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Short communication

Differentially expressed genes in *Populus simonii* × *Populus nigra* in response to NaCl stress using cDNA-AFLP

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ABSTRACT

Salinity is an important environmental factor limiting growth and productivity of plants, and affects almost every aspect of the plant physiology and biochemistry. The objective of this study was to apply cDNA-AFLP and to identify differentially expressed genes in response to NaCl stress vs. no-stress in *Populus simonii* × *Populus nigra* in order to develop genetic resources for genetic improvement. Selective amplification with 64 primer combinations allowed the visualization of 4407 transcript-derived fragments (TDFs), and 2027 were differentially expressed. Overall, 107 TDFs were re-sequenced successfully, and 86 unique sequences were identified in 10 functional categories based on their putative functions. A subset of these genes was selected for real-time PCR validation, which confirmed the differential expression patterns in the leaf tissues under NaCl stress vs. no stress. Differential expressed genes will be studied further for association with salt or drought-tolerance in *P. simonii* × *P. nigra*. This study suggests that cDNA-AFLP is a useful tool to serve as an initial step for characterizing transcriptional changes induced by NaCl salinity stress in *P. simonii* × *P. nigra* and provides resources for further study and application in genetic improvement and breeding. All unique sequences have been deposited in the Genbank as accession numbers GW672587–GW672672 for public use.

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1. Introduction

Salinity is a major environmental factor limiting plant growth and productivity. Salinity leads to osmotic stress, reactive oxygen damage, and ion toxicity resulting in irreversible cellular damage and photo-inhibition [1,2]. During exposure to salt stress conditions, almost every aspect of a plant's important life processes are affected [3]. Salt stress not only causes physiological changes in plants (phenotypic variation), but also affects plant gene expression levels (genotypic variation).

Populus simonii × *Populus nigra*, which is the hybrid of *P. simonii* and *P. nigra*, widely distributes in the northern region of the Yellow River Basin in China. The early studies of *P. simonii* × *P. nigra* were focused on germplasm introduction and cultivation [4,5]. In recent years, the research has been focusing on using transgenic technology to enhance disease resistance [6], insect resistance [7] and salt tolerance [8,9]. However, there still lacks genomic information in *P. simonii* × *P. nigra* for molecular characterization of stress tolerance and breeding.

The advent of next-generation sequencing has made sequence based gene expression analysis an increasingly common. Gene expression profiling is the measurement of the activity and the expression of thousands of genes at the same time. DNA microarray technology measures the relative activity of previously identified target genes. Sequence based techniques, like serial analysis of gene expression (SAGE, SuperSAGE) are also used for gene expression profiling. However, the cost and complexity of these experiments are also concerns to many research laboratories. We decided to apply a simple and quick RNA fingerprinting method described by Bachem et al. [10] in *P. simonii* × *P. nigra* gene expression analysis in responding to salt stress. RNA fingerprinting method, based on AFLP (amplified fragment length polymorphism) or called cDNA-AFLP, does not require prior sequence information and allows the detailed characterization of gene expression in a wide range of biological processes [10]. Comprehensive and systematic analysis can be carried out on the organism transcriptome by cDNA-AFLP, which can then be applied successfully to study gene expression characteristics [11,12], genetic marker analysis [13] and separation of differentially expressed genes [14]. The objective of this study was to apply cDNA-AFLP and to identify differentially expressed genes in response to NaCl stress vs. no-stress in *P. simonii* × *P. nigra* in order to develop genetic resources for genetic improvement, even though there are other genomic resources available

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Table 1
Numbers of transcript-derived fragments (TDFs) and different primer combination.

Primer name	T-AG	T-CA	T-CT	T-AC	T-TC	T-TG	T-GA	T-GT	Total
M-AC	4[a]/3[b] (16[c])	38/20 (42)	30/9 (23)	3/10 (29)	25/7 (41)	16/21 (48)	11/40 (43)	23/1 (4)	150/111 (246)
M-AG	34/5 (65)	21/37 (43)	18/5 (32)	32/14 (48)	7/6 (5)	14/20 (41)	30/9 (65)	18/24 (49)	174/120 (348)
M-CA	5/13 (16)	29/8 (38)	1/12 (28)	10/19 (40)	5/1 (19)	38/6 (46)	22/11 (77)	11/34 (59)	121/104 (323)
M-CT	37/19 (77)	13/10 (51)	26/10 (27)	7/16 (62)	7/4 (16)	13/24 (53)	17/27 (49)	13/16 (35)	133/126 (370)
M-TC	0/80 (20)	28/18 (30)	5/5 (23)	14/12 (43)	2/13 (14)	33/9 (45)	22/14 (46)	17/3 (24)	121/154 (245)
M-TG	0/44 (42)	36/4 (42)	11/4 (52)	(49)	(11)	0/7 (18)	(62)	7/27 (39)	101/146 (315)
M-GT	25/29 (19)	14/26 (44)	4/20 (28)	3/8 (5)	11/8 (19)	15/13 (42)	28/17 (43)	8/8 (7)	108/129 (207)
M-GA	4/25 (57)	20/12 (54)	(43)	28/15 (63)	7/5 (18)	22/2 (11)	22/25 (70)	8/12 (10)	123/106 (326)
Total	109/218 (312)	199/135 (344)	107/75 (256)	108/131 (339)	79/47 (143)	151/102 (304)	173/163 (455)	105/125 (227)	1031/996 (2380)

[a] Down-regulated gene.
[b] Up-regulated gene.
[c] Constitutive expressed gene.

such as http://www.phytozome.net/poplar. We carried out cDNA-AFLP analysis in leaf tissues under salt stress vs. no stress in order to identify differentially expressed genes, which were validated by real-time PCR analysis. The differentially expressed genes could be used in further study in characterization and breeding for salinity tolerance and understanding the response of *P. simonii* × *P .nigra* to NaCl stress.

2. Materials and methods

2.1. Plant materials

The branches of *P. simonii* × *P. nigra* from the same clone were grown under hydroponic conditions in a phytotron at 26 °C/22 °C (day/night) with 75% relative humidity, 16 h photoperiod and 175 μmol/(m^2 s) light intensity. New leaves and roots were grown out after 40 days. The branches with new leaves and roots were divided into two groups. One group was grown under normal condition as control and the other was stressed with 200 mM NaCl. After two days, the leaf tissues of these two groups were harvested and frozen immediately in liquid nitrogen. Tissues were then stored at −80 °C until use.

2.2. cDNA-AFLP analysis and TDFs isolation

Total RNA was extracted from frozen leaf tissues using Trizol reagent (Invitrogen) according to the manufacture's instructions. Two micrograms of total RNA was used initially for the first-strand cDNA synthesis, followed by the second-strand cDNA sythesis using a M-MLV RTase cDNA Synthesis Kit (Takara) according to the manufacture's instructions. cDNA-AFLP analysis was carried out using AFLP Expression Analysis Kit (LI-COR). One hundred nanograms of double-stranded cDNA was digested with *Taq*I and *Mse*I, and the fragments were ligated to adapter for amplification (*Taq*I-F: 5′-CTCGTAGACTGCGTAC-3′; *Taq*I-R: 5′-CGGTACGCAGTCT-3′; *Mse*I-F: 5′-GACGATGAGTCCTGAG-3′; *Mse*I-R: 5′-TACTCAGGACTCAT-3′). Pre-amplification was performed with a *Taq*I primer (TPPC: 5′-GTAGACTGCGTACCGA-3′), combined with a *Mse*I primer (MPPC: 5′-GATGAGTCCTGAGTAA-3′). Pre-amplification PCR conditions were as follows: denaturation at 94 °C for 30 s, annealing at 56 °C for 60 s, extension at 72 °C for 60 s, total 20 cycles. After preamplification, the selective amplification with 64 primer *Taq*I/*Mse*I (+2, +2) combination (Table 1) was carried out using a touchdown program. The PCR conditions were as follows: denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 60 s (12 cycles, scaledown of 0.7 °C per cycle); denaturation at 94 °C for 30 s, annealing at 56 °C for 60 s, extension at 72 °C for 60 s (23 cycles). The *Taq*I primers were labeled with IRD700 fluorescent dye (LI-COR, Lincoln, Nebraska). The selective amplification products were separated on a 6% polyacrylamide gel with a LI-COR 4300 DNA analyzer under 1500 V and 40 W condition. The transcript-derived fragments (TDFs) were isolated using a LI-COR Odyssey® Infrared Imaging System. The bands of interest were cut from the gel with a surgical blade and eluted in 60 μl sterile distilled water. Two microliters of eluted DNA was used as template for re-amplification using selective amplified primers. PCR products were purified with a PCR purification kit (Takara, Dalian), and cloned into pUC 119 vector (Takara, Dalian) and sequenced.

2.3. Sequence analysis

Sequencing results were analyzed using BLASTX searches against the GenBank non-redundant public sequence database. The TDFs sequences were manually assigned to functional categories based on the analysis of scientific literature and also with the aid of the information reported for each sequence by Gene Ontology consortium.

2.4. Real-time PCR and data analysis

Leaf tissues in the stressed group were sampled at 2 days after the treatment with 200 mM NaCl, as well as the control group. All samples were examined in three independent biological replications. To decrease replicated experimental variation at each sample, the three purified RNA from each biological replicate were pooled equally for qRT-PCR. Three experimental technical replications were performed for each pooled sample to assess the reproducibility, and the mean of the three replications was used to calculate relative expression quantitation. First strand cDNA was synthesized from 1 μg DNase-treated total RNA using Reverse Transcriptase M-MLV (Takara). The reverse transcription reaction was diluted to a final volume of 100 μl, and 2 μl was used as template for PCR using SYBR *Premix ExTaq*™. Threshold values (C_T) generated from DNA Engine Opticon™ 2 (MJ Research) were employed to quantify relative gene expression using the comparative $2^{-\Delta\Delta C_T}$ method [15]. Cycling parameters were set up according to the recommendation of QuantiTect SYBR green RT-PCR kit. Melting curves were run immediately after the last cycle to examine if the measurements were influenced by primer–dimer pairs.

The amplification curve was generated after analyzing the raw data, and the cycle threshold (C_T) value was calculated based on the fluorescence threshold as 0.01. *Populus* actin (EF418792) gene expression was used as an internal control to normalize all data. The expression of *Populus* actin was constant using real-time PCR. The "delta–delta C_T" ($2^{-\Delta\Delta C_T}$) mathematical model was used for description and comparison of the relative quantification of gene expressions between samples. Therefore, the amount of target gene in test sample was given by $R = 2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T\text{test sample}} - \Delta C_{T\text{control sample}}$, $\Delta C_{T\text{sample}} = C^{T}{}_{\text{test gene}} - C_{T\text{reference gene}}$. The final value of relative

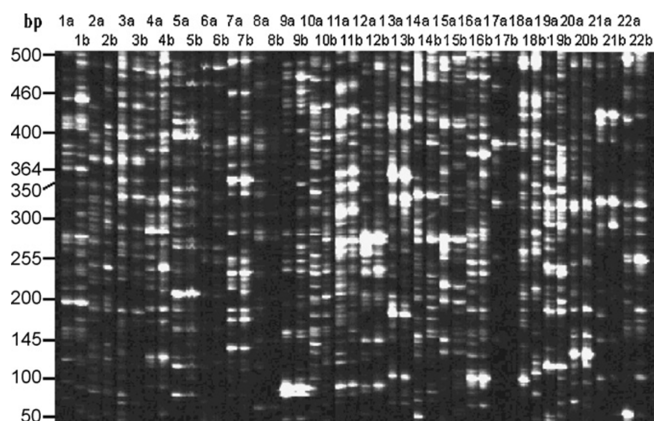

Fig. 1. Expression of *Populus simonii* × *P. nigra* transcripts under NaCl stress displayed by cDNA-AFLP. An example showing selective amplication with different primer combinations; a = water control leaves; b = NaCl treated leaves; 1–22 = different primer combination: T-TG/M-AC, T-TG/M-AG, T-TG/M-CA, T-GA/M-AC, T-TG/M-TC, T-TG/M-TG, T-TG/M-GT, T-TG/M-GA, T-TG/M-CT, T-GA/M-AG, T-GA/M-CA, T-GA/M-CT, T-GA/M-TC, T-GA/M-TG, T-GA/M-GT, T-GA/M-GA, T-GT/M-AC, T-GT/M-AG, T-TG/M-CA, T-TG/M-CT, T-GT/M-TC, and T-GT/M-TG. This study had three technical replicates of equally pooled samples of three biological replicates.

quantitation was described as fold change of gene expression in the tested sample compared with the control sample.

3. Results and discussion

To isolate differentially expressed transcripts, we carried out cDNA-AFLP analysis on total RNA samples from leaves under normal growth and salt stress. Selective amplification with 64 primer combinations allowed the visualization of 4407 TDFs, 2027 of which were differentially expressed, corresponding to about 46% of all visualized transcripts. Of the 2027 TDFs, 996 were up-regulated and 1031 down-regulated (Fig. 1). A total of 161 differentially expressed TDFs were recovered from gels and 121 were re-amplified, cloned and sequenced.

The differentially expressed TDF band was excised from the gel, eluted, re-amplified and purified for direct sequencing, which yielded 107 cDNA fragments that gave rise to useable sequence data. Among these sequences, 86 were unique sequences and searched for homologous to known databases, and 70 sequences were annotated with database matches and 16 sequences had no database matches. There were some unique sequences homologous to various *Populus* sequence databases, either as tentative consensus sequences or expressed sequence tags (EST) without known functional annotations. Seventy were homologous to known function genes and listed in Table 2, while majority were homologous to *Arabidopsis* sequences (Table 2) which have annotated functions. These TDFs might be homologous to *Populus* sequences but these sequences were not annotated yet and, therefore, these TDFs were annotated to the species with known annotations (Table 2).

All 86 TDFs isolated from NaCl stressed *P. simonii* × *P. nigra* were deposited in the Genbank under accession numbers from GW672587–GW672672, while a selection of the TDFs with known functions is shown in Table 2. Each transcript was functionally annotated through careful analysis of the scientific literature and the Gene Ontology Database. Fig. 2 shows the percentages of *P. simonii* × *P. nigra* genes assigned to different functional categories. Approximately 17.4% of the annotated sequences have primary metabolic roles, 11.6% are involved in signal transduction, and a further 12.79% in transcription regulation. There are about 18.6% with unknown proteins. Interestingly, there are about 5.8% have roles in response to stresses. Other relevant groups of differentially expressed TDFs include cellular biosynthesis (10.5%), transport (4.7%), cellular catabolism (4.7%), photosynthesis and redox (7%), and development process (6.98%). Most of the differentially-expressed *P. simonii* × *P. nigra* transcripts were down regulated in response to salt stress. There were two exception categories, response to stresses and transcription regulation where 60% and 64% of the differentially expressed genes were up-regulated (Table 2).

To verify cDNA-AFLP identified genes by real-time PCR, 10 genes with induced or repressed patterns in cDNA-AFLP study were selected for specific primer design for qRT-PCR. Relative quantitative method delta–delta C_T ($2^{-\Delta\Delta C_T}$) was used to describe expression patterns of selected genes by comparing the gene expression levels at 2 days after NaCl treatment with control. The relative quantitation comparisons based on C_T values from the treated samples and the control samples were calculated as the algorithm $R = 2^{-\Delta\Delta C_T}$. Generally, R value > 2.00 was described as induced, R value < 0.50 as repressed, and $2.00 \geq R$ value ≥ 0.50 as no-change. The results indicated that the expression levels measured by qRT-PCR reproduced the cDNA-AFLP study very well (Table 3). One exception was TDF C-2, repressed in cDNA-AFLP but classified as no-change in qPCR. Therefore, the results showed that the cDNA-AFLP technique was effective in identifying differentially expressed genes in *P. simonii* × *P. nigra*.

Although DNA microarrays are currently the standard tool for genome-wide expression analysis, their application also is limited to organisms for which the complete genome sequence or large collections of known transcript sequences are available [16,17]. Other differential cDNA screening methods, such as the suppression subtractive hybridization technique may allow such previously unidentified genes to be isolated. Here, we applied our LI-COR system and tested AFLP-based transcript profiling method, cDNA-AFLP, that allows genome-wide expression analysis without the need for prior sequence knowledge. This method has utility in tree study like *P. simonii* × *P. nigra* for gene discovery on the basis of fragment detection and for temporal quantitative gene expression analysis.

Brinker et al. [16] carried out transcriptome study to investigate early salt-responsive genes in early salt treatment after 24 h in a salt-tolerant poplar species *Populus euphratica* using microarray containing ESTs representing about 6340 genes from *P. euphratica*. They revealed that the leaves suffered initially from dehydration, which resulted in changes in transcript levels of mitochondrial and photosynthetic genes. Initially, decreases in stresses in stress-related genes were found, whereas increases occurred only when leaves had restored the osmotic balance by salt accumulation. In our study, after 2 days salt treatment, we also found that in the photosynthesis group, majority (4 out of 6) genes were repressed (Table 2), indicating adjustment of energy metabolism.

Ding et al. [17] studied salt-induced expression of genes related to Na/K and ROS homeostasis in leaves of salt-resistant and salt-sensitive *Populus* species using the Affymetrix poplar genome array after 24 h short-term exposure to 150 mM NaCl and 28 days long-term exposure to 200 mM NaCl. We studied salt-induced expression of genes in response to 200 mM NaCl after 2 days exposure and successfully identified 86 unique genes which will be used in further study, such as the highly expressed genes TDF D-10 (putative Cupin family proteins) and TDF 88-1 (putative Zinc finger protein) and the repressed gene TDF 109-2 (WRKY transcription factor). Cupin was germin-like and plant storage proteins, which regulated seed germination and early seedling development [18]. The expression level of the cupin gene (GW672616) was very high under salt stress than under control conditions using qPCR (Table 3), which will be further studied. A C3HC4-type RING finger protein was involved in protein–protein interaction and ubiquitination [19]. Most ring finger proteins were E3 ubiquitin ligases that mediate the transfer of the ubiquitin to target proteins and play important roles in diverse aspects of celluar regulations in

Table 2
Function classification of NaCl salt stress related transcript-derived fragment (TDF) in *P. simonii* × *P. nigra*.

TDF	Primer combination	Genbank accession	Length (bp)	I/R	Annotation (species)	Blast score (Blastx/Blastn[a])
					Regulation of transcription	
109-2	T-GA/M-GT	GW672671	311	–	WRKY transcription factor [*Populus tremula* × *Populus alba*]	4.00E−31
N-3	T-AG/M-AG	GW672667	437	+	TCP family transcription factor [*Arabidopsis tha liana*]	7.00E−42
N-11	T-AG/M-AG	GW672664	108	+	Bel1 homeotic proteine [*Ricinus communis*]	2.00E−06
M-21	T-CA/M-TG	GW672654	221	–	Zinc knuckle (CCHC-type) family protein [*Arabidopsis thaliana*]	0.58
E-5	T-CA/M-AG	GW672623	288	–	ARR12(Arabidopsis response regulator 12; transcription factor) [*Arabidopsis thaliana*]	6.00E−04
82-2	T-TG/M-AC	GW672595	350	+	Mitochondrial transcription termination factor [*Arabidopsis thaliana*]	0.74
F-3	T-CA/M-CT	GW672629	379	+	AP2/ERF domain-containing transcription factor [*Populus trichocarpa*]	5.00E−36
G-19	T-GA/M-CA	GW672636	143	–	ATP binding/DNA binding/DNA-dependent ATPase [*Arabidopsis thaliana*]	0.41[a]
20-2	T-AG/M-AG	GW672590	334	+	RDR6 (RNA-directed RNA polymerase6) [*Arabidopsis thaliana*]	0.087[a]
N-8	T-AC/M-CT	GW672671	391	+	DEAH box helicase[*Arabidopsis thaliana*]	1.00E−19
N-6	T-AC/M-GT	GW672670	117	+	ATP binding/DNA binding/helicase [*Arabidopsis thaliana*]	9.00E−15
					Response to stress	
M-4	T-TG/M-TC	GW672662	100	–	CPHSC70-1 (chloroplast heat shock protein 70-1) [*Arabidopsis thaliana*]	2.00E−07
H-2	T-AC/M-CT	GW672647	166	+	Osmotin precursor [*Ricinus communis*]	3.00E−21
B-4	T-CT/M-CA	GW672608	240	+	Disease resistance protein (CC-NBS-LRR class) [*Arabidopsis thaliana*]	9.00E−21
N-5	T-AC/M-AC	GW672669	295	+	Peroxidase 12 (PER12) [*Arabidopsis thaliana*]	4.00E−07
A-5	T-GT/M-CA	GW672602	108	–	ADH1 (Alcohol dehydrogenase 1) [*Arabidosis thaliana*]	2.00E−04
					Transport	
E-1	T-AG/M-AG	GW672620	310	–	ADNT1 (adenine nucleotide transporter 1) [*Arabidopsis thaliana*]	6.00E−48
G-23	T-AC/M-CT	GW672637	107	–	ATPase, coupled to transmembrane movement of substances[*Arabidopsis thaliana*]	1.00E−11
H-12	T-AC/M-CT	GW672644	154	+	Xenobiotic-transporting ATPase[*Arabidopsis thaliana*]	1.00E−19
G-1	T-GA/M-CA	GW672631	151	–	ATARLA1C (ADP-ribosylation factor-like A1C) [*Arabidopsis thaliana*]	3.00E−22
					Photosynthesis and redox	
A-4	T-CA/M-GC	GW672601	71	–	Photosystem II protein D1 [*Arabidopsis thaliana*]	1.00E−07
G-6	T-AC/M-GA	GW672639	125	–	LHCB4.2 (light harvesting complex PSII) [*Arabidopsis thaliana*]	2.00E−16
C-1	T-AG/M-AG	GW672611	218	–	LHCB3 (light-harvesting chlorophyll binding protein 3) [*Arabidopsis thaliana*]	6.00E−36
D-6	T-CG/M-CA	GW672619	147	+	NADH dehydrogenase subunit K [*Populu trichocarpa*]	8.00E−11
H-22	T-CA/M-AG	GW672648	160	+	Cytochrome P450 [*Populus trichocarpa*]	1.00E−20
C-2	T-CA/M-CA	GW672612	266	–	Malate dehydrogenase [*Clusia uvitana*]	3.00E−29
					Development process	
30-2	T-AG/M-GT	GW672591	413	+	Senescence-associated protein[*Arabidopsis thaliana*]	1.00E−31
M-34	T-TG/M-TC	GW672660	340	–	TPR1 (topless-related 1) [*Arabidopsis thaliana*]	1.00E−56
D-10	T-CT/M-CA	GW672616	238	+	Cupin family protein [*Arabidopsis thaliana*]	9.00E−18
10-1	T-AC/M-AC	GW672588	546	+	Cysteine proteinase[*Arabidopsis thaliana*]	2.00E−04
F-7	T-TC/M-CT	GW672630	359	–	Cytokinin oxidase [*Populus trichocarpa*]	5.00E−56
43-3	T-CA/M-TG	GW672593	455	–	Cinnamyl alcohol dehydrogenase-like protein [*Populus trichocarpa*]	5.00E−68
					Cellular catabolism	
E-9	T-CT/M-AC	GW672627	130	–	UBP5 (Ubiquitin-specific protease 5) [*Arabidopsis thaliana*]	2.00E−18
F-1	T-CT/M-GA	GW672628	167	+	Chitinase [*Ricinus communis*]	8.00E−11
M-26	T-CT/M-TG	GW672657	299	–	Ubiquitin-conjugation enzyme [*Glycine max*]	8.00E−40
88-1	T-TG/M-CT	GW672596	326	+	Zinc finger (C3HC4-type RING finger) family protein[*Arabidopsis thaliana*]	5.00E−37
					Cellular biosynthesis	
E-12	T-CA/M-AC	GW672621	324	–	Serine palmitoyl transferase subunit [*Nicotiana benthamiana*]	5.00E−54
E-8	T-GA/M-CA	GW672626	129	–	EIF4A1 (eukaryotic translation initiation factor 4A-1) [*Arabidopsis thaliana*]	3.00E−19
89-2	T-TG/M-TC	GW672597	388	–	S-adenosylmethionine decarboxylase 1 [*Populus maximowiczii* × *Populus nigra*]	6.00E−44
C-4	T-GA/M-GT	GW672614	180	–	Ribosomal protein S3[*Flacourtia jangomas*]	3.00E−26
G-15	T-GA/M-CT	GW672635	140	–	GAUT3 (Galacturonosyl transferase 3) [*Arabidopsis thaliana*]	2.00E−09
M-28	T-AG/M-AG	GW672658	269	–	Ferrochelatase II[*Arabidopsis thaliana*]	1.00E−23
N-16	T-AC/M-AC	GW672666	282	+	CARB (Carbamoyl phosphate synthetase B) [*Arabidopsis thaliana*]	8.00E−08
G-7	T-GA/M-CT	GW672640	146	–	Trehalose-6-phosphate synthase [*Ricinus communis*]	8.00E−18
D-2	T-CT/M-GT	GW672617	205	+	2-isopropylmalate synthase [*Arabidopsis thaliana*]	1.00E−28
					Metabolism	
E-6	T-AG/M-GA	GW672624	237	–	Radical sam protein [*Ricinus communis*]	3.00E−11
E-7	T-CA/M-AG	GW672625	270	–	Adenosine kinase [*Ricinus communis*]	2.00E−43
43-1	T-CA/M-TG	GW672592	526	–	Lactoylglutathione lyase[*Arabidopsis thaliana*]	4.00E−60
C-3	T-AG/M-AG	GW672613	342	–	4-coumarate–CoA ligase family protein [*Arabidopsis thaliana*]	0.75
M-6	T-TG/M-TC	GW672663	158	–	Serine carboxypeptidase [*Ricinus communis*]	4.00E−18
H-10	T-AC/M-TC	GW672642	351	+	Lactoylglutathione lyase family protein/glyoxalase I family protein[*ArabidopsisThaliana*]	2.00E−23

Table 2 (*Continued*)

TDF	Primer combination	Genbank accession	Length (bp)	I/R	Annotation (species)	Blast score (Blastx/Blastn[a])
47-1	T-CA/M-GA	GW672594	350	–	Glycine decarboxylase P-protein 1 [*Arabidopsis thaliana*]	9.00E–60
N-4	T-AG/M-AC	GW672668	360	+	Acetate-CoA ligase[*Arabidopsis thalian*]	1.00E–52
N-10	T-AG/M-AG	GW672587	437	+	Shock protein binding protein [*Ricinus communis*]	7.00E–30
D-5	T-CT/M-CA	GW672618	216	+	Carbonate dehydratase[*Arabidopsis thaliana*]	2.00E–19
H-15	T-TG/M-CT	GW672645	101	+	FKBP-type peptidyl-prolyl cis–trans isomerase family protein[*Arabidopsis thaliana*]	4.00E–07
H-24	T-AG/M-CT	GW672649	180	+	PDC3(Pyruvate decarboxylase-3) [*Arabidopsis thaliana*]	1.00E–28
H-11	T-AC/M-CT	GW672643	169	+	NAD+ ADP-ribosyltransferase[*Arabidopsis thaliana*]	0.0003[a]
M-31	T-CT/M-GT	GW672659	228	–	Nicotinamide phosphoribosyl transferase [*Aeromonas phage 44RR2.8t*]	9.00E–19
M-35	T-AC/M-TC	GW672661	86	–	Trehalose/maltose hydrolase or phosphorylase [*Capnocytophaga ochracea*]	5.00E–06
					Signal transduction	
A-7	T-TG/M-CT	GW672603	108	–	G-H2AX/GAMMA-H2AX/H2AXB/HTA3; DNA binding[*Arabidopsis thaliana*]	9.10E–02
G-12	T-AC/M-CT	GW672633	90	–	FTSZ2-2 structural molecule[*Arabidopsis thaliana*]	3.00E–09
H-25	T-TG/M-CT	GW672650	187	+	Calmodulin[*Arabidopsis thaliana*]	0.088
M-13	T-AG/M-AC	GW672652	179	–	Leucine-rich repeat transmembrane protein kinase[*Arabidopsis thaliana*]	0.15[a]
B-1	T-CT/M-CT	GW672605	123	+	Kinase family protein[*Arabidopsis thaliana*]	0.097[a]
G-11	T-CT/M-AC	GW672632	158	–	Cpk-related protein kinase 3 [*Populus trichocarpa*]	4.00E–21
M-14	T-CT/M-TG	GW672653	305	–	F-box family protein[*Arabidopsis thaliana*]	0.57
H-4	T-AG/M-GA	GW672651	163	+	SIT4 phosphatase-associated family protein [*Arabidopsis thaliana*]	2.00E–17
M-23	T-AG/M-TC	GW672655	226	–	Phosphate-responsive protein[*Arabidopsis thaliana*]	8.00E–24
C-9	T-AG/M-GA	GW672615	328	–	Serine–threonine protein kinase, plant-type [*Ricinus communis*]	1.00E–49

I/R: induced or repressed in cDNA-AFLP studies.
[a] Blast scores with asterisk were from Blastn, otherwise from Blastx.

plants [20,21]. The expression level of the C3HC4-type RING finger gene TDF 88-1 was higher under salt stress than under control conditions. WRKY proteins are newly identified transcription factors involved in many plant processes including plant responses to biotic and abiotic stresses. To regulate gene expression, the WRKY domain binds to the W box in the promoter of the target gene to modulate transcription [22,23]. In plants, many WRKY proteins are involved in the defense against attacks from pathogens [24,25], and abiotic stresses of wounding, the combination of drought and heat stress, and cold stress [26]. The expression of putative WRKY TDF 109-2 was repressed in this study, which we will study this gene further in broad germplasm to characterize the expression in response to salt/drought stress.

In summary, we present a method that could be used for synthesizing cDNA from salt stressed *P. simonii* × *P. nigra* vs. control, which gives broad genome coverage; this study also provides genomic information on the differentially expressed TDFs by cDNA-AFLP in *P. simonii* × *P. nigra* under NaCl salt stress. Adaptation of plants to their

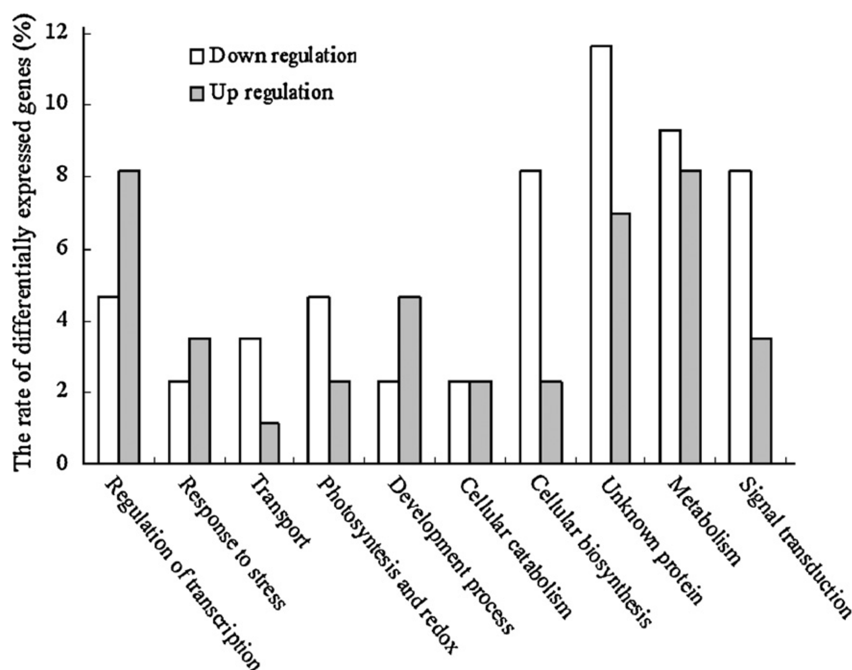

Fig. 2. Functional classification of expressed genes or TDFs (transcript-derived fragments) in *P. simonii* × *P. nigra* under NaCl stress displayed by cDNA-AFLP. The percentage of up-regulated (in grey) and down-regulated (in white) transcripts within each functional category, which was primarily based on the data displayed in Table 2.

Table 3
Validation of expression patterns of selected genes from cDNA-AFLP using real-time qRT-PCR.

TDF ID[a]	Expression pattern in cDNA-AFLP[b]	qRT-PCR[c] (mean ± SE)
C-1	–	0.32 ± 0.22
C-2	–	0.61 ± 0.31
10-1	+	4.57 ± 1.53
30-1	+	2.65 ± 0.90
47-1	–	0.25 ± 0.04
88-1	+	10.55 ± 6.06
D-2	+	2.34 ± 0.51
D-5	+	7.21 ± 4.53
D-6	+	2.98 ± 1.48
D-10	+	113.3 ± 59.5

[a] ID: TDF identification number in Table 2.
[b] cDNA-AFLP, results of the expression patterns of selected genes at 2 days after NaCl treatment compared with no stress control; +/− used to show gene expression trends in cDNA-AFLP, +, induced, −, repressed.
[c] Real time qRT-PCR, results of relative quantitative qRT-PCR ($R = 2^{-\Delta\Delta C(T)}$) of selected genes at 2 days after NaCl treatment compared to no stress control. R value > 2.00 as induced, R value < 0.50 as repressed, $2.00 \geq R$ value ≥ 0.50 as unchanged. Three experimental technical replications were performed for each equally pooled sample from three biological samples to assess the reproducibility, and the mean of the three replications was used to calculate relative expression quantitation.

environment can be highly efficient, involving many metabolic and physiological changes. This study shows that it is possible to reproduce the profiles of gene expression in a salt stressed *P. simonii* × *P. nigra* and to isolate differentially regulated sequences using a modification of the cDNA-AFLP protocol of Bachem et al. [10]. Therefore, these data suggest that cDNA-AFLP is a useful tool to serve as an initial step for characterizing transcriptional changes induced by NaCl salinity stress in *P. simonii* × *P. nigra* and provides resources for further study and will contribute to the genetic improvement of *P. simonii* × *P. nigra.* This is because prior sequence data is not required for the visual identification of differentially expressed transcripts, in contrast to other approaches.

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References

[1] R. Munns, Comparative physiology of salt and water stress, Plant Cell Environ. 25 (2002) 239–250.
[2] Widodo, J.H. Patterson, E. Newbigin, M. Tester, A. Bacic, U. Roessner, Metabolic responses to salt stress of barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance, J. Exp. Bot. 60 (2009) 4089–4103.
[3] E. Darwish, C. Testerink, M. Khalil, O. El-Shihy, T. Munnik, Phospholipid signaling responses in salt-stressed rice leaves, Plant Cell Physiol. 50 (2009) 986–997.
[4] C.Z. Cui, C.L. Huang, G.M. Yu, Y. Cui, J.M. Zhao, Research of nursery growth rule of *Populus xiaohei* planting by cutting, J. Sci. Teachers' Coll. Univ. 19 (1999) 37–39.
[5] H.Q. Ren, X.E. Liu, Z.H. Jiang, Y.H. Wang, H.Q. Yu, Effects of planting density on wood anatomical properties of *Populus xiaohei*, Forest Res. 19 (2006) 364–369.
[6] Z.B. Wang, F.L. Zhang, Z.Y. Wang, S.P. Xie, Study on Poplar transgene of the fungus disease-resistance, Forest. Sci. Technol. 31 (2006) 22–24.
[7] T. Lin, Z.Y. Wang, K.Y. Liu, T.Z. Jing, C.X. Zhang, Transformation of spider neurotoxin gene with prospective insecticidal properties into hybrid poplar *Populus simonii* × *P. nigra*, Acta Entomol. Sinica 49 (2006) 593–598.
[8] C.P. Yang, G.F. Liu, H.W. Liang, H. Zhang, Study on the transformation of *Populus simonii* × *P. nigr*a with salt resistance gene Bet-A, Sci. Silvae Sinicae 37 (2001) 34–38.
[9] S. Bai, Q.P. Song, G.F. Liu, Y. Jiang, S.J. Lin, The analysis of salt tolerance of transgenic *Poplus simonii* × *P. nigra* pollen plantlets with betA gene, Mol. Plant Breeding 4 (2006) 41–44.
[10] C.W.B. Bachem, R.S. Van der Hoeven, S.M. de Bruijin, D. Vreugdenhil, M. Zabeau, R.G.F. Visser, 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 (1996) 745–753.
[11] D. Milioni, P.E. Sado, N.J. Stacey, K. Roberts, M.C. McCann, Early gene expression associated with the commitment and differentiation of a plant tracheary element is revealed cDNA amplified fragment length polymorphism analysis, Plant Cell 14 (2002) 2813–2824.
[12] X.J. Wang, W. Liu, X.M. Chen, C.L. Tang, Y.L. Dong, J.B. Ma, X.L. Huang, G.R. Wei, Q.M. Han, L.L. Huang, Z.S. Kang, Differential gene expression in incompatible interaction between wheat and stripe rust fungus revealed by cDNA-AFLP and comparison to compatible interaction, BMC Plant Biol. 10 (2010) 9.
[13] M. Vuylsteke, H.V.D. Daele, A. Vercauteren, M. Zabeau, M. Kuiper, Genetic dissection of transcriptional regulation by cDNA-AFLP, Plant J. 45 (2006) 439–446.
[14] O. Rowland, A.A. Ludwig, C.J. Merrick, F. Baillieul, F.E. Tracy, W.E. Durrant, L. Fritz-Laylin, V. Nekrasov, K. Sjolander, H. Yoshioka, J.D.G. Jones, Functional analysis of Avr9/Cf-9 rapidly elicited genes identifies a protein kinase, ACIK1, that is essential for full Cf-9–dependent disease resistance in tomato, Plant Cell 17 (2005) 295–310.
[15] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method, Methods 25 (2001) 402–408.
[16] M. Brinker, M. Brosché, B. Vinocur, A. Abo-Ogiala, P. Fayyaz, D. Janz, E.A. Ottow, A.D. Cullmann, J. Saborowski, J. Kangasjärvi, A. Altman, A. Polle, Linking the salt transcriptome with physiological responses of a salt-resistant populus species as a strategy to identify genes important for stress acclimation, Plant Physiol. 154 (2010) 1697–1709.
[17] M.Q. Ding, P.C. Hou, X. Shen, M.J. Wang, S.R. Deng, J. Sun, F. Xiao, R.G. Wang, X.Y. Zhou, C.F. Lu, D.Q. Zhang, X.J. Zheng, Z.M. Hu, S.L. Chen, Salt-induced expression of genes related to Na+/K+ and ROS homeostasis in leaves of salt-resistant and salt-sensitive poplar species, Plant Mol. Biol. 73 (2010) 251–269.
[18] Y.R. Lapik, L.S. Kaufman, The Arabidopsis Cupin domain protein AtPirin1 interacts with the G protein α-subunit GPA1 and regulates seed germination and early seedling development, Plant Cell 15 (2003) 1578–1590.
[19] K. Ma, J.H. Xiao, X.H. Li, Q.F. Zhang, X.M. Lian, Sequence and expression analysis of the C3HC4-type RING finger gene family in rice, Gene 444 (2009) 33–45.
[20] K.L. Lorick, J.P. Jensen, S. Fang, A.M. Ong, S. Hatakeyama, A.M. Weissman, RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 11364–11369.
[21] S.L Stone, H. Hauksdottir, A. Troy, J. Herschleb, E. Kraft, J. Callis, Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis, Plant Physiol. 137 (2005) 13–30.
[22] T. Eulgem, P.J. Rushton, S. Robatzek, I.E. Somssich, The WRKY superfamily of plant transcription factors, Trends Plant Sci. 5 (2000) 199–206.
[23] S. Berri, P. Abbruscato, F.R. Odile, A.C.M. Brasileiro, I. Fumasoni, K. Satoh, S. Kikuchi, L. Mizzi1, P. Morandini, M.E. Pè1, P. Piffanelli, Characterization of *WRKY* co-regulatory networks in rice and *Arabidopsis*, BMC Plant Biol. 9 (2009) 120.
[24] T. Eulgem, P.J. Rushton, E. Schmelzer, K. Hahlbrock, I.E. Somssich, Early nuclear events in plant defence signaling: rapid gene activation by WRKY transcription factors, EMBO 18 (1999) 4689–4699.
[25] C.J. Park, Y.C. Shin, B.J. Lee, K.J. Kim, J.K. Kim, K.H. Paek, A hot pepper gene encoding WRKY transcription factor is induced during hypersensitive response to *Tobacco mosaic virus* and *Xanthomonas campestris*, Planta 223 (2006) 168–179.
[26] M. Kalde, M. Barth, I.E. Somssich, B. Lippok, Members of the *Arabidopsis* WRKY group III transcription Factors are part of different plant defense signaling pathways, Mol. Plant Microbe Interact. 16 (2003) 295–305.