Korean J. Plant Res. 21(3) : 210~215 (2008)

Comparative Expression of Stress Related Genes in Response to Salt-stressed Aspen by Real-time RT-PCR

Ja Jung Ku* and Yong Yul Kim
Department of Forest Genetic Resources, Korea Forest Research Institute, 44-3 Omokcheon-dong, Gwonseon-gu, Suwon 441-350, Korea

Abstract - Gene-expression analysis is increasingly important in biological research, with real-time reverse PCR (RT-PCR) becoming the method of choice for high-throughput and accurate expression profiling of selected genes. However, this technique requires important preliminary work for standardizing and optimizing the many parameters involved in the analysis. Plant stress studies are more and more based on gene expression. The analysis of gene expression requires sensitive and reproducible measurements for specific mRNA sequence. Several genes are regulated in response to abitoic stresses, such as salinity, and their gene products function in stress response and tolerance. The design of the primers and TaqMan probes for real-time PCR assays were carried out using the Primer Express™ software 3.0. The PCR efficiency was estimated through the linear regression of the dilution curve. To understand the expression pattern of various genes under salt stressed condition, we have developed a unique public resource of 9 stress-related genes in poplar. In this study, real-time RT-PCR was used to quantify the transcript level of 10 genes (9 stress-related genes and 1 house keeping gene) that could play a role in adaptation of *Populus davidiana*. Real-time RT-PCR analyses exhibited different expression ratios of related genes. The data obtained showed that determination of mRNA levels could constitute a new approach to study the stress response of *P. davidiana* after adaptation during growth in salinity condition.

Key words - Poplar, EST, *P. davidiana*, Gene expression, TaqMan probe

Introduction

Gene-Expression analysis is increasingly in many field of biological research. Understanding patterns of expressed gene is expected to provide insight into complex regulatory networks and will most probably lead to the identification of genes relevant to new biological processes. Gene expression levels were commonly determined using northern blot analysis (Gachon *et al.,* 2004; Dean *et al.,* 2002). However, this technique is time consuming and requires a large quantity RNA.

Real-time reverse transcription PCR (RT-PCR) has greatly improved the easy and sensitivity of quantitative gene expression studied (Bustin, 2002). Real-time RT-PCR offers a robust means for precisely quantifying changes in gene expression over wide dynamic range. However, selection of an appropriate normalization method is crucial for reliable quantitative gene expression results. The purpose of normalization is to correct for non-specific variation, such as differences in RNA quantity and quality, with can affect efficiencies of the RT and PCR methods.

Real-time RT-PCR provides the simultaneous measurement of gene expression in many different samples for a limited number of genes. Nevertheless, these new approaches require the same kind of normalization as the traditional methods of mRNA quantification.

Sensitive and reliable methods to monitor the environmental stress effects in plant tissue are essential to enable the study of the factors affecting the progression from the damaged tissue. Mechanisms of salt stress have been examined in many plant species. Salt stress involves morphological, physiological and metabolic changes (Evers *et al.,* 1998). In tissue exposed a given treatment, the level of expression of several change significantly, possible altering the total amount of RNA in the tissue. In studies to determine the change in the level of expression of a given gene of interest is used to estimate the number of copies of a transcript of interest in a total RNA extracted from tissue sample before and after treatment (Xu *et al.,* 2000; Maguire *et al.,* 2002). The determination is influenced, by the amount of total RNA in the tissue. For example, if total RNA increased 2-fold, and the occurrence of the target transcript increased 10-fold, estimating the number of

*Corresponding author. E-mail : jajungku@gmail.com

transcript copies in the target transcript, while a 10-fold increase had actually occurred in each cell.

Populus davidiana is the unique aspen species in Korea, belonging to section *Lecuce* Duby in genus *Populus* (Cervera *et al.,* 2005).

In this study, we used real-time RT-PCR to examine the expression of 10 genes in a 4 aspen clones at different stress response. The goal of our studies was to detect changes associated with clone character and response of salinity stress.

Materials and Methods

Plant material and salt stress treatment

Plants were grown in pot for 6 month. Plant tissue was obtained from apparently healthy *Populus davidiana* plants by cutting discs 25㎟ from fully expanded leaves. Leaflets were then cut off and subjected to NaCl treatment. Three type of samples were processed: (i) tissue from control plant; (ii) tissue from NaCl soaked plant; (iii) a sample consisting of equal amounts of tissue from control and treated plants. For NaCl treatment, leaves were soaked in 5ml of aqueous solution of 400mM NaCl, under dim light at 25℃. At different time point (0-24h), samples were harvested, transferred to a tube and frozen in liquid nitrogen.

Total RNA extraction

Each samples in tube were stored at -70℃ until RNA extraction. Detected leaves were subjected to respective stress treatment for 0, 1, 2, 4, 8 and 24h, and then frozen in liquid nitrogen foe analysis. The same mass of tissue from each treatment was pulverized in a mortar and pestle in liquid nitrogen. Total RNA was extracted by the RNAqueous (Ambion, USA) including Plant RNA isolation aid treatment according to the manufacturer' s instructions. Purity of the total RNA extracted was determined as the 260/280 nm ratio > 1.95 and the integrity was checked by electrophoresis in 1% agarose gel.

Primer design

The design of the primers and TaqMan probes for real-time PCR assays were carried out using the Primer Express™ software 3.0 (Applied Biosystems) as described in Mumfold *et al.* (Mumfold *et al.,* 2000). Nine stress-related genes were included in the study (Caruso *et al.,* 2002; Nanjo *et al.,* 2004).

Two step real-time RT-PCR

One microgram of each RNA sample was reverse transcribed to cDNA with High Capacity cDNA Reverse Transcription Kit (Ambion, USA) using random hexamers. The cDNA concentration was checked. Real-time PCR using TaqMan technology on ABI PRISM 7900HT Sequence Detection System (Applied Biosystem, USA) was performed. For each reaction, 150ng cDNA was added, giving a final volume of 20ul. Negative control reactions contained sterile water replacing the cDNA template. A master mix for each PCR run was prepared with TaqMan gene expression Master Mix (Applied Biosystem, USA). Final concentrations, in a total volume of 20ul, were 1X Taqman Universal PCR Master Mix, 1X Assay Mix. The assays were carried out at genetic cycle conditions (50℃ for 2min, 95℃ for 10min and 40 cycles of 60℃ for 1min, 95℃ for 15s) within ABI 7900HT Sequence Detection System (Applied Biosystems, USA) for TaqMan reaction. The real-time PCR efficiency was determined for each gene and each stress with the slope of a linear regression model (Pfaffl, 2001). For each gene, PCR efficiency was determined by measuring the Ct to a specific threshold (Walker, 2002) for a serial dilution of bulked cDNA.

Relative quantification was achieved using the standard curve technique. For standard curves, reactions were carried out in triplicates using 3ul of known plant cDNA. Standard curves for EF-1β and the normal gene, were generated by plotting the cDNA amounts (ng) against the Ct value exported from the ABI Prism Sequence Detection System for each well.

Data acquisition and statistical analysis

For each gene, PCR efficiency was determined by measuring the Ct to a specific threshold (Walker, 2002) for a serial dilution of balked cDNA. Subsequently, the Ct values were plotted against the log of the known starting concentration value and from the slope of the regression line (y). The amplification efficiency was estimated according to the equation: $E = [(10^{-1/y})-1] \times 100$ (Radonic *et al.,* 2004).

The Sequence Detection System software version 1.7 (ABI) calculates the ΔRn the equation $\Delta Rn = (Rn+) - (Rn-)$, where $Rn+$ is the florescence signal of the product at any given time and $Rn-$ is the florescence signal of the baseline emission during cycles 3 to 15. Expression level were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (Ct) (Walker, 2002). Results (Ct values) from ABI PRISM 7900 Sequence Detection System were analyzed in Microsoft Excel.

Results and Discussion

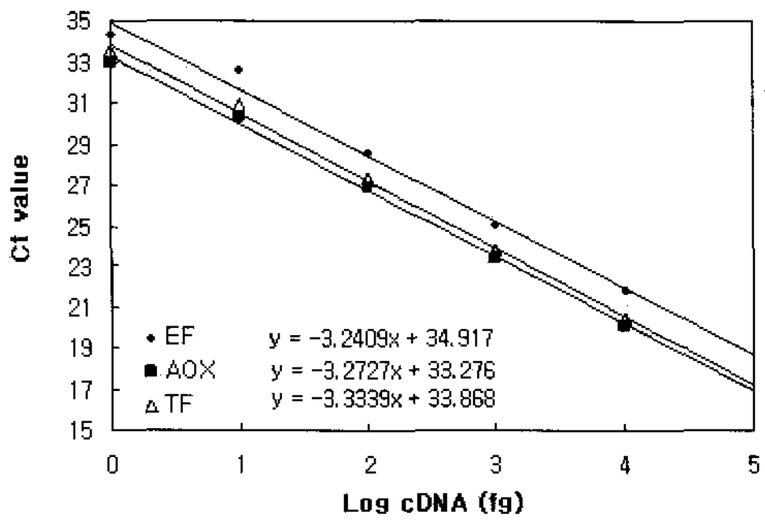
The performance of ten different genes, one internal control gene and nine stress related gene, was tested in setup to TaqMan real-time RT-PCR. Primers and probes selected for plant cDNA are given in Table 1. Specificity of RT-PCR products was documented with high resolution gel electrophoresis resulted in a single product with the desired length (data not shown). The expression level of the these genes was determined in 24 samples collected over a 24hr period from salinity stress treated leaflet (N1: Un-doo2, N2: Dae-hwa18, N3 : Pal-gong 2, N4 : Pal-gong 3).

We chose two-step RT-PCR that avoids the formation of primers dimmers observed in one-step RT-PCR (Vandesompele *et al.*, 2002). It is generally accepted that gene-expression levels should be normalized by a carefully selected stable internal control gene. To avoid bias, real-time RT-PCR is typically referenced to a housekeeping gene as the internal control gene. Ideally, the conditions of the experiment should not influence the expression of this internal gene. EF-1β, the choice of a housekeeping gene in poplar for this study, was the internal control gene. Ten genes that represent different functional genes were chosen for study. Poplar genes belonging to these gene families were identified via searches of the EST database (Table 1). The expressing level of these genes was determined in 6 treatments collected over 0, 1, 2, 4, 8 and 24hr period from 400mM NaCl treated sample.

A 10-fold dilutions series (10^{-2}-10^{-7}) of cDNA extraction from aspen was used to determine the sensitivity and linearity of TaqMan assays. The standard curves obtained showed high correlations between Ct values and cDNA quantities for all the assays; EF-1β (R^2 = 0.9995), AOX (R^2 = 0.9972), TF (R^2 = 0.9977), APP1 (R^2 = 0.9995), ERF (R^2 = 0.9989), GS (R^2 = 0.9994), HPD (R^2 = 0.9960), LAD (R^2 = 0.9994), dhn (R^2 = 0.9946), ZF (R^2 = 0.9894). Quantitation via real-time PCR is based on cycle threshold (Ct). Ct is the cycle at which a significant increase in amount of PCR product occurs, generally the middle of the exponential phase of amplification. Subsequently, by using real-time RT-PCR, the

Table 1. Description of *Populus* genes and primers for real-time RT-PCR

Gene	Gene annotation	GeneBank accession number	Length (bp)	Primer sequence
EF-1β	Elongation factor 1-beta, putative	BI125345	144	F- GGGATCCTCCAAGAAGAAAGAGAGT R-CACCACCATGTCTGTCTCATCAT
AOX	Alternative oxidase 1a, mitochondrial (AOX1a)	BP922653	376	F-AACTTCCCATCTTGTGGCATT R-ACAGTCCAATAAGCCATTTTG
APP1	Amino acid permease (APP1)	BP930782	527	F-AGAGTGTACCCTCCGAATTGA R-TATGGATTATTTGACGTCTGG
ERF	Ethylene-responsive element-binding factor 1 (ERF)	BP924591	320	F-ACCCACAGCAGTACTCAGCAC R-CTTGGACGGAAGCACCGCAGG
dhn	Dehydrin gene	AJ300524	848	F-GTTACAAGCGCAAATCACTC R-TAAATAGGAAGAAAAACAGTA
GS	Galactinol synthase (GS)	BP92497	399	F-AATTCACTCATATCCAAAACT R-CCGTCCAAACATATCATCTTG
HPD	4-Hydroxyphenylpyruvate dioxygenase	BP930649	386	F-AATTGAGAGCAAGTAAACAAT R-GTGTGGGAGAGGTTGTCCATG
LAD	Leucoanthocyanidin dioxygenaselike protein	BP934164	386	F-AATGATGAACTGCTTGCAGAG R-CCTTCATCAGTTCGTGGCTCA
TF	Transcription factor	BP930753	401	F-CATCAGGTTCAAGCCATGGTC R-ACTGATAATTATCCATTAAAA
ZF	Zinc finger protein, putative	BP932979	474	F-ATAAAGTTCACACAATTCACT R-CCACTTCCGTTAGACACGGGT



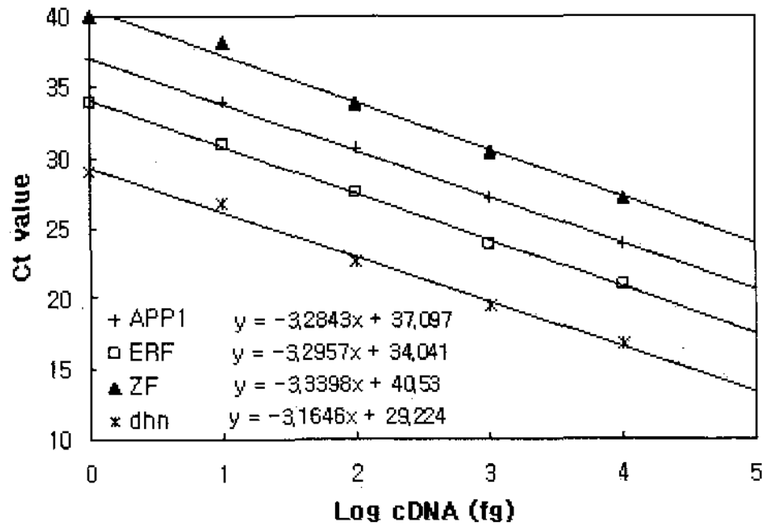


Fig. 1. Determination of real-time RT-PCR efficiencies of reference gene (EF-1β) and 9 target gene. Ct value versus cDNA (reverse transcribed total RNA) concentration input were plotted to calculate the slope. The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{[-1/slope]}$.

expression patterns of all 10 genes were analyzed and summarized (Fig. 2). All RNA samples had given threshold cycles (Ct) values between 19.2-22.8 indicating successful amplification.

To evaluation the stability of expression of housekeeping gene, RNA transcription levels for all samples were measured for each condition. Real-time PCR efficiencies were calculated from given slope in Sequence Detection System software (S.D.S 2.2). The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/slope]}$ (Rasmussen *et al.,* 2001).

All PCRs displayed efficiencies (E) between 1.90-2.07. Investigated transcripts showed high real-time PCR efficiency rates; for EF, 1.93; HPD, 2.10 and GS, 1.90 in the range from 150ng cDNA input with high linearity (correlation coefficient $r^2 > 0.95$). The mean Ct difference value observed was 1.73, which represents a maximum of only 3.38?fold variation. In tissue of stressed aspen leaflets, fold change in EF-1β gene transcript level varied from 0.48 to 3.14 (Fig. 2). Before to use the comparative Ct method, we verified that PCR efficiency of reference and target gene were approximately equal. The correlation coefficient (R^2) of amplification of the EF-1β sequence was 0.999 and the PCR efficiency (E) calculated from the slope of the standard curve was 103.5%.

After 1 hr, dhn gene expression was significantly enhanced by NaCl treatment. During salt treatment, dhn expression increased after 1hr. As 2hr of treatment, the gene expression level was the highest and increase 13.4 fold compared with the control. After 24hr, the level of expression decreased so as to reach its initial expression level. GS gene transcriptional expression increased with application of salt stress condition (Fig. 2). Salt stress includes osmotic imbalance and disturbance of the cytoplasmic K^+/Na^+ homeosis (Bhandal and Milik, 1988). Like other plant species, aspen plants under salt stress have exhibited changes in multiple genes. Salt stress and many other biotic/abiotic stresses are coordinately regulated by different interconnected pathways (Ludwig *et al.,* 2005; Ma *et al.,* 2006). Gene transcription is regulated by interplay of the positive and negative regulators. In this study, multiple genes were induced/ or repressed by salt treatment.

These results suggest that different temporal gene expression patterns exist in stress-related gene members after NaCl stress induction.

Literature Cited

Bhandal, I.S. and C.P. Malik. 1988. Potassium estimation, uptake, and its role in the physiology and metabolism of flowering plants. Int. Rev. Cyrol. 110: 205-254.

Bustin, S.A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): tends and problems. J. Mol. Endocrinol. 29: 23-29.

Caruso, A., D. Morabito, F. Delmotte, G. Kahlem and S. Carpin. 2002. Dehydrin induction during drought and osmotic stress in *Populus*. Plant Physiol. Biochem. 40: 1033-1042.

Cervera, M.T., V. Strome, A. Soto, B. Ivens, M. van Montagu, O.P. Rajora and W. Boerjan. 2005. Intraspecific and interspecific genetic and phylogenetic relationships in ghe genus *Populus* based on AFLP markers. Theoretical and Applied Genetics 111: 1140-1456.

Dean, J.D., P.H. Goodwin and T. Hsiang. 2002. Comparison of relative RT-PCR and northern blot analyses to measure

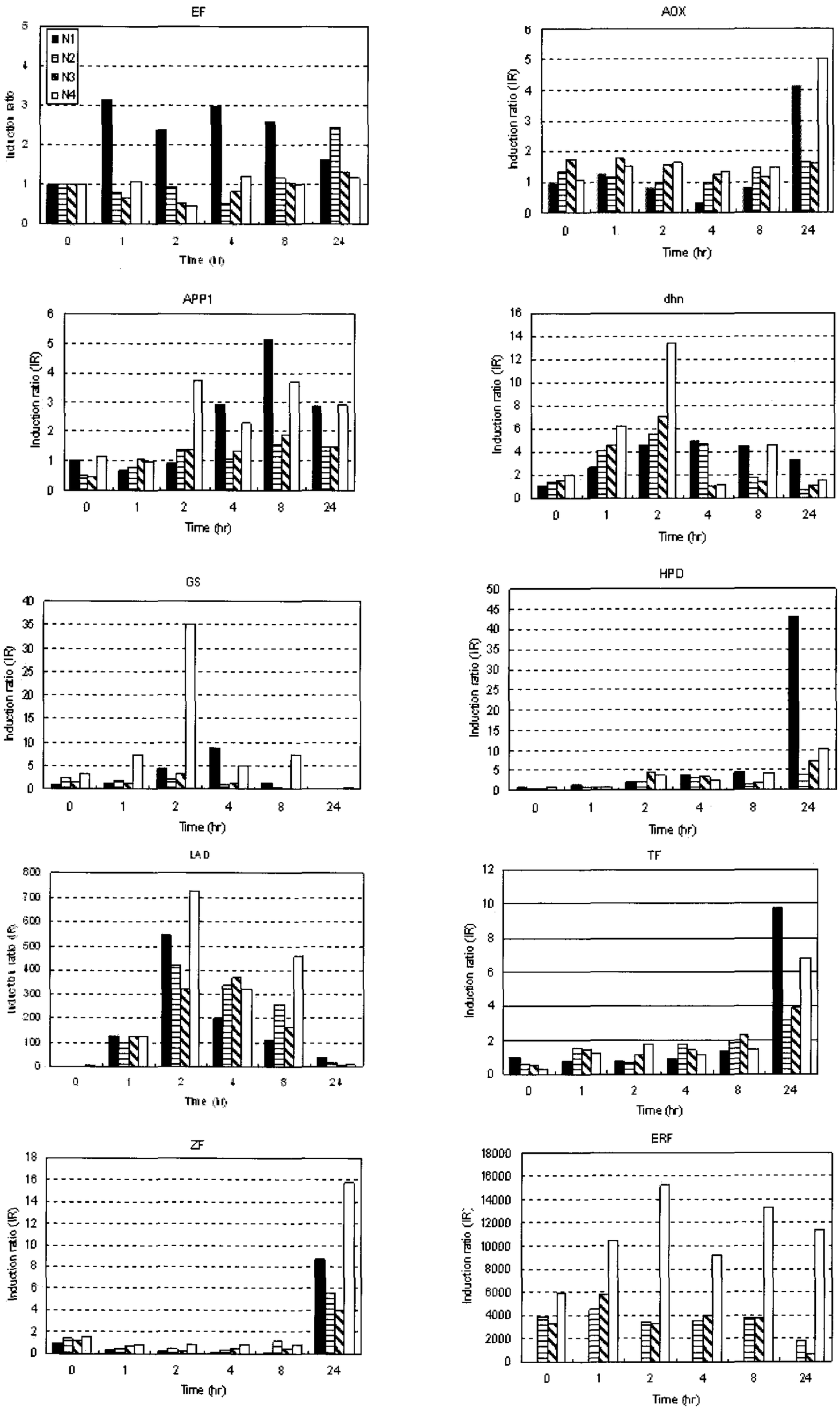


Fig. 2. Logarithmic histogram of the induction ratio (IR) of 1 internal control gene and 9 genes monitored by real-time quantitative RT-PCR in NaCl treated leaflets. The IR was calculated as recommended by the manufacturer and corresponding to $-2^{\Delta\Delta Ct}$, were $\Delta\Delta C_T = (C_T$, target gene $- C_T$, EF-1$\beta)_{stressed}$-(C_T, target gene $- C_T$, EF-1$\beta)_{control}$.

expression of β-1,3-glucanase in *Nicotiana benthamiana* infected with Colleto-trichum destructivum. Plant Molecular Biology Reporter 20: 347-356.

Evers, D., K. Hemmer and J.F. Hausman. 1998. Salt stress induced biometric and physiological changes in *Solanum tuberosum* L.cv. Bintje grown in vitro. Acta Physiologiae Plantarum 20: 3-7.

Gachon, C., A. Mingam and B. Charrier. 2004. Real-time PCR: What relevance to plant studied? Journal of Experiment Botany 55: 1445-1454.

Ludwig, A.A., H. Saitoh, G. Felix, G. Freymark, O. Miersch, C. Wasternack, T. Boller, J.D. Jones and T. Romeis. 2005. Ethylene-mediated cross-talk between calcium-dependent protein kinase and MAPK signaling controls stress responses in plants. Proc. Natl. Acad. Sci. USA. 102: 10736-10741.

Ma, S., Q. Gong and H.J. Bohnert. 2006. Dissecting salt stress pathways. J. Exp. Bot. 57: 1097-1107.

Magurite, T.L., S. Grimmond, A. Forrest, O.I. Iturbe, K. Meksem and P. Gresshoff. 2002. Tissue-specific gene expression in soybean (Glycine max) detected by cDNA microarray analysis. Journal of Plant Physiology 159(12): 1361-1374.

Mumfold, R.A., K. Walsh, I. Barker and N. Boonham. 2000. Detection of Potato map top virus and Tabacco rattle virus using a multiplex real-time florescent reverse-transcription polymerase chain reaction assays. Virology 90: 448-453.

Nanjo, T., N. Futamura, M. Nischiguchi, T. Igasaki, K. Shinozaki and K. Shinohara. Characterization of full-length enriched expressed sequence tags of stress treated poplar leaves. 2004. Plant Cell Physiol. 45(12): 1738-1748.

Pfaffl, M.W and M. Hegeleit. 2001. Validation of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol. Lett. 23: 275-282.

Radonic, A., S. Thulke, I.M. Mackay, O. Landt, W. Siegert and A. Nitsche. 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochemical and Biophysical Research Communications. 313: 856-862.

Vandesompele, J., K. De preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology. 18: 3(7).

Walker, N.J. 2002. A technique whose time has come. Science 296: 557-559.

Xu, J., J. Stolk, X. Zhang, S.J. Silva, R.L. Houghton, M. Matsumura, T.S. Vedvick, K.B. Leslie, R. Badaro and S.G. Reed. 2000. Identification of differentially expressed gene in human prostate cancer using substraction and microarray. Cancer Research 60(6): 1677-1682.

(Received 20 May 2008 ; Accepted 17 June 2008)