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**Isolation and Functional Analysis of Drought-Induced
Genes in *Pinus***

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ABSTRACT

ISOLATION AND FUNCTIONAL ANALYSIS OF DROUGHT-INDUCED GENES IN *PINUS* (BARD - US-2212-92)

Ronald Newton, Joseph Riov and John Cairney

Drought is a common factor limiting timber production in the U.S. and Israel. Loblolly (*Pinus taeda*) and alleppo pine (*Pinus halepensis*) seedling survival is reduced when out planted, and growth and reproduction are often hindered by periodic droughts during later stages of tree development. Molecular and gene responses to drought stress have not been characterized. The objectives were to characterize drought-induced gene clones from these pines, to determine the effects of a growth regulator on drought tolerance, ABA levels, and drought-induced gene expression in alleppo pine, and to develop procedures for loblolly pine transformation.

Nearly 20 cDNA clones influenced by gradual, prolonged drought stress have been isolated. Many of these have been shown to be induced by drought stress, whereas several others are down-regulated. These are the first drought-induced genes isolated from a pine species. Two genomic clones (*lp5-1* and *lp3-1*) have been sequenced and characterized, and each has been found to be associated with a gene family. Clone *lp5* appears to code for a cell wall protein, and clone *lp3* codes for a nuclear protein. The former may be associated with changing the elastic properties of the cell wall, while the latter may be involved in signal transduction and/or protection from desiccation in the nucleus. Clone *lp3* is similar to a drought-induced gene from tomato and is regulated by ABA. Several DNA sequences that are specific to induction during growth-retardation in alleppo pine by uniconazole have been identified. The active DNA species is now being identified. Promoters from genomic clones, *lp3* and *lp5*, have been sequenced. Both are functional when fused with the *gus* reporter gene and transferred to other plant tissues as well as responding to a simulated drought stress. Through exodeletion analysis, it has been established that the promoter ABRE element of *lp3* responds to ABA and that drought-induction of *lp3* expression may also involve ABA. Stable tobacco transformants carrying either the *lp5* or the *lp3* promoter fused to a reporter *gus* gene have been obtained. The *lp5/gus* fusion was expressed at several stages of tobacco development and differentiation including the reproductive stage. There was no difference in phenotype between the transformants and the wild type.

Embryogenesis procedures were developed for slash pine, but attempts to couple this process with gene transfer and plantlet transformation were not successful. Transformation of pine using *Agrobacterium* appears tractable, but molecular data supporting stable integration of the *Agrobacterium*-transferred gene are still inconclusive.

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OBJECTIVES

- Objective 1. To differentially screen a cDNA library of *Pinus taeda* and to select and characterize clones expressed under drought conditions.
- Objective 2. To isolate drought-induced genes from a genomic library of *Pinus taeda* and identify promoter regions.
- Objective 3. To determine the relationship between plant growth regulator (PGR) effects on *Pinus* drought tolerance, growth, ABA levels and expression of drought-induced genes.
- Objective 4. To conduct promoter analysis by constructing reporter plasmids bearing truncated and mutated versions of promoters and introducing these into tissue by biolistic methods and regenerating a transgenic plant.
- Objective 5. To transform *Pinus* with a reporter gene and to regenerate a transgenic tree.

DESCRIPTION OF RESEARCH ACTIVITIES AND RESULTS

Objective 1: To differentially screen a cDNA library of *Pinus taeda* and to select and characterize clones expressed under drought conditions.

Poly (A+) RNA was extracted from roots of loblolly pine seedlings subjected to a gradual and prolonged water deficit. The RNA was used to construct a cDNA library. At least 20 cDNA clones were isolated whose expression was induced or down-regulated by water deficit.

The protein encoded by *lp2* shows more than 90% identity to S-adenosylmethionine synthetase from a range of plants. This clone was expressed in all pine organs with the highest expression observed in the stems. SAM synthetase catalyzes the biosynthesis of SAM, a cofactor in numerous biochemical reactions including lignin and ethylene synthesis.

Clone *lp3* is similar to genes from tomato, rice, and citrus. It is a hydrophilic protein with a nuclear targeting signal whose function is hypothesized to be in the protection against desiccation or as a transcription factor involved in the various events leading to the drought-stress response. *lp3* is a member of a multi-gene family. It is preferentially expressed in the roots and is induced also by abscisic acid, a hormone whose synthesis is also induced by water deficit.

The *lp4* gene was induced by water deficit in needles, stems and roots of pine. The

protein encoded by *lp4* is similar to stielacyanin, a copper-binding protein in the Japanese lacquer tree and arabidopsis. It possesses a proline/serine-rich carboxy-terminus not found in other plants.

Clone *lp5* encodes a novel glycine-rich protein with similarity to both silk fibroin and the rat chondroitin core protein. The putative pine protein is distinct from previously characterized glycine-rich proteins. This gene appears to be expressed at high levels even under severe water deficit stress. In other plants, this protein is localized in the cell walls of vascular tissues and is believed to have a structural role

We have characterized other clones identified as drought responsive from the cDNA library screening. These include *lp8*, *lp9*, *lp10*, *lp15* and *lp20*. With the exception of *lp10*, all are induced by water deficit stress in more than one organ of loblolly pine seedlings. Clone *lp10* is expressed exclusively in roots and is down regulated. All these clones have been sequenced and compared with other published sequences available in Genbank.

lp8 DNA is 738 bp long and hybridizes to a message of 1.05 kb. It is induced in stems, down regulated in needles, and is constitutive in roots. The predicted translation product of *lp8* shows homology to calcium binding proteins which have 4 calcium binding sites. This clone may be involved in a signal transduction pathway related to a water deficit response in pines and perhaps in the regulation of other downstream proteins. We had originally thought that *lp8* coded for a calmodulin type protein, but now it appears that it is most likely coding for a more generic, calcium-binding protein. Southern analysis indicates that *lp8* belongs to a multigene family.

Clone *lp9* is induced in stems and roots and is down regulated in needles. The cDNA clone is 1322 bases long with the corresponding mRNA of 5.2 kb. This is the longest clone we have.

The *lp10* gene is the smallest of all the clones. It is 272 bases long and the message is about 800 bp. Since both the *lp9* and *lp10* clones are partials, no open reading frames could be detected. Genbank searches with these DNA sequences gave no homology to any published sequences.

lp15 is down regulated in stems, needles, and roots by water deficit stress. The cDNA clone is 823 bases long which hybridizes to the mRNA of 900 bp. The Genbank searches for *lp15* indicate very high homology to a histone 2A protein isolated from plants such as wheat and arabidopsis.

lp20 is induced in stems and roots whereas it is constitutive in needles. The cDNA clone is 1050 bp long with the corresponding message of 1100 bp. It is a full clone with a complete open reading frame of 244 amino acids. The predicted protein of *lp20* has very high homology to the tumor suppressor protein identified in maize, rice, arabidopsis, and

humans (Wilm's tumor suppressor gene).

Objective 2. To isolate drought-induced genes from a genomic library of *Pinus taeda* and identify promoter regions.

Once the cDNA clones had been isolated, our next objective was to isolate the genomic clones so that we could isolate the promoter and sequence it. Genomic clones for cDNA clones *lp3* and *lp5* have been isolated and sequenced. The genomic clone, *lp3-1*, of the gene family has the hydrophilicity profile similar to *lp3* except for two deletions in the 5' end. It contains 165 bases of upstream regulatory sequences besides the entire coding region and 580 bases in the 3' untranslated region. It contains a putative intron which obeys the GU-AG splicing rule of eukaryotic mRNAs. The *lp3-1* genomic clone shows an 83% homology with *lp3* cDNA at the nucleotide level and a 96% homology at the 3' end. The predicted proteins of both are highly hydrophilic. Both have a putative nuclear targeting signal at the C terminus. These results demonstrate that *lp3* cDNA and the *lp3-1* genomic clone are not exact counterparts of each other, but they belong to the same gene family of four members.

The genomic clone, *lp5-1*, has been sequenced including the 2.7 kb portion upstream of the 5' end, the coding region, and downstream from the 3' end. There are no introns. Genomic clone, *lp5-1*, shows a 100% match with cDNA clone, *lp5*. There are two members in the *lp5* gene family in the loblolly pine genome. The cloned sequence contains 2259 bp of the 5' upstream promoter region and is about 400 bases from the end of the 3' side of the cDNA sequence.

Objective 3. To determine the relationship between plant growth regulator (PGR) effects on *Pinus* drought tolerance, growth, ABA levels and expression of drought-induced genes.

DNA Preparation from Aleppo Pine. In the first year of our research, we obtained several DNA species which were specific to growth retardant-treated plants by means of differential display reverse transcription (DDRT-PCR). During the first period of the second year we devoted some time to the examination of a new method of analyzing DNA. Usually DNA analysis is performed in large sequencing gels. This method has the disadvantage of radioactive labeling and is time-consuming. In view of the need to study gene activation by growth retardants during various times after application, which means a large number of analyses, we examined a new method introduced by Pharmacia Biotech. The method involves preparation of 4% PAGE which were run on Multiphor II System of Pharmacia Biotech and visualization by silver staining. The results were obtained only 4 hours after the PCR reaction was completed, compared to the radioactive reaction which took several days. However, the silver staining caused the destruction of 70-80% of the DNA yield. Therefore, the radioactive reaction, and the common sequencing gel were preferred.

As stated above, several DNA fragments were obtained in plants treated with uniconazole, which did not appear in the untreated plants. The gel slices of the relevant cDNAs, which contained fragments of 200-400 bp, were boiled in ddH₂O for 15 min. The eluted DNA was then precipitated in 100% ethanol. The DNA fragments were reamplified using the following DDRT-PCR reaction without labeled oligonucleotide: denaturation at 94°C (the first cycle for 1 min and then for 30 sec.), annealing of the primers at 40°C for 2 min. and polymerization at 72°C for 30 sec. This cycle was repeated 40 times and the final cycle had a 5 min. extension at 70°C. The products were analyzed with 2% agarose gel and stained with ethidium bromide.

The DNA fragments obtained were eluted using a JET sorb gel extraction kit (Genomed), and cloned into pCR-ScriptTM SK+ vector (Stratagene) to be used as probes for northern blot analysis. The cloned vectors were transformed into JM109-*E. coli*, which were grown on LB medium containing 40 mg/l X-gel, 20 µg/ml IPTG and 100 mg/ml ampicillin at 37°C overnight. Complete transformation of the vector into the bacteria appeared as white colonies, in which the size of the inserts was determined by digestion with KpnI and SacI.

Although the DDRT-PCR provides an attractive tool for the isolation of genes showing regulated expression, there is a possibility that the purified cDNAs remained contaminated with unrelated DNA sequences. This may lead to artifactual isolation of false positives in the consequent steps of the method. In order to avoid this possibility, the isolated inserts (200-400 bp) were used as probes in hybridization experiments with total RNA. In this experiment, 10 µg of total RNA were analyzed on 1.5% denatured agarose gel and then transferred to a HybondTM-N+ membrane (Amersham) using northern blot capillary techniques. At present we are looking for the optimum conditions for the hybridization.

ABA Induction of *lp3* in Loblolly Pine Seedlings. Loblolly pine seedlings were treated with ABA both in the form of a foliar treatment and a soil drench. The expression of *lp3* was induced by ABA in the roots as verified by Northern analysis. Little or very low expression was observed in needles and its expression appeared constitutive in stems. ABA has also been shown to enhance the expression of *gus* when driven by the *lp3* promoter, thus it is believed that the ABRE elements on the promoter are interacting with ABA during stress (See Obj. 4 below).

Objective 4. To conduct promoter analysis by constructing reporter plasmids bearing truncated and mutated versions of promoters and introducing these into tissue by biolistic methods and regenerating a transgenic plant.

Analysis of the 5' upstream sequence for published promoter sequences of genomic clone *lp5* revealed a consensus TATAA box 25 base pairs upstream of the transcription start point and a GA response element. A 2.4 kb fragment upstream of the 5' end of the *lp5* gene was cloned in two transformation vectors, pBI221 and pBIN. This

promoter region of *lp5* was inserted either next to *gus* (B-glucuronidase) or to *gfp* (Green Fluorescent Protein). Through transient biolistic transformation studies with slash pine cell cultures, the promoter fragment of the *lp5* gene ligated in front of the *gus* gene in pBI221 was observed to be functional in driving the expression of the downstream *gus* gene. A simulated drought stress using a mannitol solution (-1.1 MPa) showed an expression of 2.5 X that of the controls. This indicated that the promoter responded to stress and was able to drive the expression of the foreign gene. The *lp5* promoter cloned in front of the *gfp* gene in the pBIN vector has been transferred into tobacco using the *Agrobacterium* mediated transformation system. The background with this particular reporter gene was high, and therefore its expression is difficult to assess. Stable, tobacco transformants carrying the *lp5/gus* constructs have been selected and transformed plantlets are being grown in the greenhouse. Expression analysis of the *lp5* promoter is now being conducted.

The *lp3-1* promoter has been sequenced, and several *cis* acting sequences such as those of ABA responsive and coupling elements and a GA responsive element are present. Analysis of this promoter is in progress to confirm its mode of expression in response to ABA and water deficit.

A 1.1 kb HindIII/MboI promoter fragment of *lp3* has also been cloned in front of either the *gus* or *gfp* reporter genes in respective plasmids, pBI221 and pBI121. Preliminary experiments indicate that the *lp3* promoter is also active in driving *gus* expression in cell suspension cultures of slash pine. Stably-transformed tobacco plants with the integrated *lp3* promoter driven *gfp* have been obtained. However, expression with *gfp* is very difficult to assess because of high background levels during fluorescence, and the use of this reporter gene has been limited. The *lp3* promoter has been cloned in front of a specific construct of the *gus* gene. Tobacco has been transformed with this construct using *Agrobacterium tumefaciens*. Under nonstressed conditions, expression of *gus* was observed in tobacco roots, root hairs, meristems, leaves, petioles, trichomes, floral pistils and placentas, and developing seeds. The *lp3p/gus* construct has also been introduced into slash pine suspension cells using particle bombardment. A simulated drought stress of -1 MPa was accomplished by adding a mannitol solution to the bombarded cells. Compared to controls, stressed cells showed more than twice as much *gus* expression, indicating that the *lp3* promoter is functional and senses the environmental signal of stress.

The *lp3* promoter is responsive to ABA treatment in bombarded transformed cells. In exodeletion analysis where the ABA responsive element (ABRE) has been removed, there was no response to ABA in bombarded transformed cells. These data indicate that ABA may be part of the drought stress response and the induction of *lp3* gene expression. Sequencing indicated that the *lp3* promoter has a gibberellic acid responsive element (GARE), however when GA was applied to bombarded transformed cells, there was no response to GA.

Objective 5. To transform *Pinus* with a reporter gene and to regenerate a transgenic tree.

Preliminary Studies with Tobacco Transformation. In an effort to study the translational products of the DNA clones and their putative functions in plants, *lp3*, *lp5*, *lp8* and *lp20* clones have been cloned in transformation vectors. The vectors constructed carry the *lp* genes behind a CaMV 35S promoter in between *nptII* (kanamycin resistance) and *gus* (*B*-glucuronidase) genes. The backbone of the vector consists of a pBI121 plasmid which has been used routinely in *Agrobacterium*-mediated transformation studies of tobacco. Stable tobacco transformants have been obtained from *lp5* constructs and the presence of the transformed genes have been confirmed in the F₂ generation. Southern analysis indicates that many of the transformed plants carry either one or two copies of the T DNA. Since *gus* expression is driven by the CaMV 35S promoter which is functional in phloem tissue, its gene product (*B*-glucuronidase) was observed only in that tissue of the transformed tobacco plants. Furthermore, since *lp5* is also driven by the same promoter in this particular construct, it is also expected to be expressed only in the phloem tissue. This has not yet been confirmed because of a lack of antibodies against the *lp5* protein which would confirm its integration into the tobacco genome through immunolocalization. However, Northern analysis indicates that both *gus* and *lp5* are expressed at similar, low levels and thus, the assumption of limited expression of these genes in phloem tissue may be a correct one. Efforts are now being directed toward obtaining the expression of *lp* genes in *E. coli*, isolating the resulting gene product protein, raising antibodies against these proteins, and using the antibodies for immunolocalization.

Gene Transfer Studies with Biolistics. We were successful in establishing procedures for embryogenesis with slash pine. We were hopeful that this procedure would be amenable for biolistic gene transfer. We conducted transformation experiments with slash pine using embryogenic and biolistic methods. We obtained transient expression of the reporter gene, *gus*, but we were unable to obtain stable transformation. Furthermore, we have been unable to obtain regenerated transformed plantlets from *Pinus* tissues transformed with biolistic methods. This is thought to be due to the use of kanamycin in the tissue selection procedure which is inhibitory to tissue differentiation and prevents subsequent plantlet regeneration.

We have established procedures for introducing promoter sequences of pine DNA into pine cell suspensions. The promoter sequences from two genomic clones (*lp3* and *lp5*) have been attached to the reporter gene *gus*. We have introduced these into loblolly pine suspensions and we have observed both *lp3p* and *lp5p* driven *gus* expression. These observations indicate that the promoter is working and functional. We are now determining the role of these promoters in response to drought stress. We also are trying to ascertain the role of abscisic acid (ABA) in the stress response and its possible interaction with the promoter sequences. (See Obj. 4 above).

Gene Transfer Studies with *Agrobacterium*. Because progress in transformation of *Pinus* using biolistic methods has not been rapid, we embarked on investigations involving loblolly pine transformation using the shoot apex method mediated by *Agrobacterium*. As a source of shoots, we used the cotyledon procedure developed in our laboratory for pines. We inoculated nearly three thousand with a 35S/*gus*/*nptII* construct. These underwent selection on kanamycin media, and within three months, we began assays for *gus* expression driven by the 35S promoter. Only 5% of these survived the kanamycin selection, and we had difficulty in establishing roots on these shoots, thus many of them did not survive. At this time we have 8 putative transformants. We initiated PCR analysis with four of these using primers to amplify DNA sequences unique to *Agrobacterium* as well as those unique to both *gus* and *nptII*. Southern analysis of genomic DNA obtained from needles of the putatively transformed shoots was also undertaken. These data are inconclusive in regard to verification of integration of the transferred DNA. More research is needed. We have inoculated afghan pine because it has a greater capability for rooting. These experiments are in progress.

EVALUATION OF RESEARCH ACHIEVEMENT

Objective 1 has been completed. We have isolated a large number of clones that have been shown to be induced under drought stress conditions. These are the first drought-induced genes isolated from a pine species. Several of these are associated with products located in the cell wall. It appears that with a prolonged moderate to severe drought stress of loblolly pine, alterations in the cell wall have occurred. Another clone (*lp3*) appears to be involved in signal transduction and/or protection against desiccation. Because of these possibilities, it would be worthwhile to continue more study of *lp3*.

Objective 2 has been completed. We have isolated 2 genomic clones (*lp5-1* and *lp3-1*) so far and we are in the process of isolating a third. The genomic clones have been sequenced and characterized, and each has been found to be associated with a gene family. Although there are many other cDNAs for which genomic clones can be derived, time and resources do not permit us to do so. Furthermore, it is recommended that research with *lp3-1* and *lp5-1* be continued. Information on the localization of their gene products in pine tissues and their role in drought tolerance is needed. Objective 3 has been completed. We have observed that one of our genomic clones, *lp3*, is similar to a gene from tomato that also is induced by drought stress and has been shown to be localized in the nucleus. We are hypothesizing that it is involved in gene regulation during drought stress. With whole plant and promoter analysis, we have determined that it is regulated by abscisic acid (ABA). Therefore, it appears that induction of this gene during drought stress may also be mediated by ABA. Furthermore, we have several DNA sequences that are specific to induction during growth-retardation in pine by uniconazole. We are working to refine our DNA analysis procedures in order to more specifically define the active species.

Objective 4 has been completed. We have sequenced two promoters obtained from two genomic clones. We have shown that both are functional when transferred to other plant tissues and that they respond to a simulated drought stress. Through exodeletion analysis, we have established that the ABRE element of *lp3* responds to ABA. We are again concluding that drought-induction of *lp3* expression can include an ABA response.

Finally, Obj. 5 has not been completed. Embryogenesis procedures were developed for slash pine, but we were unable to couple this process with gene transfer and plantlet transformation. Our research with biolistic transformation of pine has not progressed as rapidly as we had anticipated. However, we are optimistic about our work with transformation of pine using *Agrobacterium*. At this time, the molecular data supporting stable integration of the *Agrobacterium*-transferred gene are still inconclusive. More research using biolistic and *Agrobacterium* methods of gene transfer are needed.

CONCLUSIONS

1. Nearly twenty cDNA clones responding to a gradual, prolonged drought stress have been isolated from loblolly pine. Most of these are induced, but several are down regulated. These are the first drought-responsive clones isolated from this important timber species.
2. Two drought-induced genomic clones of loblolly pine have been isolated. One of these appears to code for a cell wall protein and the other a nuclear protein. The former may be associated with changing the elastic properties of the cell wall, while the latter may be involved in signal transduction and/or protection from desiccation. The gradual, prolonged drought stress that pines experienced in this study appears to result in the induction of genes whose products are involved in gene regulation and modification of cell wall properties.
3. The promoters of two loblolly pine genomic clones have been isolated and sequenced. Both are functional in driving reporter gene expression and both respond to a simulated drought stress. Stable tobacco transformants carrying either the *lp5* or the *lp3* promoter fused to a reporter *gus* gene have been obtained. The *lp5/gus* fusion was expressed at several stages of tobacco development and differentiation including the reproductive stage.
4. Along with drought stress, abscisic acid appears to be involved in the induction of expression of at least one gene from loblolly pine.
5. Stable tobacco transformants carrying *lp5* cDNA have been obtained. There is no difference in phenotype between the transformants and the wild type.
6. Embryogenesis was accomplished with slash pine, but we were unable to couple

this process with gene transfer and plantlet transformation. Shoot apical meristem procedures of gene transfer in loblolly pine via *Agrobacterium* were investigated, but molecular data supporting stable integration were inconclusive. More research concerning the transformation methodology for pines is needed.

PUBLICATIONS

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Detailed Description of Gene Expression in Plant Growth Retardant-Treated Seedlings

Plant growth retardants (PGR) have long been known to increase plant resistance against environmental stresses, including drought (Halevy 1967). Most research in this area in recent years has been conducted with the triazole group, particularly with paclobutrazol and uniconazole. A large number of species treated with triazoles showed increased water potential (Davis et al. 1988) but the underlying mechanism has not yet been clarified. Some investigators have suggested that the increased water potential results from reduced transpiration due to reduction in leaf area and/or increase in leaf conductance (Davis et al., 1988). However, increased drought resistance induced by PGR is not always accompanied by reduced transpiration (Frymire and Henderson-Cole, 1992). These observations indicate that the above mentioned suggestions cannot be the general underlying mechanism for PGR-induced drought resistance. Recent observations also suggest that ABA is not necessarily involved in PGR-induced increased resistance (Buta and Spauling, 1991; Norman et al., 1986). Since PGR have been shown to induce increased resistance to a wide range of environmental stresses (drought, low and high temperatures, salinity, diseases, etc.), it is more likely that these substances affect the expression of genes which are involved in general mechanisms of stress resistance. Therefore, we focused on studying the effect of PGR on gene expression.

I. EFFECT OF UNICONAZOLE ON DROUGHT RESISTANCE

As noted above the increase in drought resistance following treatment with PGR is well documented. However, to ensure that the plant material which we planned to use in the present study responds similarly to PGR, we studied the effect of uniconazole on two Mediterranean pines, *P. halepensis* and *P. pinea*. *Arabidopsis thaliana* was also included in this study. The reason for that was the fact that progress in developing methods for conifer transformation has been quite slow and we decided to utilize *A. thaliana* as a model system.

Material and Methods

Seeds of *P. halepensis* and *P. pinea* were sown in January in 300 ml containers filled with 2:1 (v/v) mixture of peat:vermiculite. The seedlings were grown in an open nursery in the Faculty of Agriculture, Rehovot. Seedlings were fertilized with 7N-3P-7K at 100 mg. liter⁻¹ through a drip irrigation system.

The seedlings were sprayed in July with 50 ppm uniconazole in the presence of 0.1% BB-5 surfactant. Water potential was measured 15 days or 9 months after treatment. During the period of water potential measurements, half of the plants were subjected to water stress by withholding irrigation. Water potential was measured by means of a pressure chamber using small branches. Immediately following excision, the branch was mounted inside the chamber with its cut end protruding through an airtight seal to the open air. Nitrogen pressure in the chamber was gradually increased until free water appeared at the cut surface. The pressure was lowered slightly, just enough to prevent flow, and the reading at that point was taken as the water potential of the seedlings.

Seeds of *A. thaliana* cv. Columbia were germinated in peat containing 0.25 ppm of uniconazole. This concentration was selected after testing the effect of a wide range of uniconazole concentrations. Following germination, the seedlings were grown at 24°C under a-16 h photoperiod. Four weeks after germination the seedlings were subjected to drought stress by withholding irrigation. Drought resistance was determined by following the appearance of wilt symptoms and finally death of plants. In some experiments, uniconazole was also applied by spraying.

Results and Discussion

Fifteen days after treatment with 50 ppm uniconazole water potential was measured in both unstressed (irrigated) and water-stressed (withholding irrigation) pine seedlings. Water potential of unstressed seedlings of both pines pretreated with uniconazole was significantly higher than that of control plants at the two measuring dates (0 and 3 days after the initiation of the experiment) (Fig. 1). Withholding irrigation decreased the water potential in all plants, and again the water potential of uniconazole-treated seedlings of both pines was significantly higher than that of untreated stressed seedlings at the two measuring dates (3 and 5 days after the initiation of stress).

An additional experiment was conducted with *P. pinea* in which the water potential was measured 9 months after treatment with 50 ppm uniconazole. At that period the growth retardation effect of uniconazole was very significant. Water potential in unstressed seedlings did not differ between control and uniconazole-treated seedlings (Fig. 2). In stressed uniconazole-treated seedlings, the water potential was significantly higher than that in untreated seedlings 5 and 7 days after the initiation of water stress.

The above observations indicate that the increased drought resistance induced by uniconazole results probably from specific effects on resistance mechanisms and not from a decrease in leaf area. Two types of observations support this assumption: (a) increased water potential was detected in uniconazole-treated seedlings shortly after treatment with the growth retardant, at a time when no significant growth retardation was evident and (b) 9 months after application of uniconazole, when seedling growth was significantly retarded, the difference in water potential between uniconazole-treated and untreated seedlings was lower than that observed shortly after uniconazole application. The above assumption is supported by recent observations showing specific effects of triazoles on resistance mechanisms against environmental stresses (Lurie et al., 1994).

Similar to pine, uniconazole increased resistance of *Arabidopsis* to water stress. Control plants showed severe wilting symptoms 5 days after withholding irrigation whereas uniconazole-treated seedlings were turgid at that time (Fig. 3). The latter seedlings showed wilting symptoms after 7 days but recovered after re-watering (data not shown). At that time, control plants were already dead.

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Figure 1. Effect of 50 ppm uniconazole on xylem water potential in water-stressed and unstressed *P. helepensis* (A) and *P. pinea* (B) seedlings. The experiment was initiated 15 days after treatment with uniconazole.

(*) = indicate a significant difference between control and treated seedlings at the same sampling date at P = 0.05.

Figure 2. Effect of 50 ppm uniconazole on xylem water potential in water-stressed and unstressed *P. pinea* seedlings. The experiment was initiated 9 months after treatment with uniconazole.

(*) = indicate a significant difference between control and treated seedlings at the same sampling dates at P. 0.05.

Figure 3. Effect of uniconazole on tolerance to drought stress of *Arabidopsis thaliana*. Seedlings grown in a medium containing 0.25 ppm uniconazole for 4 weeks were subjected to water stress by withholding irrigation. Left: uniconazole-treated plant; right: control plant 5 days after withholding irrigation.

II. EFFECT OF UNICONAZOLE ON GENE EXPRESSION

As noted above we focused on the effect of uniconazole on gene expression using *Arabidopsis* as a model system. We employed the differential display technique developed by Liang and Pardee (1992) to compare, identify and isolate genes expressed as mRNA following treatment with uniconazole. The strategy adopted was as follows: isolate of undegraded cellular RNA; reverse transcription of the RNA using a primer in order to produce single stranded DNA complements of a subset of the mRNA; rounds of polymerase chain reactions using other primers to amplify a set of cDNAs corresponding to a smaller subset of the mRNAs; display of the cDNAs produced in the form of bands on a matrix gel such as a DNA sequencing gel; extract bands of interest from the gel, reamplify and then clone and sequence them, and compare the sequence to data bank and use them to isolate longer sequences from a cDNA library; finally, study the function of the isolated gene(s).

Material and Methods

Plant Material - *A. thaliana* plants were grown and treated with uniconazole as described above or by spraying with a 1-ppm solution. At various dates after treatment, leaves were harvested, ground in liquid nitrogen, and immediately used for RNA purification.

RNA purification - Total RNA was purified using a phenol/chloroform-sarcosyl extraction/LiCl precipitation procedure. This procedure is similar to the one published by Sambrook et al. (1989) except that sarcosyl replaces SDS. mRNA was purified from total RNA by the PloyATtract System III Kit (Promega) according to the manufacturer's instructions.

Differential display - To identify differentially expressed genes, we employed the differential display technique developed by Liang and Pardee (1992) with some modifications. Briefly, purified total RNA (0.5-10.0µg) was reverse transcribed using oligo dT primers (dT₁₂VA, dT₁₂VC, dT₁₂VG, dT₁₂VT). Reverse transcription reactions (50µl total volume) containing 25µM oligo dT were carried out in a thermocycler as follows: reaction mixtures were heated at 70°C for 5 min, cooled to room temperature and then 200µM of dNTPs and 300 units of

MMLV reverse transcriptase were added. The reaction mixtures were heated at 37°C for 60 min, 95°C for 5 min and then brought to -20°C until used for PCR reactions.

A portion of the reverse transcription mixture was used as a template in the PCR reaction containing two arbitrary 10-base primers (OP-DDRT, Operon). When cDNA was detected by autoradiography, [α -³⁵S]dATP was included in the reaction mixtures. The reaction mixtures were heated at 95°C for 1 min, and then 40 cycles of PCR were performed with cycle times of 30 s at 94°C, 2 min at 40°C and 30 s at 72°C. At the end of the PCR cycles the reaction mixtures were heated at 72°C for 5 min.

The cDNAs were analyzed by three different methods: (1) 6% denaturing polyacrylamide ("sequencing gel"), (2) 4% non-denaturing polyacrylamide with silver staining (Doss 1966), and (3) 2% agarose with ethidium bromide staining.

Cloning cDNA - Gel regions containing the differentially expressed cDNAs were excised from the dried gels and cDNA was extracted from polyacrylamide by heating in ddH₂O for 15 min followed by precipitation with ethanol and from agarose by the Jet-Sorb Kit (Genomed). The isolated cDNA fragments were reamplified using the appropriate pair of primers. Six μ l of the PCR product were cloned into pCRTM II vector, using The Original TA Cloning Kit (Invitrogen). The ligation mixture was transfected into *E. coli* INVaF⁺ and positive clones were selected using the blue/white phenotype. DNA plasmid was extracted by the alkaline lysis method (Sambrook et al., 1989). DNA from each clone was digested with *Eco*RI or with *Bam*HI + *Xba*I (Boehringer) to ensure the existence of the DNA insert.

Northern blot analysis

Northern analysis was performed as follows: 10 μ g of total RNA were separated by electrophoresis on a 1.5% denaturing agarose gel. Fractionated RNAs were blotted onto HybondTM N+ membrane (Amersham) according to the manufacturer's instructions. Oligolabeling of the cDNAs used as probes was carried out with Amersham Oligolabeling Kit. Blots were probed with ³²P-labeled cDNA probes at 60°C in hybridization buffer. After hybridizing overnight, the membranes were washed twice with 2 x saline sodium citrate/0.1% SDS at 60°C for 10 min. Autoradiography was performed with an X-ray film.

Construction of a cDNA library - A cDNA library was constructed according to Stratagene Instruction Manual. In the following we present a brief description of the procedures employed: isolation of mRNA from total RNA; preparation of first and second strand cDNA; blunting the cDNA termini; ligating the *EcoRI* adaptor; kinasing the *EcoRI* ends; *XhoI* digestion; size fractionation; ligating the cDNA into Uni-ZEP XR vector arms; packaging with Gigapack III Packaging Extract; Plating and titering; amplification of the Uni-ZAP XR library; DNA screening using the cDNA product as a probe.

Results and Discussion

A. Technical aspects of differential display

Since the differential display method developed by Liang and Pardee (1992) was first described a short time before the initiation of this project, we devoted a significant part of the research to examine some technical aspects of the method.

(1) Starting RNA material

The initial procedure recommended the use of mRNA (Liang and Pardee 1992). The isolation of intact RNA that is free of chromosomal DNA has been regarded as crucial for the success of differential display, since arbitrarily primed PCR, which was originally developed to detect genomic polymorphism, detects DNA. In fact, this contamination is believed to be a major source of false positives or negatives (see below) (Liang and Pardee 1995). The isolation of poly(A) RNA turned out to be unnecessary or even undesirable for differential display. The reason for this is oligo-dT primer contamination, which results in smearing on the DNA sequencing gel (Liang et al. 1993).

In our hands, DNase-treated total RNA was significantly superior to mRNA. Other researchers also utilized total RNA for differential display, particularly for plant tissues (cf. for example Sharma and Davis 1995). Therefore, in all the following described results, the starting material was total RNA.

(2) Reverse transcription

The original scheme of differential display used non-degenerate (Liang and Pardee 1992) or degenerate two-base-anchored oligo-dT primers (Liang et al. 1993) to subdivide mRNA populations. We, however, used one-base anchored oligo-dT primers (see the list in material and methods), based on a report on their successful use in differential display (Liang et al. 1995). This type of primers has the advantage of cutting down the redundancy on the number of reverse transcription reactions, and at the same time it avoids the use of degenerate primers which tend to under-represent certain populations of mRNAs.

(3) Polymerase chain reaction

The use of primers for amplification of the cDNAs has been subjected to many changes since the introduction of differential display. The original method employs a two base-anchored oligo-dT primer in combination with an arbitrary decamer primer to amplify the 3' termini of mRNAs.

Among the variations introduced since then, we found that a combination of 2 decamer arbitrary primers gives the best results, particularly in reproducibility and number of bands obtained. Others have also recommended the use of this combination (Sokolov and Prockop 1994). Liang and Pardee (1995) mention that a potential problem with this procedure is that the more abundant rRNAs and even tRNAs may be amplified, since mRNAs are not exclusively selected in the reverse transcription step. We, however, believe that the higher reproducibility of this procedure surpasses this problem.

(4) DNA separation

Various gel electrophoresis methods were examined in the present research for separating amplified cDNAs. The methods included denaturing polyacrylamide gel commonly used for DNA sequencing (Fig. 4), non-denaturing polyacrylamide gel with silver staining (Fig. 5), and agarose gel with ethidium bromide staining (Fig. 6).

Although denaturing polyacrylamide gel seems to be the preferred separation method for differential display (Liang and Pardee 1995), the other two alternatives have been employed

successfully by several research groups. We and others have examined the non-denaturing acrylamide gel since the method employing a long sequencing gel has the disadvantage of radioactive labeling and is time consuming. This alternative method offers resolution of cDNA fragments over a larger size than that observed with labeled products, and cloning of or probe preparation from differentially displayed cDNA fragments does not require careful alignment of an autoradiograph with the gel. Although the silver staining is believed to leave enough unreacted cDNAs, we repeatedly failed to recover enough cDNA from the gel, and therefore did not employ this method in the later stages of the research. Others, however, used the method successfully (cf. Doss 1996).

Agarose gel has most of the above mentioned advantages of non-denaturing acrylamide gel in addition to the ease of cDNA recovery from the gel. However, it is characterized by low resolution and low sensitivity of ethidium bromide staining. We have found that using a relatively high level of starting total RNA greatly improves the sensitivity. The method has been selected by us and other labs (cf. Sokolov and Prockop 1994) for differential display and it seems to gain increasing popularity.

(5) Northern blot confirmation of differentially expressed mRNAs

It is now well recognized that many of the PCR products obtained after purification from the resolving gel contain a significant proportion of contaminating sequences, even after gel purification of the reamplified products (Liang and Pardee 1995). This may lead to an artifactual isolation of false positives in the subsequent steps of the method. Northern blotting seems to be the standard method for confirmation that a cDNA band apparently differentially expressed on the gel indeed represents a true difference between tested samples. There are still some questions left to how this should be done, particularly whether to use reamplified cDNA probes directly from the gel or only after cloning. During the first stage of the research we performed the northern blot analysis after cloning, as suggested by Liang and Pardee (1992). However, based on experience gathered in neighboring labs, we later used reamplified cDNAs directly from the gel for northern blot analysis. Liang and Pardee (1995) mentioned that this procedure has some drawbacks such as obtaining no signal because of low abundance of mRNA or a high chance to detect false positives. From our experience it is obvious that the use of a high level of starting RNA when agarose gel is used for resolving cDNA greatly decrease the chances not to get a signal in northern blot analysis. In addition, the chances to detect false

positives can be decreased by running samples from each RNA preparation several times and selecting only those cDNA bands that are repeatedly differentially expressed.

An additional method was adopted by us to aid in selecting the correct cDNA for cloning. The method is based on reamplification of the cDNA probe directly from the northern blot membrane on which it was shown to be differentially expressed. This method is also recommended by other researchers (Li et al. 1994).

B. Gene expression in uniconazole-treated plants

Total RNA isolated from *Arabidopsis* plants treated with uniconazole by spraying or by soil treatment was compared to untreated control using differential display. Several differentially expressed bands which appeared on display gels were excised, reamplified by PCR and cloned into pCR™ II vector. We focused mainly on a 1.6 kb band obtained on an agarose display gel from RNA isolated from *Arabidopsis* plants 2 h after spraying with uniconazole (Fig. 6). We randomly picked several positive clones for further analysis. Digestion of the DNA extracted from positive clones with *EcoRI* revealed that except for clones 3 and 4, all clones contained the correct DNA insert (Fig. 7). All 8 clones were proved to contain the correct DNA insert by digestion with *BamHI*+*XbaII* (data not shown).

Only clone 8 was shown to be differentially expressed by northern blot analysis and it was used further as a probe to determine the relative transcript levels in control and uniconazole-treated plants. As shown in Figure 8, it hybridized to a ca. 2kb transcript which accumulated to significantly high levels shortly after treatment and declined gradually thereafter.

Other cloned differentially expressed cDNAs extracted from denaturing polyacrylamide gel did not give any signal in northern blot analysis. We, however, intend to use them as probes for screening a cDNA library.

Translation of the 1.6 kb DNA revealed several open reading frames intercepted by a stop codon. The open reading frames are 140 and 120 amino acids in length, indicating that the cloned DNA encodes for a real gene. It is quite possible that the cloning method (i.e. PCR) was responsible for the introduction of mutation that caused a frame shift. Gene bank search revealed no significant homology with translated DNA. In order to clone the correct full length

cDNA, we constructed a Uni-ZAP XR cDNA library from control and uniconazole-treated seedlings and screened the library with the 1.6 kb DNA probe. Several positives plaques were isolated and their characterization is underway. As mentioned above, the library will also be screened by cDNA probes reamplified directly from northern blot membranes on which differentially expressed mRNAs were detected.

Conclusions

Reports on increased resistance to environmental stresses in PGR-treated plants are still being published in large numbers. However, the underlying mechanism has not yet been elucidated. Without having any clue what direction to follow, we employed the differential display technique aimed to isolate genes which are induced following treatment with the growth retardant, uniconazole. Since this method was introduced only a short time before the initiation of this project, we had to devote many efforts to overcome technical problems and introduce new improvements. For this reason, the aim to isolate differentially expressed genes in uniconazole-treated seedlings has not been reached during the 3-year period of the research. We, however, are convinced that the data obtained until now provide us with a sound basis to continue this project, and we are indeed continuing it with other sources of money which we raised.

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Figure 4. Differential display of total RNA isolated from control and uniconazole-treated *A. thaliana* seedlings on denaturing polyacrylamide gel. Total RNA samples isolated either from control (lane 1) or from seedlings grown in a medium containing uniconazole for 3 weeks (lane 2) were reverse transcribed, and the resulting cDNAs were amplified using polymerase chain reaction in the presence of [α - 35 S]dATP according to Liang and Pardee (1992). The samples were size fractionated on 6% sequencing gel, and the dried gel was exposed to X-ray film. The figure shows a section of the gel. Each 3-lane group represents a different combination of oligo dT and an arbitrary decamer primer. Arrows indicate differentially expressed cDNAs extracted for further analysis.

Figure 5: Differential display of total RNA isolated from control and uniconazole-treated *A. thaliana* seedlings on non-denaturing polyacrylamide gel. Total RNA samples isolated either from seedlings grown in a medium containing uniconazole for 3 weeks (lane 1, two replicates of the same sample) or from control (lane 3) were processed as described in Figure 4 but without radioactivity. The samples were size fractionated on 4% non-denaturing polyacrylamide gel with silver staining. Each 3-lane group shows a different combination of oligo dT and an arbitrary decamer primer. Molecular markers are indicated next to selected markers in lane L (ladder).

Figure 6: Differential display of total RNA isolated from control and uniconazole-treated *A. thaliana* seedlings on agarose gel. Total RNA samples were processed as described in Figure 4 except that two arbitrary decamer primers were used for amplification and no radioactivity was included in the reaction mixtures. The samples were size fractionated on 2% agarose gel with ethidium bromide staining. Lanes 1-4: 2, 24, 48 and 72 h after spraying with uniconazole, respectively; lane 5: seedlings grown in uniconazole-treated medium for 3 weeks; lane 6: control; L:ladder. The arrow indicates a 1.6 kb cDNA that was differentially expressed 2 h after treatment with uniconazole.

Figure 7: Restriction analysis of 8 positive clones of the 1.6 kb cDNA (Fig. 6) with *EcoRI*. L: ladder.

Figure 8: Northern blot analysis of control and uniconazole-treated *A. thaliana* seedlings. Total RNA of the various treatments was subjected to RNA blot hybridization analysis using the 1.6 kb cDNA (Fig. 6) as a probe. The size of the hybridizing mRNA was estimated by comparison to RNA size markers (Life Technologies). Lanes 1-4: 2, 24, 48 and 72 h after spraying with uniconazole, respectively; lane 5: seedlings grown in a medium containing uniconazole for 3 weeks; lane 6: control.