

Identification of transcription factors involved in root apex responses to salt stress in *Medicago truncatula*

Véronique Gruber · Sandrine Blanchet · Anouck Diet · Ons Zahaf ·
Adnane Boualem · Klementina Kakar · Benoît Alunni · Michael Udvardi ·
Florian Frugier · Martin Crespi

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Abstract The root apex contains meristematic cells that determine root growth and architecture in the soil. Specific transcription factor (TF) genes in this region may integrate endogenous signals and external cues to achieve this. Early changes in transcriptional responses involving TF genes after a salt stress in *Medicago truncatula* (Mt) roots were analysed using two complementary transcriptomic approaches. Forty-six salt-regulated TF genes were identified using massive quantitative real-time RT-PCR TF profiling in whole roots. In parallel, Mt16K+ microarray analysis revealed 824 genes (including 84 TF sequences) showing significant changes ($p < 0.001$) in their expression

in root apices after a salt stress. Analysis of salt-stress regulation in root apices *versus* whole roots showed that several TF genes have more than 30-fold expression differences including specific members of AP2/EREBP, HD-ZIP, and MYB TF families. Several salt-induced TF genes also respond to other abiotic stresses as osmotic stress, cold and heat, suggesting that they participate in a general stress response. Our work suggests that spatial differences of TF gene regulation by environmental stresses in various root regions may be crucial for the adaptation of their growth to specific soil environments.

Keywords Abiotic stress · Legumes · Root meristem · Transcription factors · Transcriptomics · Spatial expression

V. Gruber and S. Blanchet have contributed equally to this work.

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V. Gruber · S. Blanchet · A. Diet · O. Zahaf · A. Boualem ·
B. Alunni · F. Frugier · M. Crespi (✉)
Institut des Sciences du Végétal, C.N.R.S.,
91198 Gif-sur-Yvette, France
e-mail: crespi@isv.cnrs-gif.fr
URL: <http://www.isv.cnrs-gif.fr/mc/index.html>

V. Gruber
e-mail: gruber@isv.cnrs-gif.fr

V. Gruber · A. Diet · B. Alunni
Université Paris Diderot Paris 7, Les Grands Moulins,
16 rue Marguerite Duras, 75205 Paris Cedex 13, France

K. Kakar · M. Udvardi
Max-Planck Institute of Molecular Plant Physiology,
14476 Potsdam, Germany

Introduction

Abiotic stresses impact plant growth and development to diminish stress exposure (Potters et al. 2007) leading to stress-induced morphogenetic responses such as the adaptation of root architecture to specific soil conditions. The root system is the primary site of perception of the soil environment. Development of the root system depends on the activity of the apical meristem, which produces all cell types in a highly defined pattern of cell divisions (Benfey and Scheres 2000; Brady et al. 2007). Complex regulatory mechanisms in these cell types have been identified by analysing expression patterns in 15 different zones of the *Arabidopsis thaliana* root (Birnbaum et al. 2003). Among 577 transcription factor (TF) transcripts detected in the root, 331 were differentially expressed in different zones, allowing tentative links between gene activity and cell fate/tissue specialization. This suggests that spatial expression of TF genes is a critical aspect of the networks involved in

transcriptional regulations (Lee et al. 2006). Moreover, a recent study demonstrates that developmental parameters highly constrained the transcriptional response to high salinity of different cell layers and developmental stages of the *Arabidopsis* root. This reveals the crucial role the environment plays in defining the transcriptional outcome of cell-fate decisions (Dinnyen et al. 2008).

One critical step controlling stress responses involves transcriptional regulation (Wang et al. 2003). Plant genomes dedicate a large number of their coding sequences to TFs reaching about 6% (>1,500 TF genes) in the fully sequenced *Arabidopsis* genome (Riechmann et al. 2000). Among these TFs, a number of them, such as AP2/EREBP (Apetala2 / Ethylene Response Element Binding Protein), bZIP/HD-ZIP (basic leucine ZIPper/HomeoDomain—leucine ZIPper), MYB (MYeloBlastosis), HSF (Heat Shock Factor) and WRKY TF families, have been implicated in plant stress responses (Shinozaki and Yamaguchi-Shinozaki 2000; Jiang and Deyholos 2006). Very few TFs have been implicated in abiotic stress tolerance in legumes. Among them, the soybean SCOF-1 TF belonging to the C₂H₂ zinc-finger family was induced by low temperature and abscisic acid but not by dehydration or high salinity (Kim et al. 2001). The Medicago *MtZpt2-1* gene from the same family was shown to be required for both symbiotic nodule development and root growth recovery after salt stress (Frugier et al. 2000; Merchan et al. 2003, 2007). Furthermore, overexpression of the C₂H₂ zinc-finger alfalfa *Alfin1* and the chickpea AP2-type TF *CAP2* in transgenic plants enhanced growth and tolerance to salt (Winicov 2000; Shukla et al. 2006). Nevertheless, the molecular links between perception of the environment and activation of developmental adaptive responses remain largely unknown.

Transcriptomic studies have been used to identify TFs regulated by abiotic stresses potentially linked to specific genetic programs activated to cope with stress (Chen et al. 2002; Seki et al. 2002). In model legumes, extensive sequencing highlighted around 2,000 TFs per genome, of which less than 1% have been genetically characterized (reviewed by Udvardi et al. 2007). In *Medicago truncatula*, Mt16K+ microarrays covering 16,086 tentative consensus sequences derived mainly from 164,000 *M. truncatula* ESTs collected in the TIGR *M. truncatula* Gene Index 5 (<http://www.tigr.org/tdb/mtgi>) were recently used to monitor changes in the transcriptome of desiccation-sensitive radicles of *M. truncatula* seeds (Buitink et al. 2006). In this work, more than 1,300 genes were differentially expressed during embryo desiccation and several regulatory genes (including TFs) were up-regulated during maturation. This showed a partial overlapping between ABA-dependent and independent regulatory pathways in both drought stress and desiccation tolerance in seeds. Homologs of up-regulated TFs during the re-establishment of desiccation tolerance

were shown to be involved in both abiotic stress and seed development in other species (Buitink et al. 2006). Recently, transcription profiling revealed 912 genes differentially expressed during salt acclimation in *Lotus japonicus* (Sanchez et al. 2008). These transcriptional changes were presumably coordinated by specific TFs modulated during this process such as AP2/ERF and MYB (24 and 20% of the total number of TFs, respectively). An alternative high throughput approach for profiling TF genes is massive real-time RT-PCR (qRT-PCR). The sensitivity of this approach has been validated in *A. thaliana* revealing novel root- and shoot-specific lowly expressed TF genes not previously linked to these organs (Czechowski et al. 2005), as well as TF genes that respond to sugars and nutrients (Scheible et al. 2004; Osuna et al. 2007). Recently, this technology was exploited to identify putative TF genes expressed at different stages of seed development in *M. truncatula* (Verdier et al. 2008).

In root cells, overlapping stress perception and signal transduction pathways may be most critical at very early times after stress exposure (Swindell 2006). To this end, we have identified, in this work, TFs rapidly regulated in root apices by salt stress in the model legume *M. truncatula*. A massive real time RT-PCR approach allowed us to identify 46 TF genes responding after a 1-h salt treatment in roots. Several of these TFs displayed even higher salt-inductions in root apices. We then performed a microarray expression profiling directly on root apices submitted to salt stress and identify 84 regulated TF sequences. We propose that these TF genes, strongly regulated in root meristematic regions, are good candidates to be involved in transcriptional networks related to the adaptation of root architecture to the soil environmental conditions.

Materials and methods

Plant material, growth conditions, and stress treatments

Medicago truncatula cv. Jemalong A17 seeds (Barker et al. 1990) were scarified, surface-sterilised by immersion for 20 min in Bayrochlore (3.75 g/L active Chlore, Novelty Chlor, France), and washed six times with sterilised water. Seeds were sown on 1% water-agar plates and stored for 2 days at 4°C before incubating overnight at 24°C in the dark to ensure uniform germination.

For analysing stress responses in root apices, germinated seedlings were transferred to pots without bottom containing perlite:sand (3:1, v/v) placed on a grid in a container filled with liquid medium ([Soluplant 18.6.26]:water 1:1, Duclos, France). Nine pots containing nine seedlings each were used per experiment. Seedlings were grown for 10 days at 22°C on a 16-h light/8-h dark cycle in a growth

chamber with 60% relative humidity until roots passed through the grid and were immersed in the medium. Then, liquid plant growth media imbibing the root apices was replaced by fresh medium with or without salt (100 mM NaCl). Four biological replicates per salinity treatment were performed (nine plants/replicate). Root tips (1 cm) were harvested after a 1-h salt-stress treatment, immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction.

For the analysis of stress responses in whole roots, 40 germinated seedlings were grown in a container filled with perlite:sand (3:1) for 2 weeks at 24°C on a 16-h light/8-h dark cycle in a greenhouse. Then, plants were immersed into a glass container containing plant growth media, with or without 100 mM NaCl supplement, for 1 h under agitation (to prevent anoxia). Whole roots were collected, immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction.

For the analysis of temporal expression patterns, 30 germinated seedlings were grown in Magenta pots and placed for 3–4 days on a grid (to allow only the immersion of the roots in liquid plant growth medium), under agitation in a growth chamber (24°C and a 16 h-light period). In this setup, plants were submitted to a 100-mM NaCl stress and roots were collected at various time points (0, 15, 30, 60, and 360 min). For the different abiotic stress treatments, similarly grown seedlings were treated for 1 h with 100 mM NaCl or 200 mM mannitol, whereas alternatively the pots were placed for 1 h at 4 or 37°C . Root tips (1 cm) were harvested after different treatments, immediately frozen and stored at -80°C for RNA extraction. Two biological replicates per treatment were performed.

Real-time RT-PCR

Total RNA was isolated with Trizol reagent (Gibco-BRL), treated with DNase (RQ1 RNase-free DNase, Promega, France), purified using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions and stored at -80°C . RNA quality was controlled using a bioanalyzer and RNA was quantified using a Nanodrop spectrophotometer. RNA was tested for the absence of genomic DNA by PCR before proceeding to the RT step to avoid background PCR signal due to genomic DNA.

Massive real time RT-PCR TF profiling was carried out using a set of 710 RT-PCR primer pairs targeting TF genes derived from *M. truncatula* genome annotation (Kakar et al. 2008). Consistent values were selected as those having less than a cycle of difference between technical replicates.

For other real-time RT-PCR, two micrograms of total RNA were retrotranscribed for 1 h at 42°C using the

“SUPERScript® II first-strand synthesis system” (Invitrogen, Cergy Pontoise, France) and subsequently denatured for 10 min at 75°C . These cDNAs were diluted to 60 μL in sterile water and 2.5 μL was amplified using the “LC Fast-Start DNA Master SYBR Green I” (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. Real time PCR were done on a Light Cycler apparatus, using the following conditions: 95°C for 10 s, 50 amplification cycles at 95°C for 5 s, 60°C for 5 s and 72°C for 15 s. A negative control without cDNA template was always included for each primer combination. The Mt *UBI*, Mt *H3L* and Mt *RBP1* genes were used as internal controls and behave similarly all through the salt treatment kinetic. Mt *UBI* was then selected to normalize the expression data for each gene in all figures. Primer sequences for the different TFs and internal control analysed are given in Electronic supplementary material Table S6.

Syntheses of two independent cDNA preparations from the same RNA sample (technical duplicates) were performed for each experiment. Ratios against a constitutive control (Mt *UBI* gene) were used to normalize gene expression between different biological conditions. The ratio value of the experimental control condition was set up to 1 as a reference to determine relative induction/repression factors. Except for Fig. 6, a representative example out of the two biological replicates performed is shown for each figure.

Hybridization of Mt16K+ microarrays

Four independent biological replicates of salt-treated root apices and controls were used. Two micrograms of total RNA from each sample was extracted as described for RT-PCR experiments and used to synthesize Cy5/Cy3-labelled cDNA using the Amino Allyl MessageAmp™ aRNA Kit (Ambion, TX, USA) according to the manufacturer's instructions.

Cy5/Cy3-labelled cDNA were hybridized with the 70-mer Mt16K+ oligonucleotide microarrays (<http://www.ebi.ac.uk/arrayexpress>; accession number A-MEXP-138) for 16 h at 60°C . Microarray hybridizations have been deposited and are described at <http://www.ebi.ac.uk/arrayexpress> (ArrayExpress accession: E-MEXP-1441).

Microarray data analysis and statistics

Microarray slides were scanned with a Genepix two-laser scanner (Axon Instruments, Inc.) and the resulting images were analysed with the GenePix 6.0 software (Molecular Devices). Data transformations and normalization, performed with the MAnGO R script (version 1.0; Marisa

et al. 2007), consisted in a local background correction, omitting flagged spots, and successively an intensity-dependent print-tip loess normalization and a scale between array normalization (Yang et al. 2002). Differential analysis was based on an empirical Bayes moderated *t* test adjusted with the false discovery rate (FDR, Benjamini and Hochberg 1995) multiple test correction. Differentially expressed genes were defined based on thresholds on adjusted *P*-values ($P < 0.001$), fold-change (FCI > 1.8), and mean intensity of the two channels (A-mean > 7 on a log₂ scale; threshold defined using negative controls available on the microarray).

Bioinformatic analyses

Annotation of differentially expressed TFs was systematically verified based on BLASTX searches on the TIGR database (<http://www.tigr.org/tdb/tgi/mtgi/>; release 8.0, January, 2005) for ESTs, and on the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST/>) for available genomic sequences.

MapMan display

Data were visualized using the MapMan software (downloadable at <http://gabi.rzpd.de/projects/MapMan/>; Thimm et al. 2004; Tellström et al. 2007), following instructions provided on the website. To predict BINs that exhibit a different bias of expression profile among all constituting BINs, we applied the statistical analysis provided in Mapman (Benjamini and Hochberg corrected Wilcoxon rank sum test or BHW test). Significant differences were defined based on a *p*-value < E-02 and the BIN size (element number > 10).

Results

Identification of transcription factor genes differentially expressed in *M. truncatula* roots under salt stress

An analysis based on a massive qRT-PCR TF profiling approach was carried out using set of 710 PCR primer pairs targeting TF genes. As large quantities of RNA were required for this approach, whole root systems, either untreated or submitted to a 1-h salt treatment (100 mM NaCl) were used. We retained 401 TF transcripts (56%) having consistent values in the two technical replicates for each condition. Among these, 46 TF transcripts were salt-regulated and showed a difference of at least one PCR cycle between normalized untreated control and 1-h salt-treated

samples (a cut-off threshold of 1.8-fold was considered; Electronic supplementary material Table S1). To further validate these expression analyses, the efficiency and specificity of 26 primer pairs (corresponding to various TF gene families) were tested and 15 (58%) of them gave efficient and specific amplification for the expected TF gene (Electronic supplementary material Table S2). Using these oligonucleotides, the results obtained were validated in 80% of the cases using a replicated biological experiment.

Refined temporal expression patterns were analysed for six TF genes (randomly selected from ESM Table S2) and named AP/EREBP1207, DOF207, MYB119, NAC969, NAC1081 and NAC1126 (based on their best homology to known TF domains), in *M. truncatula* roots submitted to a 100-mM NaCl treatment for various incubation times (from 15 min to 6 h; Fig. 1). Different patterns of gene expression were observed. AP2/EREBP1207 and MYB119 transcript levels were rapidly and transiently induced, reaching maximum values after 15 or 30 min, respectively, and then decreased after 1 h and 6 h of salt treatment. For NAC1081 and NAC1126, the highest transcript levels were detected after 6 h of salt treatment, indicating a late activation of these genes, whereas NAC969 transcript levels were maximal at 1 h. Hence, several new legume TFs regulated in response to salt stress have been identified in whole *M. truncatula* roots.

Salt regulation of several TF encoding transcripts is enhanced in root apices

To determine TF genes that might be linked to the activation of specific genetic pathways in root apices, we analysed the expression of TF genes in root apices *versus* whole roots (Fig. 2). We first analysed ten TF genes having specific and optimised real time RT-PCR conditions

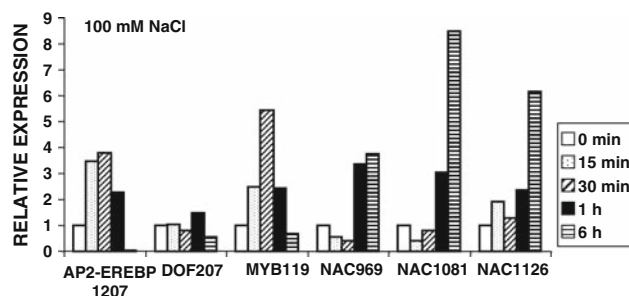


Fig. 1 Expression analysis of whole roots submitted to salt stress for different times. Real-time RT-PCR expression analysis of selected *M. truncatula* transcription factors was performed. Total RNAs were obtained from *M. truncatula* whole roots treated with 100 mM NaCl for 15 min to 6 h. Histograms represent quantification of the products normalized to the constitutive control Mt *UBI*. The value for untreated control roots is set to 1 as reference. A representative example out of two biological replicates is shown

(Fig. 2a) including seven TFs identified from our initial approach (DOF207, DOF891, HD953, MYB119, NAC969, NAC1126, WRKY1219, Electronic supplementary material Table S1) and three other genes (AP/EREBP1207, and NAC1081 and MYB1070).

Weak changes of expression levels were observed in whole roots for the ten tested TFs (induction factors were up to 3.4, Fig. 2 black bars) similar to those observed in the massive real time RT-PCR approach albeit their magnitude sometimes differed (ESM Table S1). In contrast, several TF genes such as AP2/EREBP1207 and MYB119 showing minor variation in whole root RNA samples were strongly induced in root apices (with induction factors > 30; Fig. 2 white bars). Similar trends in favour of higher transcript levels in root apices were observed for NAC1081, WRKY1219 and DOF207, with induction factors ranging between 5 and 13. Interestingly, larger decreases in transcript levels of DOF891 and HD953 were observed in root apices in contrast to whole roots. Hence, specific members of TF gene families showed an enhanced transcriptional regulation in root apices challenged with salt stress. In contrast, MYB1070, NAC969 and NAC1126 transcriptional induction was higher in whole roots compared to root apices, suggesting ubiquitous role of these TF genes. Hence, large spatial differences of TF gene regulation by salt stress were found between complete root systems and root apices, allowing us to define a specific set of TF genes rapidly regulated by salt in the root meristematic and elongation zones.

Identification of early salt-regulated transcription factors expressed in *M. truncatula* root apices using a microarray-based approach

In order to investigate more broadly changes in gene expression induced by salt stress in *Medicago* root apices, a transcriptome analysis based on *M. truncatula* 16K+ microarrays (Mt16KOLI1) was performed using four independent biological replicates of salt-treated root apices and controls. Normalization and statistical analysis ($P < 0.001$, Amean > 7, IFCI > 1.8, see [Materials and methods](#)) resulted in 824 genes that were differentially expressed between untreated control and 1-h salt-treated root apices (Electronic supplementary material Table S3), 558 of them being up-regulated (Fig. 3). The salt-regulated genes were functionally assigned using the GeneBin database of MapMan software (Thimm et al. 2004) initially designed for studying the *Arabidopsis* transcriptome and adapted for *M. truncatula* (Tellström et al. 2007). This automatic annotation may limit the relevance of *Medicago* MapMan assignments, but has been used as an initial approach for *Medicago* transcriptomes.

Global analysis of transcriptome changes indicated that the largest group of up-regulated genes (besides the non-assigned category representing about 30% of total number of differentially regulated genes), corresponded to genes related to secondary metabolism genes (13 and 2% of the up- and down-regulated genes respectively, Figs. 3, S1). Benjamini Hochberg corrected Wilcoxon rank sum test

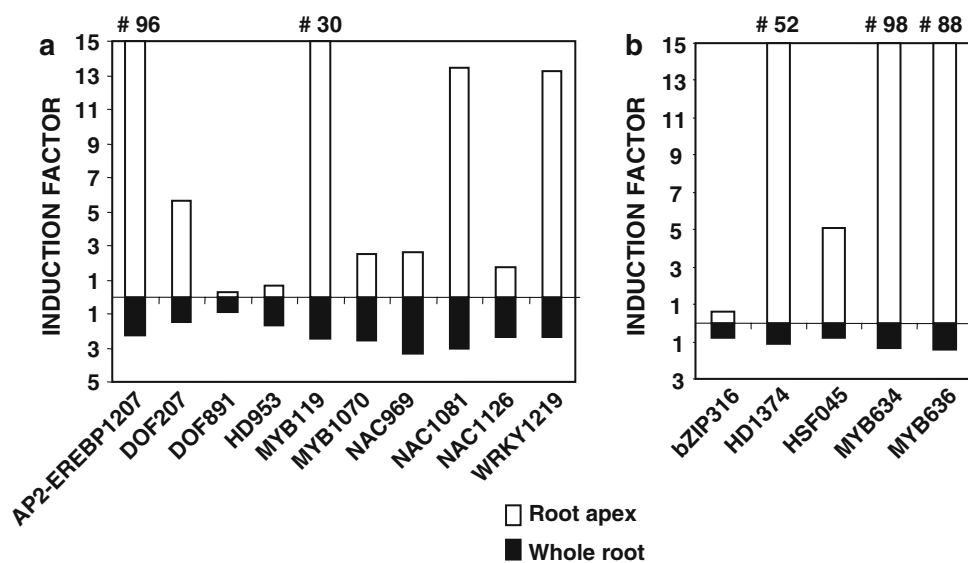
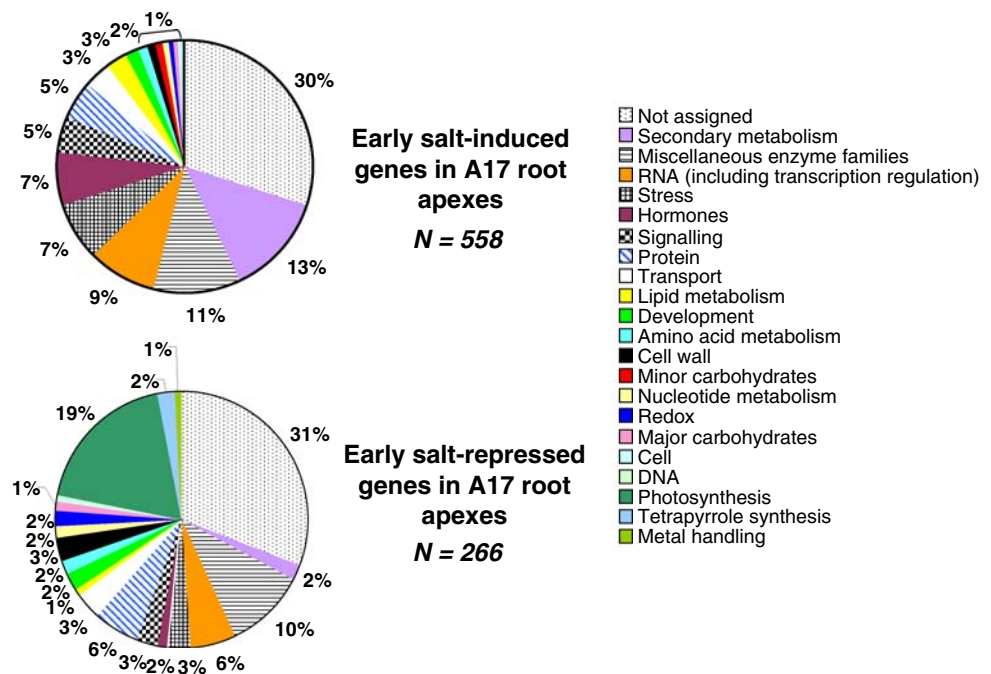


Fig. 2 Comparison of transcription factor (TF) expression in response to salt stress in whole roots versus root apices. Real-time RT-PCR analysis was performed for 15 TF genes identified using the two transcriptomic approaches. Total RNAs were obtained from *M. truncatula* whole roots and root apices treated with 100 mM NaCl for 1 h.

Histograms represent quantification of the products normalized to the constitutive control Mt *UBI*. **a** Selected TF genes identified through the massive RT-PCR approach or **b** through microarray analysis. The value for untreated control roots is set to 1 as reference. A representative example out of two biological replicates is shown

Fig. 3 Functional categories distribution of the 824 salt-regulated genes in *M. truncatula* root apices based on Mapman BIN assignments. A complete list of the classified genes is given in ESM Table S3. *N* denotes the number of regulated genes ($A_{\text{mean}} > 7$, $P < 0.001$, $|FCI| > 1.8$). Upper and lower distributions correspond to induced or repressed genes, respectively



(BHW test) was used to define precisely the statistical significance of the differential expression of this functional category (BHW test; $P = 2.6 \text{ E-}11$, Electronic supplementary material Table S4). Within this category, genes involved in phenylpropanoid, flavonoid, and isoprenoid pathways were significantly induced (BHW test, $P = 9.96 \text{ E-}05$, $P = 6.27 \text{ E-}05$ and $P = 2.68 \text{ E-}02$, respectively; Electronic supplementary material Table S4), indicating likely activation of cellular protection mechanisms in *M. truncatula* root apices in response to salt stress.

In addition, respectively, 7 and 3% of the up- and down-regulated genes were significantly linked to environmental stress responses (biotic and abiotic, BHW test, $P = 9.81 \text{ E-}05$, ESM Table S4, Fig. S2), most of them being related to biotic stresses (BHW test, $P = 1.30 \text{ E-}05$, ESM Table S4, Fig. 3). Surprisingly, few up-regulated genes (11/42) were specifically related to abiotic stress and only three of them were annotated as regulated by drought or salt stress in Mapman GeneBins. This probably reflects the limits of the current annotation in the GeneBin database for *Medicago* based on analysis of abiotic stress responses detected, mainly in *Arabidopsis*. For example, GSTs are annotated as miscellaneous genes although they are well-known defence-related genes.

Another significant group included, respectively, 7 and 2% of the salt up- and down-regulated genes (BHW test, $P = 2.29 \text{ E-}04$, ESM Table S4) linked to phytohormonal pathways (Figs. 3, ESM S3). Within this group, ABA-related genes were found in our analysis as not significantly regulated at these early time points in root apices (based on BHW test). However, genes involved in jasmonate

and ethylene pathways were significantly regulated (BHW test, $P = 9.96 \text{ E-}05$ and $P = 5.86 \text{ E-}02$, respectively, ESM Table S4). This suggests a role of these phytohormones in adaptation of root apices to external environmental conditions.

Finally, the TF genes represented an important salt-regulated group in root apices as visualized on the MapMan pictorial diagram referred as “regulation overview” (Fig. S3, ESM Table S3). Their MapMan GeneBin annotation was verified by BLAST against the *M. truncatula* TIGR database (release 8.0). From the 824 salt-regulated genes corresponding to stringent statistical criteria, 84 were found to be TFs distributed into 72 salt-induced and 12 salt-repressed genes after a 1-h NaCl treatment (Fig. 4, Electronic supplementary material Table S5). Whereas the largest TF set is associated to “transcription regulation” (37 and 10 genes; salt-induced and salt-repressed, respectively), several other salt up-regulated TF genes are linked to “phytohormones” (mainly ethylene; nine genes), “stress” (six genes) or “development” (four genes) categories. As automatic annotations are derived from best homology with genes from other plants, these classifications should be interpreted with caution in the context of *M. truncatula* roots, particularly as certain gene families contain many members (e.g. the AP2/EREBP family) carrying a homologous DNA-binding domain.

In conclusion, functional assignment of the 824 differentially expressed genes revealed extensive modifications of *M. truncatula* transcriptome in root apices, including 84 TFs linked to the activation of transcriptional networks, in response to salt stress.

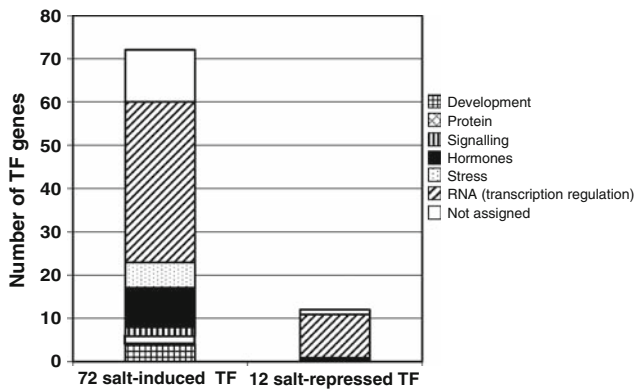


Fig. 4 Number of TF genes belonging to different functional categories based on Mapman BIN assignment. The 84 TF genes ($A_{\text{mean}} > 7$, $P < 0.001$, $\text{IFCI} > 1.8$) regulated in *M. truncatula* root apices after a 1 h salt-treatment were classified accordingly to Mapman BINs. Complete list is given in ESM Table S5

Characterization of early salt-regulated transcription factor families of *M. truncatula*

TF MapMan GeneBin annotation and putative functional domains of the 84 salt-regulated TF genes expressed in root apices were systematically determined using BLAST comparisons (Fig. 5). The overall view highlighted a distribution of 16 TF families grouped into three categories according to their regulation. A first category exhibited exclusively salt-induced TF genes and is constituted by eight TF families [named WRKY, GAI, RGA, SCR (GRAS), Zinc-finger protein expressed in Inflorescence Meristem (ZIM), basic Helix-Loop-Helix (bHLH), HD, HSF, NAM, ATAF1, CUC2 (NAC) and MYeloCytomatosis (MYC)]. In the second group, four TF families [named AP2/EREBP (Zinc-finger), MYB and bZIP] showed a majority of salt-induced but a few salt-repressed TF members. Among them, the predominant AP2/EREBP family included 15 members of which 14 were up-regulated by salt and a salt-repressed DREB-like gene (Dehydration Responsive Element Binding, MT004883, Supplementary Table S5). The Zn-finger TF family included nine members salt-induced and one down-regulated. The third category grouped four TF families showing gene repression after a salt treatment [response regulator (RR), lateral organ boundaries (LBD), Bric-a-brac, tramtrack, and broad/POxvirus zinc finger (BTB/POZ) and TB1, CYC, PCF (TCP)]. Only the WRKY TF family was statistically significantly regulated by salt (BHW test, $P = 7.52 \text{ E-}02$, ESM Table S4).

The spatial regulation of salt-regulated TFs identified in root apices was similarly analysed as the previously identified TFs (in the massive real time RT-PCR approach, Fig. 2). Four highly induced [HD1374 (MT008226), HSF045 (MT016090), MYB634 (MT014860) and

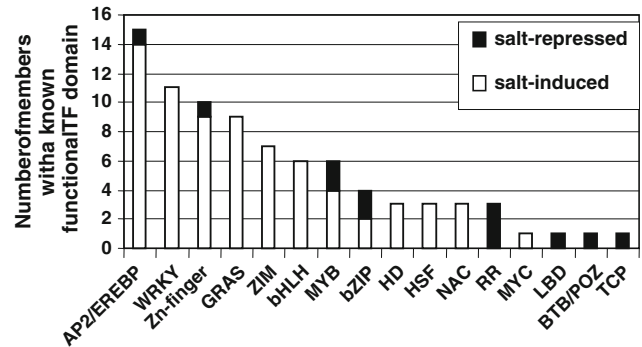


Fig. 5 Number of gene family members with a known functional TF domain. The collection of 84 salt-regulated TF gene identified in *M. truncatula* root apices was individually analysed to score the number of members for each gene family that is either up-regulated (white) or down-regulated (black). Names are: AP2/EREBP apetala2/ethylene response element binding protein, AS2 asymmetric 2, bHLH basic helix-loop-helix, bZIP basic leucine ZIPper, Zn-finger zinc-finger, BTB/POZ bric-a-brac, tramtrack, and broad/POxvirus zinc finger, GRAS GAI, RGA, SCR, HD HomeoDomain, HSF heat shock factor, LBD lateral organ boundaries, MYB myeloblastosis, MYC MyeloCytomatosis, NAC NAM, ATAF1, CUC2, RR Response regulator, SBP squamosa binding protein, TCP TB1, CYC, PCF, ZIM zinc-finger protein expressed in inflorescence meristem, Zn-finger zinc-finger

MYB636 (MT014600)] and one salt-repressed [bZIP316 (MT015835)] TF genes were chosen to evaluate their expression in root apices versus whole roots submitted to salt stress (Fig. 2b). HD1374, MYB634 and MYB636 were strongly transcriptionally induced in root apices (induction factors of 52, 98 and 88, respectively) whereas changes in transcript abundance using whole root RNA samples were very weak (induction factors of 1.1, 1.3 and 1.4, respectively). Less differential induction was observed for HSF045 (Fig. 2b). In contrast, the salt-repressed bZIP316 showed similar transcript levels in both RNA samples indicating a salt-regulation independent of the root zone. These results suggest that rapid increase in transcriptional response of some specific TF genes in meristematic regions after a salt stress may be crucial for adaptation of root growth in response to changes in the soil environment.

To evaluate the specificity of the salt response, eight selected salt-induced TFs (DOF207, HD1374, MYB634, MYB636, MYB1070, NAC969, NAC1081 and NAC1126) in *Medicago* root apices were submitted to three additional stresses: osmotic stress (mannitol 200 mM), cold (4°C) and heat stress (37°C) for 1 h (Fig. 6). These other stresses also induce the expression of the TF genes at variable levels. Furthermore, differential regulation by these stresses was observed among the three MYB or the three NAC genes belonging to the same TF family. Globally, these genes seem to be general stress-responsive. Nevertheless, several TF genes (e.g. NAC1081 and NAC 969) are induced at much higher levels by salt stress than by any of the other

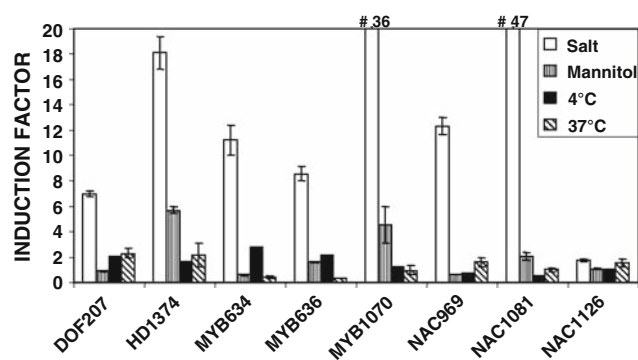


Fig. 6 Expression of TF genes in response to different abiotic stresses in *Medicago* root apices. Real-time RT-PCR analysis was performed for eight selected TF genes in root apices subjected to four abiotic stresses [NaCl 100 mM, mannitol 200 mM, cold (4°C) and heat (37°C)] for 1 h. Total RNAs were obtained from root apices after the different treatments. Histograms represent quantification of the products normalized to the constitutive control *Mt UBI*. The mean of two biological replicates is shown

treatments, suggesting certain specificity of the salt response.

Discussion

The development of genomic resources for *M. truncatula* has enabled to combine Mt16k+ microarrays and massive quantitative real-time PCR approaches to identify a large number of differentially expressed TF genes in early salt stress conditions. The massive TF profiling approach may be more sensitive to monitor changes in TF expression when compared to arrays (Czechowski et al. 2005; Kakar et al. 2008), but may require large amounts of RNA, a limitation to analyse expression specifically in root apices. Another difference between both transcriptomic approaches is that the genes targeted by the RT-PCR resource were selected from IMGAG (International Medicago Genome Annotation Group) annotated-genes derived from genome sequences (including potential annotation errors), while the microarray contained probes designed mostly from TC/ESTs generally corresponding to highly expressed genes. Further, oligo-based microarray hybridization is more prone to cross-hybridization than PCR-derived techniques, even though in our case we were able to confirm by RT-PCR the microarray results in all cases. Finally, gene expression patterns in whole roots and root apices revealed large differences between these regions, preventing a direct comparison of the identified TF genes from these different approaches. Although globally a large overlapping of TF genes exist, these methodologies are complementary for TF gene identification to dissect regulatory pathways involved in salt stress responses in legume roots.

As an initial step in responding to salt stress, plants must sense and transduce appropriate stress-derived signals into physiological processes leading to changes in root growth and development. It has been speculated that the early responsive genes may provide initial protection and amplification of signals (Ramanjulu and Bartels 2002). Consistent with this idea, our results revealed global transcriptional changes in response to salt stress in the secondary metabolism including phenylpropanoid, flavonoid, and isoprenoid pathways in agreement with the stress-triggered production of defence-related secondary metabolites previously reported for rice (Walia et al. 2005). Moreover, large-scale genomic studies realized in the legume *L. japonicus* subjected to long-term salt acclimatization also reinforce this observation (Sanchez et al. 2008). In addition, phytohormones such as abscisic acid, jasmonic acid, ethylene and salicylic acid appear to be critical components of complex signalling networks and are being incorporated into current models of stress responses (Zhu 2002). In *Medicago* root apices, we significantly identified early salt-regulated genes mainly linked to jasmonate and ethylene signalling pathways. Multiple interferences between both pathways may occur when tissues are submitted to environmental stresses (Devoto and Turner 2005; Ludwig et al. 2005). Moreover, microarray experiments in *A. thaliana* have previously demonstrated that the majority of ethylene- and jasmonic acid- related transcripts were responsive to salt treatment (Jiang and Deyholos 2006; Ma et al. 2006).

Stress perception and signalling pathways are critical components of plant adaptative response to survive under environmental constraints. General-stress responses may arise from the activities of various TFs interacting among distinct abiotic stress response pathways (Chen et al. 2002; Chen and Zhu 2004). We have identified a collection of TF genes that are rapidly induced by salt treatment in roots of the model legume *M. truncatula*. This finding is consistent with large-scale genomic studies realized in several plant species as *A. thaliana* (Kreps et al. 2002; Seki et al. 2002; Jiang and Deyholos 2006), tomato (Ouyang et al. 2007), rice (Chao et al. 2005), *L. japonicus* (Sanchez et al. 2008) and moss (Cuming et al. 2007) which support the strong implication of TF genes in early salt stress responses.

The TFs of *M. truncatula* identified in this study could be classified into TF families based on their functional domains and fits well with the recent report of Udvardi et al. (2007). Expression of members of these families has been reported in *Arabidopsis* roots (Shinozaki and Yamaguchi-Shinozaki 2000; Jiang and Deyholos 2006; Dinneny et al. 2008). Nevertheless, little is known about spatial control of transcriptional complexity in plant organs in response to stress. Previous studies showed diverse responses in different maize root regions using a kinematic analysis to characterize spatial and temporal patterns of cell

expansion within the root growth zone (Sharp et al. 2004). Furthermore, Sun et al. (2008) reported that salt inhibits root elongation, but also greatly affects root tip growth direction in *A. thaliana*, indicating that salt-induced changes in root apical cells may contribute to alter gravitropism in order to enable plants adapt to saline environments. Recently, stress responses including salt, osmotic, drought, cold and hormone treatments were placed within the context of tissues and cell lineages in the *Arabidopsis* root using the fuzzy k-means clustering method to identify conditionally co-expressed genes (Ma et al. 2006; Ma and Bohnert 2007). In these studies, the gene expression map previously established for the *Arabidopsis* root (Birnbaum et al. 2003) was integrated with clusters defining stress specificity of the responses. This analysis provided intersections between stress-responsive and cell-specific profiles and identified distinct cell lineages and developmental stages affected by abiotic stresses. In addition, the diversity of *Arabidopsis* transcriptional patterns in different cell-types exposed to salinity was recently further analysed (Dinneny et al. 2008). These analyses revealed that the transcriptional state of a cell is largely a reaction to environmental conditions regulated by a small core set of genes that stably determines cell identity. Hence, the environment plays a crucial role in defining the transcriptional outcome of cell-fate decisions in roots. Our data are in agreement with these results, showing large spatial differences of TF gene regulation by salt stress between whole root systems and root apices in *Medicago*. TF genes encoding AP2/EREBP1207, HD1374, MYB119, MYB634, MYB636, NAC1081, WRKY1219, DOF207 and HSF045 showed a considerable enhancement of expression in root apices in response to salt stress when compared to whole roots. In *Arabidopsis*, most of the TF families examined including AP2-like, HD-ZIP, MYB and WRKY expressed specific members in multiple cell types and tissues of the root at progressive developmental stages (Birnbaum et al. 2003). Furthermore, the analysis of the publicly available Affymetrix *Arabidopsis* gene-chip data on abiotic stress displayed a cluster of 171 genes only or mostly up-regulated by salt-stress, among which nearly 20% corresponded to TFs (Ma et al. 2006). The regulation of specific members of TF families in *Medicago* root tips supports the hypothesis that these genes may intersect root developmental pathways and salt-related transcriptional networks also in legumes. The AP2/EREBP TF genes were already reported to be linked to abiotic stress in many plants (Kizis et al. 2001; Yamasaki et al. 2004; Zhang et al. 2005; Shukla et al. 2006; Kumari et al. 2008). In addition, drought and salt tolerance in *Arabidopsis* are conferred by the related *HARDY* gene (Karaba et al. 2007). In our study, the AP2/EREBP1207 was one of the most highly salt-induced TF genes in *Medicago* root apices, consistent with the involvement of *DREB* genes of

the AP2/EREBP family in abiotic stress tolerance notably in the soybean and chickpea legumes (Li et al. 2005; Shukla et al. 2006). Moreover, spatial differences in the expression of AP2/EREBP in *Medicago* root meristems and differentiated roots were previously observed under non-stress conditions (Holmes et al. 2008). Our data also revealed different expression levels for two *Medicago* HD-ZIP members (HD1374 and HD953) in root apices versus whole roots in response to salt stress. In agreement, Ni et al. (2008) have recently reported that the cotton homeodomain-leucine zipper *GhHBI* transcripts accumulated during early root development but decreased to very low levels as roots developed further. The expression of the *GhHBI* gene was dramatically increased in roots under salt treatment, further supporting the involvement of HD-ZIP TF members in response to environmental conditions (Ariel et al. 2007). Three *Medicago* MYB members were highly induced in root apices, a superfamily known to be involved in numerous processes, including stress responses (Jin and Martin 1999; Ito et al. 2001; Stracke et al. 2001; Chen et al. 2006). Finally, NAC1081 was highly induced in root apices in contrast the other selected NAC TFs. Members of this TF family have previously been associated with multiple stress responses in *Arabidopsis* (Tran et al. 2004) and rice (Nakashima et al. 2007). AtNAC2, a NAC-type TF from *A. thaliana*, has been proposed to integrate environmental and endogenous stimuli into the process of plant lateral root development (He et al. 2005).

The productivity and yield of plant crops is often limited by the joint influence of several stress combinations (Mittler 2006). Identification of genes exhibiting expression responses to several stresses may provide insights into the functional basis of multiple stress tolerance in plants. Genome wide correlations between transcriptional responses to different stress treatments peaked in *Arabidopsis* roots at 1 h of stress exposure (Swindell 2006), suggesting a general-stress response at this time point. Our analysis of transcriptional changes in *Medicago* root apices subjected for 1 h to salinity, osmotic stress, heat and cold shocks is in agreement with this report. Indeed, the selected eight salt-induced TF genes were also induced in *Medicago* root apices under one or several additional stresses, even though the salt induction was quantitatively higher in most cases. As these TF genes respond to various environmental stresses, they may represent possible intersection points among otherwise independent stress-response pathways (Swindell 2006). Indeed, osmotic adjustment is a response involved in salt stress, and several TF genes were also regulated by this stress.

In conclusion, we characterized TF genes issued from large-scale transcriptomic analyses of *Medicago* roots subjected to salt stress. Analysis of salt-stress responses in root apices versus whole roots linked these TFs to the spatial

regulation of gene expression after salt stress. Using four short-term applications of abiotic stresses, we conclude that these TF genes may be part of a general stress response of root apices even though their induction is much higher under salt stress conditions. These data contribute to elucidate the complex regulatory mechanisms associated with stress responses in legume roots. Further functional studies are, however, required to understand the role of specific TF networks activated. It will be particularly challenging to decipher how root developmental pathways may intersect specific salt-related transcriptional networks in the root apex to control salt tolerance and lead to the adaptation of root growth to the soil environment.

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