Expression profiling of *Arabidopsis* TF genes by qPCR

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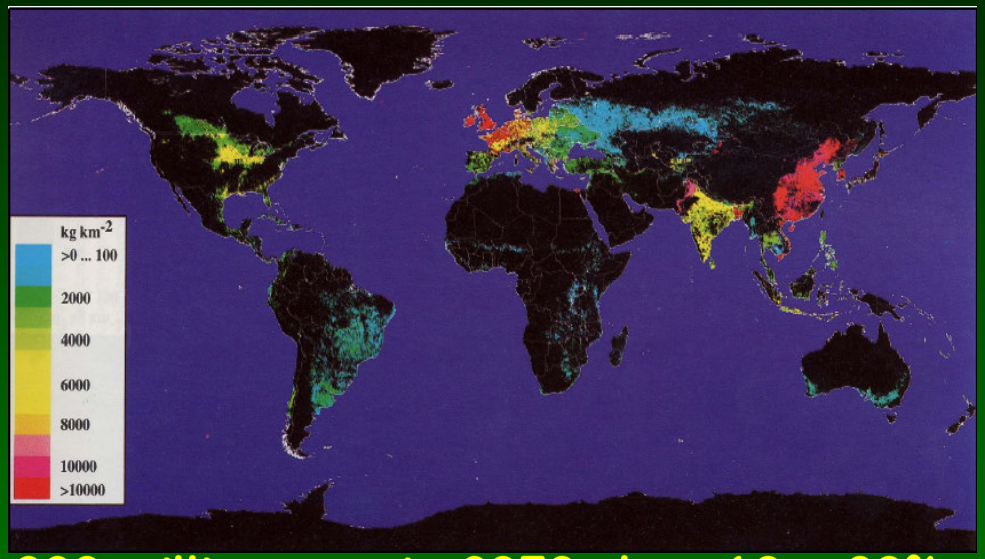
Nitrogen metabolism in plants

Nitrogenous fertilizers on earth



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....

***Arabidopsis thaliana* (L.)**

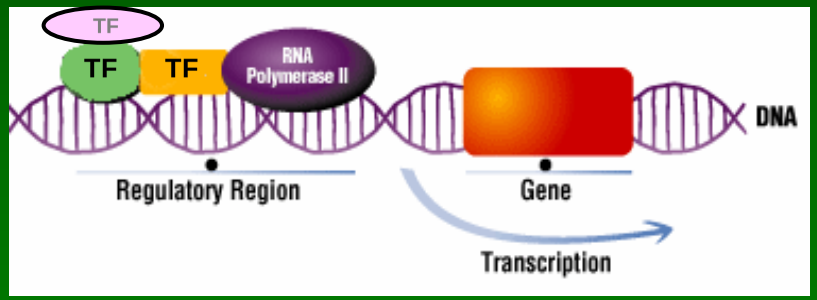
130 Mb full genome sequence (~24000 genes)

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 ABUNDAND TRANSCRIPTS !



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

(AGRIS database (http://arabidopsis.med.ohio-state.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

The Plant Journal (2004) **38**, 366–379 doi: 10.1111/j.1365-313X.2004.02051.x

TECHNICAL ADVANCE

Real-time RT-PCR profiling of over 1400 *Arabidopsis* transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes

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qPCR Workflow

equal weight, usually Trizol method

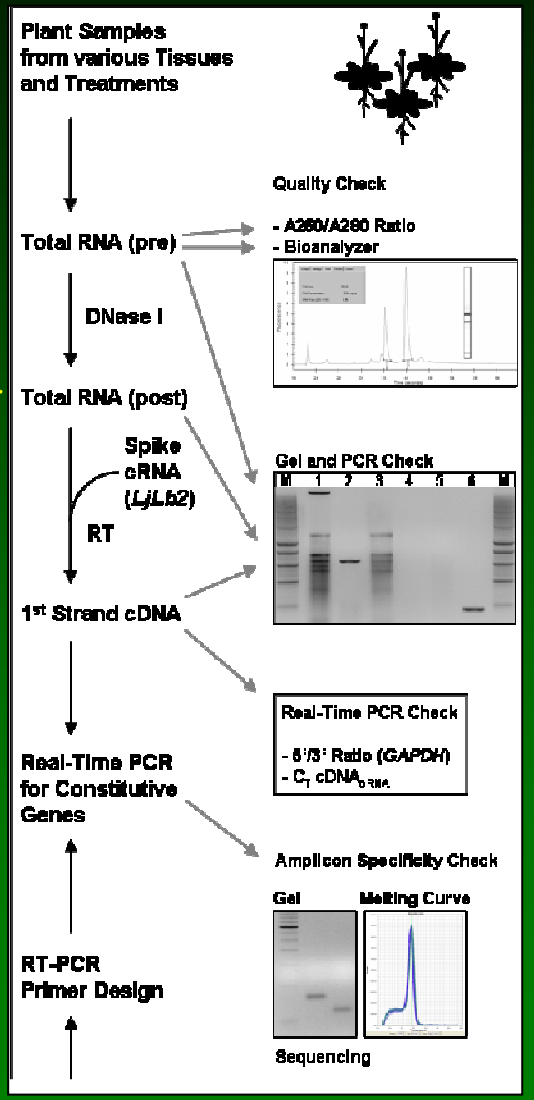
RNA quality and quantity- NanoDrop spec., Agilient 2100

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



Reaction set-up - high throughput

Evolution P3 Liquid Handling system (PE):

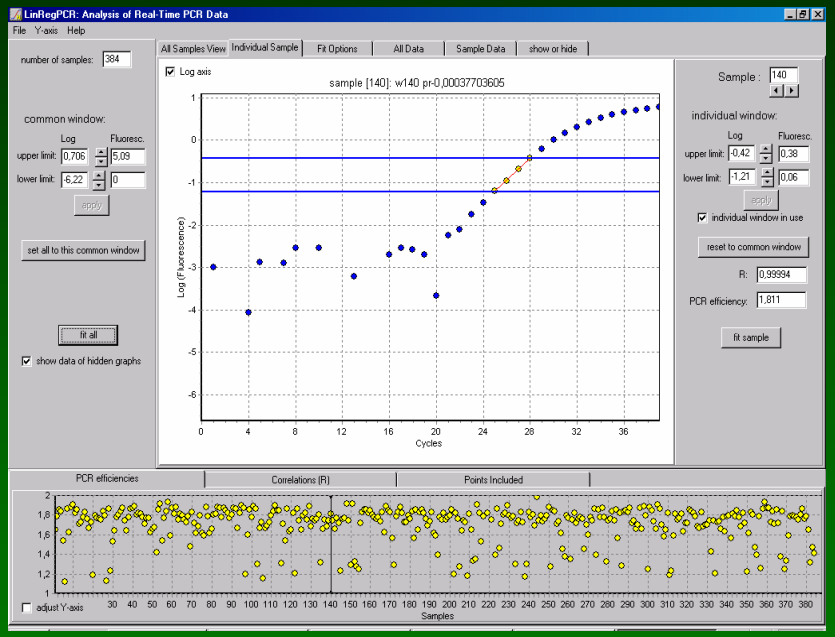
- 6 x 384 well plates with TF and HK gene primers (4 or 2µl)
- cDNA + SYBRGreenI (6 or 3µl)

2 7900HT systems (AB):

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

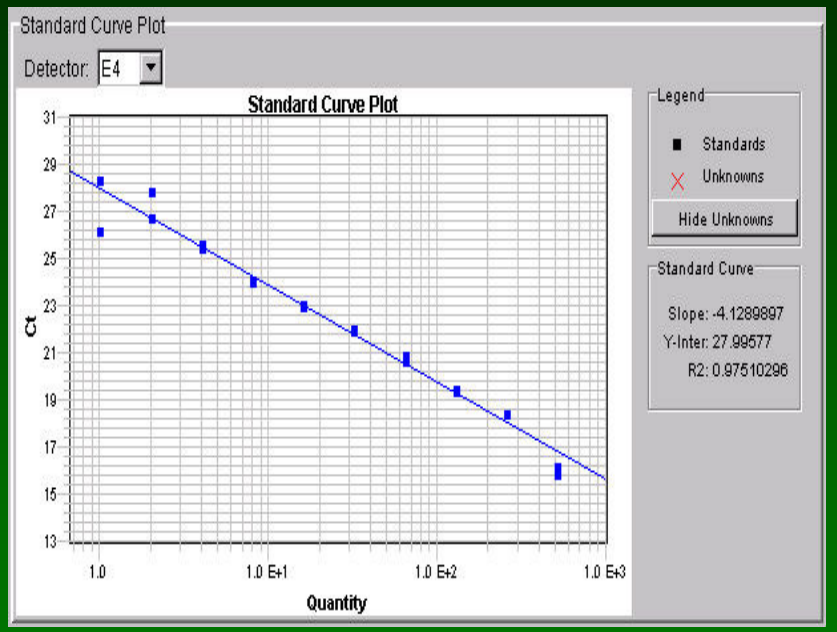
Efficiency of PCR reactions

Efficiency – LinRegPCR (Ramakers et al. 2003)



Vs.

Efficiency – Dilution method (Pfaffl et al. 2001)

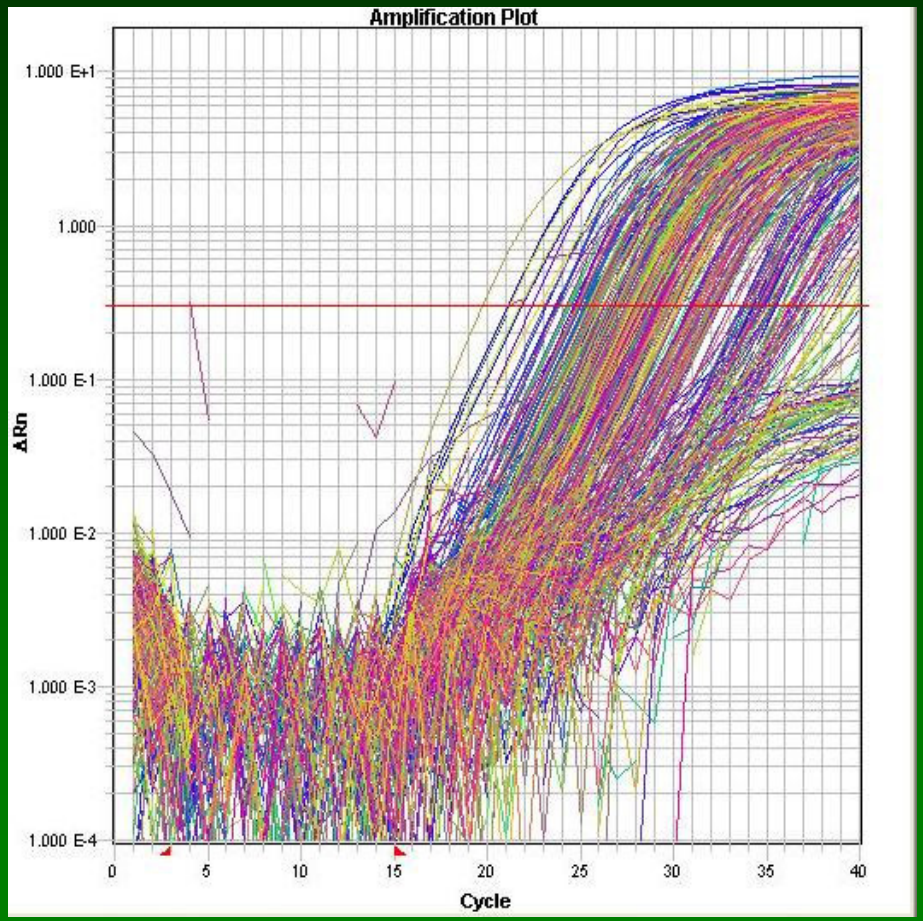


Very similar results for 46 primer pairs tested – usually higher E values estimated by dilution method

Czechowski et al, 2004; The Plant Journal

Calculation of Expression Ratios

Modified delta C_T method



1. $\Delta C_T = C_{T\ (GOI)} - C_{T\ (HK/cRNA)}$

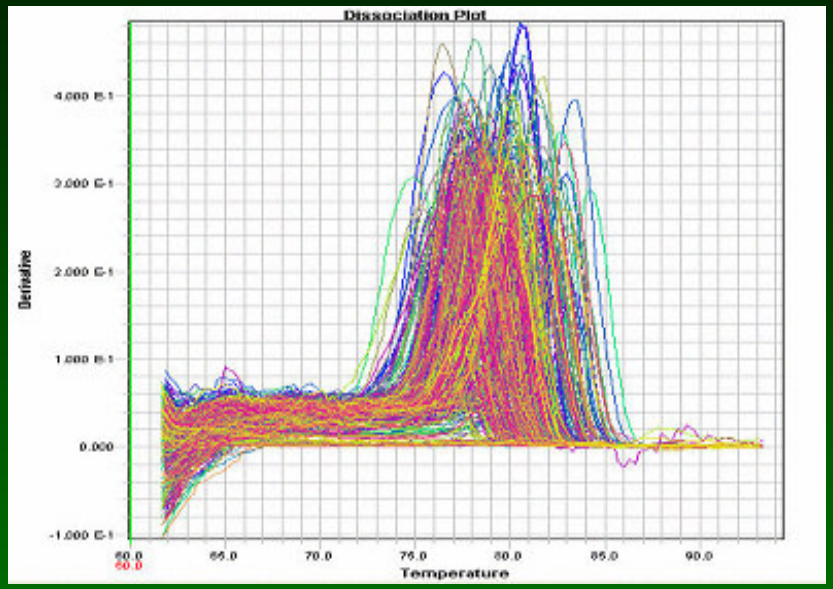
2. $\Delta\Delta C_T = \Delta C_{T\ (B)} - \Delta C_{T\ (A)}$

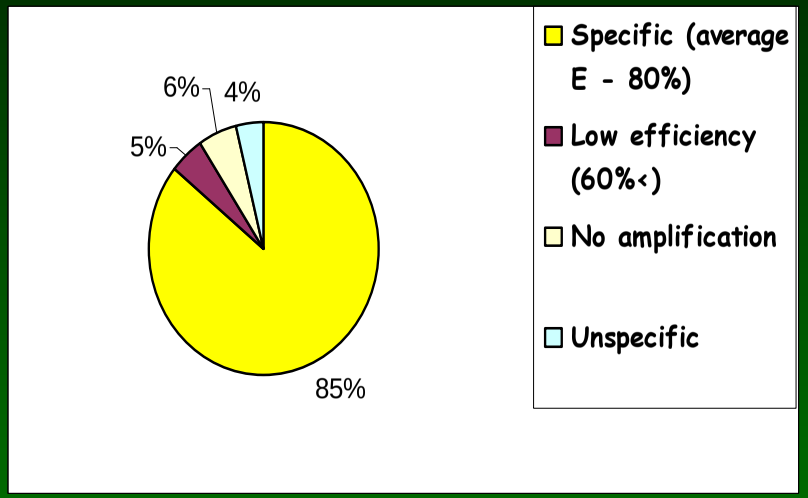
3. B/A ratio = $(1+E)^{-\Delta\Delta CT}$

Czechowski et al, 2004; The Plant Journal

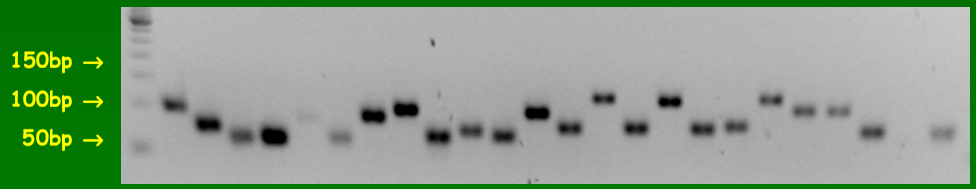
Primers quality

1. Dissociation curve of amplicons





2. PCR products on 4% agarose gels

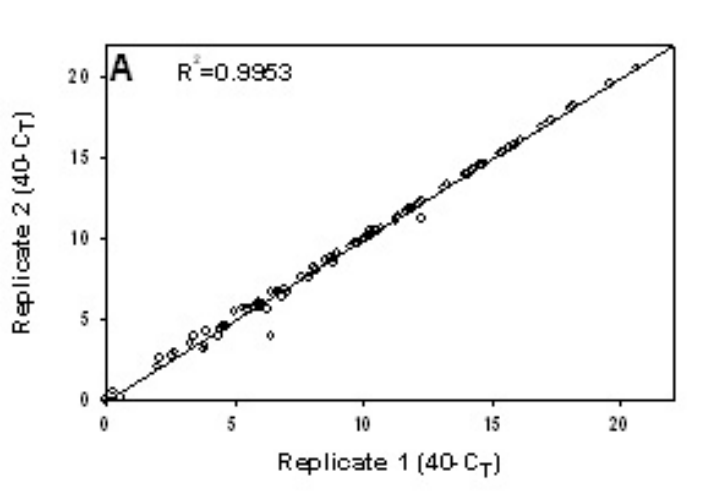


3. Direct sequencing from PCR reaction: for 17 close homologues all sequences gave specific hits.

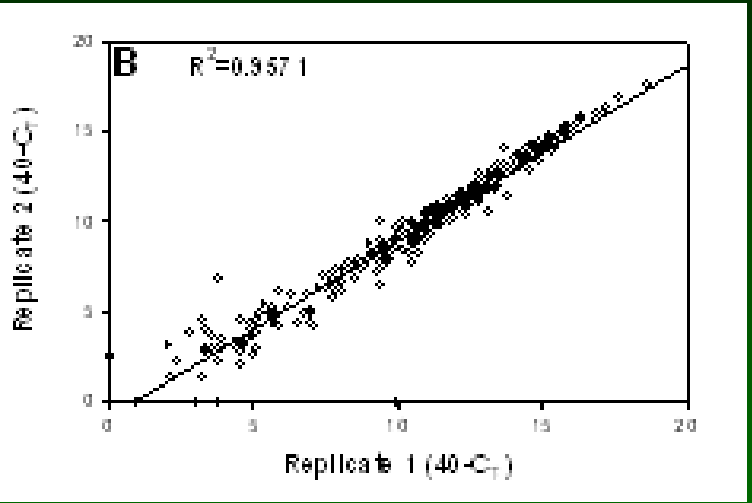
Czechowski et al, 2004; The Plant Journal

Technical precision

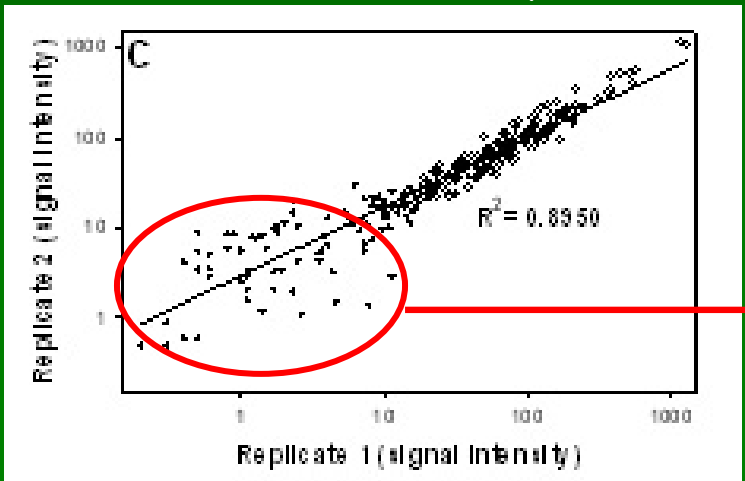
Intra-assay variation: 2 times the same cDNA for 101 genes



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



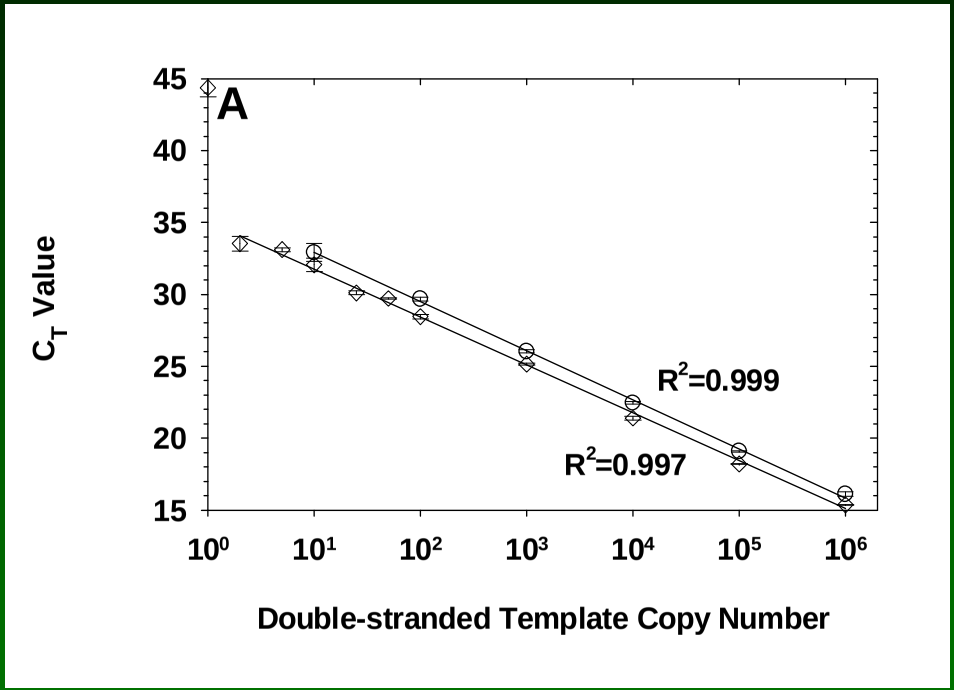
Inter-assay variation for Affymetrix DNA chip for 277 genes



„Absent"

Czechowski et al, 2004; The Plant Journal

Sensitivity



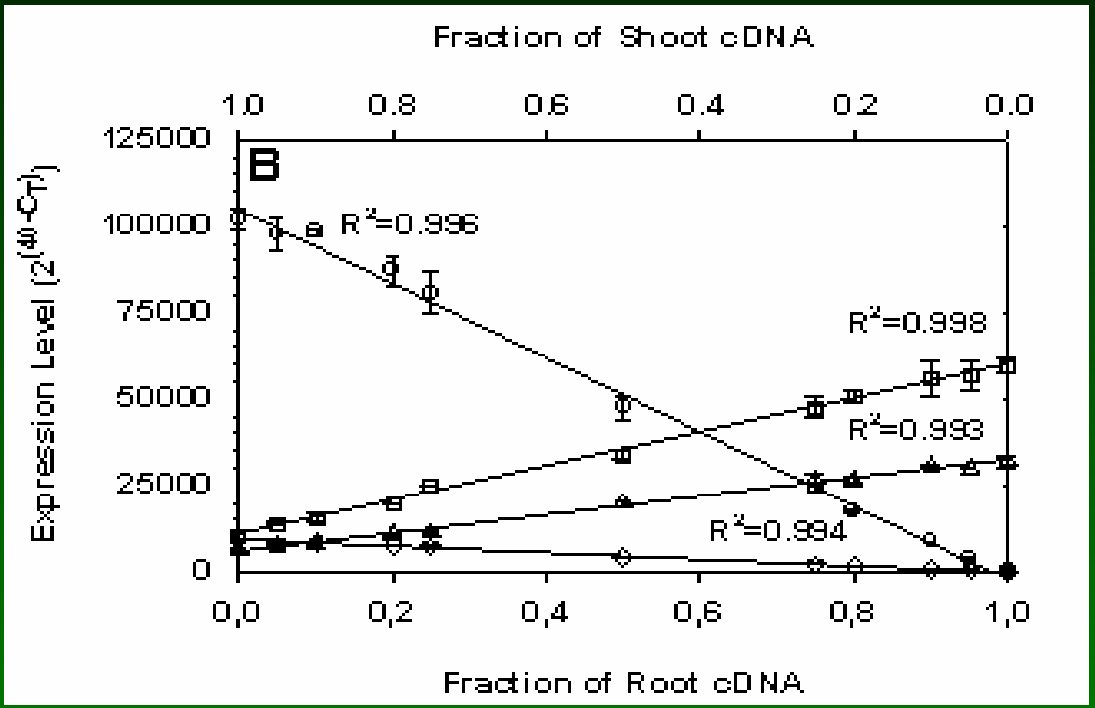
(A) Relationship between amplification kinetics (C_t) and copy number of a luciferase gene (o) and an intragenic DNA fragment (◊) in reactions containing a complex pool of 1ng *Arabidopsis* cDNA.

Detection limit = one transcript in 1000 cells

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

Czechowski et al, 2004; The Plant Journal

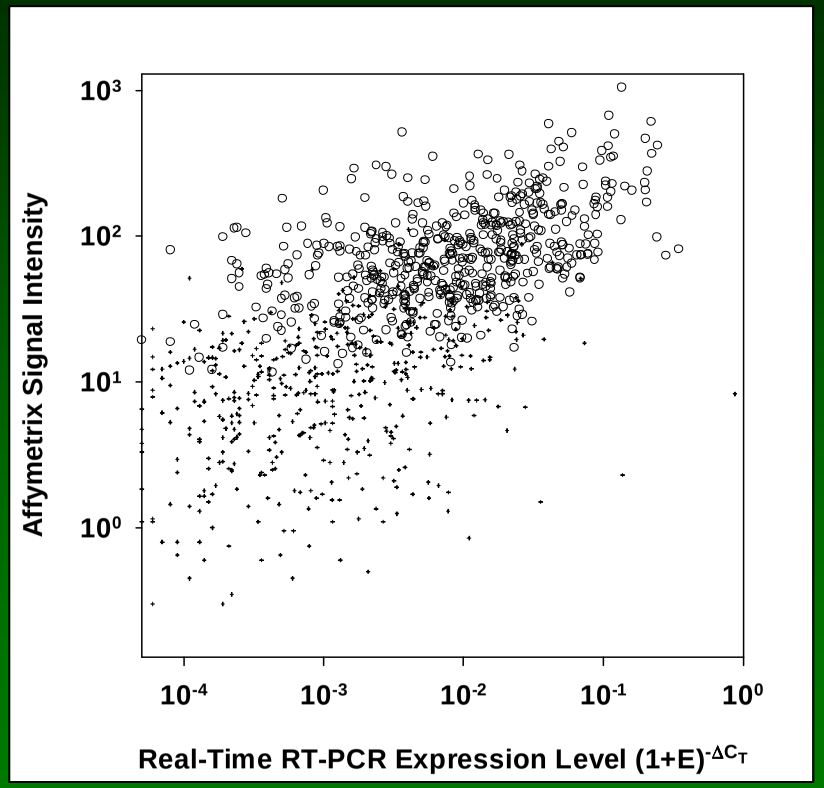
Robustness



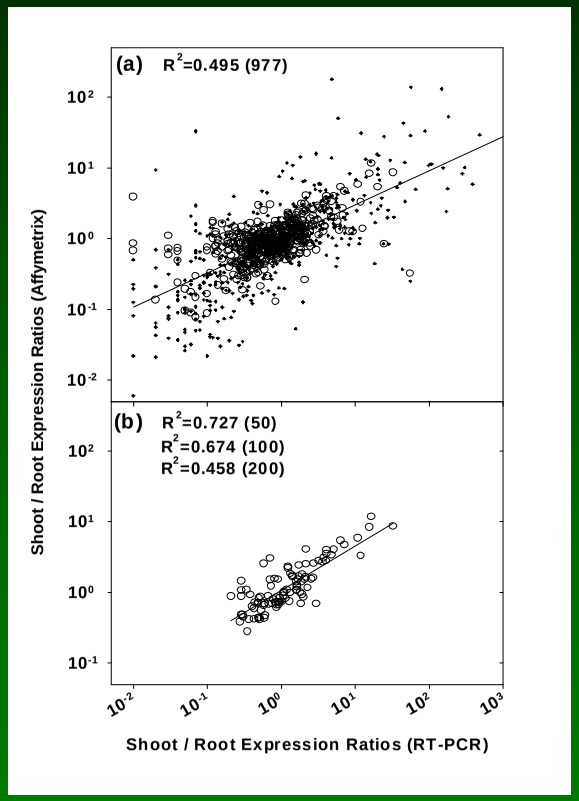
Linear Relationship between the expression level, $2^{(40-Ct)}$, and the fraction of root or shoot cDNA in a mixture of the two totalling 1 ng, for the four TF genes

Czechowski et al, 2004; The Plant Journal

Comparison to Affymetrix ATH1 array hybridisation



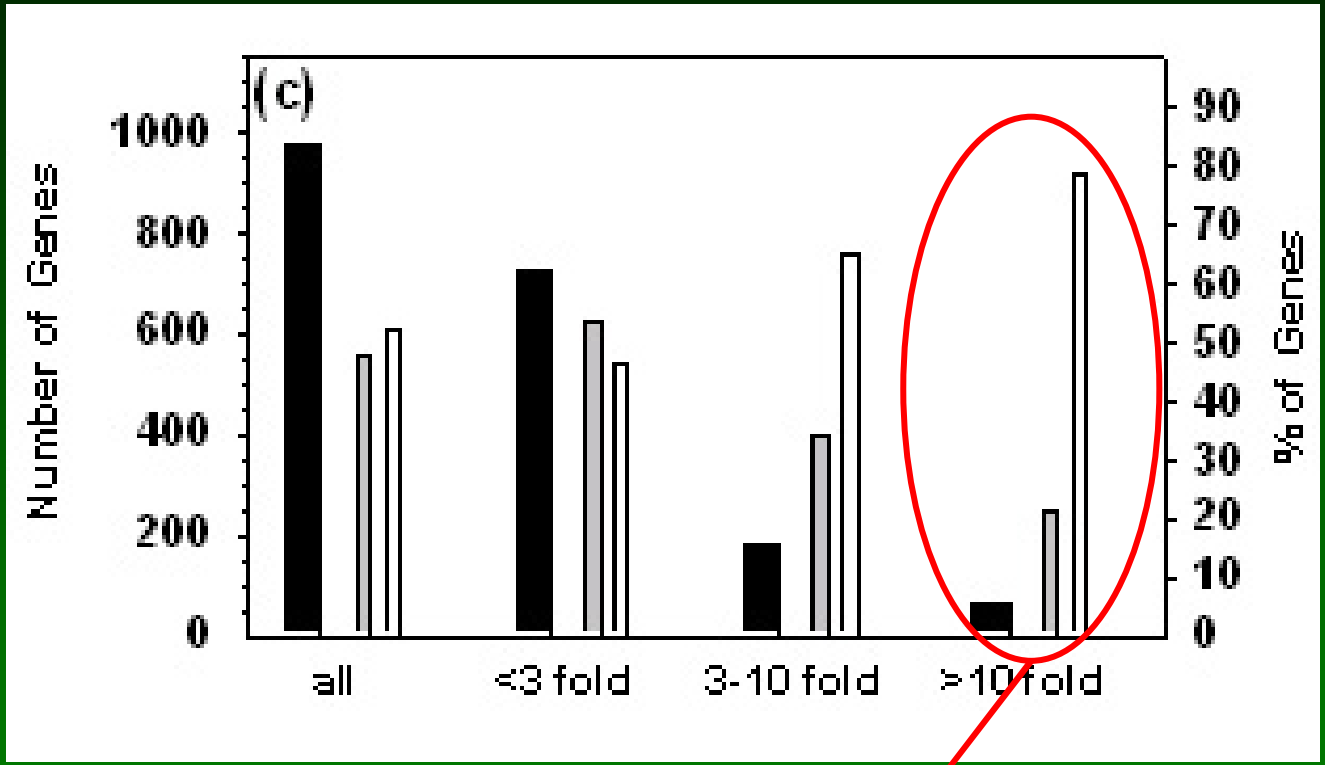
Raw, normalised signals compared (1083 genes)



Expression ratios (shoot/root)

Czechowski et al, 2004; The Plant Journal

Comparison to Affymetrix ATH1 array hybridisation

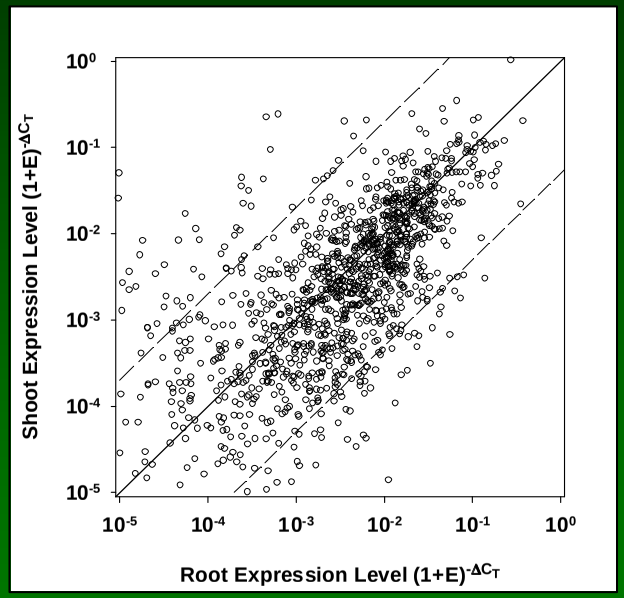


The overlap between 2 techniques rapidly decreases when the fraction of genes that were called 'absent' by Affymetrix technology, increases.

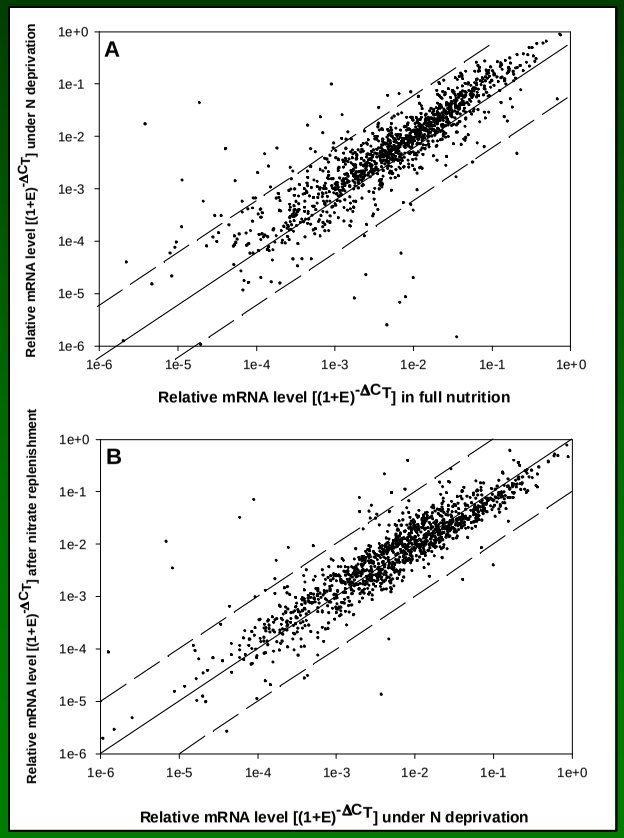
Czechowski et al, 2004; The Plant Journal

Example results

A – different plant organs (1243 genes)



B – different growth conditions, whole seedlings (1243)



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)

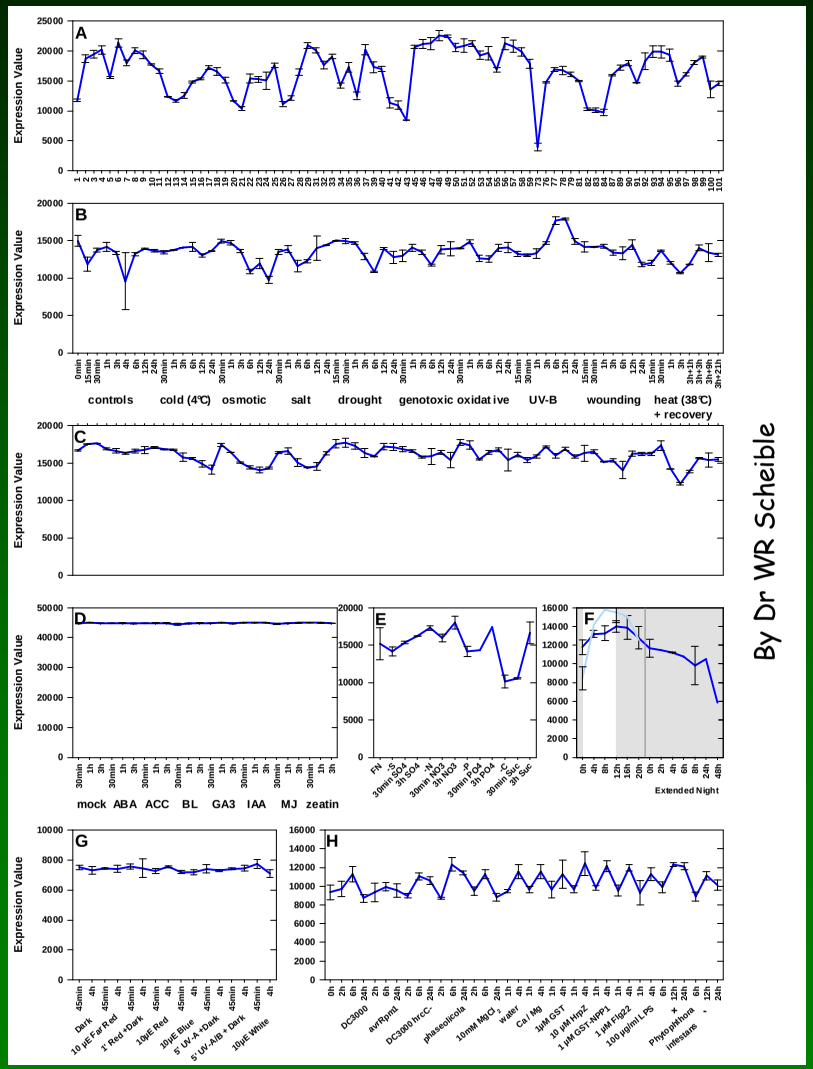
Future qRT-PCR development for other model plant species

Oryza sativa (Rice) ~2500 TFs

Lotus japonicus ~3000 TFs

Medicago truncatula ~ 3000 TFs

AtGenExpress Initative – *Arabidopis* 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)

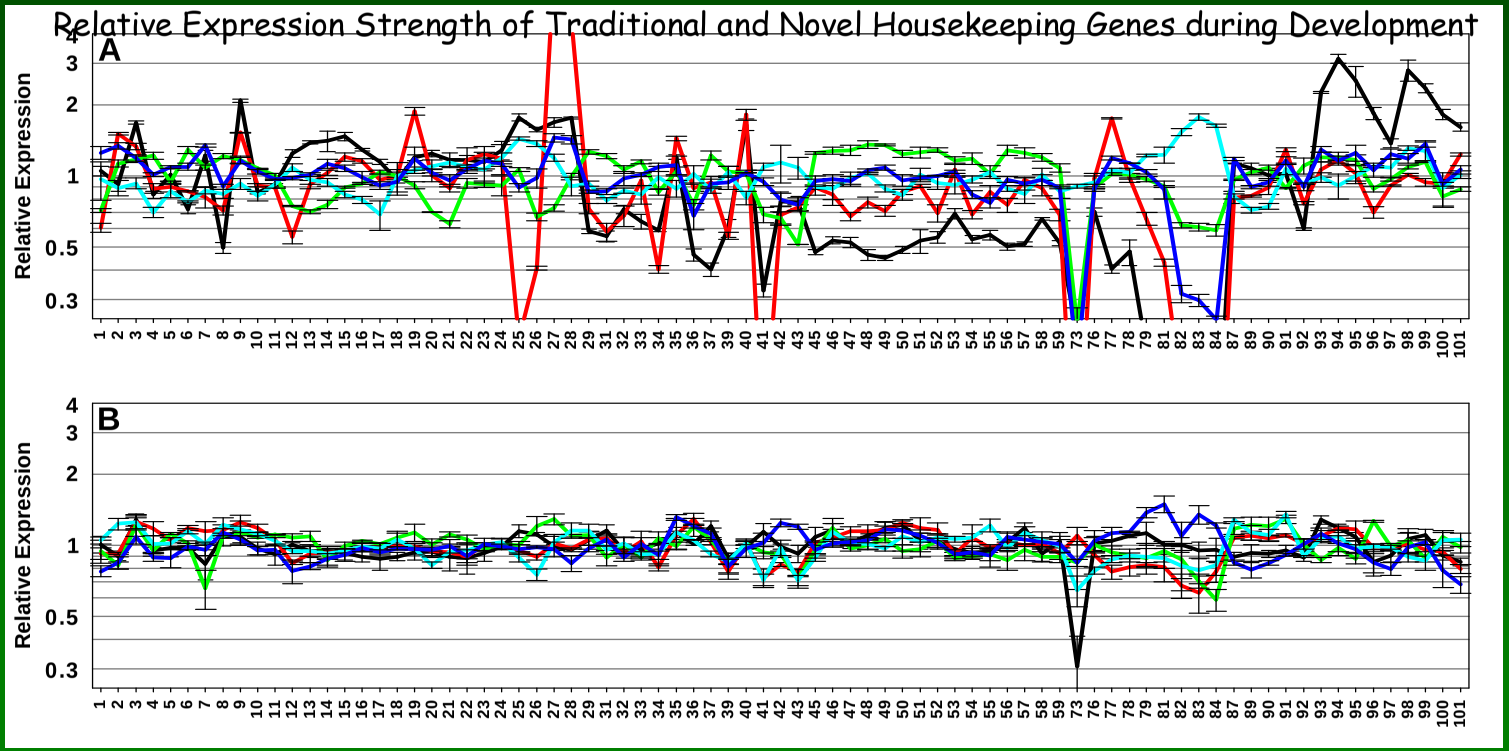
(Altmann *et al.*, 2004;
http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm)

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

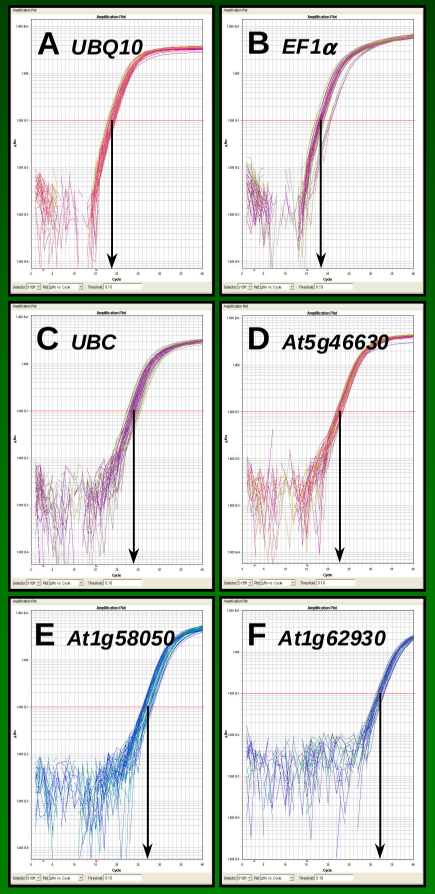
(WR Scheible, manuscript in preparation)



(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

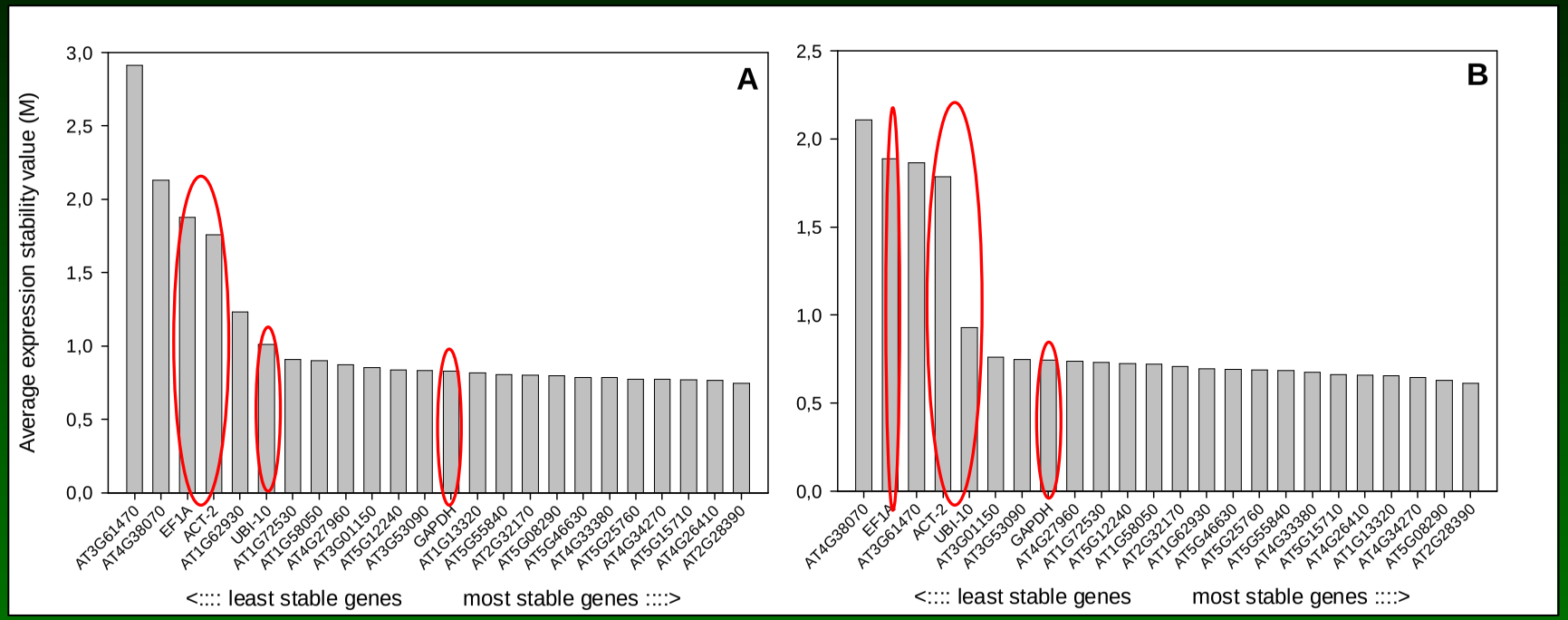
Primer design
20 RNA samples – various organs, abiotic stresses,
RT with spiked cRNA (*LjLb2* transcript),
$\Delta Ct = Ct\ (HK) - Ct\ (cRNA)$



AGI	Annotation	Mean ΔC_T [b]	Mean $(1+E)^{-\Delta CT}$ $(x10^6)$	Relative Expression	SE (n=20)
AT4G05320	*UBQ*10	8.76	4270	1	6.9 E-04
AT5G60390	*EF*-1α	9.07	3660	0.858	6.7 E-04
AT1G13440	*GADPH*	9.50	2488	0.583	3.5 E-04
AT4G27960	*UBC*9	10.98	1160	0.272	1.5 E-04
AT3G18780	*ACT*2	11.74	917	0.215	1.9 E-04
AT5G46630	Clathrin adapter complex subunit	12.71	421	0.099	3.9 E-05
AT5G08290	*YLS8*	13.60	343	0.081	3.9 E-05
AT4G33380	Expressed	13.45	342	0.080	3.2 E-05
AT2G32170	Expressed	14.47	246	0.058	3.3 E-05
AT4G34270	TIP41-like	13.68	243	0.057	2.9 E-05
AT5G25760	*UBC*	13.82	190	0.044	2.6 E-05
AT1G13320	*PDF*2	13.41	189	0.044	2.7 E-05
AT2G28390	SAND family	14.69	167	0.039	1.9 E-05
AT4G26410	Expressed	14.11	132	0.031	1.7 E-05
AT3G01150	*PTB*	18.56	40	0.009	4.7 E-06
AT5G55840	PPR repeat	18.59	38	0.009	4.4 E-06
AT1G58050	Helicase	16.81	36	0.008	3.9 E-06
AT3G53090	*UPL7*	19.70	22	0.005	2.1 E-06
AT5G15710	F-box family	17.53	15	0.003	1.9 E-06
AT4G38070	bHLH	22.46	6	0.001	1.1 E-06
AT5G12240	Expressed	21.91	1.6	0.00038	1.8 E-07
AT1G62930	PPR repeat	24.02	0.4	0.00009	6.3 E-08
AT3G32260	Hypothetical	NA	NA	NA	NA
AT2G07190	Hypothetical	NA	NA	NA	NA
AT1G47770	Hypothetical	NA	NA	NA	NA

(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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Supervision of the project:

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- Dr. Wolf-Rüdiger Scheible (also for providng ATH1 data for the analysis)

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- Monika Bielecka (Amino Acids and Sulfur Metabolism Group)
- Dr. Wenming Zheng and Anna Blacha (Molecular Plant Nutrition Group)

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AG Udvardi
Molecular Plant Nutrition

Thanks for Your attention !!!