qPCR Reference Genes for Barley





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Introduction

Many traditional reference genes for qPCR are actually differentially regulated under some conditions, so efficient methods for selecting new stable reference genes are needed.

Selection based on microarray

Using available datasets from the Affymetrix Barley 1 GeneChip we identified **38 stably expressed barley genes** from the ~ 20.000 genes present on the microarray.

Six datasets from public databases and one unpublished dataset were combined. This resulted in a combined dataset with 28 different sample types covering nine genotypes, three tissues, and several types of biotic and abiotic stresses.

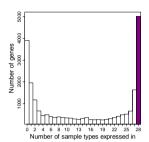


Figure 1. Number of genes (Y) expressed in X out of 28 samples.

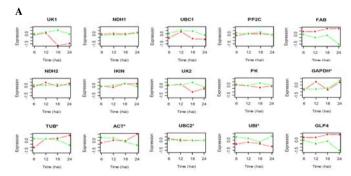
- 5 014 expressed in all sample types (Fig. 1)
- 2 507 of these had CVs < 0.1
- 38 of these had stable expression

Testing potential new reference genes by qPCR

From the 38 identified candidate genes, the top ten were selected for further testing together with 5 traditional reference genes (marked with an asterisk) as potential reference genes (Table 1). The expression of these genes was examined in barley material challenged with powdery mildew (Fig. 2).

Table 1. Potential reference genes applied in qPCR

Gene title	Gene description	Affymetrix ID
UK1	Unknown	Contig14037_at
NDH1	NADH dehydrogenase	Contig3802_at
UBC1	Ubiquitin-conjugating enzyme	Contig10533_at
PP2C	Protein phosphatase 2C	Contig15653_at
FAB	Fatty acid β-oxidation	Contig3702 s at
NDH2	NADH dehydrogenase	Contig4663 s at
IKIN	Inositol kinase	Contig2297_at
UK2	Unknown, expressed protein	Contig14966 at
UK3	Unknown (excluded)	HY08N07u s at
PK	Protein kinase	Contig3529_at
GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	Contig3529_at
ACT*	Actin	Contig14605 at
TUB*	α-tubulin	Contig5567 s at
UBC2*	Ubiquitin-conjugating factor	Contig17401_at
UBI*	Ubiquitin	M58754 e at



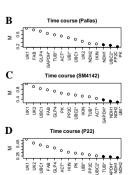


Figure 2. qPCR was applied to samples representing 3 barley cultivars (Pallas, P22 and SM4142), ±inoculation with powdery mildew and a timecourse covering 6, 12, 18 and 24 HAI. A) Expression levels in Pallas for the potential new reference genes for inoculated (red) and uninoculated controls (green). UK3 was excluded and GLP4, a mildew induced gene, was included as a positive control. The geNorm software [1] was used to rank the genes according to stability as displayed for Pallas (B), SM4142 (C) and P22 (D). The lower the M-value, the more stable is a gene across all samples.

Conclusions:

- Different genes ranked most stable in the 3 genotypes
- NDH1 is the only gene ranking top 4 in all genotypes
- Large variation for some of the traditional reference genes

Validation of recommended reference genes

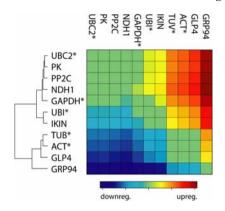


Figure 3. A matrix showing the regulation of each (columns) depending on which gene it has been normalised to (rows). Expression levels were determined for 9 potential reference genes and 2 genes known to be upregulated by powdery mildew infection (GLP4 and GRP94) in 5 replicates from Pallas material with and without mildew inoculation. The means the inoculated uninoculated groups were calculated using normalisation to each of the included genes.

The top 5 genes in Fig. 3 are least likely to be differentially regulated, and using the NormFinder software [2] they are ranked as follows: UBC*, NDH1, PP2C, GAPDH and PK, from most to least stable. However, GAPDH is highly induced at 12 HAI (Fig. 1 A).

Table 2 shows that not only GLP4 and GRP94, but also the four reference genes, GAPDH, IKIN, TUB and ACT, are significantly upregulated (p > 0.001) by powdery mildew infection.

Table 2. The expression ratio (inoculated vs. controls) and p-values (t-test) of the difference, using UBC2*, NDH1, and PP2C for normalisation.

Gene title	Regulation	P-value
UBC2*	1.1	0.26
PK	1.1	0.40
PP2C	1.0	1.00
NDH1	0.9	0.26
GAPDH*	1.2	0.08
UBI*	1.8	2.3×10 ⁻⁵
IKIN	2.0	2.8×10-6
TUB*	4.3	3.3×10 ⁻⁸
ACT*	5.2	5.7×10-9
GLP4	6.6	4.3×10 ⁻⁴
GRP94	14.3	8.5×10 ⁻⁷

Conclusions:

- · UBC*, NDH1 and PP2C are recommended
- for normalising qPCR experiments in mildew-infected Pallas.
- One new and 3 traditional reference genes were shown to be significantly upregulated during infection with powdery mildew.

Overall Conclusions

$\ensuremath{\mathbf{qPCR}}$ reference genes need to be selected and validated for the specific experimental setup!

In our experiment different reference genes ranked as most stable for different genotypes of barley.

High-throughput selection is a valuable alternative to using traditional reference genes!

Two out of three recommended genes in this study came from the high-throughput selection.

The 38 stably expressed genes identified are a good starting point for selection of reference genes in barley

Our work sets an example of how selection can be made:

- 1) Use microarray data dealing with the experimental parameters that you want to investigate in your qPCR experiment to rank the genes.
- Test ten top-ranking genes + some traditional reference genes in a qPCR experiment including cDNA samples representing relevant treatments.
- 3) Analyse the data and select stable genes using available software (e.g. geNorm [1] or NormFinder [2]).
- 4) Preferably include biological replicates.

Traditional reference genes can be highly inappropriate!

We show that tubulin, actin and ubiquitin are significantly up-regulated in barley when infected with powdery mildew. Using these to normalise qPCR data could lead to erroneous conclusions.