Gene expression profiling of key enzymes in azalea flower colour biosynthesis

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Introduction

Azalea flower colour is mainly determined by two groups of pigments, anthocyanins and flavonols. Genes coding for two key enzymes in the biosynthesis of these pigments, *chalcon synthase* (*chs*) and *dihydroflavonol-4-reductase* (*dfr*), were isolated from an azalea cDNA library and fully characterised. The expression of these two genes in the petals of 8 azalea flower colour sports of the 'Hellmut Vogel' sporting series will be used as a model system for the development of a real-time PCR protocol for gene expression analysis in azalea.

Optimisation

Relative quantitative RT-PCR is currently the most sensitive method for expression analysis, especially suitable because very little amounts of RNA are sufficient. Optimisation was needed at all crucial steps from RNA isolation up to the final quantification. Since no intron-spanning primers could be developed, DNase treatment of mRNA samples appeared to be a good alternative for preventing the co-amplification of contaminating DNA, as shown by the low or non-existing amplification in the noRT-samples (Fig. 1). There exist many different quantification strategies, but, although most labour-intensive, the use of standard curves remains the most reliable method. However, reproducibility and stability of these dilution series was a major problem. This problem could be circumvented by linearization of the used plasmids and by diluting them in a yeast tRNA solution (50 ng/µl).

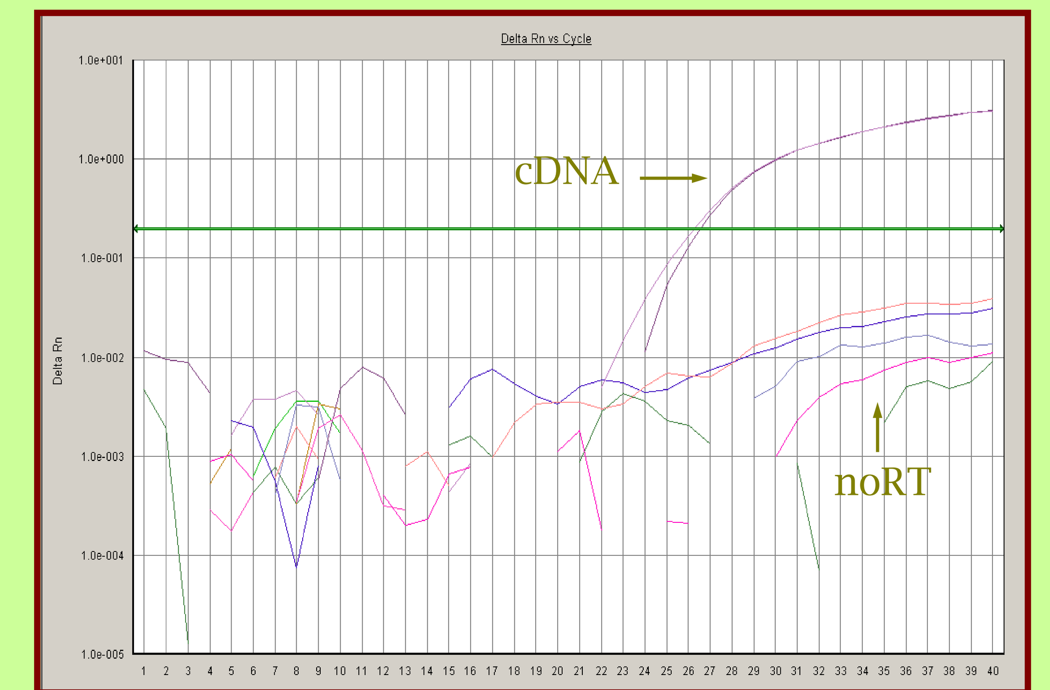

Fig. 1: Real-time PCR amplification plot of 5 noRT and 2 cDNA samples

cDNA fragment	Putative function
HK5	Histone H3
HK47	Nucleosome assembly protein
HK65	AKIN gamma mRNA
HK92	Heterotrimeric G-protein, α-subunit
HK96	Expansin
HK112	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase
HK129	Protein phosphatase 2C
HK134	Chorophyll a/b binding protein CP24 precursor
HK156	Cytochrome P450 mRNA
HK164	Chorophyll a/b binding protein
HK173	Pyruvate dehydrogenase
HK190	Protein dislphide isomerase mRNA

Table 1: Putative function of 12 candidate housekeeping genes

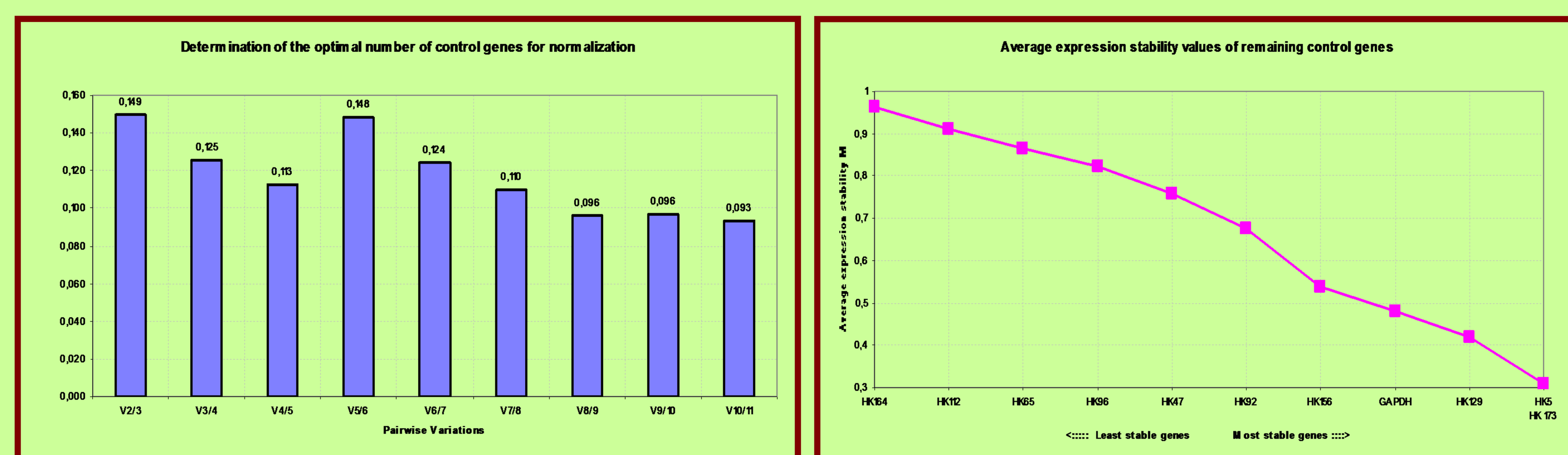

Fig. 2: GeNorm output for evaluation of the optimal number of housekeeping genes. Applying a cut off value of 0.15, the use of 2 housekeeping genes is sufficient (left panel); HK5 and HK173 are the best for this assay (right panel).

Housekeeping genes

The most critical step for the whole quantification process was the selection of the right housekeeping genes. In previous analysis, only GAPDH was used for this purpose, but, not only GAPDH is reported to be unreliable as a housekeeping gene, also the use of multiple housekeeping genes is recommended. Starting from the 'Flamenco' cDNA library, 200 cDNA fragments were randomly picked, sequenced and putative functions were determined by comparison with EMBL accessions. In this way, 60 azalea genes could be identified, of which 12 potential housekeeping genes (Table 1). Primers were developed and tested on cloned cDNA fragments. One primer pair produced no amplicon (HK 190), another one (HK134) was not specific. The remaining 10 genes were, together with GAPDH, used in a real-time PCR assay on cDNA of 8 azalea flower colour sports. Using the geNorm software (Vandesompele *et al.*, 2002) we could state that the use of two housekeeping genes, HK5 and HK173 (Fig. 2), will be sufficient for expression analysis in flower petals of the 'Hellmut Vogel' sporting series.

Expression analysis

Aiming to find a correlation between flower colour and the expression level of *chs* and *dfr* in the petals of 8 azalea flower colour sports, the real-time PCR protocol was applied to these samples. For calculation and normalisation, qBase software (Hellemans *et al.*) was used. Expression levels are shown in figure 3. Between sports, differences can be detected, but there appears to be no correlation with flower colour.

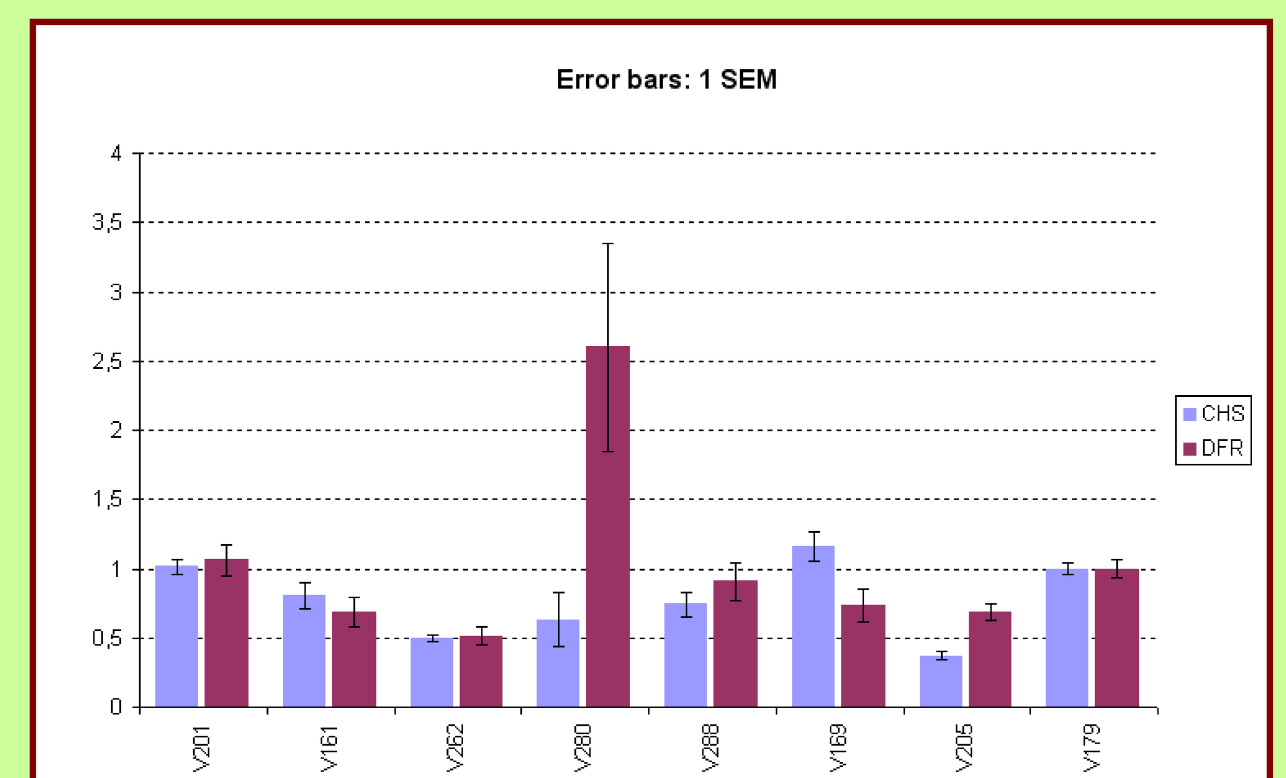

Fig. 3: *Chs* and *dfr* expression levels in white (V201), pink (V161, V262, V280 and V288), red (V169 and V205) and brick red (V179) flower colour sports.

Future perspectives

The expression of other genes of the flavonoid biosynthesis pathway (*fls*, *ans*, *f3h*, *f3'h* and *f3'5'h*) will now be determined. Expression profiles can then also be compared to flower colour.

References

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR by geometric averaging of multiple internal control genes. Genome Biology, 3: 1-11

Hellemans, J. and Vandesompele, J. (in preparation). http://medgen.ugent.be/qbase

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