Small deletion in a photosynthesis-related gene is involved in anthocyanin accumulation in the mesocarp of bf blood-flesh peaches

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Small deletion in a photosynthesis-related gene is involved in anthocyanin accumulation in the mesocarp of *bf* blood-flesh peaches

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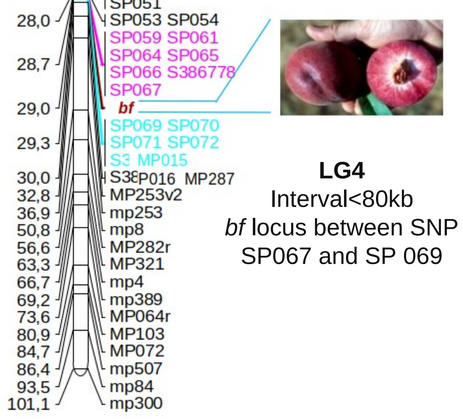
Little is known about mechanisms by which light controls anthocyanin biosynthesis in fruit-flesh. Here, we studied the molecular bases of a photosynthesis related gene segregating with the recessive blood-flesh trait (*bf*) in peach (*Prunus persica*). This gene is furthermore linked with red midrib of leaves and a reduced height of tree. For this study, a fine mapping (positional cloning) approach was coupled with a candidate gene strategy to gather a bundle of converging evidences [**A**]. The sequence polymorphism analysis of the genes present in this interval allowed the identification of only one candidate gene (CG) in the LG4 with a deletion of 21-bp in exon 3. A deletion KASP[tm] marker was designed in the CG and its link to *bf* phenotype validated in different genetic backgrounds [**B**]. A comparative RNAseq study [**C**] of fruit flesh from *bf* and *non-bf* cultivar was revealed co-expressed genes and this results was validated par qPCR [**D**].The cg was overexpressed in flesh of the *bf* cultivar compared to the *non-bf* cultivar and this overexpression was especially correlated with expression level of *HY5* (photosynthesis-related gene), *PpWD40* (regulatory gene) and *PpUFGT* (structural gene). Yeast two-hybrid analysis was performed to confirm physical interactions between proteins detected by RNAseq study [**E**].

A _ Genetic and physical mapping of the *bf* locus and candidate gene approach by NGS-Illumina sequencing



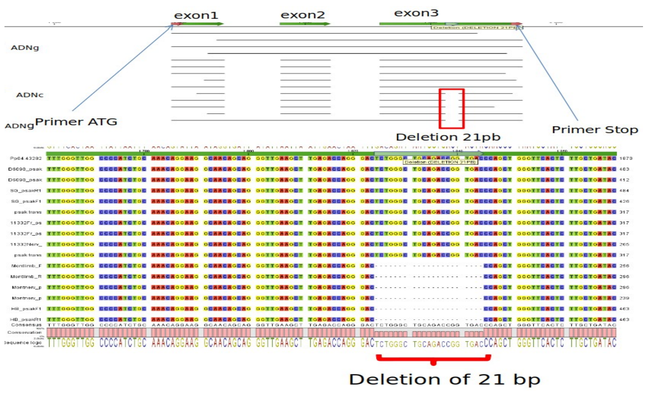
LG4
Interval<80kb
bf locus between SNP SP067 and SP 069

v.2 genes	Gene start position	Position of the variation	reference Lovell	Montarsa clone 7634	seq. quality
gene1	variation 2	aaa0060	GGACTCTGGGCTGCAGACCG GTGAC	GGAC	228
gene6	variation 3	aaa3602	TGAGAGAGAGAGAGAGAGAG AGAGAGAGAGAGAGAG	TGAGAGAGAGAGAGAGAGAGAG AGAGAGAGAGAGAG	21.5
gene8	variation 4	aaa4407	ATCTCTCTCTCTCTCTCTCTCT CTCTCTCTCTCTCT	ATCTCTCTCTCTCTCTCTCTCT CTCTCTCTATCTCTCTCTCTCTCTCT CTCTCTCTCTCT	157

1600 trees comprising a F2 segregating population dubbed 173² (cross O'Henry [non-blood-flesh] x Super Tardive [blood-flesh] [1,2]), at INRAE GAFL, were used to fine map the *bf* locus

- the bf region < 80kb included 8 predicted genes
- the predicted genes were checked in v.2 with Augustus (http://bioinf.uni-greifswald.de/augustus/)
- according to the annotation file, all were potentially good candidate genes for *bf*

B _ A 21 pb mutation is present in the candidate gene (cg) for all *bf* cultivars tested

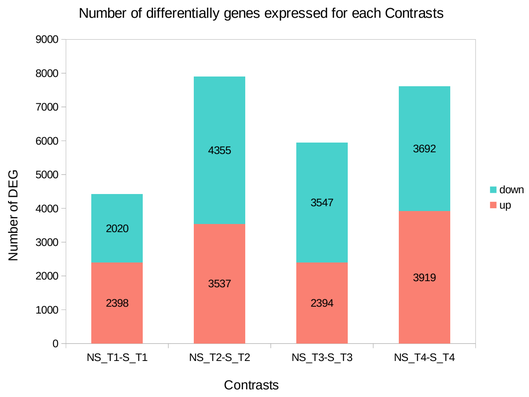


A bioinformatic analysis of the genomic sequence of the blood-flesh nectarine Montarsa® (NECTAVIGNE® Montarsa clone S7634) was performed to study the nucleotidic and proteic sequences of the 8 genes.

- among the 8 genes, 3 had a sequence polymorphism
- only 1 (CG) of the 3 genes had a variation (INDEL) in the coding sequence
- a deletion of 21 bp in exon3 was observed in CG by IGV software

A KASP[tm] marker was designed in CG and its link to *bf* phenotype validated on 2000 individuals

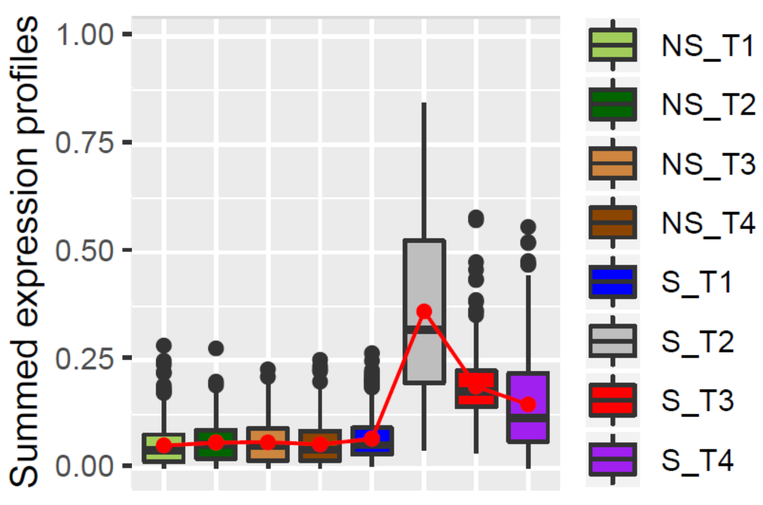
C_ RNA-Seq data generation and expression analysis



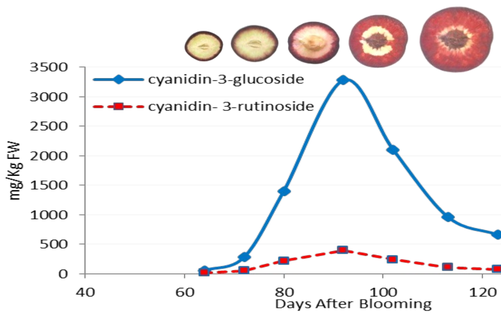
RNAseq study was carried out on flesh from 2 blood-flesh (*bf)* and non blood-flesh (non-*bf*) cultivars at 4 fruit development stages, from 60 days after blooming up to fruit maturity :
2 cultivars x 4 times (T) x 5 biological replicates (fruit lots) = 40 samples
We employed a RNAseq approach to collect genome wide expression data and identify differentially expressed genes.
40 librairies including biological and technical replicates were sequenced by Illumina plateform (GetPlaGe, Toulouse) which generated 145 Gbp
Data has been implemented using the Snakemake workflow and post analyses were performed by DiCoExpress pipeline [3]

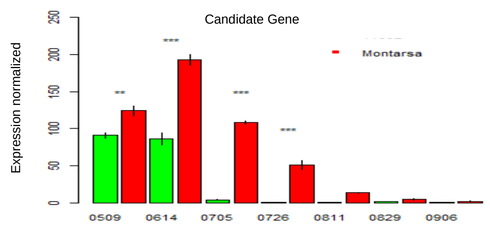
Histogram represent the number of genes differentially expressed for each contrast (non-*bf* (NS) versus *bf* (S)) studied at each fruit development stage (T1 to T4)

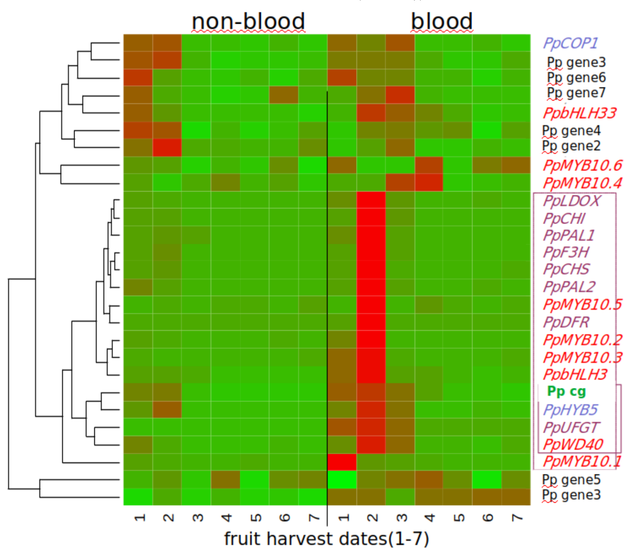
One cluster of genes, composed of 604 genes including the CG, is linked to the Photosystem I and II overexpressed at times 2 and 3 in MONTARSA (*bf*).
It followed the pattern of expression of the CG and of genes involved in the pathway of anthocyanin biosynthesis according to the stage of development fruit.



D _ Validation by qRT-PCR







We observed a similar variation between anthocyanin variation and expression level of CG

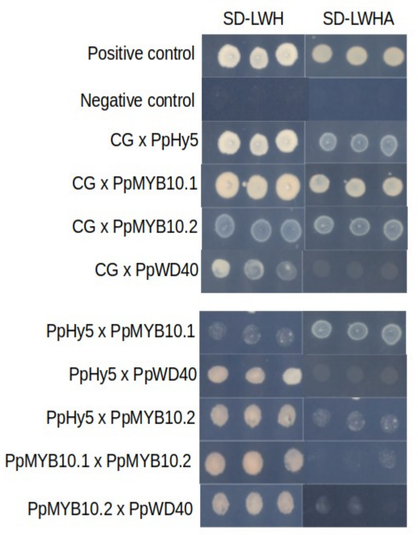
- accumulation of two anthocyanins into fruit flesh during fruit growth from 60 DAB to fruit maturity (extraction and identification by HPLC)

- expression level of CG in Montarsa (red) and non blood-flesh (green) cultivar according a kinetic of development of fruit from 60 DAB to fruit maturity

Elevation of anthocyanin levels was correlated with the expression of the CG, as well as with *PpMYB10,1,2,3,5* transcription factors in the mesocarp

- structural genes of anthocyanin biosynthesis pathway
- regulatory genes of anthocyanin biosynthesis pathway
- photosynthesis-related genes involved in anthocyanin biosynthesis
- CG
- other genes present in the bf region (<80Kb)

E _ Protein-protein interaction study



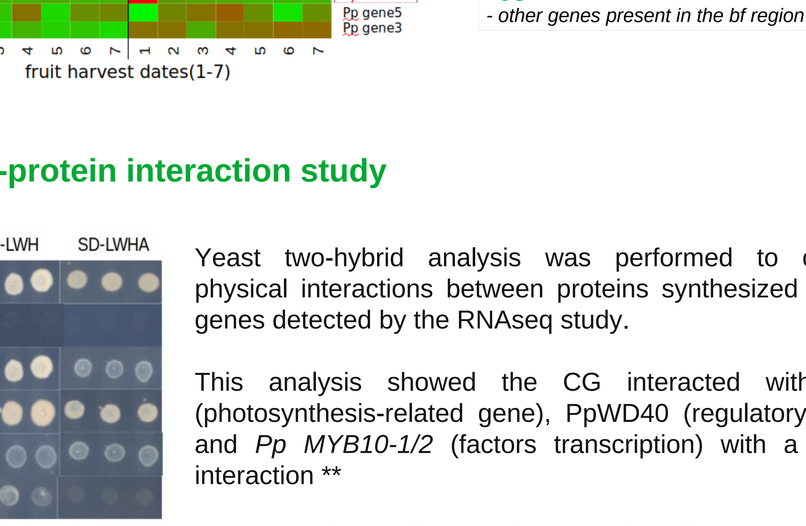
Yeast two-hybrid analysis was performed to confirm physical interactions between proteins synthesized by the genes detected by the RNAseq study.

This analysis showed the CG interacted with HY5 (photosynthesis-related gene), PpWD40 (regulatory gene) and *Pp MYB10-1/2* (factors transcription) with a strong interaction **

We also observed *HY5* interacted with *MYB10.1* and *MYB10.2* and with *pPWD40* with a less strong interaction than CG *
PpWD40 interacted with *MYB10.2*.

** LWH = medium without Leucine, Histidine and Tryptophan to interaction*
*** LWHA = medium without Leucine, Histidine, Tryptophan and Adenin, specific to strong interaction*

Hypothesis of regulation genes



Conclusion

We observed an accumulation of anthocyanins in mesocarp and midvein of blood-flesh genotypes. Fine mapping and resenquencing showed that a small deletion in a unique candidate gene was linked to anthocyanin accumulation in the mesocarp of *bf* blood-flesh peaches. We validated the deletion KASP[tm] marker (diagnostic marker) in various backgrounds. In the RNAseq study, we observed an over-expression of CG in the mesocarp and midvein of blood-flesh cultivars compared to non blood-flesh cultivars (Lovell for example). In addition, we found a cluster of genes linked to the Photosystem I and II overexpressed in times 2 and 3 in blood-flesh, which corresponds to the peak of synthesis of anthocyanins in the mesocarp of *bf* cultivars. The protein-protein interaction study confirmed the links between the product of the CG and the product of the photosynthesis-related genes and of transcription factors.

[1] Chaparro et al. (1995). Inheritance, genetic interaction and biochemical characterization of anthocyanin phenotypes in peach. *J Hered* 86: 32-36.
[2] Werner et al. (1998). Inheritance of the blood-flesh trait in peach. *Hortscience 33(7):* 1213-1216.
[3] Lambert et al. 2020. DiCoExpress: a tool to process multifactorial RNAseq experiments from quality controls to co-expression analysis through differential analysis based on contrasts inside GLM models. *Plant Methods*, 16:68-68.