THE USE OF RELATIVE QUANTITATIVE RT-PCR FOR EXPRESSION ANALYSIS IN AZALEA FLOWER COLOR SPORTS

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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 color biosynthesis, chalcon synthase (CHS) and dihydroflavonol 4-reductase (DFR).

Housekeeping genes
The use of housekeeping genes (constitutive expressed genes) for quantification is necessary to normalise the expression of the flower color genes. Two genes were found to be expressed equally in four azalea bud stages, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the chloroplast gene maturase K (matK).

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, there was no difference seen between cDNA and DNA amplicons (Fig. 2). For these primers, noRT samples (cDNA synthesis without reverse transcriptase enzyme) will be included to control for DNA contamination.

Primer dimers
To set up a reliable real-time experiment, primer-dimer artefacts must be avoided (Vandesompele et al., 2002). Different concentrations of primer sets of the housekeeping genes (GAPDH and matK) and flower color genes (DFR and CHS) were tested with real-time RT-PCR followed by melting curve analysis on water (no template control, NTC) and cDNA. Primer dimers could be seen as an additional peak in the first derivative of the melting curve (Fig. 1). No primer dimers were detected when a 300 nM primer concentration was used.

Standard curve stability
Amplified fragments of all genes were cloned using the TOPO TA Cloning Kit (Invitrogen). Transformed cells were screened for the presence of the cloned fragments and finally plasmids were diluted to a stock concentration of 0.04 ng/µl. Standard curves were constructed as 10-fold serial dilutions and used for no longer as 24 hours. When the same standard curve was used in three assays, Ct values of almost all dilutions changed significantly (Fig. 3), but when the plasmids were linearised by a restriction enzyme cut and BSA (0.1 µg/µl) was added as a carrier, standard curve amplification appeared to be reproducible (Fig. 4).

References

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