Identification of drought responsive genes and promoters in *Musa* by using RNA-seq.

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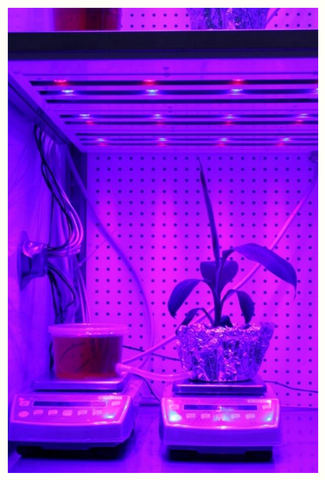
Introduction

Bananas and plantains (*Musa* spp.) are a major staple food with a worldwide production of over 135 million tonnes per year (FAO, 2012). However, water is one of the most limiting abiotic stress factors in the production. Thus, a better understanding of the biodiversity and genetic basis of drought tolerance is needed. In our lab, we have performed an RNA-seq experiment on 3 different banana cultivars known for their contrasted response to mild-drought stress. Read mapping was performed on the double haploid *Musa acuminata* (AA) reference genome [1] as a template. Since the cultivars used are triploids and have variable genome composition (AAA or ABB), mRNA-seq results had to be analyzed in a special manner. More than 803 million out of 1.2 billion reads were uniquely mapped on the reference genome. Applying various statistical methods, we have identified a set of candidate genes differentially expressed under stress. A number of them are tissue-specific and appropriate for identification and cloning of promoter regions able to drive expression of drought responsive genes. Currently, these candidate genes/promoters are being validated with alternative approaches (qRT-PCR) and in different experiments carried out in the lab, greenhouse and under field conditions.



In vitro osmotic stress test. Three triploid banana genotypes with contrasting drought tolerance (ABB Cachaco: tolerant; AAA Grande Naine: intermediate; AAA Mbwazirume: sensitive) were grown autotrophically under osmotic-stress (5% PEG 8000=-0.5 Bars) and control conditions (0% PEG). Samples from root and leaf tissues were collected at 3 days and used for the RNA-seq experiment (with 3 biological replicates).

Results

Characterizing drought- stress reaction in banana

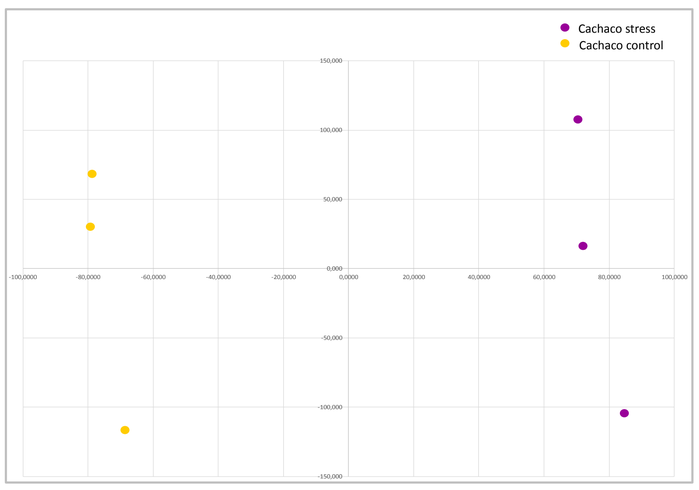


Figure 1. Multivariate Partial Least Square (PLS) analysis. Results indicate that the transcriptome is significantly different after 3 days of osmotic stress in the tissues analyzed. Example for Cachaco root (similar results were obtained for the other genotypes and tissues).

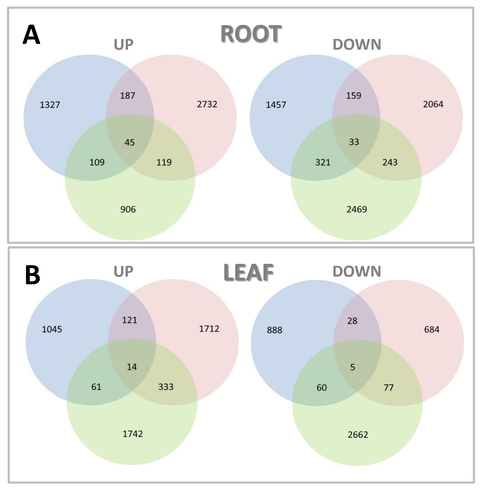


Figure 2. Venn diagrams of differentially expressed genes. Kolmogorov-Smirnov (K-S) test (P<0.1) was used to identify unique transcripts with significantly altered expression in stress vs. control conditions. **A)** genes up regulated (left) and down regulated (right) in root. **B)** Idem for leaf. Color code: blue=Cachaco; pink=Grande Naine; green= Mbwazirume. To characterize the general drought-stress reaction, we focused on the forty five genes upregulated in roots of the 3 genotypes. From those, 11 were also detected by the multivariate method edgeR-RLE. They were selected for validation by qRT-PCR together with the 5 most important genes only detected by PLS+K-S and and the 5 most important only detected by edgeR-RLE.

Abbreviation[a]	Gene annotation	KEGG pathway[b]	Involved in abiotic stress[c]
DUF872	Unknown	-	-
ETHE-1	ETHE-1 like protein	Sulfur metabolism	Drought, osmotic, salt
GT61	Glycosyltransferase 61	-	Biotic and Abiotic
PFK3	6-Phosphofructokinase 3	Glycolysis	Drought
AILP1	Unknown	-	-
P4H1sub	Prolyl 4-hydroxylase subunit alpha-1-like	-	Drought, Anoxia
HB1	Non-symbiotic hemoglobin	-	Hypoxia
CB5-B	Cytochrome b5	-	Drought, others
ALAAT2	Alanine aminotransferase 2	Aminoacids metabolism	Hypoxia, oxidative stress
UMAMIT19	Usually multiple acids move In and out transporters 19	-	Biotic and Abiotic
CYTC-2	Cytochrome C-1	-	Drought, salt
HAD	Haloacid dehalogenase-like hydrolase	-	Heavy metals (Cd)
CPO-1	Coproporphyrinogen-III oxidase	Porphyrin and chlorophyll metabolism	Drought, Temperature
MSD1	Superoxide dismutase [Mn] 3.1	-	Salt, Heat
PDC2	Pyruvate decarboxylase isozyme 2	Glycolysis	Drought, Anoxia
HIG_1_N	Hypoxia-responsive family protein	-	Hypoxia
P4H-1	Oxidoreductase, proline 4 hydroxilase	-	Drought, Anoxia
SAD6	Fatty acid desaturase6	-	Drought, Hypoxia
PK	Pyruvate kinase	Glycolysis	Drought
B12D	B12D protein	-	-
DUF3774	unknown function		Wound

Table 1. Selected genes for qRT-PCR validation. List of 21 unique transcripts induced after 3 days of osmotic stress in banana roots. [a] Abbreviation according to the orthologous gene in *Arabidopsis*. [b] KEGG pathway assigned using Blast2Go (http://www.blast2go.com/b2ghome). [c] involvement in drought or other abiotic stresses based on previous studies.

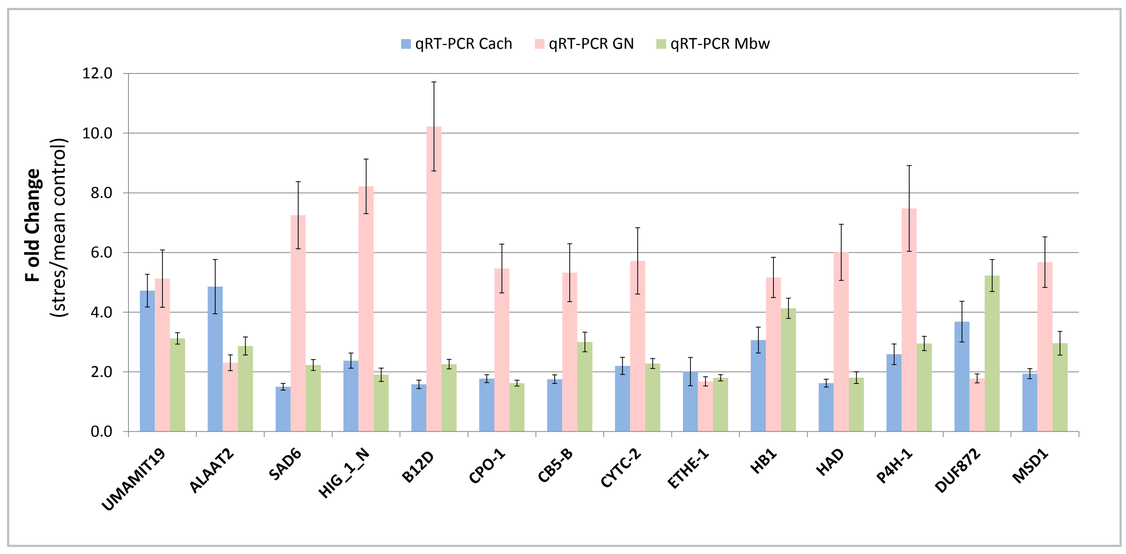


Figure 3. Verification of RNA-seq results by qRT-PCR. Comparison of expression levels (Fold Change of log-transformed data) of unique transcripts between RNA-seq and qRT-PCR in root after 3 days of stress. For the validation, a repetition of the PEG experiment was carried out with 6 biological replicates and 3 time points (6h, 3 days and 7 days). qRT-PCR data were normalized using three reference genes (ribosomal protein L8, Actin-1 and Tubulin beta-1 chain). Cach= Cachaco; GN= Grande Naine; Mbw=Mbwazirume.

Analysis of tissue and stress-specific promoters

Abbreviation[a]	Gene annotation	Leaf/Root[b]	Root/Leaf[b]	Stress/Control[b]	Size of cloned fragment (bp)[c]	Involved in abiotic stress[d]
NCED3	9-Cis-epoxycarotenoid dioxygenase 3	8.9×10^{0}	1.1×10^{-1}	1.6	2380	Drought, osmotic, cold
MYB94	Myb-type transcription factor 94	11.2×10^{1}	8.9×10^{-3}	3.1	2416	Salt
YABBY5	YABBY-type transcription factor 5	74.7×10^{2}	1.3×10^{-4}	1.5	1598	Several stresses
COL2	Putative zinc finger protein CONSTANS-LIKE 2	15.8×10^{0}	6.3×10^{-2}	1.7	2947	Drought, cold
ALAAT2	Alanine aminotransferase 2	9.4×10^{-2}	10.5×10^{0}	1.8	2492	Hypoxia, oxidative stress
biLTP	Bifunctional inhibitor, lipid transfer family protein	1.0×10^{-4}	98.0×10^{2}	1.7	975	Drought, cold, salt
TPPI	Trehalose-6 phosphate phosphatase	1.2×10^{-2}	80.5×10^{0}	1.7	2582	-
Per59	Class-III peroxidase Per59	2.6×10^{-5}	38.7×10^{3}	2.7	810	Oxidative stress
LTP	Lipid transfer family protein	5.0×10^{-5}	19.8×10^{3}	1.6	2167	Drought, cold, salt
ETHE-1	ETHE-1 like protein	6.2×10^{-2}	16.2×10^{0}	2.6	1666	Drought, osmotic, salt

Table 2. Tissue and stress-specific genes selected for promoter isolation and cloning. List of 10 unique transcripts expressed ≥ 8 times in one tissue vs. the other and induced after 3 days of osmotic stress in at least one genotype. [a] Abbreviation according to the orthologous gene in *Arabidopsis*. [b] Ratios based on normalized RNA-seq data. [c] Promoter fragment cloned in vector pETKUL16 driving GUS expression. [d] involvement in drought or other abiotic stresses based on previous studies.

Conclusions

- This study characterizes the drought-stress reaction in banana using RNA-seq and it proved to be a valid method to select genes significantly altered during stress and with tissue specificity.
- The upregulation in the 3 genotypes of 14 (~67 %) out of the 21 selected candidates has be verified by qRT-PCR in an independent lab experiment. Further validations in field experiments are needed to confirm their involvement in drought–stress response.

References

[1] D'Hont A. et al. (2012). The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. Nature, 488, 213-219.

The authors would like to thank Hien Do, Els Thiry, Saskia Windelinckx and Edwige André for technical assistance, and Hugues Parrinello and Emeric Dubois (MGX-Montpellier) for the RNA sequencing and raw data analyses. Financial support from the Bioversity International project 'ITC characterization' (research project financed by the Belgian Directorate-General for Development Cooperation (DGDC)) is gratefully acknowledged.