From phenotype to genotype: flower colour in azalea as a model for integration with gene expression

Ellen De Keyser[1], Peter Lootens[1], Jan De Riek[1] and Erik Van Bockstaele[1,2]

[1] Institute for Agricultural and Fisheries Research (ILVO) – Plant Unit, Caritasstraat 21, 9090 Melle, Belgium

E-mail: Ellen.dekeyser@ilvo.vlaanderen.be

[2] Department for Plant Production, Ghent University, Coupure links 653, 9000 Gent, Belgium

Flower colour

Flower colour is inherited as a semi-qualitative trait in azalea and is mainly determined by differences in anthocyanins and flavonols. A two-gene model explains the phenotypic variation between white, brick red and carmine red colour: W in case the flower petals contain anthocyanins and Q if flavonols are present as co-pigments in carmine red flowers. However, the presence of flavonols in white flowers cannot be detected visually. Also, the existence of pink flowers is not explained by this two-gene model (Table 1).

Image Analysis

Flower colour was determined on 161 plants of the mapping population ('Sima' x 98-13-4 (seedling)) with image analysis using the RGB colour model (Lootens *et al.* 2007). RGB scores were used as input for discriminant analysis, that was used for classification (Fig. 1). Carmine red, brick red and white flowers could clearly be separated. Pink flowers were distributed in between the other groups, suggesting that pink can be split up in different classes depending on the pigment types present (pink flowers with or without flavonols). This classification could not be made visually. Only pale pink flowers grouped close to white, can easily be distinguished from the other pink flowers.

Genetic map

A genetic map of 16 linkage groups was constructed using a framework of anonymous AFLP and SSR markers. Besides, a set of 8 functional EST-markers, developed on random sequences (De Keyser *et al.* 2008), and also 4 specific EST markers for genes of the flavonoid biosynthesis pathway (FLS, ANS, DFR and UFGT; Fig. 2) were used for mapping. MYB-profiling, a sequence directed technique targeting the conserved MYB-motif, generated 15 dominant markers functionally related to the MYB gene family. This family is a large group of transcription factors involved in a wide array of cellular processes and also in flavonoid biosynthesis.

Gene expression

Genes coding for key enzymes in the flavonoid biosynthesis pathway in azalea (Fig. 2) were isolated and consistent qPCR primer sets could be developed for five genes (CHS, F3H, ANS, DFR and FLS). The expression level of these genes in the petals of 20 members of the mapping populations was determined using 3 housekeeping genes for normalisation.

Table 1: Genotype of the flower colour trait in azalea

Locus\colour	Carmine red	Brick red	White	Pink
W (colour)	W-	W-	ww	?
Q (co-pigmentation)	Q-	qq	Q- or qq	?

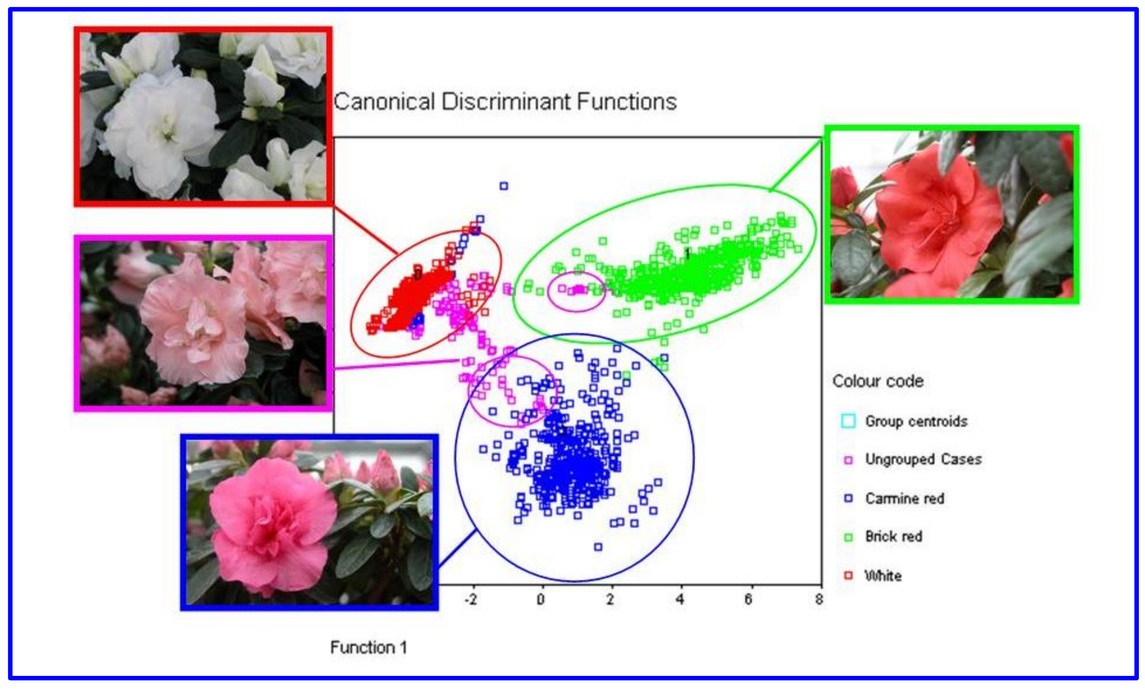

Figure 1: Result of the discriminant analysis on carmine red, red and white flowers. Pink flowers were ungrouped and are dispersed in between the other colour groups.

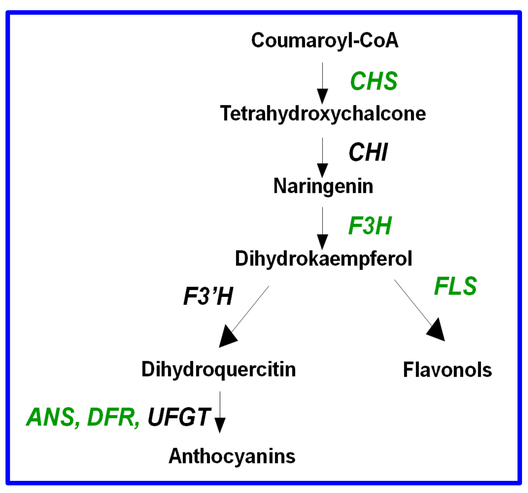

Figure 2: Flavonoid biosynthesis pathway. Genes marked in green were used for expression analysis.

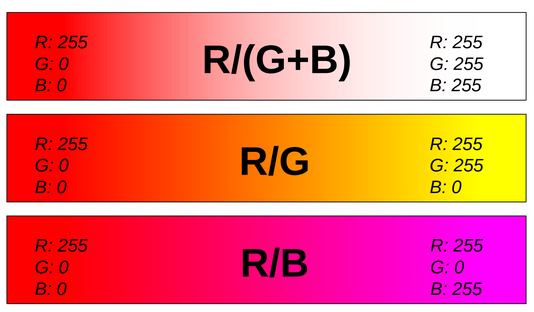

Figure 3: Gradual effect of G and B values on the red colour (R). When both G and B are high, flowers appear to have white petals

Table 2: Integration of image analysis and gene expression data as QTL on the genetic map

Linkage Group	Colour (Image Analysis)	Gene Expression	Locus
LG2	R	CHS, F3H, ANS	MYB11
LG3	-	DFR	-
LG6	R/B(col), B_high, B_low	FLS	Q
LG12	R, B_high	CHS, F3H, ANS	ANS, MYB10, MYB15, MYB12
LG14	-	F3H	FLS
LG16	R, G_low, G_high, B_high, R/(G+B)(col), R/G(col)	-	W, DC011

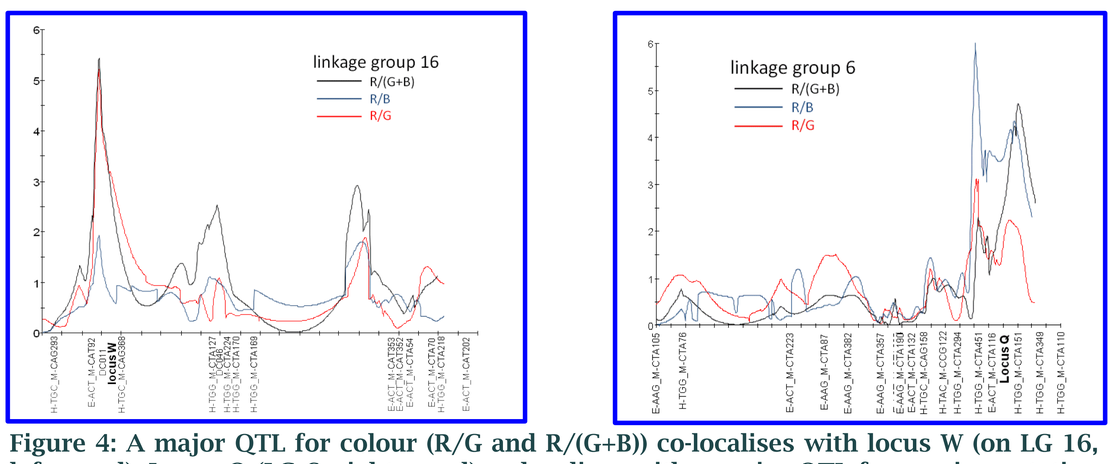

Figure 4: A major QTL for colour (R/G and R/(G+B)) co-localises with locus W (on LG 16, left panel). Locus Q (LG 6, right panel) co-localises with a major QTL for co-pigmentation (R/B) of the carmine red flowers.

QTL mapping of RGB and gene expression data

To elucidate the 'pink' problem, integration of the image analysis data as QTLs on a genetic map of the mapping population was evaluated by Interval Mapping and permutation testing ($p < 0.01$).

1. the loci W and Q were mapped as qualitative traits. W was mapped on linkage group 16; Q on linkage group 6 (Fig. 4);
2. RGB values (after Log- or Power-transformation to normally distributed values) were mapped as QTL loci (Table 2). The G and B scores were split into high and low scores (B_high, B_low, G_high, G_low);
3. white flowers were omitted from the data set (high score for R, G and B; Fig. 3);
4. derived values R/(G+B), R/G and R/B were mapped only for coloured flowers (Fig. 4)

By omitting the white flowers from the analysis and only including coloured flowers, we tried to localise the QTL loci, apart from W and Q, that are controlling colour intensity differences ranging from pink to (carmine) red. Apart from linkage group 6 and 16 where W and Q are located, QTL for RGB values were also mapped on linkage group 2 and 12 (Table 2). Gene expression data for key enzymes of the flavonoid biosynthesis pathway were mapped using Kruskal-Wallis tests (after Log-transformation to normally distributed values). Only loci showing significant correlation for different neighbouring markers (at least 1 marker at $p < 0.05$ or 0.01) were recorded (Table 2).

On linkage group 2, co-localised to the MYB11 marker, a QTL for R values integrates with expression of CHS and F3H which are located in the common part of the anthocyanin and flavonol biosynthesis pathway (Fig. 2). On linkage group 12, a comparable QTL co-localises to the ANS locus. It can be speculated that both QTL function as feedforward (linkage group 2) and feedback (linkage group 12) controlling regions for the level of dihydrokaempferol.

Expression of FLS co-localises on linkage group 6 with the locus Q and RGB QTLs for carmine red, indicating a high flavonol content. The FLS locus itself was mapped on linkage group 14, in a region controlling F3H expression. This could be a feedback loop from flavonol levels to dihydrokaempferol synthesis. No expression of a key enzyme could be related with the locus W; unfortunately the best candidate for this, F3'H, failed in primer development in azalea. Similar to FLS, we postulate F3'H can control anthocyanin levels. ANS and DFR are occurring further in the chain and are also converting several substrates. Therefore, they are less obvious candidates to regulate the total level of anthocyanins.

References

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