. Range Manage. 45: 454-459.

Bachvarova, R.F. 1992. A maternal tail of poly(A): the long and the short of it. Cell 69: 895-897

Broglie KE., Gaynor JJ, Broglie RM: Ethylene-regulated gene expression: molecular cloning of the genes encoding an endochitinase from Phaseolus vulgaris. Proc Natl Acad. Sci USA 83:6820-6824 (1986).

Cairney J., S. Chang , M.A.D.L. Dias, E.A. Funkhouser and R.J. Newton. 1993. cDNA Cloning and Analysis of Loblolly Pine Genes Induced by Drought Stress. Proceedings, 22nd Southern Forest Tree Improvement Conference 14-17 June, Atlanta, Georgia. pp357-369

Chang S, J.D. Puryear, M.A.D.L. Dias, E.A. Funkhouser, R.J. Newton, and J.Cairney . 1995. Gene expression under water deficit in loblolly pine *(Pinus taeda L.):* Isolation and characterization of cDNA clones. (Physiol. Plant. in press)

Chang S, Puryear J, Cairney J: A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter. 11: 114-117 (1993)

Chrispeels M.J., and D.E. Sadava. 1994. Plant genetic engineering: new genes in old crops. in Plant, Genes and Agriculture. Jones and Bartlett Publishers. ISBN 0-86720-871-6.

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, and K.Vad. 1993. Plant chitinases. Plant J. 3:31-40.

De Jong, A.J., Cordewener, J., Schiavo, F.L., Terzi, M., Vandekerckhove, J., Van Kammen, A., and S.C. De Vries. 1992. A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4: 425-433.

Denarie, J., and J.,Cullimore. 1993. Lipo-oligosaccharide nodulation factors: a minireview. New class of signaling molecules mediating recognition and morphogenesis. Cell 74:951-954.

Futterer, J,, Kiss-Laszlo, Z., and T. Hohn. 1993. Nonlinear ribosome migration on cauliflower mosaic virus 35S RNA. Cell 73: 789-802.

Gallie, D.R. 1993. Posttranscriptional regulation of gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44: 77-104.

Green, P. 1993. Control of mRNA stability in higher plants. Plant Physiol. 102: 1065-1070.

Keen, N.T. 1992. The molecular biology of disease resistance. Plant Mol. Biol. 19: 109-122.

Matzke, M.A. and A.J.M. Matzke. 1995. Homology-dependent gene silencing in transgenic plants: what does it really tell us? Trends Genet. 11: 1-3.

Meier, C.E., Newton, R.J., Puryear, J.D. and S. Sen. 1992. Physiological responses of loblolly pine *(Pinus taeda* L.) seedlings to drought stress: osmotic adjustment and tissue elasticity. J. Plant Phusiol. 140: 754-760

Parsons, T.J., Bradshaw, H.D. and M.P Gordon,: Systemic accumulation of specific mRNAs in response to woudning in poplar trees. Proc Natl Acad. Sci USA 86:7895-7899 (1989).

Raikhel, N.V., Lee H-I., and W.F. Broekaert: Structure and function of chitin-binding proteins. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44:591-615 (1993).

Sambrook, J., Fritsch, E.F., and T. Maniatis. 1989. Molecular Cloninng: A laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc.Natl. Acad. Sci. USA 74: 5463

Shinishi, H., Mohnen, D., and F Meins Jr: Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc. Natl. Acad. Sci. USA 84:89-93 (1987).

Shinshi, H., Neuhaus, J-M., Ryals, J., and F. Meins Jr.1990. Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol. Biol. 14:357-368

Skriver, K. and Mundy, J. 1990. Gene expression in response to abscisic acid and osmotic stress Plant Cell 2: 503-512.

Stiekema, W.J., Heidekamp, F., Dirkse, W.G., Van Beckum, J., de Haan, P., Bosch, C, and Louwerse. (1988). Molecular cloning and analysis of four potato tuber mRNAs. Plant Mol. Biol. 11, 255-269.

Yamaguchi-Shinozaki, K., and Shinozaki, K. 1993a. Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol. Gen. Genet. 236: 331-340.

Yamaguchi-Shinozaki, K., and Shinozaki, K. 1993b. The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of *rd22,* a gene responsive to dehydration stress in *Arabidopsis thaliana.* Mol. Gen. Genet. 238: 17-35.