Expression profiling of *Arabidopsis* TF genes by qPCR

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Nitrogen metabolism in plants

Nitrogenous fertilizers on earth

*Arabidopsis thaliana* (L.)

220 million tons in 2050, loss 10 - 80%

Primary N acquisition and assimilation in plants are both tightly regulated on transcriptional level, but NONE factors involved in those processes are identified so far....

130 Mb full genome sequence (~24000 genes)
Arabidopsis transcription factors

- TFs - sequence specific DNA-binding proteins capable to repress/activate transcription of their target genes
- Most are regulated in spatial and/or temporal manner by internal or environmental signals

• LOWLY ABUNDANT TRANSCRIPTS!

2200 TF genes (9% of the genome) grouped into 53 different families

(AGRIS database (http://arabidopsis.med.osu.edu/AtTFDB/) and http://genetics.mgh.harvard.edu/sheenweb/AraTRs.html)

• Homo sapiens (6.1%)
• D. melanogaster (4.6%)
• C. elegans (3.5%)
• S. cerevisiae (3.5%)

Just about 7% of them characterised functionally, mostly by forward genetics approaches
Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes

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equal weight, usually Trizol method

RNA quality and quantity- NanoDrop spec., Agilent 2100

qPCR with intron-designed primers - no product

mRNA (optional, Oligotex, batch protocol)

cDNA quality - qPCR with one of the HK genes / spiked cRNA
Ct +/- 1 for all samples; qPCR to check 3'/5' ratio,
both primer pairs for GAPDH - 1,3kb; acceptable ratio: 1- 4

Tm 60 +/- 2 C; GC content- 45 to 55%, amplicon length 60-150bp, spanning exon/exon junctions;
specific - TAIR Blast - single hits
Evolution P3 Liquid Handling system (PE):

- 6 x 384 well plates with TF and HK gene primers (4 or 2µl)
- cDNA + SYBRGreenI (6 or 3µl)

27900HT systems (AB):

- full TF screen (2256 genes) for one sample in just one working day
- costs = 250€ (5µl) or 500€ (10µl)
Efficiency of PCR reactions

Efficiency - LinRegPCR (Ramakers et al. 2003)

Efficiency - Dilution method (Pfaffl et al. 2001)

Very similar results for 46 primer pairs tested - usually higher E values estimated by dilution method

Czechowski et al., 2004; The Plant Journal
Calculation of Expression Ratios

Modified delta $C_T$ method

1. $\Delta C_T = C_T (GOI) - C_T (HK/cRNA)$

2. $\Delta\Delta C_T = \Delta C_T (B) - \Delta C_T (A)$

3. $B/A \text{ ratio} = (1+E)^{-\Delta\Delta C_T}$

Czechowski et al, 2004; The Plant Journal
Primers quality

1. Dissociation curve of amplicons

2. PCR products on 4% agarose gels

3. Direct sequencing from PCR reaction: for 17 close homologues all sequences gave specific hits.

Czechowski et al, 2004; The Plant Journal
Technical precision

Intra-assay variation: 2 times the same cDNA for 101 genes

Inter-assay variation: 2 different cDNA synthesis for 298 genes

Inter-assay variation for Affymetrix DNA chip for 277 genes

„Absent“

Czechowski et al, 2004; The Plant Journal
Sensitivity

(A) Relationship between amplification kinetics ($C_{t}$) and copy number of a luciferase gene (o) and an intragenic DNA fragment ($\textcircled{o}$) in reactions containing a complex pool of 1ng *Arabidopsis* cDNA.

Detection limit = one transcript in 1000 cells

(Ruan et al., 1998 AGI, 2000; Haas et al., 2002)

*Czechowski et al., 2004; The Plant Journal*
Robustness

Linear Relationship between the expression level, $2^{(40-Ct)}$, and the fraction of root or shoot cDNA in a mixture of the two totalling 1 ng, for the four TF genes

Czechowski et al, 2004; The Plant Journal
Comparison to Affymetrix ATH1 array hybridisation

Raw, normalised signals compared (1083 genes)

Expression ratios (shoot/root)

Czechowski et al, 2004; The Plant Journal
The overlap between 2 techniques rapidly decreases when the fraction of genes that were called 'absent' by Affymetrix technology, increases.

Czechowski et al, 2004; The Plant Journal
Example results

A – different plant organs (1243 genes)

B – different growth conditions, whole seedlings (1243)

Relative mRNA level \([ (1+E)^\Delta C_T ]\) under N deprivation

- \(10^{-6}\)
- \(10^{-5}\)
- \(10^{-4}\)
- \(10^{-3}\)
- \(10^{-2}\)
- \(10^{-1}\)
- \(10^{0}\)

Relative mRNA level \([ (1+E)^\Delta C_T ]\) in full nutrition

- \(10^{-6}\)
- \(10^{-5}\)
- \(10^{-4}\)
- \(10^{-3}\)
- \(10^{-2}\)
- \(10^{-1}\)
- \(10^{0}\)

Relative mRNA level \([ (1+E)^\Delta C_T ]\) after nitrate replenishment

- \(10^{-6}\)
- \(10^{-5}\)
- \(10^{-4}\)
- \(10^{-3}\)
- \(10^{-2}\)
- \(10^{-1}\)
- \(10^{0}\)

Relative mRNA level \([ (1+E)^\Delta C_T ]\) under N deprivation

- \(10^{-6}\)
- \(10^{-5}\)
- \(10^{-4}\)
- \(10^{-3}\)
- \(10^{-2}\)
- \(10^{-1}\)
- \(10^{0}\)
Current applications

- Shoot - , root - , silique - , seed - specific TF genes
- Seed development
- Heterosis
- Macronutrient signalling: N, P, S, CHO
- Salt and osmotic stress
- Biotic stress
- Seed dormancy

(AG Udvardi, AG Scheible, and international collaborators)
Future qRT-PCR development for other model plant species

*Oryza sativa* (Rice) ~2500 TFs

*Medicago truncatula* ~3000 TFs

*Lotus japonicus* ~3000 TFs
AtGenExpress Initiative - Arabidopsis gene expression atlas

Over 700 ATH1 arrays (23,500 genes)

- Developmental series
- Shoot and root abiotic stress series
  - Hormone series
  - Photomorphogenic Light series
  - Biotic stress series
- Nutrient stress series
  (MPI-MPP Scheible et al., 2004, Scheible et al., unpublished)
- Diurnal rhythm
  (MPI-MPP, Bläsing et al. Unpublished)

(Altmann et al., 2004; http://web.uni-frankfurt.de/fb15-botanik/mcb/AFGN/atgenex.htm)
Newly identified HK genes outperform „traditional“ ones w.r.t. expression stability

Gene selection from ATGenExpress series:

1. Mean expression value (MV)
2. Standard deviation (SD)
3. Coefficient of variation (COV) = SD/MV
4. At least 80% „Present“ calls within series
5. Select gene with lowest COV
6. Sort according to ATH1 expression values

(WR Scheible, manuscript in preparation)

(A) Traditional genes: ACT2 (black); TUB6 (red); EF-1a (green); UBQ10 (cyan); and GAPDH (blue).
(B) Novel genes, i.e. At4g34270 (black); At1g13320 (red); At1g59830 (green); At4g33380 (cyan) and At2g28390 (blue).
Validation by qPCR for 18 selected genes

Primer design
20 RNA samples - various organs, abiotic stresses,
RT with spiked cRNA (LjLb2 transcript),
\[ \Delta C_t = C_t (HK) - C_t (cRNA) \]

<table>
<thead>
<tr>
<th>AGI</th>
<th>Annotation</th>
<th>Mean ( \Delta C_t )</th>
<th>Mean ((1+E)^{\Delta C_t} )(x10^6)</th>
<th>Relative Expression</th>
<th>SE (n=20)</th>
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<tbody>
<tr>
<td>AT4G05320</td>
<td>UBIQ10</td>
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<td>4270</td>
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<td>AT5G46630</td>
<td>Clathrin adapter complex subunit</td>
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<td>AT4G34270</td>
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<td>AT2G28390</td>
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</table>

(WR Scheible manuscript in preparation)
**geNorm analysis** (Vandesompele *et al.*, 2002)

Average expression stability of control genes, measured using GeNorm software

(A) all
(B) without mature seed sample

(WR Scheible, manuscript in preparation)
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Thanks for Your attention !!!