Identification and characterization of water-stress-responsive genes in hydroponically grown maritime pine (Pinus pinaster) seedlings

Christian Dubos, Grégoire Le Provost, David Pot, Franck Salin, Céline Lalanne, Delphine Madur, Jean-Marc Frigerio, Christophe Plomion

Tree Physiology 23, 169–179

Identification and characterization of water-stress-responsive genes in hydroponically grown maritime pine (*Pinus pinaster*) seedlings

CHRISTIAN DUBOS,[1] GREGOIRE LE PROVOST,[1] DAVID POT,[1] FRANCK SALIN,[2] CELINE LALANE,[1] DELPHINE MADUR,[1] JEAN-MARC FRIGERIO[1] and CHRISTOPHE PLOMION[1,3]

[1] *Equipe de Génétique et Amélioration des Arbres Forestiers, INRA, 69 route d'Arcachon, 33612 Cestas Cédex, France*

[2] *Laboratoire de Chimie des Substances Végétales, Institut du Pin, 351, Cours de la libération, 33405 Talence Cédex, France*

[3] *Author to whom correspondence should be addressed (plomion@pierroton.inra.fr)*

Received July 9, 2002; accepted August 30, 2002; published online January 31, 2003

Summary Growth, development and productivity of long-lived organisms such as forest trees are continuously challenged by abiotic stresses, and may also be greatly affected by predicted climatic change. As a first step toward creating stress-resistant maritime pine (*Pinus pinaster* Ait.) varieties by marker-assisted breeding, we describe the identification and characterization of water-stress-responsive genes in hydroponically grown seedlings that were well watered (–0.08 MPa) or subjected to water deprivation (–0.45 MPa) by the addition of polyethylene glycol. The cDNA amplified fragment-length polymorphism (cDNA-AFLP) technique was used to identify genes regulated by water deprivation. Approximately 4000 transcript-derived fragments (TDFs) were screened, of which 28 increased and 20 decreased in seedlings subjected to water deprivation. Of these 48 TDFs, 62.6% corresponded to proteins of known function, which indicate the main mechanisms involved in the osmotic stress response (photosynthesis, carbohydrate metabolism, cell wall synthesis and plant defense). We found that 16.6% of the 48 TDFs were similar to *Arabidopsis thaliana* (L.) Heynh gene products, 10.4% were similar to *Pinus taeda* L. expressed sequence tags (ESTs) and 10.4% did not match any sequences in the public databases. The relative abundance of these transcripts was quantitatively analyzed by reverse northern of both needle and root tissues, confirming the effectiveness of the cDNA-AFLP technique in detecting differentially expressed genes. The identification and characterization of water-stress-responsive genes provide new insights into the nature of the machinery involved in the response to water deprivation in a forest tree.

Keywords: cDNA-AFLP, osmotic stress, polyethylene glycol, reverse northern.

Introduction

Changes in gene expression leading to the synthesis and activation of novel proteins in plants subjected to water deficits have been well documented in crops and model plant systems such as *Arabidopsis thaliana* (L.) Heynh and *Craterostigma plantagineum* (Ingram and Bartels 1996, Shinozaki and Yamaguchi-Shinozaki 1996, Riccardi et al. 1998, Cushman and Bohnert 2000). However, little is known about the biochemical and molecular mechanisms involved in drought tolerance in forest trees (Chang et al. 1995, Pelah et al. 1995, 1997, Mayne et al. 1996, Padmanabhan et al. 1997, Costa et al. 1998, Richard et al. 2000, Dubos and Plomion 2001). To our knowledge, no comprehensive study on water-stress-responsive genes and proteins in forest trees has been published.

Given the characteristics of forest trees (e.g., large size, long life span), it is possible that the problems posed by water stress in annual plants have been solved by adjustments of specific biochemical pathways in forest trees. Similarly, if the same water-stress-responsive genes are expressed in annual plants and forest tree species, it is possible that they differ in the way that they are regulated. For example, except for phase change (Jarvis et al. 1996) and somatic embryo development (Dong and Dunstan 1997), few studies on drought-stressed conifers have described the involvement of genes or proteins corresponding to lea or dehydrin, even though these proteins are frequently reported to be linked to the water-stress response in annual species. Similarly, no abscisic acid-responsive (*rab*) genes and few drought-inducible genes have been reported in drought-stressed conifers to date, although they have been well documented in crop plants (Vernon and Bonhert 1992, Nelson et al. 1998).

We believe that the genetic improvement of tree growth and productivity in drought-prone environments cannot be derived solely from studies of crops and model organisms, but requires an understanding of the molecular mechanisms involved in stress responses and stress adaptation in trees. A response to this challenge is underway with maritime pine (*Pinus pinaster* Ait.), the main conifer species used for reforestation in south-western Europe where contrasting climatic conditions prevail. In France, where maritime pine represents 10% of the forested area, this species often faces severe drought in summer, limiting growth and productivity. To elucidate the processes that determine its adaptability to dry sites, molecular (Costa et al. 1998, Dubos and Plomion 2001, Dubos and Plomion 2002),

genetic (Costa 1999, Brendel et al. 2002) and physiological (Nguyen-Queyrens et al. 1998, 2003*a*, 2003*b*, Fernandez et al. 2000) studies are currently being undertaken.

The main objective of this study was to identify and characterize candidate genes involved in the water-stress response of maritime pine seedlings raised in hydroponic solution. Among seedlings subjected to a combination of osmotic stress duration and osmotic stress severity, we selected those from two contrasting conditions to examine the molecular response to water deprivation. We used the cDNA-amplified fragment-length polymorphism (cDNA-AFLP) technique (Bachem et al. 1996) to examine gene expression regulation at the transcriptional level in developing needles. Reverse northern was used to quantitatively analyze the isolated clones from the needles and roots of maritime pine seedlings.

Materials and methods

Plant material and water deficit treatment

Pinus pinaster Ait. seeds were sterilized and placed on moist filter paper until germination (N'Guyen and Lamant 1989). Five groups of 100 randomly chosen seedlings were selected and transferred to an aerated nutrient solution (Seillac 1960). After 3 weeks of growth, the treatments were applied. One group of seedlings grown in Seillac solution (–0.08 MPa) served as controls. Water stress treatments were applied to the remaining seedlings by lowering the osmotic potential of the nutrient solution to –0.15, –0.30, –0.45 and –0.60 MPa with polyethylene glycol (PEG3350, Sigma-Aldrich, St. Louis, MO). Different osmotica have been tested to induce osmotic stress in hydroponically grown maritime pine seedlings, including sorbitol, mannitol and different molecular weight PEGs. Triboulot (1996) showed that PEG3350 had the advantage of little to no toxicity and good aeration of the nutrient solution. The PEG3350 from Sigma is also free of heavy metal contamination, reducing risk of toxicity of this compound. In contrast, low molecular weight PEGs (< 1000 g mol^{-1}) are highly absorbed by the plant and induce toxicity and eventually mortality, whereas high molecular weight PEGs (> 6000 g mol^{-1}) are too viscous and can induce anoxia when they are used in long-term experiments. An additional group of unstressed plants was raised in a 1:1:1 (v/v) mixture of bark:peat:sand and used as a second control group. The experiment was conducted in a growth chamber providing a 16-h photoperiod, a day/night temperature of 24/18 °C, a day/night relative humidity of 45/70% and a quantum flux of 260 µmol m^{-2} s^{-1}.

All seedlings were harvested after 21 days of treatment, immediately frozen in liquid nitrogen and stored at –80 °C. Media were changed once a week to avoid fungal contamination. The –0.45 MPa treatment resulted in 50% seedling mortality, indicating that the stress was substantial, but that adaptation occurred in the surviving seedlings.

Determination of chlorophyll concentration

Chlorophyll a and b concentrations were measured on Day 21 in seedlings in all treatments. Chlorophylls were extracted in 80% (v/v) acetone and quantified as described by Arnon (1949).

mRNA fingerprinting

Total RNA was extracted by the procedure of Chang et al. (1993) from needles and roots of two samples each of 50 seedlings harvested after 21 days in the control (–0.08 MPa) and stress (–0.45 MPa) treatments. Purification and cDNA synthesis were performed according to the method described by Dubos and Plomion (2002). The nonradioactive cDNA-AFLP procedure described by Dubos and Plomion (2002) was used to identify differentially expressed fragments. Briefly, in the selective amplification step, primers were complementary to the adaptors PstI and MseI, with one or two additional selective 3′-end nucleotides (PstI primers: 5′-GACTGCGTACAT GCAGX-3′, where X corresponds to G, AG, AT, CA, or CC; MseI primers: 5′-GATGAGTCCTGAGTAAX-3′, where X corresponds to A, C, G, AC, AT, CC, GA and GT). After electrophoresis, gels were silver-stained and dried, and informative fragments were eluted in 5 µl of distilled water as described by Chalhoub et al. (1997) and used as the matrix for PCR re-amplification, under the same conditions as in the selective PCR reaction. The transcript-derived fragments (TDFs) were fractionated on 2% agarose gels and purified using a PCR product purification kit (Promega, Madison, WI). Cloning of the TDFs was performed using the TOPO TA-cloning® kit (Invitrogen, Carlsbad, CA) and purified plasmids were used as the template for sequencing. Sequences were determined with a Li-Cor 4000 sequencer (Li-Cor, Lincoln, NE). Nucleotide sequences were loaded onto the Infobiogen Resource Centre (http://www.infobiogen.fr/) form for BLAST searching. Scores and E-values from BLAST-X were considered when determining significant similarities: we used a score greater than 45 and an E-value of less than $5e^{-4}$. Sequences were also compared to maritime pine expressed sequence tags (ESTs) (1900 entries available in the EMBL database) using BLAST-N software. Highly significant matches were obtained for five TDFs (Pp.ap12, Pp.ap31, Pp.ap35, Pp.ap37 and Pp.ap41), resulting in longer consensus sequences at the 5′- or 3′-end or both.

Characterization of differentially expressed TDFs

Reverse northern and data analysis Inserts of sequenced cDNA clones were amplified by PCR in 50 µl reaction volumes, using 2 µM of primers (M13 forward: 5′-TGACCGG CAGCAAAATG-3′ and reverse: 5′-GGAAACAGCTATGA CCATG-3′) that are complementary to vector sequences flanking both sides of the cDNA inserts. The thermal cycling parameters were 94 °C (5 min) followed by 40 cycles of 94 °C (45 s), 55 °C (45 s) and 72 °C (45 s), and a final extension step at 72 °C (10 min). The PCR products were diluted to a final concentration of 5 ng $µl^{-1}$, denaturated with NaOH (0.2 M final concentration) for 10 min, and spotted onto GeneScreen Plus® nylon membrane filters (8 × 12 cm, NEN Life Science, Boston, MA) using a Millipore 96-well dot-blot apparatus. Each clone was spotted on 12 different membranes. Membranes were baked

for 1 h at 80 °C. Controls consisting of distilled water, and 15 replicates of a cDNA corresponding to a *Pisolithus tinctorius* receptor tyrosine Kinase/Ras (kindly provided by S. Duplessis, INRA-Nancy, France) as an internal quantification standard (i.e., the cDNA is not expressed in plants; Arabidopsis Genome Initiative, 2000) were spotted at specific positions on the nylon membranes.

Total RNAs used for the cDNA-AFLP procedure were used to prepare complex probes from unstressed and stressed needles and roots by reverse transcription using the SMART-PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA). Labeling of the cDNA probes was done in the presence of 50 µCi of γ^{32}P-dATP and 50 µCi of γ^{32}P-dCTP with the Prime-it® II Random Primer Labeling kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

Arrayed filters were pre-incubated in hybridization solution (5× SSC, 5× Denhardt's solution, 0.5% SDS, 500 µg ml^{-1} shared salmon sperm DNA) for 4 h at 65 °C. The filters were incubated in the same solution with radiolabeled probes at 65 °C for 24 h. Half of the membranes were hybridized with the probe (aerial part and root) corresponding to the control condition, the other half with the probe corresponding to the drought-stressed condition (aerial part and root). The hybridized filters were washed successively for 5 min in 2× SSC containing 0.5% SDS at room temperature, 20 min in 2× SSC containing 0.1% SDS (65 °C), 20 min in 1× SSC containing 0.1% SDS (65°C), 20 min in 0.5× SSC containing 0.1% SDS (65 °C), 40 min in 0.1× SSC containing 0.1% SDS (65 °C) and then 10 min in 0.1% SDS (65 °C). Konica (Tokyo, Japan) medical X-ray film was exposed to the filters for 1 to 3 days.

After hybridization and exposure of X-ray film, the raw images were digitized with LabScan software (Amersham-Pharmacia Biotech). Detection and quantification of the signals representing hybridized DNA were performed with the ImageMaster 2D Elite software Version 3.0 (Amersham-Pharmacia Biotech). The local background value for each membrane was calculated based on 16 positions with no DNA-spotted area. The net signal was determined by subtraction of the mean background from the intensity of each spot. Interfilters normalization was performed based on the signal intensities of the receptor tyrosine Kinase/Ras control spotted at 15 locations on each filter.

The intensity, Y_{if}, of each spot i belonging to filter f was corrected by multiplying Y_{if} by a linear scaling factor K_f ($K_f = Z/Z_f$, where Z is the mean intensity of all control spots on all filters and Z_f is the mean intensity of all control spots on filter f). Before analysis of variance, the distribution of the corrected spot intensity was normalized by transforming (log base 10) the data. For each tissue (needle and root), the effect of the treatment (control versus stress) was tested with the following model: $Y_{ijk} = \mu + C_j + E_{ijk}$, where Y_{ijk} is the intensity value of the spot i ($i = 1$ to 48) in condition j ($j = 1$ to 2) for replicate k ($k = 1$ to 3), μ is the mean intensity of the spots in all conditions, C_j is the effect of condition j and E_{ijk} is the residual term.

3′-End RACE cloning Amplification of the 3′-end was performed using the 3′ RACE (rapid amplification of cDNA ends) kit (Gibco BRL). Reverse transcription was carried out using total RNA (5 µg) from stressed needles in the presence of an adaptor poly(T) tail (AP: 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′) and SuperScript™ II reverse transcriptase as described by the manufacturer. The second strand was amplified by PCR (35 cycles of 94 °C for 60 s, 55 °C for 60 s, 72 °C for 90 s, with a final extension at 72 °C for 10 min) with the AUAP adapter primer on the poly(A) tail (5′-GGCCACGCGTCGACTAGTAC-3′) and 5′ proximal primers designed for Pp.ap1, Pp.ap14, Pp.ap46 and Pp.ap54. The PCRs were performed in 50 µl of 1× buffer (Gibco BRL) containing 0.1 mM dNTPs, 0.2 µM of each primer, 2 mM $MgCl_2$, 0.5 U Taq DNA Polymerase (Gibco BRL) and 2 µl of diluted single-strand cDNA. The reaction products were purified, cloned and sequenced as described above for the isolation of TDF from silver-stained polyacrylamide gel.

5′-End PCR screening of the cDNA library An oriented cDNA library constructed in the Uni-Zap® XR vector (Stratagene) from control needles was screened. The PCR was performed directly on the library using the T3 universal primer (5′-AATTAACCCTCACTAAAGGG-3′) and 3′ proximal primers designed for Pp.ap3 and Pp.ap21, with the PCR parameters described for the 3′-end RACE cloning. Reaction products were purified, cloned and sequenced as previously described.

Results and discussion

Effect of water deprivation on chlorophyll concentration

Water deprivation, like other environmental stresses including cold, salinity or extreme temperatures, induces numerous complex biochemical and physiological responses in plants (Lichtenthaler 1998). For example, drought stress can induce several events such as the adjustment of stomatal conductance and carbon allocation (Borghetti et al. 1998), and modifications in photosynthetic pigments and the efficiency of the photochemical photosystem II (Loggini et al. 1999), resulting in altered plant growth (Figure 1). The production and proportions of metabolically important carbohydrates (sugars, starch and sugar alcohols) can be modified (Volaire and Thomas 1995) and the fatty acid composition of membrane lipids can also be changed (Zuniga et al. 1990, Maldonado et al. 1997).

Osmotic stress induced 25 and 30% decreases in the concentrations of chlorophyll a and chlorophyll b, respectively (Figure 2). A decrease in chlorophyll concentration is a commonly reported response of plants facing abiotic stresses (Jagtap et al. 1998, Sutinen et al. 2000) and is linked to stomatal closure and a subsequent reduction in photosynthesis. Chlorophyll loss is the consequence of increased production of superoxide, H_2O_2 and hydroxyl radicals (Asaka 1996). These active oxygen species are extremely damaging to proteins, pigments and lipids and can lead to chlorophyll degradation, photodamage and lipid photo-oxidation (Munné-Bosch et al. 1999). A decrease in chlorophyll concentration can also be considered an adaptive feature in plants grown un-

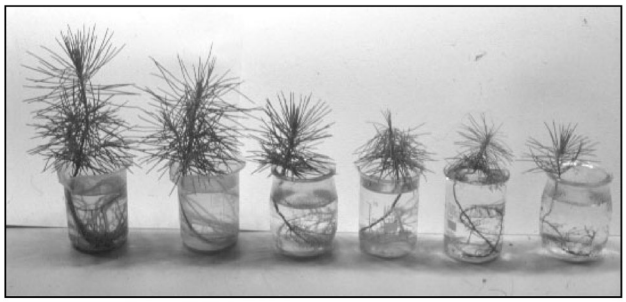

Figure 1. Morphology of control (C, G) and stressed (S) seedlings 21 days after the osmoticum (PEG = polyethylene glycol) was added in treatments S1–S4. Abbreviations: C = control seedling raised hydroponically without PEG (–0.08 MPa); G = seedling raised in the ground with a normal watering regime; S1 (–0.15 MPa), S2 (–0.30 MPa), S3 (–0.45 MPa), and S4 (–0.60 MPa) are seedlings raised hydroponically in the presence of different concentrations of the osmoticum PEG3350.

der stress, because it reduces both the amount of light intercepted by needles and the formation of activated oxygen, thereby limiting the possibility of damage to the photosynthetic machinery (Gilmore and Ball 2000).

Analysis of gene expression using the cDNA-AFLP technique

The cDNA-AFLP technique (Bachem et al. 1996) identified differentially expressed genes in needles of maritime pine seedlings growing under control and stress conditions. The cDNA fragments were amplified with 80 primer–enzyme combinations (PECs) and revealed by silver staining. An average of 49 TDFs per PEC was obtained, resulting in approximately 3950 screened cDNAs, ranging from 50 to 800 bp.

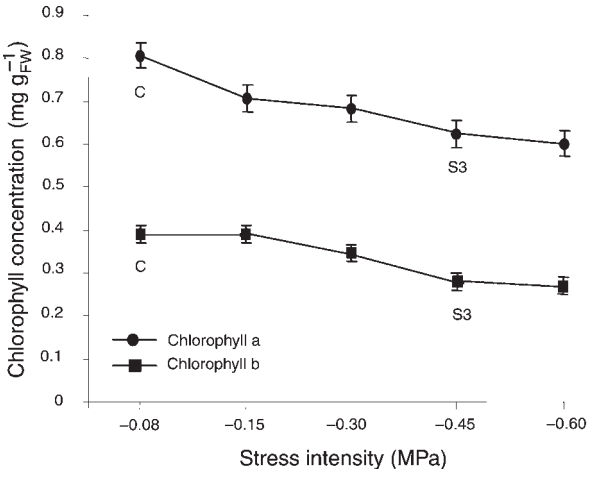

Figure 2. Variations in chlorophyll a and chlorophyll b concentrations in needles of maritime pine seedlings subjected to different osmotic stresses (–0.08, –0.15, –0.30, –0.45, –0.60 MPa) for 21 days. Abbreviations C and S3 correspond to the control and stress conditions used in the cDNA-AFLP assay, respectively.

About 7.6% (300) of the TDFs were differentially expressed between both conditions, of which 16% (48 fragments) displayed clear and reproducible presence/absence variation. Of these 48 TDFs, 28 increased and 20 decreased in response to water stress (Table 2). These 48 TDFs were isolated from the gel, re-amplified, cloned and sequenced.

Blast analysis showed that 30 of the 48 TDFs corresponded to genes of known function, whereas eight corresponded to hypothetical proteins, five corresponded to *Pinus taeda* ESTs and five did not match any sequences in the public databases. Additional sequence information toward the 3′- or 5′-ends, or both, was obtained for 11 fragments and, for five of them (Pp.ap3, Pp.ap19, Pp.ap21, Pp.ap35 and Pp.ap37), the full coding sequence was obtained. Fragment Pp.ap3 encoded a 251 amino acid protein (M_r = 26.9 kDa, pI = 7.78) corresponding to a *Pinus palustris* Mill. chlorophyll a/b binding protein type 4, with 96% identity and similarity. Fragment Pp.ap19 encoded a 73 amino acid protein (M_r = 8.2 kDa, pI = 5.1) corresponding to a hypothetical protein from *A. thaliana* with 69 and 84% identity and similarity, respectively. Fragment Pp.ap21 encoded a 235 amino acid protein (M_r = 26.1 kDa, pI = 8.3). It had 70% identity and 80% similarity with an *A. thaliana* nucleoside diphosphate kinase III. Fragment Pp.ap35 encoded a 143 amino acid protein (M_r = 15.3 kDa, pI = 5.1). It had 41 and 57% identity and similarity, respectively, with a cytokinin-repressed gene from *Cucumis sativus* L. Fragment Pp.ap37 encoded a 113 amino acid protein (M_r = 12.6 kDa, pI = 8.8), with an amino acid sequence that matched the *A. thaliana* translation initiation factor SUI1, with 88 and 96% identity and similarity, respectively.

Quantitative analysis of gene expression by reverse northern

The 48 isolated TDFs were analyzed by reverse northern to measure the relative abundance of the transcripts in needles and roots. Overall, the data obtained with this technique agreed with those obtained by the cDNA-AFLP technique, validating the results obtained by cDNA-AFLP, and confirming its effectiveness in detecting differentially expressed genes. We note that the statistical design of the reverse northern experiment allowed the detection of small changes in gene expression that would have been undetected by the commonly used ± twofold ratio (Figure 3).

The qualitative variants (presence/absence) observed in the cDNA-AFLP assay always showed a quantitative variation in the reverse northern assay. This result demonstrates that none of the studied genes were specifically expressed in either the control or the stress conditions and that the cDNA-AFLP technique tends to yield "false condition-specific genes." On the one hand, the cDNA-AFLP may be unable to detect low-expressed transcripts, because the assay involves a series of dilutions (digested and ligated cDNA fragments are first diluted 10-fold, then the preamplification product is diluted 35-fold before being used for the selective amplification). On the other hand, low-expressed transcripts would show up clearly on reverse northern (or RT-PCR), because the cDNA synthesis kit (SMART-PCR cDNA Synthesis Kit) allows all mRNAs to be

Table 1. Homologies of sequences of AFLP fragments to sequences in the databases. Abbreviations: P = photosynthesis; GD = growth and development; T = transduction, transcription and translation; CM = carbohydrate metabolism; OM = other types of metabolism; CW = cell wall related genes; D = plant defense related genes; and S = significant at $P < 0.05$. Symbols: + = presence of the AFLP fragment; – = absence of the AFLP fragment; ↑ = increase in intensity in the reverse northern; and ↓ = decrease in intensity in the reverse northern.

Functional category	Clone	Pst/Mse*	Size (bp)	Accession no.	Behavior in needles				Behavior in roots			Homology	Accession no.	Species	BLAST Score/E-value
					cDNA-AFLP	Reverse northern			Reverse northern						
P	Pp.ap30	AG/GT	309	AJ297299	–	↓	(0.44)	S	↓	(0.52)	S	Preprotein translocase precursor (secA)	S65668	*Pisum sativum*	$192/1e^{-48}$
P	Pp.ap3[c, d]	GT/GA	751	AJ309094	–	↓	(0.50)	S	↓	(0.41)	S	Chlorophyll a/b binding protein type 4 (LHCII)	CAA78901	*Pinus palustris*	$472/e^{-132}$
P	Pp.ap39	AT/G	363	AJ309119	–	↓	(0.54)	S	↓	(0.36)	S	Chlorophyll a/b binding protein type 1 (LHCI)	Q00984	*Pinus palustris*	$83/2e^{-15}$
P	Pp.ap41[a]	AT/C	333	AJ309102	–	↓	(0.45)	S	↓	(0.13)	S	Chlorophyll a/b binding protein type 1 (LHCI)	AAB19040	*Pinus palustris*	$18/7e^{-48}$
P	Pp.ap17	G/AC	445	AJ309096	–	↓	(0.67)	S	↓	(0.82)		Rubisco small subunit (EC 4.1.1.39)	CAA31774	*Pinus thunbergii*	$211/4e^{-54}$
GD	Pp.ap8	GT/CC	193	AJ309121	–	↓	(0.48)	S	↓	(0.54)	S	EST, gibberellin-regulated protein (GAST1)	AW226116	*Pinus taeda*	$72/2e^{-10}$
GD	Pp.ap35[a, d]	AG/AC	291	AJ309087	+	↓	(0.91)		↓	(0.22)	S	Cytokinin-repressed gene (CR9)	BAA06153	*Cucumis sativus*	$61/3e^{-09}$
GD	Pp.ap38	AT/AC	317	AJ309101	–	↑	(1.21)	S	↓	(0.75)	S	RNA helicase/RNAse III protein (CAF)	AAF03534	*Arabidopsis thaliana*	$47/8e^{-05}$
T	Pp.ap29	AG/GT	319	AJ309099	–	↓	(0.60)		↓	(0.88)		ADP-ribosylation factor (ARF1)	BAA04607	*Oryza sativa*	$96/2e^{-19}$
T	Pp.ap21[c, d]	AG/GA	397	AJ309098	–	↓	(0.39)	S	↑	(1.05)		Nucleoside diphosphate kinase III (EC 2.7.4.6)	NDK3_SPIOL	*Spinacia oleracea*	$86/2e^{-34}$
T	Pp.ap12[a]	G/CC	525	AJ309112	+	↓	(0.21)	S	↓	(0.52)	S	Putative wall-associated protein kinase (WAK)	AAG50588	*Arabidopsis thaliana*	$59/3e^{-08}$
T	Pp.ap37[a, d]	AT/AC	223	AJ309088	+	↑	(1.06)		↑	(1.14)		Translation initiation factor (SUI1)	AAD25609	*Arabidopsis thaliana*	$94/2e^{-19}$
CM	Pp.ap6	GT/G	429	AJ309083	+	↓	(0.50)	S	↑	(1.53)		Glucose regulated repressor protein (GRR1)	AAB70660	*Glycine max*	$109/1e^{-23}$
CM	Pp.ap40	AT/G	242	AJ309089	+	↑	(1.33)	S	↓	(0.88)		Glucose regulated repressor protein (GRR1)	AAB70660	*Glycine max*	$59/1e^{-08}$
CM	Pp.ap42	CA/AT	545	AJ309090	+	↑	(1.38)	S	↓	(0.51)	S	Sucrose-phosphate synthase (EC 2.4.1.14)	BAA19241	*Saccharum officinarum*	$291/3e^{-78}$
CM	Pp.ap54[b]	AT/CC	348	AJ309093	+	↑	(1.29)	S	↑	(2.19)	S	Sucrose synthase (EC 2.4.1.13)	BAA88904	*Citrus unshiu*	$203/8e^{-52}$
CM	Pp.ap51	CA/GT	449	AJ419595	+	↑	(1.18)	S	↑	(3.27)	S	Pyrophosphate-dependent phosphofructokinase (EC 2.7.1.90)	BAB11328	*Arabidopsis thaliana*	$231/5e^{-60}$

Continue on next page.

Table 1 cont'd. Homologies of sequences of AFLP fragments to sequences in the databases. Abbreviations: P = photosynthesis; GD = growth and development; T = transduction, transcription and translation; CM = carbohydrate metabolism; OM = other types of metabolism; CW = cell wall related genes; D = plant defense related genes; and S = significant at $P < 0.05$. Symbols: + = presence of the AFLP fragment; – = absence of the AFLP fragment; ↑ = increase in intensity in the reverse northern; and ↓ = decrease in intensity in the reverse northern.

Functional category	Clone	Pst/Mse*	Size (bp)	Accession no.	Behavior in needles				Behavior in roots			Homology	Accession no.	Species	BLAST Score/E-value
					cDNA-AFLP	Reverse northern			Reverse northern						
OM	Pp.ap4	GT/A	606	AJ309082	+	↓	(0.35)	S	↓	(0.19)	S	Acid phosphatase (EC 3.1.3.2)	AAA34135	*Lycopersicon esculentum*	$190/8e^{-48}$
OM	Pp.ap14[b]	G/CC	200	AJ309084	+	↑	(1.80)	S	↓	(0.57)	S	Nodulin-like protein (MtN3)	BAA96219	*Oryza sativa*	$79/7e^{-15}$
OM	Pp.ap31[a]	AG/AT	215	AJ300718	+	↑	(1.37)	S	↑	(1.73)	S	Glycolate oxidase (EC 1.1.3.15)	BAB01334	*Arabidopsis thaliana*	$66/4e^{-11}$
OM	Pp.ap36	AG/AC	317	AJ309118	+	↑	(1.16)		↑	(1.41)	S	EST, putative plasma membrane associated protein	BF516652	*Pinus taeda*	$147/6e^{-33}$
OM	Pp.ap44	G/AT	258	AJ309103	–	↓	(0.67)	S	↓	(0.51)	S	Metallothionein-like protein (EMB30)	AAB01564	*Picea glauca*	$56/1e^{-07}$
CW	Pp.ap13	G/CC	446	AJ309095	–	↓	(0.56)	S	↓	(0.04)	S	Pectin methylesterase-like protein (EC 3.1.1.11)	BAB01985	*Arabidopsis thaliana*	$173/1e^{-42}$
CW	Pp.ap20	G/AC	178	AJ309097	–	↓	(0.60)	S	↓	(0.31)	S	Glycine-rich protein homolog (PtaGRP2)	AAF75823	*Pinus taeda*	$78/2e^{-14}$
CW	Pp.ap32	AG/AC	466	AJ309100	–	↓	(0.58)		↓	(0.20)	S	Arabinogalactan/proline-rich protein (PtaGRP4)	AAF75826	*Pinus taeda*	$48/5e^{-05}$
D	Pp.ap1[b]	G/C	294	AJ309081	+	↑	(1.27)		↑	(2.09)	S	Flavonoid 3′-hydroxylase (EC 1.14.13.21)	AAD56282	*Petunia hybrida*	$151/1e^{-36}$
D	Pp.ap26	AG/GA	314	AJ309086	+	↑	(1.95)	S	↑	(2.40)	S	Dihydroflavonol 4-reductase (EC 1.1.1.219)	BAA84940	*Camellia sinensis*	$57/5e^{-08}$
D	Pp.ap48	AT/C	326	AJ309091	+	↑	(2.02)	S	↑	(1.18)		Pathogenesis related protein (NTPRP27)	BAA81904	*Nicotiana tabacum*	$113/1e^{-30}$
D	Pp.ap49	AT/CC	253	AJ309092	+	↑	(1.23)		↑	(1.20)		Allene oxide synthase (EC 4.2.1.92)	CAB88032	*Lycopersicon esculentum*	$71/2e^{-12}$
D	Pp.ap16	G/AC	369	AJ309085	+	↑	(2.14)	S	↑	(1.73)	S	Glucosidase-like protein (EC.3.2.1.-)	CAB62327	*Arabidopsis thaliana*	$136/1e^{-31}$
	Pp.ap19[d]	G/AC	334	AJ309108	+	↑	(1.11)		↓	(0.50)	S	Hypothetical protein (NP_568201)	AY035161	*Arabidopsis thaliana*	$147/2e^{-35}$
	Pp.ap10	G/GT	439	AJ309106	+	↑	(1.67)		↓	(0.57)	S	Hypothetical protein (T22E19.7)	AAF26483	*Arabidopsis thaliana*	$45/5e^{-04}$
	Pp.ap15	G/AC	593	AJ309107	+	↑	(1.57)		↓	(0.70)		Hypothetical protein (F27D4.1)	AAD03372	*Arabidopsis thaliana*	$251/6e^{-66}$
	Pp.ap23	AG/GA	364	AJ309109	+	↑	(1.38)		↓	(0.68)	S	Hypothetical protein (F15K9.5)	AAC72109	*Arabidopsis thaliana*	$44/6e^{-04}$
	Pp.ap46[b]	CA/GA	483	AJ309110	+	↑	(1.31)		↓	(0.64)		Hypothetical protein (F18O22_220)	CAB87782	*Arabidopsis thaliana*	$131/5e^{-30}$

Continue on facing page.

Table 1 cont'd. Homologies of sequences of AFLP fragments to sequences in the databases. Abbreviations: P = photosynthesis; GD = growth and development; T = transduction, transcription and translation; CM = carbohydrate metabolism; OM = other types of metabolism; CW = cell wall related genes; D = plant defense related genes; and S = significant at $P < 0.05$. Symbols: + = presence of the AFLP fragment; – = absence of the AFLP fragment; ↑ = increase in intensity in the reverse northern; and ↓ = decrease in intensity in the reverse northern.

Functional category	Clone	Pst/Mse*	Size (bp)	Accession no.	Behavior in needles				Behavior in roots			Homology	Accession no.	Species	BLAST Score/E-value
					cDNA-AFLP	Reverse northern			Reverse northern						
	Pp.ap47	AG/GT	254	AJ309111	+	↑	(1.79)	S	↑	(1.13)		Hypothetical protein (F25A4.23)	AAD55296	*Arabidopsis thaliana*	$91/3e^{-28}$
	Pp.ap2	G/C	289	AJ309104	–	↓	(0.34)	S	↑	(3.48)	S	Hypothetical protein (F5K20_280)	CAB88360	*Arabidopsis thaliana*	$57/4e^{-08}$
	Pp.ap28	AG/GT	499	AJ309105	–	↓	(0.39)	S	↓	(0.66)		Hypothetical protein (F6E13.26)	AAC23417	*Arabidopsis thaliana*	$83/1e^{-15}$
	Pp.ap7	G/CC	231	AJ309114	+	↑	(1.33)	S	↑	(2.06)	S	EST	BF518235	*Pinus taeda*	$105/1e^{-20}$
	Pp.ap18	G/AC	255	AJ309122	–	↓	(0.67)	S	↓	(0.06)	S	EST	AW011420	*Pinus taeda*	$72/2e^{-10}$
	Pp.ap25	AG/GA	348	AJ309123	–	↓	(0.65)		↓	(0.38)	S	EST	BG526931	*Pinus taeda*	$591/1e^{-166}$
	Pp.ap33	AG/AC	319	AJ309124	–	↓	(0.86)		↓	(0.85)		EST	BF610233	*Pinus taeda*	$311/2e^{-82}$
	Pp.ap34	AG/AC	326	AJ309125	–	↓	(0.64)		↓	(0.78)		EST	BF610233	*Pinus taeda*	$294/3e^{-77}$
	Pp.ap9	GT/GT	477	AJ309115	+	↑	(1.24)		↑	(1.42)		No hit			
	Pp.ap22	AG/GA	369	AJ309116	+	↑	(1.18)		↑	(1.11)		No hit			
	Pp.ap27	AG/GA	278	AJ309117	+	↑	(1.77)		↑	(1.20)		No hit			
	Pp.ap50	CA/GT	542	AJ309120	+	↑	(1.02)		↑	(1.07)		No hit			
	Pp.ap43	CA/AT	350	AJ309126	–	↓	(0.06)	S	↓	(0.44)	S	No hit			

* Selective bases of each Pst and Mse primer enzyme combination.

[a] Match to the maritime pine EST database.
-Pp.ap41 (684 bp) hits to *Pinus thunbergii* chlorophyll a/b binding protein type 1 (score/E-value: $363/1e^{-99}$; Accession number: CAA43907).
-Pp.ap35 (707 bp) hits to *Cucumis sativus* cytokinin-repressed gene CR9 (score/E-value: $88/9e^{-18}$; Accession number: BAA06153).
-Pp.ap37 (635 bp) hits to *Arabidopsis thaliana* translation initiation factor SUI1 (score/E-value: $213/2e^{-54}$; Accession number: AAD25609).
-Pp.ap31 (527 bp) hits to *Arabidopsis thaliana* glycolate oxidase (score/E-value: $124/1e^{-27}$; Accession number: BAB01334).
-Pp.ap12 (757 bp) hits to *Arabidopsis thaliana* putative wall-associated protein kinase (score/E-value: $43/4e^{-03}$; Accession number: AAG50588).

[b] 3′-RACE: match to the non-redundant database.
-Pp.ap54 (787 bp) hits to *Citrus unshiu* Sucrose synthase (score/E-value: $382/1e^{-105}$; Accession number: BAA88904).
-Pp.ap14 (588 bp) hits to *Medicago truncatula* MtN3-like protein (score/E-value: $94/2e^{-18}$; Accession number: BAA96219).
-Pp.ap1 (707 bp) hits to *Pelargonium hortorum* flavonoid 3′-hydroxylase (score/E-value: $179/3e^{-44}$; Accession number: AAG49315).
-Pp.ap46 (665 bp) hits to *Arabidopsis thaliana* hypothetical protein F18O22_220 (score/E-value: $134/7e^{-36}$; Accession number: CAB87782).

[c] 5′-end amplification from library: match to the non-redundant database
-Pp.ap3 (819 bp) hits to *Pinus sylvestris* Chlorophyll a/b binding protein type 4 (score/E-value: $504/1e^{-142}$; Accession number: CAA78932).
-Pp.ap21 (914 bp) hits to *Arabidopsis thaliana* Nucleoside diphosphate kinase III (score/E-value: $310/2e^{-83}$; Accession number: NDK3_ARATH).

[d] Full coding sequence available.

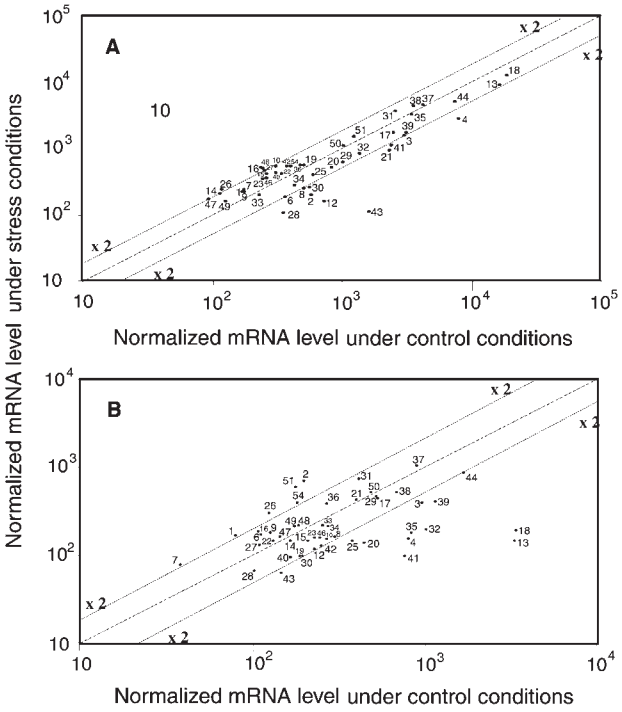

Figure 3. Scatter plots showing expression levels of the 48 transcript-derived fragments isolated by cDNA-AFLP from needles (A) and roots (B) of seedlings grown in control and osmotic stress conditions. For each clone, numbered as in Table 1, transcript levels are expressed in arbitrary units and plotted on a $\log_{10}$-$\log_{10}$ scale. Dotted lines indicate twofold expression differences between control and stress conditions.

amplified and subsequently detected. In addition to the sensitivity of the technique, it is possible that different members of a gene family cross-hybridized in the reverse northern assay, resulting in less contrasted differential expression. However, because all genes showed the same trend in terms of the degree of variation between the two assays, this hypothesis is less likely.

Gene function and expression in needles

Only major modulations of mRNA abundance in array assays are discussed in the next two sections.

In needles (Figure 3A), the nature of the variation (increase or decrease) detected by the cDNA-AFLP and reverse northern assays was similar for most (43 of 48) of the clones (Table 1). Of these 43 clones, 25 displayed significant differences between the two conditions. Of these 25, 11 and 14 increase or decrease by a factor ranging from 1.18 to 2.14 and 1.49 to 16.6, respectively. For the remaining clones (5 of 48), the nature of the variation differed between assays, and four clones (Pp.ap4: acid phosphatase; Pp.ap6: glucose regulated repressor protein; Pp.ap12: putative plasma membrane protein; and Pp.ap38: RNA helicase/RNAse III protein) showed a significant difference in spot intensity based on the ANOVA model. An intriguing finding from recent genomic and EST sequencing projects is the omnipresence of large gene families in plant genomes, especially conifer genomes (Kinlaw and Neale 1997). Therefore, it is possible that the differences we observed between assays arise because different members of these genes were revealed by the two techniques.

Among genes with unknown function, clones were found to increase by a factor of up to 1.79 (Pp.ap47) or decrease by a factor of up to 16.6 (Pp.ap43). Among genes with known function, the highest and lowest differential expressions were for plant defense and photosynthesis-related genes, respectively. All five genes associated with plant defense increased their expression in stressed tissues. Clone Pp.ap1 corresponded to flavonoid 3′,5′-hydroxylase (F3′H). Clone Pp.ap26 was similar to dihydroflavol 4-reductase (DFR). Induction of F3′H mRNA and protein has already been described in *Catharanthus roseus* (L.) G. Don in response to UV radiation (Kaltenbach et al. 1999). Induction of DFR mRNA was observed during nitrogen deficiency and drought stress (Bongue-Baetelsman and Philips 1995, Iuchi et al. 1996). Clone Pp.ap48 was similar to a pathogenesis-related protein NtPRP-27. Fragment Pp.ap49 corresponded to allene oxide synthase, a cytochrome P450 protein. Fragment Pp.ap16 corresponded to glucan endo-1,3-β-glucosidase, a β-1,3-glucanase-like protein. In other studies, this gene was induced during pathogen infection (Vogeli-Lange et al. 1994), ozone treatment (Pell et al. 1997), freezing (Hincha et al. 1997) and water deprivation (Cui et al. 2000). Here we report its accumulation in osmotically stressed seedlings of a pine species. The unambigous increase in defense-related genes in stressed maritime pine seedlings agrees with the general behavior of this functional category of genes in plants subjected to abiotic stresses.

Five clones were classified in the functional category of photosynthesis-related genes. Clone Pp.ap30 was similar to the preprotein translocase SecA. Clones Pp.ap3 on the one hand, and Pp.ap39 and Pp.ap41 on the other hand, corresponded to chlorophyll a/b-binding proteins type 4 and type 1, respectively. These three proteins are involved in the antenna system of the photosynthetic apparatus. Clone Pp.ap17 corresponded to the small subunit of the ribulose-biphosphate carboxylase. Their general decrease in expression in needles and roots (Table 2) is consistent with a previous study in maritime pine, where a decrease in protein expression for other photosynthesis-related genes such as Rubisco activase and an increase in RbcS protein degradation during drought were found (Costa et al. 1998). Drought has been reported to modify the expression level of photosynthesis-related genes in other plant species (Bartholomew et al. 1991).

Gene function and expression in roots

The expression pattern of the water-stress-responsive genes detected in needles by cDNA-AFLP was also studied in roots by reverse northern (Figure 3B). In root tissue, a total of 30 clones (62.5%) displayed significant differences between the control and stress conditions, of which 9 and 21 increased or decreased by a factor ranging from 1.41 to 3.48 and 1.33 to

25.0, respectively. Among genes of unknown function, clones were found to increase by a factor of up to 3.48 (Pp.ap2) or decrease by a factor of up to 16.7 (Pp.ap18). Among known function genes, down- and up-regulations were much more notable in roots compared with needles, although this was not selected for in the original cDNA-AFLP assay. As exemplified by Pp.ap1 (F3′H) and Pp.ap26 (DFR), defense-related genes were also up-regulated in the root system. The three clones belonging to cell-wall category genes showed a strong decrease in expression in roots (to a much greater extent than in needles) when subjected to water stress. Clone Pp.ap13 was identical to a member of the pectin methylesterase multigene family. It was strongly repressed in roots (25-fold decrease). Clones Pp.ap20 and Pp.ap32 corresponded to glycine-rich (GRP) and arabinogalactan/proline-rich proteins (AGP), respectively.

Comparative gene expression between needles and roots

Most clones (75%) showed a similar pattern of expression in both control and stress conditions in needles and roots. However, although the nature of variation appeared to be conserved between needles and roots, the degree of variation was characteristic of each organ. Clones belonging to carbohydrate metabolism were of particular interest. Three of them (Pp.ap6, Pp.ap40 and Pp.ap42) showed contrasting behavior between needles and roots, suggesting tissue-specific regulation of the corresponding genes, and two of them (Pp.ap54 and Pp.ap51) were more enhanced in roots. Clones Pp.ap6 and Pp.ap40 were similar to the glucose-regulated repressor protein GRR1, a central component of a glucose signal transduction mechanism responsible for glucose-induced gene expression. The expression patterns in roots contrasted with those found in needles. Although these two clones were similar to the GRR1 gene, the translation of their common sequence showed only 65% identity and 81% similarity. These results may indicate the presence of at least two members whose expression seems to be tissue specific. Clones Pp.ap42 and Pp.ap54 corresponded to sucrose-phosphate synthase (SPS) and sucrose synthase (Susy), respectively. On cDNA-AFLP profiles, both were enhanced in stressed needles. Increased SPS activity has been described in many plant species subjected to drought (e.g., Geigenberger 1999). Induction of Susy was also reported during various stresses such as cold, oxygen deficiency or drought (Hesse and Willmitzer 1996, Zeng et al. 1998, Kleines et al. 1999). Both clones displayed opposite trends in the roots: a twofold decrease for SPS and a twofold increase for Susy. Clone Pp.ap51 was similar to a pyrophosphate-dependent phosphofructokinase and was found to increase in both tissues, but particularly in roots (3.27-fold increase). The regulation of these five genes demonstrates the central role of soluble carbohydrate in osmoregulation of water-stressed maritime pine seedlings.

Conclusion and perspectives

In most cases where the differentially expressed TDFs were analyzed by reverse northern, the result obtained by cDNA-AFLP was confirmed, demonstrating the ability of the cDNA-AFLP technique to identify differentially expressed genes. However, in a few cases, a strong divergence was observed between assays, suggesting that full characterization of these genes is needed before any conclusion can be drawn. Most genes of known function that were affected by water deficit in maritime pine seedlings corresponded to categories already described as being affected by abiotic stresses in other plants (photosynthesis, cell wall, defense and carbohydrate metabolism). However, we detected no dehydration tolerance genes corresponding to dehydrin, lea and rab.

We identified some genes of known function that have not previously been reported as being affected by water deficit in any organism (e.g., translocase secA, nucleoside diphosphate kinase III and nodulin-like protein). This finding may indicate that pine trees respond to osmotic stress by strengthening or repressing specific genes that are unaffected by drought in annual plants.

It is unknown if the water-stress-responsive genes that we identified play important roles in drought tolerance. We are currently using the Single Strand Conformation Polymorphism technique to map these genes (Plomion et al. 1999) on genetic maps where quantitative trait loci (QTLs) for physiological traits (e.g., carbon isotope discrimination) have been localized (Costa 1999, Brendel et al. 2002). Any co-location between a gene and a QTL could be further validated by association studies in natural populations (e.g., Remington et al. 2001, Thornsberry et al. 2001), genetic transformation in maritime pine or the phenotypic evaluation of the corresponding *Arabidopsis* mutants.

The relatively high proportion of isolated sequences corresponding to hypothetical proteins, ESTs or presenting no hit in public databases (37.4%) underscores how little is known about the function of genes regulated by water deprivation. Characterization of expression patterns of unknown genes using microarrays will facilitate the functional analysis of these genes. This is the goal of the "Lignome" project (http://www.pierroton.inra.fr/Lignome/) that has been launched by INRA for several woody plants including maritime pine. Additionally, investigations will also be needed to determine the nature of the differentially expressed gene pool that is expressed transiently at an early stage of the drought response.

Acknowledgments

C.D. was supported by a Doctoral Scholarship from the French Ministry of Research and Technology. This work was funded by the European Union (INCO-IC18-CT97/0200), Région Aquitaine (n°98030 7005) and INRA (ECOGENE and LIGNOME).

References

Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–815.

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*. Plant. Physiol. 24:1–15.

Asaka, K. 1996. Radical production and scavenging in the chloroplast. *In* Photosynthesis and the Environment. Ed. N.R. Baker. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 123–150.

Bachem, C.W.B., R.S. Van der Hoeven, S.M. de Bruijn, D. Vreugdenhil, M. Zabeau and R.G.F. Visser. 1996. Visualisation of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J. 9:745–753.

Bartholomew, D.M., G.E. Bartley and P.A. Scolnik. 1991. Abscisic acid control of *rbc*S and *cab* transcription in tomato leaves. Plant Physiol. 96:291–296.

Bongue-Baetelsman, M. and D.A. Philips. 1995. Nitrogen stress regulates gene expression of enzymes in the flavonoid biosynthetic pathway of tomato. Plant Physiol. Biochem. 33:539–546.

Borghetti, M., S. Cinnirella, F. Magnani and A. Saracino. 1998. Impact of long-term drought on xylem embolism and growth in *Pinus halepensis* Mill. Trees 12:187–195.

Brendel, O., D. Pot, C. Plomion, P. Rozenberg and J.-M. Guehl. 2002. Genetic parameters and QTL analysis of $\delta^{13}C$ and ring width in maritime pine. Plant Cell Environ. 25:945–953.

Chalhoub, B.A., S. Thibault, V. Laucou, C. Rameau, H. Höfte and R. Cousin. 1997. Silver staining and recovery of AFLP amplification products on large denaturing polyacrylamide gels. Biotechniques 22:216–220.

Chang, S., J. Puryear and J. Cairney. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11:113–116.

Chang, S., J. Puryear and J. Cairney. 1995. Gene expression under water deficit in loblolly pine (*Pinus taeda* L.): isolation and characterization of cDNA clones. Physiol. Plant. 95:139–148.

Costa, P. 1999. Réponse moléculaire, physiologique et génétique du pin maritime à une contrainte hydrique. Ph.D. Thesis, Université Henri Poincaré, Nancy, 116 p.

Costa, P., N. Bahrman, J.-M. Frigerio, A. Kremer and C. Plomion. 1998. Water-deficit-responsive proteins in maritime pine. Plant Mol. Biol. 38:587–596.

Cui, Y.X., A.A. Bell, O. Joost and C. Margill. 2000. Expression of potential defense response genes in cotton. Physiol. Mol. Plant. Pathol. 56:25–31.

Cushman, J.C. and H. Bohnert. 2000. Genomic approaches to plant stress tolerance. Curr. Opin. Plant Biol. 3:117–124.

Dong, J.Z. and D.I. Dunstan. 1997. Characterization of cDNAs representing five abscisic acid-responsive genes associated with somatic embryogenesis in *Picea glauca*, and their responses to abscisic acid stereostructure. Planta 203:448–453.

Dubos, C. and C. Plomion. 2001. Drought differentially affects expression of a PR-10 protein in needles of maritime pine (*Pinus pinaster* Ait.) seedlings. J. Exp. Bot. 358:1143–1144.

Dubos, C. and C. Plomion. 2003. Identification of water-deficit responsive genes in maritime pine (*Pinus pinaster* Ait.) roots. Plant Mol. Biol. 51:249–262.

Fernandez, M., L. Gil and J.A. Pardos. 2000. Effects of water supply on gas exchange in *Pinus pinaster* Ait. provenances during their first growing season. Ann. For. Sci. 57:9–16.

Geigenberger, P., R. Reimholz, U. Deiting, U. Sonnewald and M. Stitt. 1999. Decreased expression of sucrose phosphate synthase strongly inhibits the water stress-induced synthesis of sucrose in growing potato tubers. Plant J. 19:119–129.

Gilmore, A. and M. Ball. 2000. Protection and storage of chlorophyll in overwintering evergreens. Proc. Natl. Acad. Sci. 97: 11,098–11,101.

Hesse, H. and L. Willmitzer. 1996. Expression analysis of a sucrose synthase gene from sugar beet (*Beta vulagaris* L.). Plant Mol. Biol. 30:863–872.

Hincha, D.K., F. Meins, Jr. and J.M. Schmitt. 1997. Beta-1,3-glucanase is cryoprotective in vitro and is accumulated in leaves during cold acclimation. Plant Physiol. 114:1077–1083.

Ingram, J. and D. Bartels. 1996. The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47:377–403.

Iuchi, S., K. Yamaguchi-Shinozaki, T. Urao, T. Terao and K. Shinozaki. 1996. Novel drought-inducible genes in the highly drought-tolerant cowpea: cloning of cDNA and analysis of the expression of the corresponding genes. Plant Cell Physiol. 37:1073–1082.

Jagtap, V., S. Bhargava, P. Streb and J. Feierabend. 1998. Comparative effect of water, heat and light stresses on photosynthetic reactions in *Sorghum bicolor* (L.) Moench. J. Exp. Bot. 49:1715–1721.

Jarvis, S.B., M.A. Taylor, J. Bianco, F. Corbineau and H.V. Davies. 1996. Dormancy-breakage in seeds of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco). Support for the hypothesis that LEA gene expression is essential for this process. J. Plant Physiol. 151:457–464.

Kaltenbach, M., G. Schroder, E. Schmelzer, V. Lutz and J. Schroder. 1999. Flavonoid hydroxylase from *Catharanthus roseus*: cDNA, heterologous expression, enzyme properties and cell-type specific expression in plants. Plant J. 19:183–193.

Kinlaw, C.S. and D.B. Neale. 1997. Complex gene families in pine genomes. Trends Plant Sci. 2:356–359.

Kleines, M., R.C. Elster, M.J. Rodrigo, A.S. Blervacq, F. Salamini and D. Bartels. 1999. Isolation and expression analysis of two stress-responsive sucrose-synthase genes from the resurrection plant *Craterostigma plantagineum* (Hochst.). Planta 209:13–24.

Lichtenthaler, H.K. 1998. The stress concept in plants: an introduction. Ann. N.Y. Acad. Sci. 851:187–198.

Loggini, B., A. Scartazza, E. Brugnoli and F. Navari-Izzo. 1999. Antioxidative defense system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant Physiol. 119:1091–1099.

Maldonado, C.A., G.E. Zuñiga, L.J. Corcuera and M. Alberdi. 1997. Effect of water stress on frost resistance of oat leaves. Environ. Exp. Bot. 38:99–107.

Mayne, M.B., J.R. Coleman and E. Blumwald. 1996. Differential expression during drought conditioning of a root-specific *S*-adenosylmethionine synthetase from jack pine (*Pinus banksiana* Lamb.) seedlings. Plant Cell Environ. 19:958–966.

Munné-Bosch, S., K. Schwarz and L. Alegre. 1999. Enhanced formation of alpha-tocopherol and highly oxidized abietane diterpenes in water stressed rosemary plants. Plant Physiol. 121:1047–1052.

Nelson, D.E., G. Rammesmayer and H.J. Bohnert. 1998. Regulation of cell-specific inositol metabolism and transport in plant salinity tolerance. Plant Cell 10:753–764.

N'Guyen, A. and A. Lamant. 1989. Variation in growth and osmotic regulation of roots of water-stressed maritime pine (*Pinus pinaster* Ait.) provenances. Tree Physiol. 5:123–133.

Nguyen-Queyrens, A. and F. Bouchet-Lannat. 2003*a*. Osmotic adjustment in three-year-old seedlings of five geographic races of maritime pine (*Pinus pinaster*) in response to drought. Tree Physiol. In Press.

Nguyen-Queyrens, A., P. Costa, D. Lousteau and C. Plomion. 2003*b*. Osmotic adjustment in *Pinus pinaster* cuttings in response to a soil drying cycle. Ann. For. Sci. In Press.

Nguyen-Queyrens, A., A. Ferhi, D. Loustau and J.M. Guehl. 1998. Within $\delta^{13}C$ spatial variability and interannual variations in wood cellulose of two contrasting provenances of *Pinus pinaster*. Can. J. For. Res. 28:766–773.

Padmanabhan, V., D.M.A.L. Dias and R.J. Newton. 1997. Expression analysis of a gene familly in loblolly pine (*Pinus taeda* L.) induced by water deficit stress. Plant Mol. Biol. 35:801–807.

Pelah, D., O. Shoseyov and A. Altman. 1995. Characterization of BspA, a major boiling-stable, water-stress-responsive protein in aspen (*Populus tremula*). Tree Physiol. 15:673–678.

Pell, E.J., C.D. Schlagnhaufer and R.N. Arteca. 1997. Ozone-induced oxidative stress: mechanisms of action and reaction. Physiol. Plant. 100:264–273.

Plomion, C., P. Hurme, J.-M. Frigerio et al. 1999. Developing SSCP markers in two *Pinus* species. Mol. Breed. 5:21–31.

Remington, D.L., J.M. Thornsberry, Y. Matsuoka, L.M. Wilson, S.R. Whitt, J. Doebley, S. Kresovich, M.M. Goodman and E.S. Buckler. 2001. Structure of linkage disequilibrium and phenotypic associations in the maize genome. Proc. Natl. Acad. Sci. USA 98: 11,479–11,484.

Riccardi, F., P. Gazeau, D. de Vienne and M. Zivy. 1998. Protein changes in response to progressive water deficit in maize. Plant Physiol. 117:1253–1263.

Richard, S., M.J. Morency, C. Drevet, L. Jouanin and A. Seguin. 2000. Isolation and characterization of a dehydrin gene from white spruce induced upon wounding, drought and cold stresses. Plant Mol. Biol. 43:1–10.

Seillac, P. 1960. Contribution à l'étude de la nutrition du pin maritime: variation saisonnières de la teneur des pseudophylles en azote, potassium et acide phosphorique. Ph.D. Thesis, Université de Bordeaux, 105 p.

Shinozaki, K. and K. Yamaguchi-Shinozaki. 1996. Molecular responses to drought and cold stress. Curr. Opin. Biotechnol. 7: 161–167.

Sutinen, M.L., T. Repo, S. Sutinen, H. Lasarov, L. Alvila and T.T. Pakkanen. 2000. Physiological changes in *Pinus sylvestris* needles during early spring under sub-arctic conditions. For. Ecol. Manage. 135:217–228.

Thornsberry, J.M., M.M. Goodman, J. Doebley, S. Kresovich, D. Nielsen and E.S. Buckler. 2001. Dwarf8 polymorphisms associate with variation in flowering time. Nat. Genet. 28:286–289.

Triboulot, M.-B. 1996. Effets du stress osmotique et de la carence en potassium sur la croissance racinaire du pin maritime. Etude biomécanique du grandissement cellulaire. Ph.D. Thesis, Université Henri Poincaré, Nancy, 85 p.

Vernon, D.M. and H.J. Bohnert. 1992. A novel methyl transferase induced by osmotic stress in the facultative halophyte *Mesembryanthemum crystallinum*. EMBO J. 11:2077–2085.

Vogeli-Lange, R., C. Frundt, C.M. Hart, F. Nagy and F. Meins, Jr. 1994. Developmental, hormonal, and pathogenesis-related regulation of the tobacco class I beta-1,3-glucanase B promoter. Plant Mol. Biol. 25:299–311.

Volaire, F. and H. Thomas. 1995. Effects of drought on water relations, mineral uptake, water-soluble carbohydrate accumulation and survival of two contrasting populations of cocksfoot (*Dactylis glomerata* L.). Ann. Bot. 75:513–524.

Wang, P.D.O., O. Shoseyov, A. Altman and D. Bartels. 1997. Differential accumulation of water-stress related proteins, sucrose synthase and soluble sugars in *Populus* genotypes which differ in their water-stress response. Physiol Plant. 99:153–159.

Zeng, Y., Y. Wu, W.T. Avigne and K.E. Koch. 1998. Differential regulation of sugar-sensitive sucrose synthases by hypoxia and anoxia indicate complementary transcriptional and post-transcriptional responses. Plant Physiol. 116:1573–1583.

Zuniga, G.E., J. Fernandez, R. Cristi and M. Alberdi. 1990. Lipid changes in barley seedlings subjected to water and cold stress. Phytochemistry 29:3087–3090.