Accurate quantification of mRNAs and housekeeping gene selection for qRT-PCR normalization in European beech (*Fagus sylvatica* L.) during abiotic and biotic stress

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Issue

• The most important deciduous tree in Central Europe

• Limited genetic and genomic information is available

• High genomic diversity

• Long generation times

• Identification of genes specifically up- or down-regulated in plants upon environmental stimuli

• Represents a key step in understanding of ecotoxicological processes and the systemic results of abiotic and biotic stress
Issue

- Generated a microarray with ozone-responsive EST’s

- Ozone is an abiotic elicitor of plant defence, well studied in herbaceous plants

- Investigation of transcriptional regulation upon environmental stress, with microarray-technology

Materials, Methods and Results
Reference Genes

- 18S rRNA
- GAPDH2
- Actin
- α-tubulin
- GAPDH1
- Ubiquitin-like protein
Stress Scenarios

2x ambient O₃

2x ambient CO₂

Sun leaves

Shade leaves

Phytophthora citricola infection

Picture from tree: Frank Fleischmann
RNA-Isolation and RT-PCR

- RNA isolation from leaves according to Kiefer et al. 2000

- Yield and quality of RNA using Nano Drop system (Kisker, Steinfurt, Germany)

- RT-PCR: oligo dT and Superscript III (Invitrogen, Karlsruhe, Germany)

- Digest with RNAse H and A

qRT-PCR

- Primerdesign: Primer Express 2.0® (Applied Biosystems)
- qRT-PCR with Sybr Green PCR kit (ABgene, Hamburg, Germany)
- 7500 Real-Time PCR System, Applied Biosystems (Darmstadt, Germany)
Statistical analysis

- Analysis of variance, using a linear model

- Specific structure of data (no identical genotypes of beech trees) the data were grouped with respect to trees (technical replicates)

- Linear mixed-effects model, nlme package of R-system

- Testing of variance homogeneity (diagnostic plot), normal and independent distribution of residuals

PCR efficiency

- Efficiencies of target and reference genes should be approximately equal.
- 2-fold dilution series display ΔCt values of 1 between adjacent dilution, reflecting a PCR efficiency of 100%.
- Increasing cDNA concentration on the axis result in a graph with a slope -1.
qPCR efficiency

- **18S rRNA**: $y = -1.039x + 24.341$, $R^2 = 0.9999$
- **Actin**: $y = -1.0274x + 23.386$, $R^2 = 0.9999$
- **GAPDH1**: $y = -0.9131x + 22.346$, $R^2 = 0.9975$
- **α-Tubulin**: $y = -0.929x + 10.01$, $R^2 = 0.9968$
Phytophthora and CO$_2$

- Three year old European Beech (*Fagus sylvatica* L.)

- Climate chamber: 360 and 780 ppm CO$_2$

- Direct inoculation of soil with *P. citricola*, flooding of the container

- Samples were taken at 14h, 31h, 12d after infection (F. Fleischmann, W. Oßwald, TU München, Ökophysiologie)

Exposure of European beech trees with 2x ambient O$_3$
Kranzberg Forest – sun and shade leaves

- Sampling of sun and shaded leaves

Conclusion

- Actin appears to be the best reference gene for European beech under the conditions tested

- Transcriptional level of plant genes is influenced by many factors

- For a single experiment a reference gene must be validated
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