Quantification of mRNAs and Housekeeping Gene Selection for Quantitative Real-Time RT-PCR Normalization in European Beech (*Fagus sylvatica* L.) during Abiotic and Biotic Stress

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Analyses of different plant stressors are often based on gene expression studies. Quantitative real-time RT-PCR (qRT-PCR) is the most sensitive method for the detection of low abundance transcripts. However, a critical point to note is the selection of housekeeping genes as an internal control. Many so-called 'housekeeping genes' are often affected by different stress factors and may not be suitable for use as an internal reference. We tested six housekeeping genes of European beech by qRT-PCR using the Sybr Green PCR kit. Specific primers were designed for 18S rRNA, actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH1, GAPDH2), α-tubulin, and ubiquitin-like protein. Beech saplings were treated with increased concentrations of either ozone or CO_2. In parallel, the expression of these genes was analyzed upon pathogen infection with *Phytophthora citricola*. To test the applicability of these genes as internal controls under realistic outdoor conditions, sun and shade leaves of 60-year-old trees were used for comparison. The regulation of all genes was tested using a linear mixed-effect model of the R-system. Results from independent experiments showed that the only gene not affected by any treatment was actin. The expression of the other housekeeping genes varied more or less with the degree of stress applied. These results highlight the importance of undergoing an individual selection of internal control genes for different experimental conditions.

Key words: Abiotic/Biotic Stress, European Beech (*Fagus sylvatica*), Housekeeping Genes, Quantitative Real-Time RT-PCR

Introduction

The identification of genes specifically up- or down-regulated in plants upon environmental stimuli represents a key step in understanding ecotoxicological processes and systemic results of abiotic and biotic stress. On a global scale, tree species dominate some of the largest ecosystems on earth and are critically important for carbon fixation and human health. Limited genetic and genomic information is available for European beech, the most important deciduous tree in Central Europe (Schütt *et al.*, 1992). This is caused by the genomic diversity and slow growth rate of European beech, as well as its long generation times. The analysis of gene expression requires sensitive and reproducible methods for specific transcripts. Quantitative real-time RT-PCR (qRT-PCR) is widely used to quantify changes in gene expression and verify data from microarray analysis; it represents an excellent method because of its outstanding accuracy, broad dynamic range and high sensitivity (Bustin, 2002; Ginzinger, 2002; Radonic *et al.*, 2004). However, all technical steps (*e.g.* RNA isolation, cDNA preparation, RT-PCR) have to be carefully optimized; this includes the very important statistical analysis (Stürzenbaum and Kille, 2001; Bustin, 2002). Furthermore, many additional conditions must be taken into consideration, including: the purity of the RNA, primer design and primer synthesis for both RT-PCR and qRT-PCR reactions, enzyme efficiency for RT-PCR and qRT-PCR, stability and efficiency of qRT-PCR, and maintenance of identical condi-

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tions for all samples analyzed (Stürzenbaum and Kille, 2001; Bustin, 2002).

A first step of normalization is established by using the same amount of RNA. A critical step is the RT reaction, because the amount of cDNA produced by the reverse transcriptase must accurately represent the mRNA input concentration (Stürzenbaum and Kille, 2001; Bustin, 2002). Therefore the range, specificity, and sensitivity of the enzyme used is important (Bustin, 2002); also, different mRNAs must be transcribed with the same efficiency (Malboeuf *et al.*, 2001). In addition, mRNAs should not contain GC-rich or secondary rich structures, because the reverse transcriptase can stop or skip over these looped-out regions (Malboeuf *et al.*, 2001; Bustin, 2002).

Primer choice for RT-PCR seems to be another important factor. Common primers for cDNA synthesis are random hexamers, oligo-dTs, or gene-specific primers. Hexamers will generate cDNAs with no bias and will give a better distribution between coding and non-coding regions, whereas oligo-dTs and specific primers will result in a potential bias (Ginzinger, 2002).

It cannot be excluded that different amounts of cDNAs are synthesized; these differences are caused by the quality of RNA, differences in RNA preparation, and manual handling of cDNA preparation. To solve this problem normalization is necessary. Normalization against external standards is not sufficient, because the RT-PCR efficiency might be influenced by internal inhibitors (Malboeuf *et al.*, 2001). Therefore RT-PCR is typically referenced to an internal control gene to avoid bias (Huggett *et al.*, 2005). The most commonly reference genes used for normalization were actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal genes, and to a more less extent cyclophilin and elongation factor 1-α (Stürzenbaum and Kille, 2001). In contrast to animal or human tissue, little is known about suitable reference genes in plants. In some studies, adenine phosphoribosyltransferase, elongation factor 1-α, α-tubulin 2 or actin were used (Oztruk *et al.*, 2001; Bézier *et al.*, 2002; Dean *et al.*, 2002; Orsel *et al.*, 2002). A more systematic search for reference gene selection was carried out in bell pepper (Pozueta-Romero *et al.*, 1997), rice (Kim *et al.*, 2003), poplar (Brunner *et al.*, 2004), sugarcane (Iskandar *et al.*, 2004), barley (Burton *et al.*, 2004) and potato (Nicot *et al.*, 2005). Furthermore Czechowski *et al.* (2005) were looking for reference genes with stable expression levels in *Arabidopsis* within a large set of data generated from an Affymetrix ATH1 whole-genome GeneChip.

In the present study, European beech was treated with different types of abiotic and biotic stress such as elevated levels of ozone and CO_2, and infection with *Phytophthora citricola*; following these stressors, a search for reference genes was conducted. In addition, reference gene selection between sun and shade leaves of a 60-year-old European beech grown under free air conditions was performed. The expression level of several prominent genes that belong to different functional classes like α-tubulin, actin, 18S rRNA, GAPDH1, GAPDH2, and ubiquitin-like protein was measured by qRT-PCR and analysis of variance was used to assess their functionality as internal control genes.

Materials and Methods

Plant material, CO_2 treatment, and P. citricola infection

In April 2003, one-year-old European beech saplings (*Fagus sylvatica* L.; Bavarian state nursery Laufen, provenience Kempten, Germany) were planted in containers ($40 \times 30 \times 35$ cm), that had been filled with natural forest soil (site Höglwald, Bavaria, Germany; Grams *et al.*, 2002), and transferred to the climate-controlled greenhouse of the Helmholtz Zentrum München, Germany; the greenhouse is covered with UV-transparent glass to allow photo-biological processes to occur as they do in nature under a free sky (http://www.helmholtz-muenchen.de/eus/index.php). Saplings were kept under ambient climatic conditions with regard to relative humidity and temperature. Furthermore, half of the plants were kept under ambient and ambient + 300 ppm CO_2 from April until October of 2003 and 2004, respectively. During the winter, the saplings were placed under an outdoor pergola. At the beginning of March 2005, the saplings were transferred to the greenhouse [Technische Universität München (TUM), Horticulture, Freising, Germany] at a temperature of 15 °C during the day-time and 5 °C at night. Two weeks before bud broke, the containers were transferred into four climate chambers (TUM, Grassland Science, Freising, Germany) at a constant temperature of 20 °C with 80% relative humidity and 14 h of light (255 μE m^{-2} s^{-1}). Two of the chambers were treated with either 380 ppm or 680 ppm CO_2. At

the end of March, the soil of one chamber from each of the CO_2-treated chambers was directly inoculated with *P. citricola* as described by Fleischmann *et al.* (2002). The other two chambers were mock-inoculated with a sterile growth substrate. To trigger the production of zoospores of *P. citricola*, the containers were flooded with water for 38 h. For each treatment, leaves were sampled at 14 h, 31 h and 12 d after infection.

Plant material and ozone treatment

In July 2003, beech saplings (Schauinsland, Freiburg, Germany) were planted in 14-l pots in habitat soil. Until 2004, the trees were kept under free air conditions. During the vegetation period of 2004 the beech trees were treated with ozone (150 nl l^{-1}); sampling of leaves was carried out at different time points. Over the winter, the trees were kept under a pergola in free air. At the beginning of May 2005 the saplings were transferred to the above-mentioned climate-controlled greenhouse under ambient conditions. In early June the saplings were treated with 150 nl l^{-1} ozone for 8 h per day (8 a. m.–4 p. m.). After 14 d, the ozone concentration was increased to 190 nl l^{-1}. Leaf samples were taken at the 27th of July and the 29th of August 2005.

Sun and shade leaves of adult beech trees under free air conditions

Five leaves were taken from both the shade and sun crown of approx. 60-year-old beech trees (*Fagus sylvatica* L.) (Kranzberg Forest, Freising, Germany; Pretzsch *et al.*, 1998) at the 12th of May and the 19th of September 2005.

RNA isolation

Total RNA was isolated from leaves according to Kiefer *et al.* (2000). The yield and quality of the RNA was determined by spectral photometry at 260 and 280 nm using the NanoDrop system (Kisker, Steinfurt, Germany).

Quantitative real-time RT-PCR (qRT-PCR)

For qRT-PCR, 10 μg of total RNA were used. Oligo-$dT_{(12-18)}$ primers (5 $\mu g\,\mu l^{-1}$) were used for first strand cDNA synthesis. Reverse transcription was carried out at 42 °C using superscript III reverse transcriptase according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). Following RT-PCR, the cDNA was treated with ribonuclease H (Amersham, Freiburg, Germany) and RNase cocktail A (Ambion, Frankfurt, Germany).

The obtained cDNA was diluted 1 : 10, and PCR was performed in a volume of 14.5 μl of Sybr-Green PCR kit (Abgene, Hamburg, Germany) and 10.5 μl of diluted cDNA, using the Applied Biosystems (Darmstadt, Germany) 7500 Real-Time PCR System. The PCR conditions were as follows: 1 cycle at 50 °C for 1 min, 1 cycle at 95 °C for 15 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min.

In order to find an internal standard for normalization, we generated specific primer sets with Primer Express 2.0® (Applied Biosystems). The design was based on available ESTs of beech (Table I). Primers were obtained from MWG (Ebersberg, Germany).

Analysis of variance

To test for the effects of various treatments (*P. citricola*, ozone, CO_2 and sun vs. shade leaves) on gene expression values, an analysis of variance was performed using a linear model. However, the specific structure of the data that were grouped with respect to trees (technical replicates) required application of linear mixed effects models (lme-models). The package nlme (Pinheiro *et al.*, 2005) of the R-system (R Development Core Team, 2005) was used to generate ANOVA tables and to check the assumptions of variance homogeneity (diagnostic plot) and normal and independent distribution of residuals. If the premises of homogeneity of variance, independent residuals, and normal distribution of the residuals were fulfilled, the lme-model could be used.

Results

Gene expression of reference genes

Specific primers were designed for six commonly used reference genes using available sequences of European beech: 18S rRNA, actin, GAPDH1, GAPDH2, α-tubulin, ubiquitin-like protein (Table I). For plant material, leaves of beech saplings that were stressed using different conditions were used: (i) increased CO_2 contents (680 ppm); (ii) 150–190 nl l^{-1} ozone (8 h d^{-1}; up to 3 months); (iii) *P. citricola* infection. Three to five leaves of a single sapling were combined and 5–6 individual saplings were analyzed. As a con-

Table I. Sequences of primers used for quantitative real-time RT-PCR (qRT-PCR).

Gene	Acc. No.	Forward primer	Reverse primer
18S rRNA		5′-AAACGGCTACCACATCCAAG-3′	5′-CCTCCAATGGATCCTCGTTA-3′
Actin	AM063027	5′-AGAGATTCCGTTGCCCAGAA-3′	5′-TGGATTCCAGCAGCTTCCA-3′
GAPDH1	AM062861	5′-TCAACCTTGCTGTGTCGAAGA-3′	5′-GAGGCTTGAGCACGATGGA-3′
GAPDH2	AM063022	5′-GCATGATTCCTCTTCAGATGGAA-3′	5′-TCGCGGCCGAGGTACA-3′
α-Tubulin	AM062873	5′-TGAGTTGCTCAGGGTGGAAAA-3′	5′-CGAGCCCACTGTCATCGAT-3′
Ubiquitin-like protein	AJ972496	5′-TGATGTGGTCCTTGTAGATGTTGTAC-3′	5′-CGGCCGCGAATTCACTAGT-3′

trol, the same number of untreated saplings was used. To compare reference gene expression patterns under realistic outdoor conditions, five leaves of either the sun or shade crown of an adult tree were combined, and five trees were analyzed. For each sample analyzed, three technical repetitions within a single measurement were performed and aliquots of the same cDNA synthesis were used.

Real-time PCR efficiency calculation

For determining the relative gene expression, the amplification efficiencies of target and reference genes should be approx. equal (Soong *et al.*, 2001). A sensitive method for an assessment of the same efficiency of two amplicons is given by the cycle threshold (Ct) variation within a template dilution. In addition, serial dilution reflects the efficiency of the primers; this is important for gene comparison (Applied Biosystems, User Bulletin #2, 2001). Ideally a two-fold dilution series displays Ct-values of 1 between adjacent dilutions, reflecting a PCR efficiency of 100%. That means that an increasing cDNA concentration on the *x*-axis will result in a graph that has slope −1. In order to test the efficiency of qRT-PCR, a two-fold dilution series of a control sample (*P. citricola* infection study after 14 h) was created. Genes coding for 18S rRNA, actin, GAPDH1 and α-tubulin demonstrated elevated efficiency on a high statistical level because all regression coefficients were about 1. In addition, the slopes were about −1. The other genes showed lower slopes and diminished regression coefficients.

Analysis of variance

For all Ct-values of the different genes, the premises, homogeneity of variance, independent residuals, and normal distribution of the residuals were fulfilled. Therefore, the lme-model could be used.

Comparing reference gene expression upon CO_2 treatment

To assess the expression of the housekeeping genes upon treatment with increased CO_2 levels (2× ambient) RNA samples from six biological replications were assayed in triplicate (Fig. 1A). Two different time points (31 h, 12 d) after the onset of *P. citricola* infection were analyzed. At both time points there were no differences between the Ct-values of control and CO_2-treated samples for 18S rRNA (p = 0.1079 and 0.1019), actin (p = 0.3812 and 0.1974) and GAPDH1 (p = 0.5582 and 0.3464) (Table II). All other genes were affected by the CO_2 treatment and showed significant differences between the treated and untreated samples (Table II).

Comparing reference gene expression upon P. citricola infection

Again for RNA transcription levels six biological replication and triplicate analyses were carried out (Fig. 1B). The gene coding for 18S rRNA displayed low p-values 31 h after the *P. citricola* treatment, and 12 d after the treatment control and infected samples were significantly different (p = 0.0458) (Table II). For GAPDH1, the Ct-values of the infected and control samples were significantly different at the earliest time point (p = 0.0398). Actin, GAPDH2, α-tubulin and ubiquitin-like protein were not affected by the *P. citricola* treatment (Table II).

Comparing reference gene expression upon ozone treatment

Beech saplings were fumigated with increasing ozone concentrations (150–190 nl l^{-1}) for up to

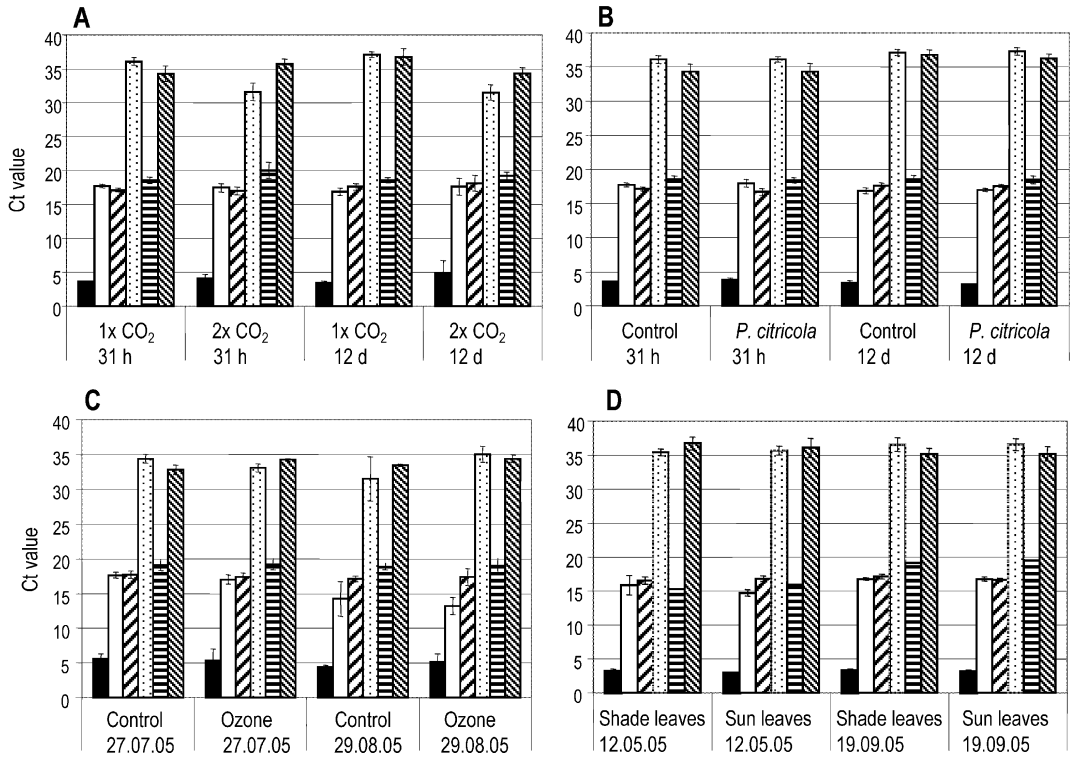

Fig. 1. RNA transcription levels of housekeeping genes tested, presented at Ct-values in the different treatments. (A) Beech saplings were grown under normal (1×) and elevated (2×) CO_2 (680 ppm) contents and analyzed 31 h and 12 d after infection with *P. citricola*. (B) Beech saplings were grown under normal CO_2 contents and analyzed 31 h and 12 d after infection with *P. citricola*. (C) Beech saplings were treated with ozone (150–190 nl l^{-1}; 8 h d^{-1}) starting in early June 2005 and samples were taken at the 27th of July and the 29th of August 2005. (D) Leaves of 60-year-old beech trees were taken at the 12th of May and the 19th of September 2005. Cycle threshold (Ct)-values are the mean of 5–9 replicates. Symbols: ■ 18S rRNA; □ actin; ▨ GAPDH1; ⁝ GAPDH2; ▤ α-tubulin; ▧ ubiquitin-like protein.

Table II. *p*-Values generated in an analysis of variance for the different treatments, time points, and genes. A *p*-value < 0.05 indicates significant differences between the treated and the untreated trees (marked in bold).

Gene	CO_2		*P. citricola*		Ozone		Sun/shade leaves	
	31 h	12 d	31 h	12 d	27.07.05	29.08.05	12.05.05	19.09.05
18S rRNA	0.1079	0.1019	0.0822	**0.0458**	0.7768	0.1819	0.2565	0.3977
Actin	0.3812	0.1974	0.2752	0.6422	0.1038	0.3249	0.1295	0.9942
GAPDH1	0.5582	0.3464	**0.0398**	0.8910	0.3586	0.6691	0.4078	**0.0255**
GAPDH2	< **0.0001**	< **0.0001**	1,0000	0.5685	**0.0082**	0.5062	0.5164	0.9726
α-Tubulin	**0.0203**	0.0572	1,0000	0.7995	0.7976	0.8549	0.2947	0.4366
Ubiquitin-like protein	**0.0304**	**0.0024**	0.9841	0.3675	0.8896	0.7622	0.3897	0.9440

3 months as given under Materials and Methods, and RNA transcription levels are given in Fig. 1C. A significant difference between ozone-treated and non-treated trees was seen for GAPDH2 on the 27th of July ($p = 0.0082$). The Ct-values of the other genes assayed showed no significant differences following ozone treatment (Table II).

Comparing reference gene expression in different leaves (sun/shade) of beech trees

To analyze the reference gene expression pattern under realistic outdoor conditions, five biological replications of sun vs. shade leaves of adult beech trees of Kranzberg Forest were assayed, and

RNA transrciption levels are given in Fig. 1D. The first sampling point was during spring; a second occurred in autumn of 2005. Interestingly, only a single gene coding for GAPDH1 ($p = 0.0255$) was significantly different in the sun vs. shadow leaves in autumn (Table II).

Discussion

The accuracy of qRT-PCR depends on the PCR conditions, the quality of RNA, the enzymes and specific primers used, and a valid reference gene for the normalization of data that finally results in the quantification of gene expression (Malboeuf *et al.*, 2001; Bustin, 2002; Nicot *et al.*, 2005). To calculate the total RNA amount, the conventional absorbance measurement at 260 nm (OD_{260}) was applied. This is less accurate than measurement with RiboGreen (Malboeuf *et al.*, 2001), but both methods are comparable if the RNA concentration is not less than 100 ng μl^{-1}. In our RNA extractions from beech leaves, the RNA amount was about 1000 ng μl^{-1}. In this study, 10 μg of total RNA and superscript III reverse transcriptase worked fine at 42 °C.

Amplification efficiency in qRT-PCR is important for conducting a comparison of data (Dean *et al.*, 2002). According to Soong and Ladányi (2003), it is imperative to realize that the comparison of Ct-values is dependent on similarities in the PCR efficiency between two genes. Ct-values of a reference gene should be < 29.07 (Soong and Ladányi, 2003), because higher Ct-values will result in a high standard deviation (Stahlberg *et al.*, 2004). The genes coding for actin, 18S rRNA, α-tubulin and GAPDH1 were efficient with Ct-values of about 20, whereas the ubiquitin-like protein and GAPDH2 were not that efficient with Ct-values above 30.

In the present study, beech trees of different ages under various environmental conditions and treatments were analyzed. In addition, the plant material was not homogeneous. Therefore gene expression levels can be very different in trees undergoing the same conditions or trees at the same age. To solve this problem we implicated the factor "tree" in the statistical analysis (Pinheiro *et al.*, 2005).

Different studies have shown that some reference genes are affected by experimental conditions (Schmittgen and Zakrajsek, 2000; Kim *et al.*, 2003). In this study, the only gene that was not affected by all treatments was actin (Table II). In different plant gene expression studies actin was used successfully as a reference gene (Rao *et al.*, 2000; Pignocchi *et al.*, 2003; Tsukamoto *et al.*, 2005). In a biotic interaction study, actin was used as reference gene in *P. citricola*-infected *Hevea* clones (Thanseem *et al.*, 2005). This demonstrates that actin can be used as a reference gene for different tissues under different stress conditions.

18S rRNA is often used as an internal control for qRT-PCR in plants. Although oligo-dT priming of RNA will not work well with rRNA as no polyA tail is present (Huggett *et al.*, 2005), the use of short oligo-dT primers$_{(10)}$ resulted in reverse transcription of rRNA from beech. In our study, the 18S rRNA was affected only by *P. citricola* treatment (Table II), whereas Thanseem *et al.* (2005) found no change in gene expression after *P. citricola* treatment of *Hevea* clones. According to Bustin (2002), 18S rRNA is a suitable control but different problems exist. 18S rRNA is highly abundant compared to most target mRNA transcripts; that will result in difficulties in accurate data analysis (Schmittgen and Zakrajsek, 2000; Brunner *et al.*, 2004). In plants, ribosomal subunit transcription is influenced by various external factors like ozone, CO_2 or UV-B (Ernst *et al.*, 2001; Gupta *et al.*, 2005; Olbrich *et al.*, 2005; Taylor *et al.*, 2005). Furthermore, 18S rRNA sequences are highly conserved, and RT-PCR of RNA of plants infected by fungi might result in an amplification of both plant and fungal 18S rRNA (Dean *et al.*, 2002).

Our results showed that GAPDH1 was affected in the early stage of the *P. citricola* infection and was different in sun and shade leaves in autumn (Table II). The transcript levels of GAPDH2 changed significantly following CO_2 treatment and early stages of ozone treatment (Table II). A regulation of gene expression of the GAPDH A subunit after ozone treatment was found in *Arabidopsis* (Mahalingam *et al.*, 2005), whereas GAPDH3 was constitutively expressed (Mahalingam *et al.*, 2003). In potato, the level of chloroplast GAPDH transcripts decreased, while cytosolic GAPDH increased following treatment with ozone (Glick *et al.*, 1995). GAPDH2 showed no optimal efficiency and is not suitable as an internal reference gene for qRT-PCR. GAPDH1 is affected by *P. citricola* stress under free air conditions.

The transcript level of the ubiquitin-like protein changed only after treatment of beech saplings

with CO_2 (Table II). The ubiquitin system may be involved in plants that are damaged by ozone (300 nl l^{-1}), because in Scots pine seedlings the contents of two polyubiquitin species were increased after ozone treatment (Wegener *et al.*, 1997). Similarly, the contents of polyubiquitin transcripts were increased in aspen trees upon ozone or CO_2 fumigation (Gupta *et al.*, 2005). Potato plants inoculated with *P. infestans* also showed an accumulation of ubiquitin transcripts in two different cultivars, one susceptible and the other partially resistant (Basso *et al.*, 1996).

Tubulins are often used as housekeeping genes (Brunner *et al.*, 2004; Iskandar *et al.*, 2004; Nicot *et al.*, 2005). However, like many other housekeeping genes, expression of tubulins can vary upon different stresses in various tissues (Iskandar *et al.*, 2004; Nicot *et al.*, 2005). The gene expression of α-tubulin was significantly influenced only after treatment of the saplings with increased CO_2 concentrations (Table II). On the basis of the other data given in Table II, α-tubulin might also be a suitable internal reference gene for qRT-PCR.

For some studies it can be helpful to use more than one gene for normalization (Brunner *et al.*, 2004). There are a number of software programs available that allow the evaluation of multiple reference genes. For example, the program geNorm allows the most appropriate reference gene to be chosen using the geometric mean of the expression of the candidate cDNA (Vandesompele *et al.*, 2002; Czechowski *et al.*, 2005; Huggett *et al.*, 2005). However in this study, for this type of data the application of geNorm would be inadequate because the calculation of standard deviations on normalized expression levels is only correct for a single sample and not for multiple samples, *e.g.* biological replicates, technical replicates (geNorm manual, August 14, 2006). Czechowski *et al.* (2005) demonstrated the application of geNorm in combination with microarrays for *Arabidopsis*. However, in contrast to *Arabidopsis* for European beech no large gene databases for a selection of potential candidate reference genes are available.

To conclude, in this study we have compared actin, α-tubulin, GAPDH1, GAPDH2, ubiquitin-like protein and 18S rRNA as reference genes for qRT-PCR analysis in European beech. Dependent on the stress applied and leaf tissue analyzed, more than one gene could be used as reference gene for normalization. Actin appeared to be the best reference gene for European beech under the conditions tested, because no significant differences between all treatments were detected. However, as pointed out by Vandesompele *et al.* (2002) an ideal and universal control gene does not exist. Therefore additional reference genes should be selected, depending on the experimental conditions (see Table II). It is important to note that the transcriptional level of plant genes is influenced by many factors, *e.g.* development stage, age, abiotic stress, and biotic stress. This implicates that for each single experiment reference genes must be validated in order to receive accurate gene expression levels.

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