PUBLISHABLE SUMMARY

Genetic and physiological regulation of skin colour development on apples under high temperature environments: genetic tools for developing heat tolerant red-skinned apples

Grant Agreement number: 295146

Period: 1st March 2012 – 28th February 2015, extended to 31th May 2015

Acronym: REDHOTGEN-2

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Project partners: FEM-IASMA (Italy) and Plant & Food Research (New Zealand)

INTRODUCTION

Major apple producing regions are located in warm areas of South European countries, such as the Ebro Valley (Northern Spain), Emilia Romagna (Italy), Midi Pyrénées and Languedoc-Roussillon (Southern France). Spain is the country where almost all the apple production is located in warm regions and this negatively affects the fruit quality obtained from economically significant cultivars such as 'Golden Delicious' (lack of firmness) and bicoloured cultivars such as 'Gala' and 'Fuji' (lack of skin colouration). The lack of apple's adaptation to warm temperature has caused a dramatic decrease of production in Spain from 1.200 k ton to 430k ton in the 1985-2014 period. Poor coloured fruit is perceived by the consumer as a lack of quality. In those warm climates hot summer temperatures cause a lack of colouration of apple skin. Consequently, the economic return to the fruit growers can be reduced by as much as 60% compared to the premier class apples. The most efficient way to counterattack these adverse climatic conditions is to obtain new selected apple cultivars adapted to this particular environments able to develop high skin colour in warm and hot climates.

OBJECTIVES

REDHOTGEN 2 **"**Genetic and physiological regulation of skin colour development on apples under high temperature environments: genetic tools for developing heat tolerant red-skinned apples", is a project approved by the EU Commission running from 1st March 2012 to 28th February 2015 extended to 31th May 2015. This project is a prolongation of the activities started in REDHOTGEN (carried out over the period 2009-2012).

The specific science objectives are to gain knowledge on the genomics of the interaction of genetic (cultivar, strain) and environmental (carbon balance and temperature) influences in the regulation of the anthocyanin pathway in apple skin. A second objective is to develop

genetic markers and implement them for marker assisted selection (MAS) in a breeding programme for new apple cultivars that can be produced under high temperatures and are characterized by the stable red skin colour and high nutraceutical content desired by consumers. The global aim of the project is use this example of the practical application of genomics delivering economic benefits to the EU as a "springboard" for further applications of this technology in an era of climate change. This project is a multidisciplinary initiative allowing the exchange of scientists among 3 research institutes with international recognition in fruit tree biology. This is a unique opportunity to ally the institutes and combine participant's skills in plant genomics, biotechnologies, plant physiology, postharvest and apple and pear breeding towards a unique outcome.

RESULTS ACHIEVED

The results obtained during the development of Redhotgen and Redhotgen-2 projects have enabled us to better understand the response of apple skin to warm temperatures. We studied the physiological control of skin coloration by comparing skin colour of the same cultivars, with low and high potential to develop red colour, grown in different environments: New Zealand (cool summer) and Spain (warm summer). We demonstrated that anthocyanin concentration in the apple fruit skin is affected by warm summer temperature. This was confirmed by *in vivo* warming fruit in New Zealand and cooling fruit in Spain. We made a complete inventory of the genes coding for the enzymes involved in the anthocyanin synthesis pathway, as well as the genes regulating these enzymes using the apple genome sequence. We paid special attention to regulatory genes as they are likely to respond to environmental conditions and then up- or down-regulate the enzymes that synthesize pigments. We studied the gene expression of such synthetic enzymes and regulatory genes in a range of cultivars with contrasting red skin colour patterns, as well as cultivars grown under warming (in NZ) and cooling (in Spain) conditions. Our results extended to two apple cultivars with different genetic control of fruit colour development ('Gala' and 'Braeburn') demonstrated that the anthocyanin biosynthetic and regulatory genes are down regulated by warm temperature. The effect of heating (in New Zealand) or cooling (in Spain) 'Braeburn' apples on gene expression of the genes involved in the anthocyanin biosynthesis pathway is illustrated in **Figures 1a 1b** and **Figures 2a, 2b**. Also the effect of cooling on RNA expression of the *MYB* repressors was determined.

Evaluating new sources of red skin colouration and GWA analysis

In REDHOTGEN project we constructed a genetic map using a population segregating for skin colour from the joint IRTA-PFR pipfruit breeding, and identified genomic regions (Quantitative Trait Loci, QTLs) that are linked to the red colouration. The QTL that explained most of the variability in in skin colouration was detected at the bottom of LG 9, where a gene coding for a regulator of the anthocyanin biosynthesis pathway enzymes is located (*MYB10*). The association between a marker located close to *MYB10* and skin colouration was confirmed in a wider set of apple germplasm.

In REDHOTGEN2 project new sources of red skin colouration, other than the ones derived from sports of 'Gala', were investigated using a Genome Wide Association Study approach

(GWAS). GWAS is a method of mapping QTLs that uses the association between markers and a phenotype of interest that has been scored across a large number of individuals (i.e. germplasm collection). GWAS can map QTLs with high resolution as it the advantage of a wider genetic pool. GWAS has been made possible in the project by the recent development of high-throughput genotyping tool in apple, the Illumina Infinium 20K SNP chip.

Skin colour phenotyping was carried out in 2013 and 2014 on fruit harvested from different apple genotypes planted at Plant & Food Research orchards in Hawke's Bay, New Zealand. Plant material included underdeveloped (raw) germplasm, and elite breeding material together with some commercial cultivars. A wide variation in colour phenotype was found for all measured variables to determine fruit colour. There was no difference between the frequency distribution of red colour coverage between raw and elite germplasm (see **Figure 3**).

The same genotypes were characterized with the Illumina Infinium 20K SNP chip. GWAS analysis was performed using a set of 10K robust single nucleotide polymorphism (SNP) markers that cover the apple genome at a very high-density (1 SNP every 58 Kbp)every 58 kilobase pair (kb). The association between these markers and red skin phenotype (coverage, intensity, colorimetric value, and anthocyanin concentration) was calculated using the TASSEL and GAPIT software. Surprisingly, only one locus at the bottom of chromosome 9 was found associated with red skin colouration across the apple germplasm (see **Figure 4**). This locus co-locates with the *MYB10* candidate gene and with the QTLs previously detected. This indicates that there is no other locus other than *MYB10* involved in the genetic control of red skin colouration in apple.

Marked assisted breeding

The *MYB10* gene is linked to a QTL that was identified using the A121R18T089 x Gala segregating population in REDHOTGEN project (2009-12). We identified a SNP marker close to *MYB10* using the Golden Delicious genome assembly (SNPs located on LG9 at positions 32,822kb and 31,964kb). The SNP markers for *MYB10* was validated in the IRTA breeding populations using a Taqman assay. Phenotypic data for red skin colouration and SNP genotypes were obtained usingfive validation families planted at IRTA plot (Gimenells, Spain).

The SNP marker located at position 31,964 kb segregated in the 5 validation populations and was linked to all parameters measured for red skin colouration. The marker is predicting extremely well the red skin phenotype, with AA genotypes being mostly non red, AB intermediate red colouration and BB intensely red (**Figure 5**). The new marker had different segregation patterns and ratio in the 5 populations (PM91 AB x BB, PM92 AB x AB, PM94 AB x AB, PM95 BB x AB and PM96 AA x AB), however the linkage was confirmed in all populations (see **Figure 6**).

Summary and conclusions

Genetic markers linked to red skin colouration in apple have been identified, including markers located close to *MYB10*, a gene controlling the anthocyanin synthesis pathway and responding to warm temperature. It is likely that allelic variants of *MYB10* are less sensitive to warm temperature. Such alleles can be selected in breeding populations to enable the identification of apple seedlings that are resistant to warm summer temperature producing red colour on the skin.

In REDHOTGEN and REDHOTGEN 2 projects, a genetic marker derived from *MYB10* has been developed and tested on breeding populations to validate its efficiency as a tool for faster breeding using marker-assisted selection. These from give us a good confidence for using MAS for selecting for red skin in apple breeding programmes. The genetic markers developed and validated in Spanish breeding populations can predict the phenotype very accurately, even under challenging warm summer temperatures. We recommend that the markers we developed be used for faster and more efficient breeding, for example for screening seedlings and predicting their skin colour years before they bear fruit, or for genotyping parents and elites and predict the performance of the breeding populations. In fact, we have already used the REDHOTGEN 2 outcomes (genetic marker) for MAS in the Plant & Food Research breeding programme, and we will start using the same marker in the Spanish and Italian breeding programmes in 2015-16.

We expect that the information gained in this project will be in the future a useful tool for breeders to improve the efficiency of breeding programs focusing on the development of new apple cultivars with high potential of fruit colour development even in warm and hot climates typical of Mediterranean areas.

PROJECT CONTACT DETAILS

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FIGURES

Figure 1a. Different views of the heating experience in Motueka (New Zealand) in March 2014. Top left: the heater to supply hot air to the apples. Top right: heated apples ('Braeburn'). Bottom: 'Braeburn' control apples (left) and heated apples (right), with different colour expression.

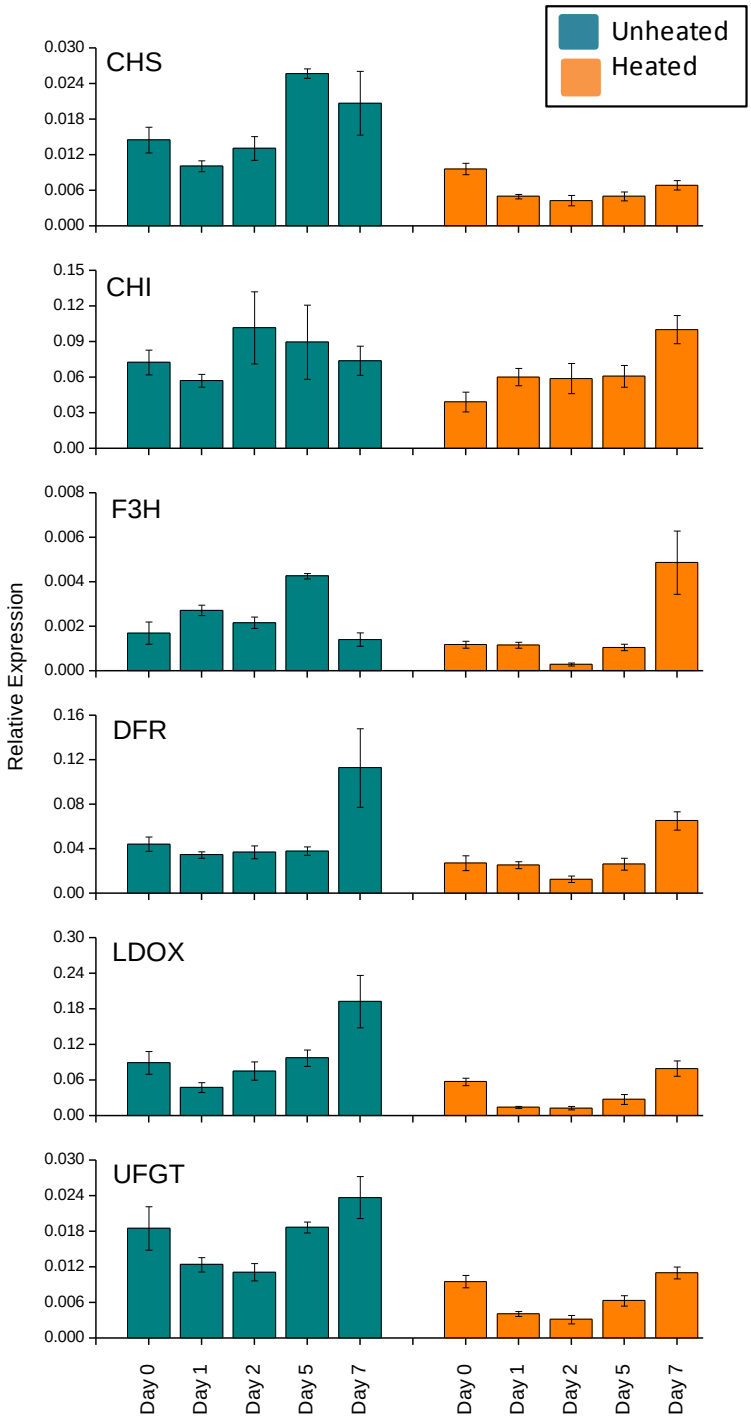

Figure 1b. RNA expression of *CHS, CHI, F3H, DFR, LDOX and UFGT* genes using real-time quantitative PCR analysis in the experiment of heating 'Braeburn' apples, summer 2014 (Nelson, New Zealand). Control (blue bars) and heated (orange bars).

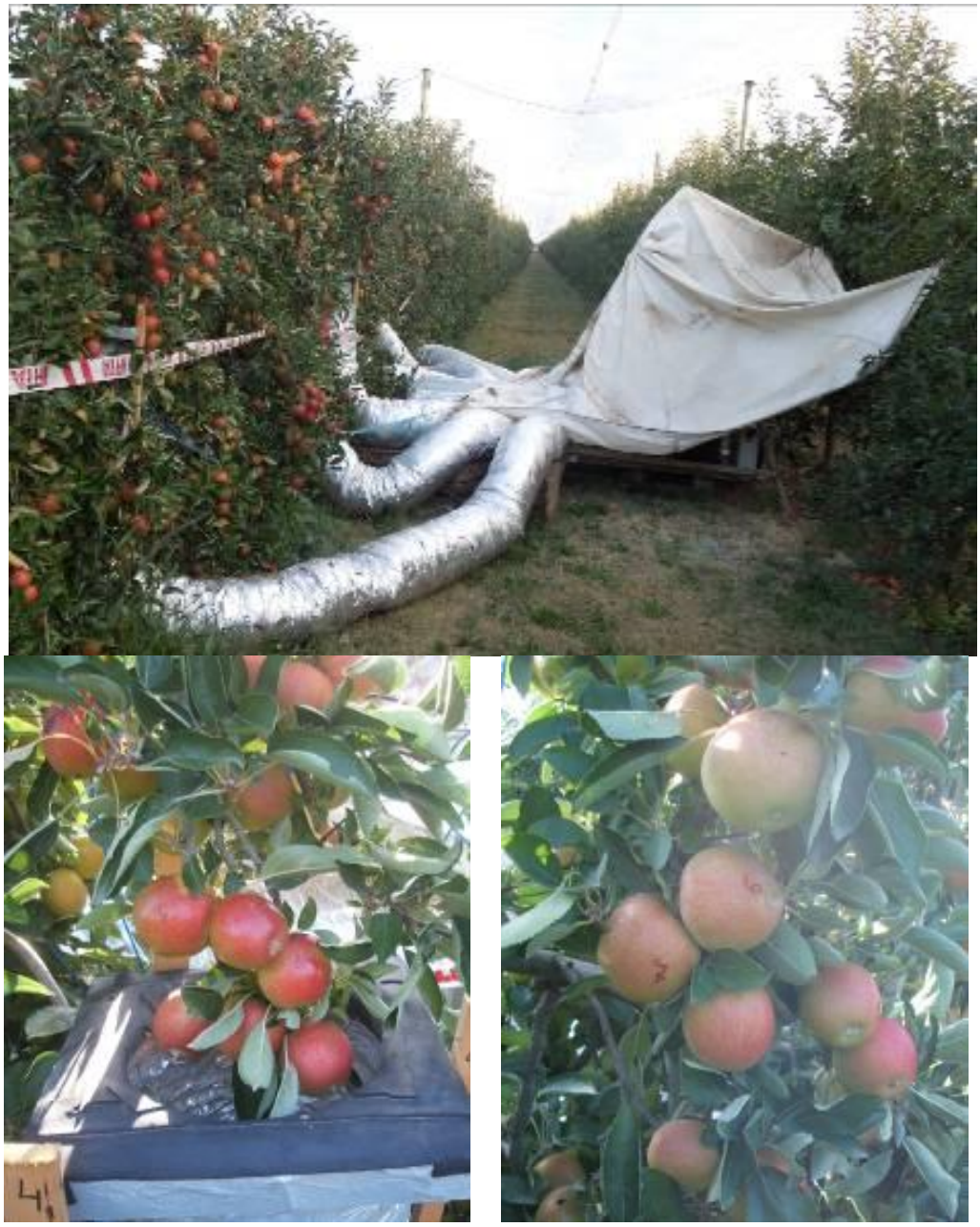

Figure 2a. Different views of the cooling experience in Mollerussa (Spain) in September 2014 (top). The effect of cooling on fruit colour development. On the bottom left 'Braeburn' cooled and right control apples on 12th September 2013.

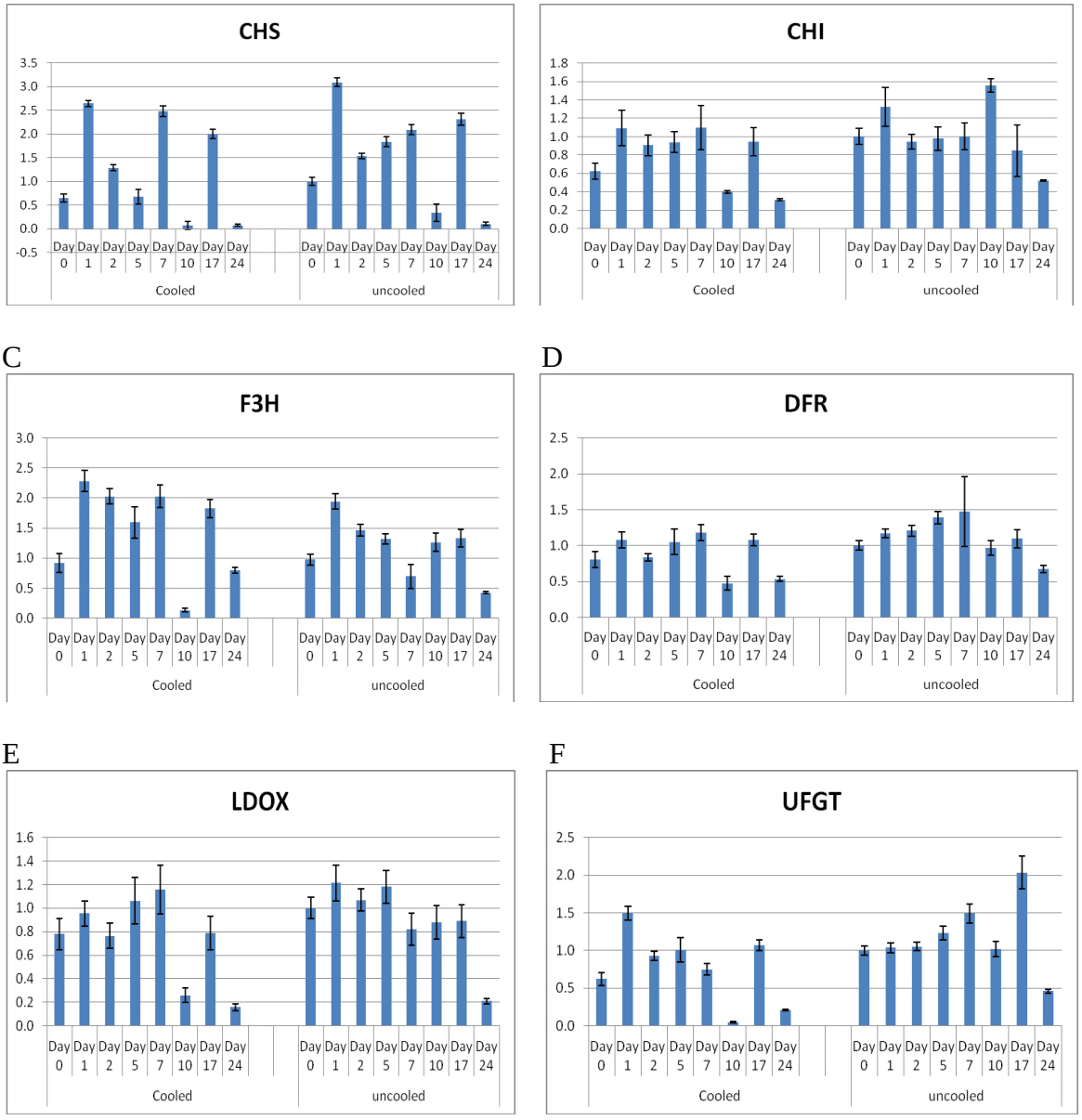

Figure 2b. RNA expression of *CHS, CHI, F3H, DFR, LDOX and UFGT* genes using real-time quantitative PCR analysis in the experiment of heating 'Braeburn' apples, summer 2013 (Mollerussa, Spain). Cooled samples on the left, un-cooled on the right.

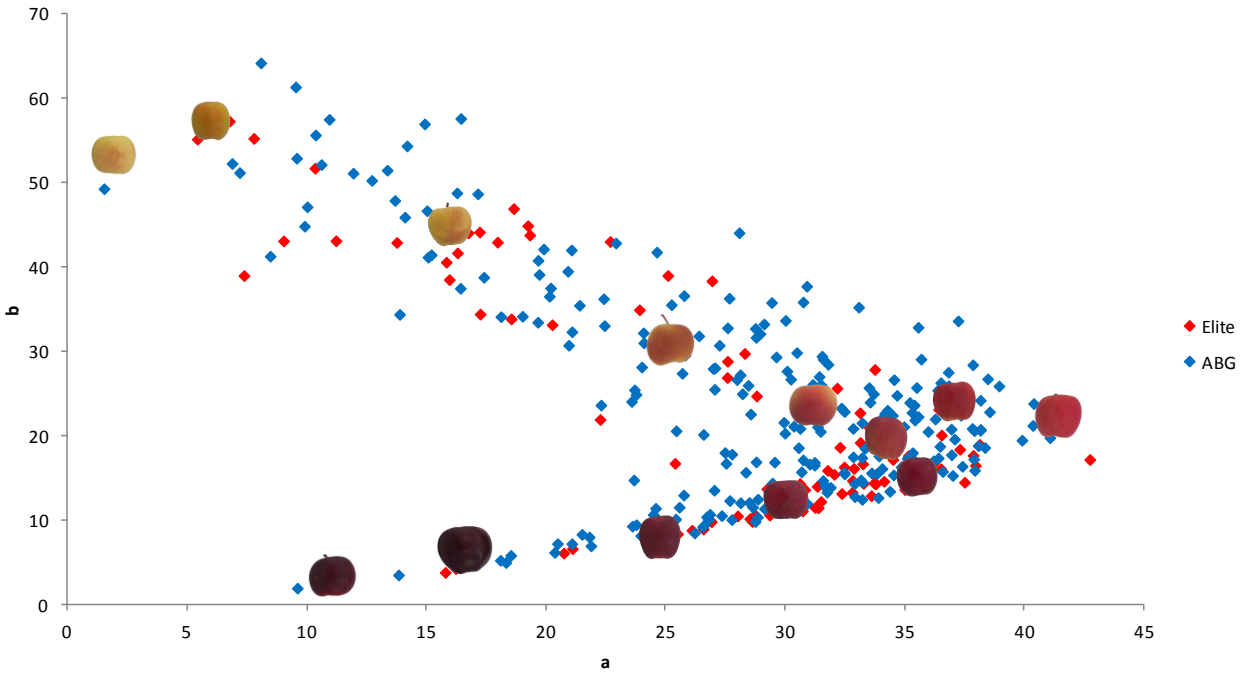

Figure 3. Plot of a* (increasing red) vs b* (increasing yellow) for fruit skin overcolour measured on raw (ABG) and elite apple germplasm in Hawke's Bay, New Zealand (2013). Each point represents a single genotype.

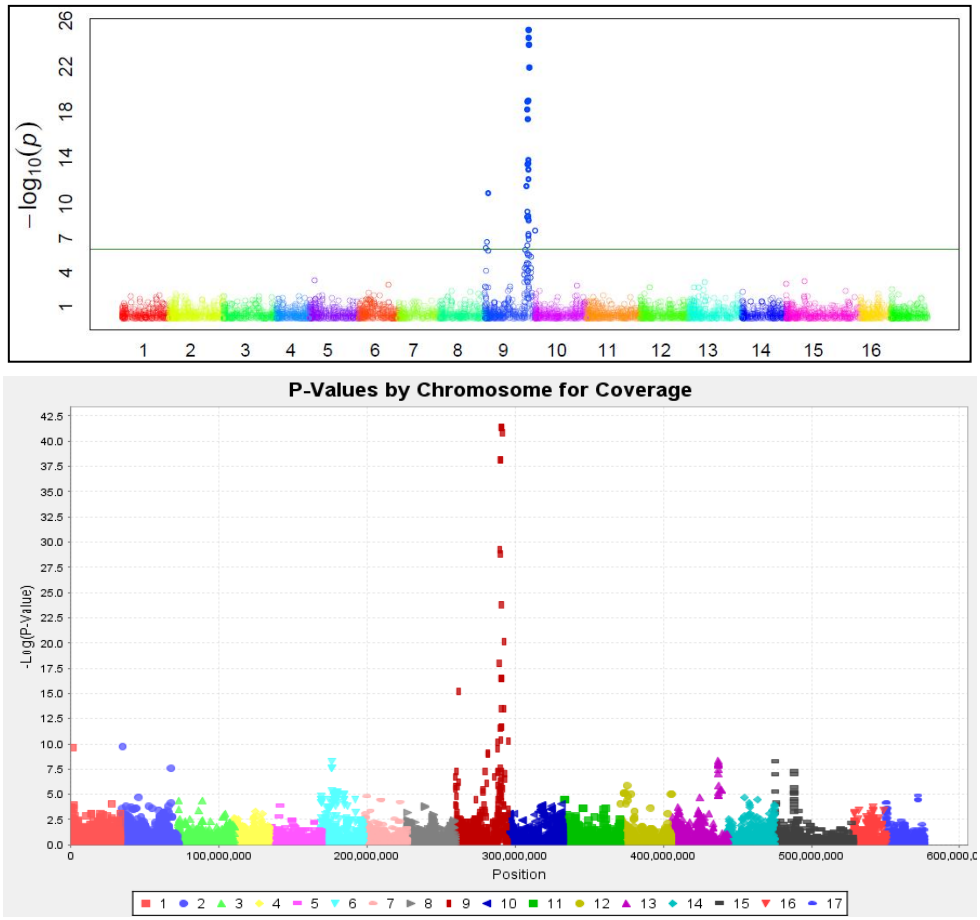

Figure 4: GWAS analysis between 10,236 genome wide SNP markers and red skin fruit coverage using the GAPIT (top) TASSEL (bottom) software. Using both analysis a significant association was only found for markers at the lower end of chromosome 9.

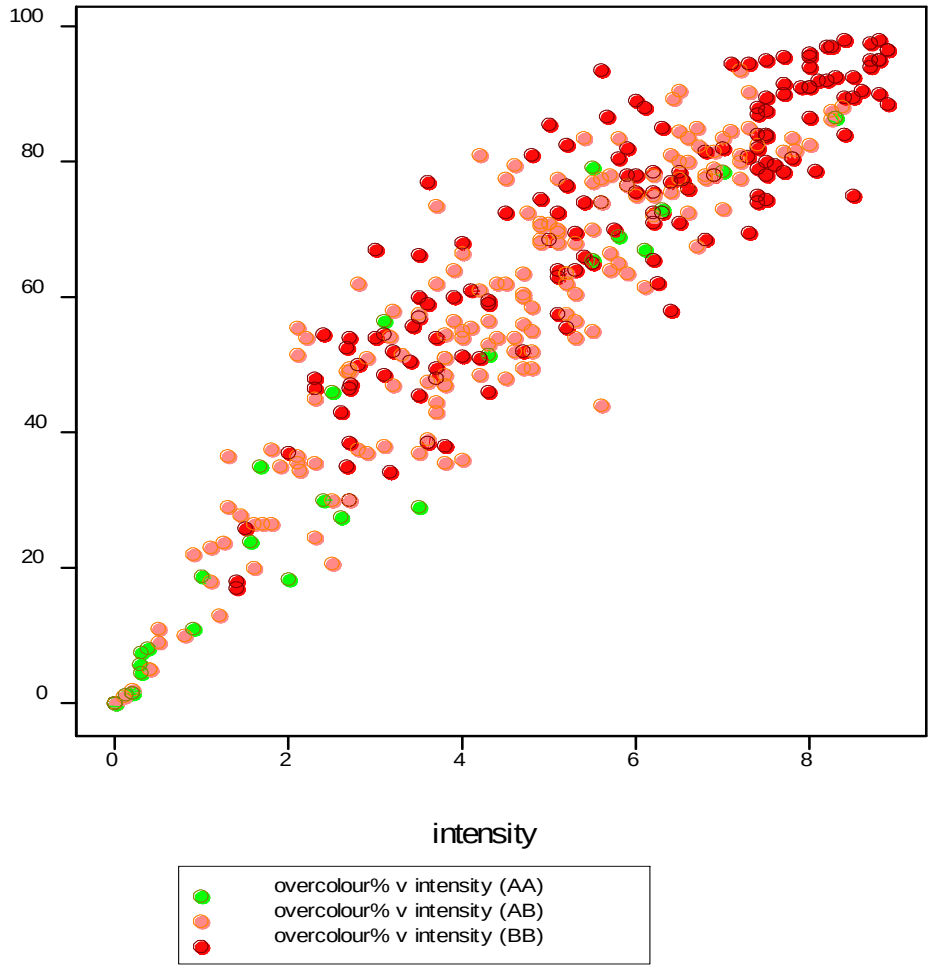

Figure 5. Genetic marker performance for red skin colouration in 5 segregating populations. Red skin is measured as % overcolour and intensity. Phenotypic data developed during 2011 and 2012.

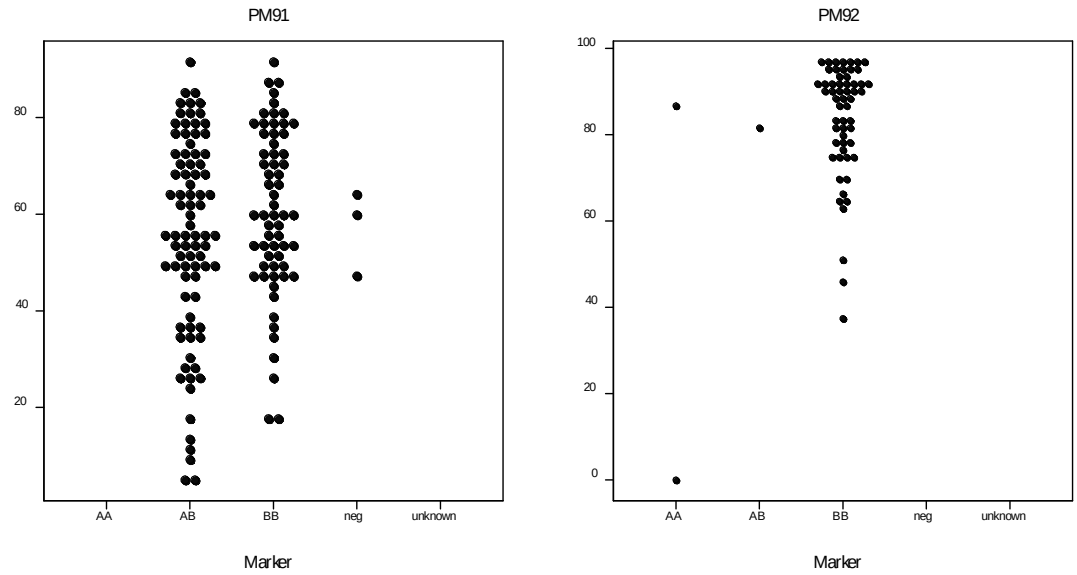

Figure 6. Red marker segregation in two validation families (Table 1) for % overcolour phenotype. One family is not presented due to too few individuals. Phenotypic data developed during 2011 and 2012.