Optimisation of Relative Quantitative RT-PCR for Expression Analysis in Azalea Flower Colour Sports

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Abstract
The fastest way to create new azalea (Rhododendron simsii hybrids) cultivars is by making use of flower colour sports, which appear spontaneously on azalea plants. Unfortunately, there is still very little known on how bud sport induction occurs. Therefore, genes coding for two key enzymes of the azalea flavonoid biosynthesis pathway, chalcon synthase (chs) and dihydroflavonol 4-reductase (dfr) that were reported before to be apt for modification by the action of bud sporting, were isolated and characterized. The expression of these two flower colour genes in the petals of azalea flowers will be compared between all ‘Hellmut Vogel’ flower colour sports. To measure the expression levels of both genes, relative quantitative RT-PCR analysis will be worked out on a real-time PCR machine. The expression of housekeeping genes, which is expected to be the same for all sports, will be used to calculate the relative expression level of the two genes of interest. The optimisation of this technique will be discussed.

INTRODUCTION
Real-time RT-PCR provides the simultaneous measurement of gene expression in many different samples. It is currently the most sensitive method, especially suitable because very little amounts of RNA are required. This technique will be fully optimised for expression analysis of two azalea genes involved in flower colour biosynthesis, chalcon synthase, and dihydroflavonol 4-reductase. For normalisation of gene expression, housekeeping genes will be selected. Primers will be developed and their ideal concentration will be determined in a primer matrix experiment. To avoid that DNA contamination tampers cDNA expression rates, primers will be used on DNA samples for finding introns. If no introns are present, noRT samples will be used to quantify DNA contamination. Finally, reproducibility of standard curves and samples will be evaluated.

MATERIALS AND METHODS

Plant Material
Flower buds in four developmental stages, stage I (closed bud), stage II (slightly coloured flower buds, scales still present), stage III (candle stage) and stage IV (fully opened flowers), were harvested on 21 bud sports of the ‘Hellmut Vogel’ sporting series: Inga, Sima, Paloma, Hector, Anja, Hellmut Vogel, Marianne Haerig, Mw. Edmond Troch, Nordlicht, Cyriel Buyssse, Marcella, Terra Nova, Ilona, Julia, Inka, Mary, Zalm Vogel, Super Nova, Luntera, Gilles, Aquarel.

cDNA Synthesis
mRNA was isolated using the MicroPoly(A)Pure™ Small Scale mRNA Purification Kit (Ambion). The mRNA was dissolved in 20 µl DEPC water/EDTA. First strand cDNA synthesis was performed by adding 9 µl RNase free water, 700 ng oligo(dT) and 20 nmol dNTP to 15 µl mRNA. As a control for DNA contamination (noRT samples), 7 µl RNase free water, 700 ng oligo(dT) and 20 nmol dNTP was added to 5 µl mRNA. Both mixtures were incubated for 5 min at 65°C. After cooling on ice, 8 µl 5x
first strand buffer, 0.4 µmol DTT, 200 U Superscript II RT and 1 µl SuperaseIn (Ambion) was added to the samples and 5 µl 5x first strand buffer, 0.4 µmol DTT and 1 µl SuperaseIn (Ambion) to the noRT samples. The mixtures were incubated for 2 h at 42°C, followed by incubating for 15 min at 70°C.

**Housekeeping Genes**

Degenerate primers were developed (Primer Express 2.0) for the amplification of 4 potential housekeeping genes: glyceraldehydes-3-phosphate dehydrogenase (GAPDH), TATA-box binding protein, citrate synthase and maturase K. All primers produced a 151 kb fragment. They were evaluated by PCR on cDNA of flower buds in 4 developmental stages. The PCR amplification mixture was composed of 2 µl cDNA solution, 10 pmol of each primer, 10 nmol dNTP, 2 µl 10xPCR buffer and 1.25 U AmpliTaq DNA polymerase. Water was added to a final volume of 20 µl. Amplifications were carried out in a PE9700 (Perkin Elmer) with the following parameters: 2 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 56°C, 1 min at 70°C and finally 10 min at 70°C. Fragments were separated on a 1.5 % agarose gel and visualised by UV illumination after staining with ethidium bromide. Amplified fragments were sequenced using an ABI 377 Sequencer and azalea specific primer sequences were determined (table 1). Primers for DFR and CHS were also developed using Primer Express 2.0 (table 1).

**Standard Curves**

Amplified fragments of the selected houdsekeeping genes were cloned using the TOPO TA Cloning™ Kit (Invitrogen) containing TOP10F chemically competent cells and the pCR®2.1-TOPO® cloning vector. For CHS and DFR, complete cDNA sequences were cloned (De Schepper et al., 2001). Transformed cells were screened for the presence of the cloned fragments by direct colony PCR according to the pMOSBlue protocol (Amersham). Plasmids were purified (GFX™ Micro Plasmid Prep Kit, Amersham) and by means of digestion, the presence of the fragments was verified once more. Plasmid concentration was determined spectrophotometrically using the Gene Quant DNA/RNA calculator (Pharmacia). Plasmids were diluted to a stock concentration of 0.04 ng/µl. Standard curves were constructed as 10-fold serial dilutions from the stock and used for no longer as 24 hours.

**Intron Identification**

All primer pairs were used on DNA isolated from the petals of azalea flowers, to evaluate for the presence of an intron in the amplified genomic fragment. The PCR amplification mixture was composed of 10 pmol of both primers, 10 nmol dNTP, 2 µl 10x PCR buffer, 1.25 U AmpliTaq DNA polymerase and 20 ng plasmid DNA or 20-150 ng DNA in a total volume of 20 µl. Amplification reactions were carried out in a PE9700 (Perkin Elmer): 2 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 56°C, 2 min at 70°C and finally 10 min at 70°C. Fragments were separated on a 2 % agarose gel and visualised by UV illumination after staining with ethidium bromide.

**Real-time RT-PCR**

The real-time RT-PCR analysis was carried out in an ABI7000 (Applied Biosystems). The amplification mixture was composed of 12.5 µl SYBR Green I Master Mix (Applied Biosystems), 7.5 pmol of both primers (300nM) and 5 µl cDNA. Water was added to a final volume of 25 µl. For the primer matrix experiment, 22.5 pmol and 1.25 pmol primer were used for 900nM and 50 nM respectively. Cycling conditions were 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C, 1 min at 60°C. For melting curve analysis, cycling conditions were 15 sec at 95°C, 15 sec at 60°C, followed by ramping from 60°C to 95°C with a ramp speed of 2 % and a final step of 15 sec at 95°C. Each sample was tested in duplicate. For analysis, threshold and baseline were set according to ABI User Guide and User bulletin #2.
RESULTS AND DISCUSSION

Housekeeping Genes
The expression of four potential housekeeping genes was evaluated by RT-PCR on flower buds of four developmental stages. No fragments were amplified for TATA-box binding protein and citrate synthase. MaturaseK and GAPDH were expressed in all stages (results not shown) and could be used for normalisation of expression in real-time RT-PCR. The use of two housekeeping genes for quantification is recommended (Vandesompele et al., 2002b; Thellin et al., 1999), since the expression of these so-called constitutive expressed genes is not always as stable in different tissues as generally assumed.

Primer Matrix Experiment
To set up a reliable real-time experiment, primer-dimer artefacts must be avoided (Vandesompele et al., 2002a). All 4 primer sets (DFR, CHS, GAPDH and maturaseK) were tested with real-time RT-PCR followed by melting curve analysis on water (NTC: no template control) and cDNA. Three different primer concentrations were used: 900 nM, 300 nM and 50 nM. Primer dimers could be seen as an additional peak in the first derivative of the melting curve. For GAPDH the 900 nM primer concentration resulted in the formation of primer dimers, but for the others, there was no difference seen between 900 nM and 300 nM. The Ct-value at 50 nM was remarkably higher, so it seemed most appropriate to use primers at a concentration of 300 nM for further experiments.

DNA Contamination
Since DNase treatment of the RNA samples resulted in major losses of RNA, primers were used on DNA samples to detect whether they amplified an intron. For GAPDH and DFR this was the case, for CHS and matK however, no difference was seen between cDNA and DNA amplicons (Fig. 1). For these two primers, noRT samples had to be included to control for DNA contamination.

A difference of seven Ct-units between noRT (DNA) and RT (cDNA) samples means there is 128-fold less contaminating DNA present, as there is cDNA. In that case, DNA contamination can be neglected. This was clearly the case for all but one samples amplified with CHS-primers (Fig. 1). For these two primers, noRT samples had to be included to control for DNA contamination.

Reproducibility
When comparing expression of samples measured in several assays, it is very important PCR is reproducible and always occurs with the same efficiency. Calculation of PCR efficiency is based on the equation of the standard curve (Eff = 10^(-1/slope) – 1), which is also used for calculating cDNA concentrations from Ct values. Therefore we evaluated the stability of our standard curves.

When the same standard curve was used in three assays in 24 hours, Ct values of almost all dilutions changed (results not shown), even more than two units for some dilutions, this would mean a 4-fold change in concentration. This is unacceptable and presumably due to supercoiling of part of the plasmids, hindering primer access to the template. This problem could be solved, by opening up the plasmids by a restriction enzyme cut (Uvstebo et al., 2003). Therefore GAPDH plasmids were cut with Hind III and BSA (0.1 µg/µl) was added as a carrier to the linearised plasmid stock. The addition of a carrier such as BSA should prevent the loss of very little quantities of plasmid DNA. When comparing the results of three successive assays of this standard curve (linear, +BSA) with the previous one (circular, -BSA), it clearly shows that only linearization
and the addition of BSA results in reproducible amplification of standard curves (Fig. 3 and 4).

Inter- and intra-assay variation of cDNA samples was also determined. Standard deviation of the Ct-value of duplicate samples in the same run should always be smaller than 0.15 (Applied Biosystems). Few samples fulfil this condition. For inter-assay variation the problem is the same. Therefore the cDNA samples will be purified and BSA will be added as a carrier.

Future Perspectives

Standard curve stability will be tested over a longer period of time. To optimise reproducibility of cDNA samples, the influence of the addition of BSA to cDNA will also be evaluated. When fully reproducible results can be obtained, real-time RT-PCR will be used for the determination of the flower bud stage with maximal expression of both genes. Finally, the technique will be used to measure expression levels of chs and dfr for all cDNA samples of material harvested in the ideal bud stage.

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Literature Cited


Tables

Table 1. Primer sequences for the amplification of housekeeping genes and genes of interest.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>Maturase K</td>
<td>CTTTGTTTTTCTCCGCAACCA</td>
<td>CAGGAAAGGCTCTTTAAACACC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCGGAATCAACGGTTTTGGA</td>
<td>CACTGACCCTGAACACTGT</td>
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<tr>
<td>CHS</td>
<td>TGGGCTCATCTCCAAGAACAT</td>
<td>CTCGGGCTTAAGGCTCAACTT</td>
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<tr>
<td>DFR</td>
<td>CGTCATGAGGCTGCTTGAAC</td>
<td>AAAGCTCCCTTCCTCGTTGAG</td>
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</tbody>
</table>
Figures

Fig. 1. Results of PCR on plasmid DNA (P) and DNA of azalea flowers (1-4) using DFR primers (right panel) and matraseK primers (left panel). In the lanes marked with W, water is used as a template. M: lambda Pst marker. The length of the cDNA fragments is 151 bp, the DFR fragment with intron is 700 bp.

Fig. 2. Amplification plot of four cDNA samples and their corresponding noRT samples using CHS-primers. All samples were tested in duplicate.
Fig. 3. Results of three successive amplifications of a GAPDH standard curve made from circular plasmid DNA and without BSA added. All dilutions (10^7–10^2) were amplified in duplicate and mean Ct values were used for linear regression.

Fig. 4. Results of three successive amplifications of a GAPDH standard curve made from linearised plasmid DNA and BSA (0.1 µg/µl) added. All dilutions (10^7–10^2) were amplified in duplicate and mean Ct values were used for linear regression.