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Project number:	CP 094
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[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

- Genetic markers have been identified which confer fruit firmness and which can be used in marker assisted strawberry breeding.

Background and expected deliverables

Strawberry is one of the most economically important fruit crops and it is essential to maintain the profitability and sustainability of this crop. Today, strawberry growers face increased production challenges, such as maintaining yield, fruit size and good fruit quality. These traits rely on good plant architecture and high levels of pest and disease resistance. In order to maintain competitiveness, the extension of cropping season and better adaptation to the particular growing environment is essential.

Strawberries produced in the UK are sold both as fresh fruit and for the processing market. Strawberry cultivars exhibit clear differences in fruit firmness. Soft fruits are not desirable for commercial production. Firmness is an important issue for commercial production so it is a major strawberry breeding target.

The objective of this study is to identify quantitative trait loci (QTL) linked to traits of major breeding targets, in this case fruit firmness in cultivated strawberry (Fragaria × ananassa). QTL regions and associated molecular markers can significantly improve the breeding efficiency of complex traits through marker-assisted breeding (MAB). However, the development of these molecular markers is usually difficult due to the complexity of the traits and environmental factors affecting QTL stability.

This project aims to cut the cost of breeding by developing novel molecular markers linked to fruit quality and disease resistance in cultivated strawberry. An example study of QTL mapping and a validation of closest linked molecular markers to QTLs responsible for fruit firmness in cultivated strawberry is reported here.

Summary of the project and main conclusions

A mapping population derived from the cross ‘Redgauntlet’ × ‘Hapil’ was used for a number of purposes:

- Phenotypic data collection over a period of 3 years (2013, 2014 and 2015)
- Linkage map construction and saturation

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- Quantitative trait loci (QTL) detection
- Validation of the molecular markers linked to fruit firmness

As previously reported in the Year 1 report, 30 different traits of ‘Redgauntlet’ × ‘Hapil’ population were recorded and correlation analysis was conducted based on three-year phenotypic data. The saturation of the existing SSR-based (simple sequence repeat) linkage map was also previously reported in the Year 2 report, however a novel SNP-based (single nucleotide polymorphism) genetic linkage map was developed and used for QTL mapping associated with traits recorded.

This report focuses mainly on the QTL identification linked to fruit firmness using the improved SSR-based genetic linkage map and validation of the closest linked SSR markers. A total of eight potential QTLs associated with fruit firmness were identified. This information led to the identification of the 16 SSRs closest linked to the loci, which were selected and screened in a wider strawberry germplasm. Four SSRs were identified showing most significant results in predicting strawberry cultivars associated with fruit firmness. These markers are therefore potentially suitable to be used in marker-assisted breeding.

In addition, the physical locations between previously reported expansin genes and strongest associated SSR markers were compared, in order to investigate whether QTLs linked to fruit firmness were overlapping expansins. The expansin gene family has been reported to be controlling fruit softening and firmness (Dotto et al. 2006).

Finally, the combined analysis of QTLs associated with plant characteristics, fruit quality, *Verticillium* wilt and powdery mildew resistance was investigated in cultivated strawberry. These results are not presented in this report. The majority of traits analysed were mapped to different locations and no consistent overlapping chromosomal regions were detected among the traits analysed. Only a single overlapping region between *Verticillium* wilt QTLs and fruit quality trait were identified. These findings suggest that breeding for different fruit quality and disease resistance traits is independent and traits are less likely to be dependent on each other.

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Financial benefits

There are no direct financial benefits to be gained by growers from this type of work. However, the results will be adopted by the East Malling Strawberry Breeding Club in its work to deliver improved strawberry fruit quality to the industry.

Action points for growers

- No direct action points for growers have arisen from this project.

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SCIENCE SECTION

Introduction

Strawberry fruits are mainly sold for the fresh market, as well as for the food processing industries worldwide. Fruit firmness is important for consumers and therefore directly influences producers, suppliers and commercial retailers. In addition, firmness is an essential attribute for fruit production and includes easier harvesting, handling and storage (Badenes, 2012). Firm fruits usually have better fruit qualities than soft fruits, and include better postharvest storage. This has been reported in a number of stone and soft rosaceous crops including cherry (Kappel, 2008; Sansavini and Lugli, 2008; Oraguzie, 2010), apple (Johnston et al. 2002; Rocha and Morais, 2003; Zude et al. 2006), peach (Gorny et al. 1999; Manganaris et al. 2007), pear (Gorny et al. 2000) and strawberry (Vicente et al. 2002). Furthermore, the disadvantages of soft fruits include increased pathogen susceptibility, significantly reduced postharvest shelf life and quality loss of fresh fruits (Dotto et al. 2006). As a result, breeding for fruit firmness in strawberry is a major goal.

Fruit firmness is a complex trait and is associated with the fruit ripening process. The process involves multiple developmental factors and includes environmental signals, age, auxins, enzymes and proteins controlling modification of cell wall and fruit maturity (Civello et al. 1999; Harrison et al. 2001). Fruit ripening changes not only fruit firmness, texture, flavour and coloration but also increases the susceptibility to microbial infections (Harpster et al. 1998). It has been reported that fruit firmness constantly declines throughout the developmental process of the fruits and is mainly due to the cell wall changes (Harrison et al. 2001). This involves the changes in the composition and the structure of the cellulose microfibrils, polysaccharides and structural proteins located within the cell wall (Carpita and Gibeaut, 1993; Harpster et al. 1998). Indeed, for a plant to grow, the cell wall must continuously modify and expand. According to Knee et al. (1977), the cell volume can increase up to 1000-fold during the plant grow stages by the extreme swelling of the cell walls resulting in the fruit softening.

A group of genes, named expansins, associated with fruit softening have been identified. Expansins are present in cell walls and are linked to cell wall expansion,

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loosening and metabolism, as well as in changes in the ripening fruit tissue (Harrison et al. 2001; Dotto et al. 2006). These genes, encode proteins and are expressed during fruit ripening stages (Dotto et al. 2006).

In strawberry, seven expansin genes, named *FaEXP1* to *FaEXP7*, regulating fruit softness have been reported (Civello et al. 1999; Harrison et al. 2001; Dotto et al. 2006). Of those, two genes (*FaEXP2* and *FaEXP5*) are fruit specific and regulate ripening, resulting in increased expression of these genes during the fruit ripening stages (Dotto et al. 2006). The remaining five genes (*FaEXP1*, *FaEXP3*, *FaEXP4*, *FaEXP6* and *FaEXP7*) are expressed in other plant tissues such as leaves, roots and runners, and thus are thought to be not fruit specific (Civello et al. 1999; Harrison et al. 2001; Dotto et al. 2006). Indeed, the study of Dotto et al. (2006), showed that although *FaEXP1* gene is expressed in fruits but the highest mRNA accumulation was found in strawberry roots. Harrison et al. (2001) demonstrated that genes *FaEXP3* and *FaEXP4* are expressed in leaves, runners and roots during fruit development, whereas the expression of genes *FaEXP6* and *FaEXP7* is enhanced in unripe fruits and these genes control the transition of ripening. Interestingly, three expansins (*FaEXP1*, *FaEXP2* and *FaEXP5*) showed correlations between their mRNA expression level and fruit firmness, confirming the function (fruit softening and firmness) of the expansin gene family (Dotto et al. 2006). Moreover, these findings also showed that although not all of the expansin genes are fruit specific, these genes might be all involved in fruit softening, especially due to the fact that they belong to the expansin gene family. This suggests that expansin genes have similar functions and were formed by duplication of an original gene.

The aims of this study were (1) to investigate the transferability of SSR markers identified significantly associated with fruit firmness QTLs, and (2) to determine whether previously reported expansin genes are underlying the QTL regions linked to firmness. The validation analysis was performed using a wider cultivated strawberry germplasm producing strawberry fruits of different known firmness level. Four SSRs were identified showing most significant results in predicting strawberry cultivars associated with fruit firmness.

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In addition, mRNA sequences of seven expansin genes (*FaEXP1*, *FaEXP2*, *FaEXP3*, *FaEXP4*, *FaEXP5*, *FaEXP6* and *FaEXP7*) were obtained from the on-line available database and were aligned to the diploid *Fragaria* reference genome sequence. Of those, three genes (*FaEXP1*, *FaEXP2* and *FaEXP5*) were the main focus in this study because these genes were reported to be correlated with fruit firmness in cultivated strawberry (Dotto et al. 2006), although the positions of the remaining genes were also investigated. The aligned gene positions were compared to the positions of the closest linked SNP markers underlying QTLs linked to firmness.

Materials and methods

Plant material

The F_1 mapping population used in this study was raised in the glasshouse from a cross between the two octoploid strawberry (*Fragaria* × *ananassa*) cultivars 'Redgauntlet' and 'Hapil' as previously reported in Year 1 report.

A total of 188 seedlings were raised from the cross and of those 120 seedlings randomly were selected and further clonally propagated twice (once during summer 2012 and once during summer 2014) by pinning down the runners of the mother plants. A total of six replicates of the 122 seedlings and parental genotypes ('Redgauntlet' and 'Hapil') were produced, thus 732 plants (including parents) were planted in the open field at East Malling Research in late September 2012 and mid-August 2014 and were used for phenotypic data collection over the three consecutive years (2013, 2014 and 2015).

The field trial plots were covered with polyethylene while plant phenotyping was on-going; this was later (late July) removed in order to avoid disease. Irrigation system was installed in each row, and plants were watered and fertilized following conventional practices and depending on weather conditions. Plants were sprayed against common pests (aphid), insects (spotted wing drosophila) and diseases (mildew and botrytis) before, during and after the phenotyping season. The spraying programme for the season was as follow: once a week for 23 weeks for mildew (March - September), once a week for ten weeks for botrytis (May - September), a single spray for spotted wing drosophila (in August) and five sprays for aphid (March - June).

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Strawberry germplasm for marker transferability analysis

An octoploid strawberry cultivars, selections and a number of individuals of 'Redgauntlet' × 'Hapil' (Rg × H) mapping progeny were selected for the validation of molecular markers linked to fruit firmness QTLs. Cultivars were chosen to represent firm and soft variability among the material tested. The sample set contained 16 plants representing fruit softness (3 cultivars, 2 selections and 11 progenies of the Rg × H population) and 17 plants representing fruit firmness (10 cultivars, 1 selection and 6 individuals from the Rg × H progeny). Strawberry samples selected and tested in this study are summarised in Table 1.

Table 1. The list of strawberry plants selected for the transferability analysis of SSR markers. Plants were selected producing either soft or firm fruits. Names of the individuals of 'Redgauntlet' × 'Hapil' mapping progeny starts with two capital letters 'RH', followed by three numbers

Soft fruits		Firm fruits	
1.Earliglow	9.RH105	1.Albion	9.Seascape
2.Gorrela	10.RH115	2.Argentera	10.Selva
3.Osmanli	11.RH122	3.Buddy	11.SDBL122
4.EM1792	12.RH130	4.Diamante	12.RH050
5.P85	13.RH153	5.Elegance	13.RH135
6.RH006	14.RH158	6.Flamenco	14.RH137
7.RH051	15.RH164	7.Florence	15.RH163
8.RH093	16.RH179	8.Holiday	16.RH168
			17.RH061

Firmness

Fruit firmness of individuals from Rg × H mapping population was evaluated by measuring up to 10 marketable fruits using Firmtech (Umweltanalytische Produkte GmbH) in g/mm (the values observed were the force which is required to deflect the fruit by 1 mm) (Figure 1). The level of fruit firmness for the strawberry cultivars and selections were already known from the phenotypic data collected from the on-going different strawberry breeding projects at East Malling Research (EMR).

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Figure 1. Harvested strawberry fruits and measuring fruit firmness using Firmtech.

Linkage map development

The saturation of the existing SSR-based genetic linkage map was previously reported in Year 2 report. This linkage map was further re-constructed using different parameters in JoinMap 4.0 (Kyazma, NL) software applying the Haldane mapping function. Novel SSR markers were mapped by analysing a single linkage group at a time. The parameters used for map construction were modified using a more stringent analysis and were as follows: a minimum of a logarithm of the odds (LOD) score of 1.0 used to assign markers to linkage groups and a maximum recombination fraction of 0.4, goodness-of-fit jump threshold of 5.0 and a triplet threshold of 1.0. Markers exhibiting segregation distortions were identified applying the Chi-square (X^2) test. Graphical presentation of an improved SSR-based genetic linkage map of the Rg ×H progeny consisting of 28 linkage groups was generated using MapChart version 2.2 software (Voorrips, 2002). The SSR-based genetic linkage map was used for QTL analysis in this study.

In addition, a novel consensus SNP-based genetic linkage map containing a total of 3,933 binned unique markers over the 28 linkage groups of the 'Redgauntlet' × 'Hapil' mapping progeny was developed using high-throughput ISraw90® genotyping array.

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QTL identification linked to fruit firmness using SSR and SNP linkage maps

The mean values of 30 plant characteristics and fruit quality traits were used for the analysis. A consensus SSR-based linkage map was used for QTL mapping linked to all phenotypic data collected. QTL identification was performed for all phenotypic traits for each year individually using MapQTL® 5 software package (Van Ooijen, 2004), although QTLs associated with fruit firmness is presented in this report only.

As a result of phenotypic means not being normally distributed after the performance of two types of transformations (log and square root), the non-parametric test of Kruskal-Wallis (KW) was used for the identification of significant associations between phenotypic traits and molecular markers. The KW test sorts individuals according to the single-dose quantitative trait loci and classifies them according to their marker genotype (van Ooijen, 2004). The closest SSR markers linked to QTLs were identified based on the significance level and the KW test statistics (K^*) value.

QTLs linked to fruit firmness graphically were presented on the linkage map using MapChart 2.2 software (Voorrips, 2002).

The same parameters, which were discussed above, were used for the identification of QTLs associated with fruit firmness using SNP-based linkage map. These QTL regions were used for the identification of the closest SNP markers and the comparison of their physical locations with expansin gene physical locations.

Expansin genes and mRNA alignment to the *Fragaria vesca* genome sequence

Seven mRNA sequences of the expansin genes were obtained from the National Center for Biotechnology Information (NCBI) database. These sequences were aligned to the diploid *Fragaria vesca* genome sequence, in order to identify physical locations of the genes. The BLAST (Basic Local Alignment Search Tool) function was applied to compare the mRNA gene sequences to the *F. vesca* genome sequence, and was performed using the on-line available genome browser - The Plant Genome portal (Phytozome 10.3) (http://phytozome.jgi.doe.gov/pz/portal.html). An example of the aligned three expansin gene mRNA sequences against *F. vesca* genome is presented in Figure 2.

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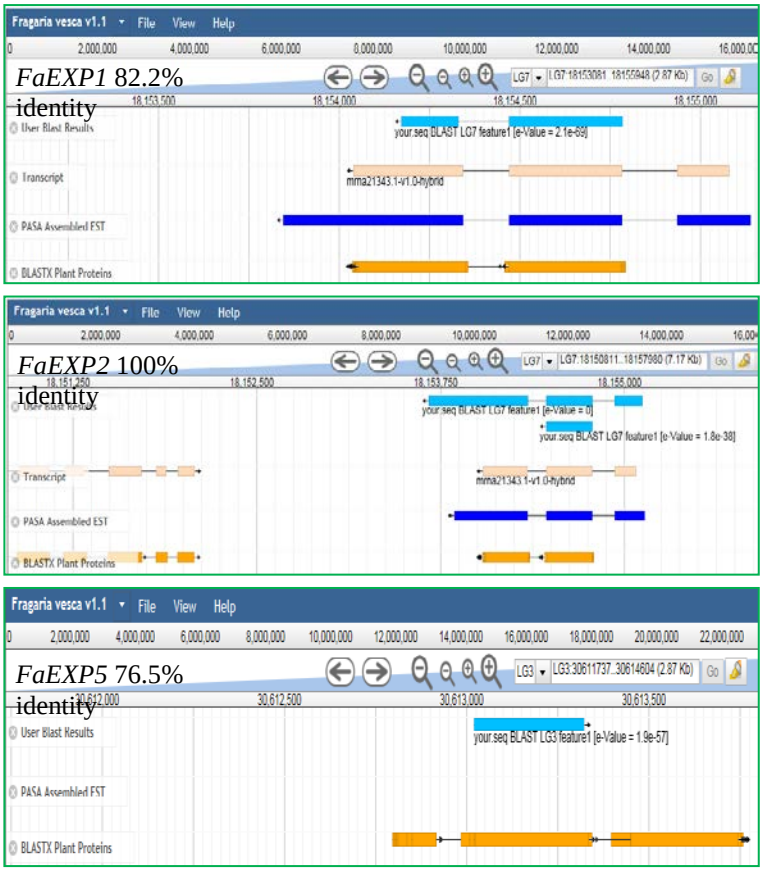

Figure 2. The expansin proteins coding gene positions aligned to the diploid strawberry (*Fragaria vesca*) genome sequence using Phytozome 10.3 genome browser.

Results

Fruit firmness

Phenotypic mean values for fruit firmness were compared between parental genotypes (‘Redgauntlet’ and ‘Hapil’) over the three years. ‘Hapil’ consistently exhibited firmer fruits than ‘Redgauntlet’ (Figure 3).

The firmness of fruits produced by ‘Hapil’ ranged from 171 (g/mm) to 211 (g/mm), whereas the fruit firmness in ‘Redaguntlet’ fruits ranged from 152 (g/mm) to 175 (g/mm). Statistically significant differences between parental genotypes for fruit firmness were observed in 2014 ($p < 0.029$) and 2015 ($p < 0.010$). Fruit firmness observed between parental genotypes in 2013, was not statistically significant ($p < 0.213$) (Figure 3).

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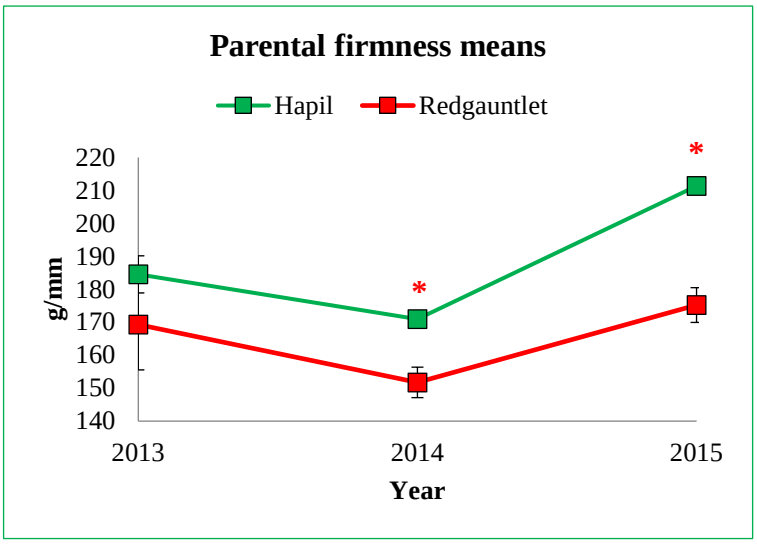

Figure 3. Phenotypic means for fruit firmness of 'Redgauntlet' and 'Hapil' for three consecutive years analysed. Red stars indicate differences ($p < 0.05$) observed between parental genotypes based on Student *t*-Test in 2014 and 2015.

Phenotypic means for fruit firmness were compared between parental genotypes and the progeny. In general, the softest fruits were observed in 2014, whereas most firm fruits were harvested in 2015 (Figure 4).

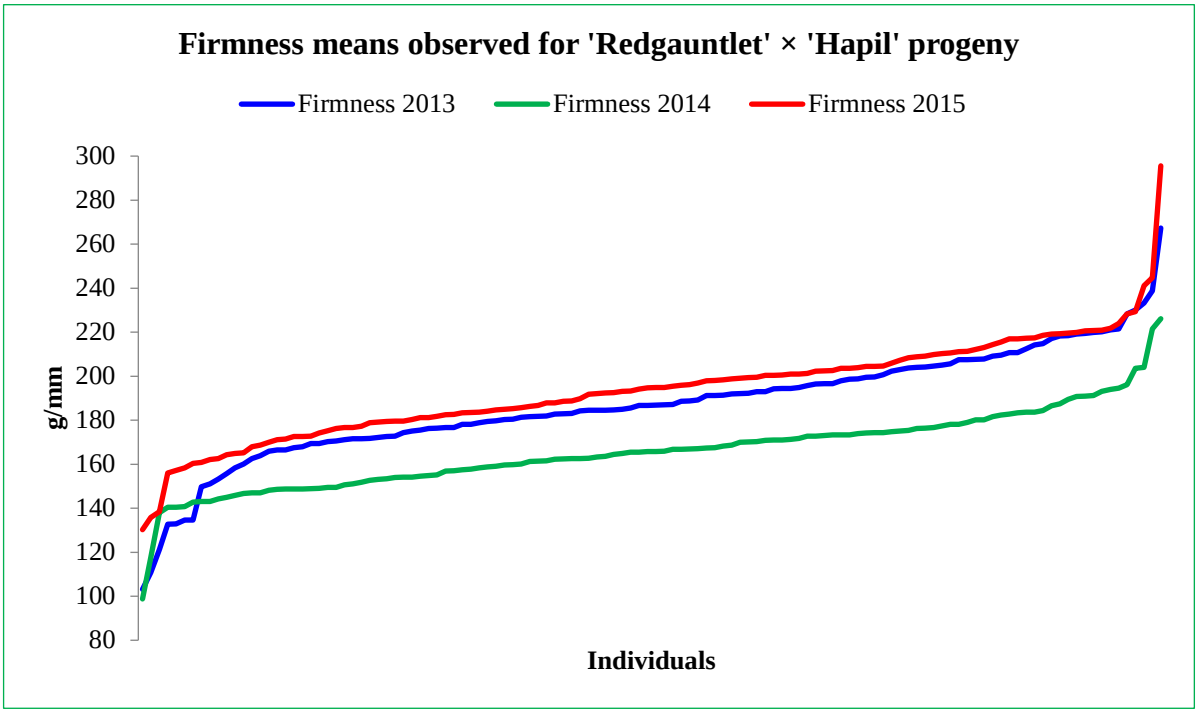

Figure 4. Distribution of the phenotypic means calculated across six replicates for fruit firmness observed among 'Redgauntlet' × 'Hapil mapping progeny for 2013, 2014 and 2015.

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The results showed that the variance within the progeny is not equally distributed among the individuals, and it is transgressive. A large number of individuals displayed higher or lower values than parental genotypes and this was consistent across 3 years. Strawberry cultivars and selections chosen for the transferability analysis was based on the existing phenotypic data information only.

QTL identification linked to fruit firmness using SSR-based linkage map and the comparison of the locations to the QTLs identified using SNP-based map

The combined phenotypic and genotypic information was used for QTL mapping linked fruit firmness. QTL identification using SSR-based linkage map was performed as previously described in Materials and methods section, pg. 8.

A total of 8 QTLs associated with fruit firmness were detected on the SSR-based genetic linkage map. When the detected QTL positions were compared between their mapping positions on SSR and SNP linkage maps, some inconsistencies were observed (Table 2, a). The results showed that 5 loci were identified on the same linkage groups (LG1A, LG1D, LG3A, LG4A and LG4B) between the two maps, whereas the remaining 3 loci were mapped to the homoeologous linkage groups (LG1B, LG2B and LG7B).

The reason behind the inconsistencies observed among QTL positions identified between the two linkage maps could be because of the incorrect annotation of the SSR-based linkage group names. The linkage groups of the SNP-based linkage map were named according to the high density octoploid strawberry genetic linkage map ‘Holiday’ × ‘Korona’, previously reported by van Dijk et al. (2014). The linkage group naming of the SSR-based linkage map was based on the identified common molecular markers between the diploid *Fragaria* reference map (*Fragaria vesca* × *Fragaria bucharica*) and the octoploid strawberry linkage map (Sargent et al. 2009). As a result, seven main linkage groups (LG1 to LG7) were identified correctly, however the homoeologous linkage groups (A, B, C and D) were named randomly. Indeed, when each of the 28 linkage groups of the SSR-based map were annotated according to the SNP-based map, only 8 linkage group names (highlighted in green in Table 2, b) were in agreement with the linkage groups of the SNP linkage map. The remaining 20 linkage groups showed inconsistent homoeologous linkage group naming.

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Table 2. QTL positions mapped to the linkage groups of SNP and SSR genetic linkage maps (a). The comparison of linkage group name annotations between SNP and SSR linkage maps (b). QTL positions on the SSR linkage map after linkage group re-naming

a) Year	Firmness		b) The comparison of the linkage group names		c) Year	Firmness	
	SNP map	SSR map[1]	SNP map	SSR map		SNP map	SSR map[2]
2013	1A	1A	1A	1B	2013	1A	1A
2014	1A	1A	1B	1C	2014	1A	1A
2015		1A	1C	1D	2015		1A
2013		1B	1D	1A	2014		1C
2014		1B	**2A**	**2A**	2015		1C
2015		1B	2B	2C	2013	1D	1D
2013	1D		2C	2B	2014		1D
2014		1D	**2D**	**2D**	2015		1D
2015		1D	**3A**	**3A**	2014	2C	2C
2014		2B	3B	3D	2015	2C	2C
2015		2B	3C	3C	2013	2D	
2014	2C		3D	3B	2013	3A	3A
2015	2C		4A	4B	2014	3A	3A
2013	2D		4B	4A	2015		3A
2013	3A	3A	**4C**	**4C**	2013	4A	4A
2014	3A	3A	**4D**	**4D**	2014		4A
2015		3A	5A	5B	2015		4A
2013	4A	4A	5B	5C	2013		4B
2014		4A	5C	5A	2014	4B	4B
2015		4A	**5D**	**5D**	2015	4B	4B
2013		4B	**6A**	**6A**	2013	4C	
2014	4B	4B	6B	6D	2013	7A	7A
2015	4B	4B	6C	6B	2014		7A
2013	4C		6D	6C	2015	7A	7A
2013	7A		7A	7B			
2015	7A		7B	7C			
2013		7B	7C	7A			
2014		7B	**7D**	**7D**			
2015		7B					

[1]QTL locations on the SSR-based linkage map.

[2]QTL locations on the SSR-based linkage map after the re-naming of the linkage groups according to the SNP-based linkage map.

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After the re-naming of the linkage groups of the SSR-based linkage map according to the SNP-based linkage map, 7 out of 8 QTLs linked to firmness were mapped to the same linkage groups. Only 1 QTL (LG1C) was mapped to the homoeologous linkage group (Table 2, c). It is worth mentioning that 9 loci were detected on the SNP-based linkage map, instead of 8 identified on the SSR-based map.

QTLs identified on SSR linkage map were more stable than QTLs detected on SNP map. Out of 8 loci, 6 were present in all three years and the remaining 2 loci were present in 2 of 3 years. In contrast, out of 9 loci identified on the SNP linkage map, 4 loci were present in 2 of 3 years and the remaining 4 loci were present in 1 year only. No QTLs were present in all 3 years on the SNP-based linkage map (Table 2, c).

Molecular markers closest linked to the QTLs associated with fruit firmness

Closest linked molecular markers were identified for each QTL associated with fruit firmness using SSR-based genetic linkage map.

A total of 16 molecular markers were identified for each of 8 QTLs and are summarised in Table 3. Of those, two were amplified fragment length polymorphism (AFLP) markers (Marker-03 and Marker-08), whereas the remaining 14 markers were simple sequence repeats (SSRs). In four cases, the same SSR marker was identified in 2 out of 3 years for the same QTL. These QTLs were mapped to LG1A, LG1B, LG4A and LG7B (Table 3, markers in bold). In one case, the same SSR marker (Marker-12) was identified for all years on LG4B.

QTL effects across years

All 16 molecular markers identified that had the strongest association with firmness QTLs were used to investigate the effect of QTLs across the three years (2013, 2014 and 2015) analysed. The results showed increasing linear distributions of fruit firmness with the increase number of molecular markers linked to fruit firmness. This is demonstrated across the three years by the gradient of slope (Figure 5).

The steepest slope was observed in 2015, which also represents the most firm fruits harvested. In contrast, the slope was least steep in 2014, which was the year of the softest fruits harvested (Figure 5 and 6).

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Table 3. A summary of 16 closest linked molecular markers associated with fruit firmness loci using re-named SSR-based genetic linkage map. The year QTL detected, marker name, the linkage group and position (cM) of closest linked marker, K value, significance level and parent from which QTL was present are presented

Year	Linkage group	Position (cM)	Marker	K*	Significance[1]	Parent
2013	LG1A	38.23	**Marker-01**	8.386	****	Redgauntlet
2014	LG1A	38.23	**Marker-01**	8.245	****	Redgauntlet
2015	LG1A	77.276	Marker-02	4.386	**	Redgauntlet
2014	LG1C	31.493	Marker-03	6.639	***	Redgauntlet
2015	LG1C	22.304	Marker-04	5.683	**	Redgauntlet
2013	LG1D	98.525	**Marker-05**	18.24	*******	Hapil
2014	LG1D	52.737	Marker-06	8.379	****	Redgauntlet
2015	LG1D	98.525	**Marker-05**	6.844	***	Hapil
2014	LG2C	83.778	Marker-07	9.361	****	Redgauntlet
2015	LG2C	83.422	Marker-08	6.845	***	Redgauntlet
2013	LG3A	28.4	Marker-09	7.932	****	Hapil
2014	LG3A	14.35	Marker-10	8.957	****	Redgauntlet
2015	LG3A	100.225	Marker-11	9.152	****	Redgauntlet
2013	LG4A	12.347	**Marker-12**	8.401	****	Hapil
2014	LG4A	12.347	**Marker-12**	4.722	**	Hapil
2015	LG4A	12.347	**Marker-12**	7.18	***	Hapil
2013	LG4B	17.035	Marker-13	7.472	***	Hapil
2014	LG4B	49.914	**Marker-14**	8.042	****	Redgauntlet
2015	LG4B	49.914	**Marker-14**	7.943	****	Redgauntlet
2013	LG7A	62.115	Marker-15	16.198	*******	Redgauntlet
2014	LG7A	66.525	**Marker-16**	7.249	***	Redgauntlet
2015	LG7A	66.525	**Marker-16**	7.867	***	Redgauntlet

[1]Significance level as observed using Kruskal-Wallis test in MapQTL and are as follow: ** = 0.05, *** = 0.01, **** = 0.005, ***** = 0.001, ****** = 0.0005 and ******* = 0.0001.

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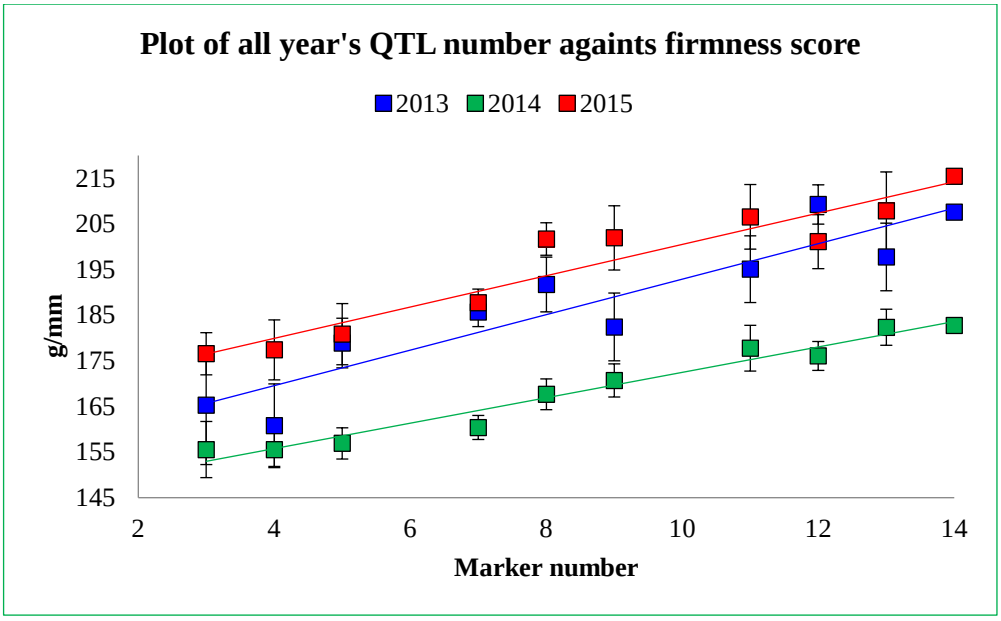

Figure 5. Plot of relationship between QTLs identified and phenotypic firmness score among individuals of 'Redgauntlet' × 'Hapil' mapping progeny for three consecutive years analysed. A consistent increase in firmness can be seen over the years. Error bars are 95% confidence intervals.

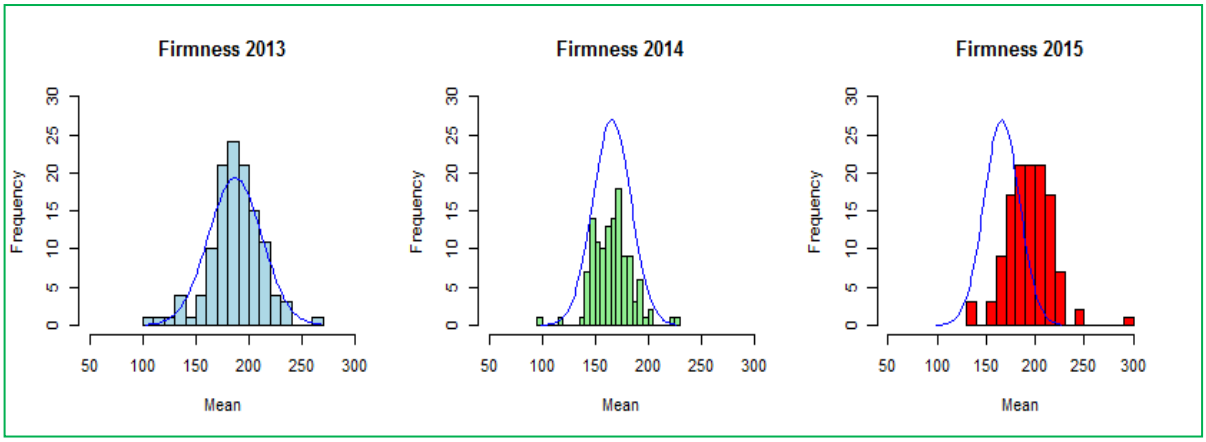

Figure 6. Distribution of the firmness means of the 'Redgauntlet' × 'Hapil' progeny for three years.

Validation of markers in strawberry germplasm

A set of strawberry cultivars, selections and individuals from 'Redgauntlet' × 'Hapil' progeny with known firmness level were chosen for marker validation study (Table 4).

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Table 4. Validation of QTLs in wider strawberry germplasm and mapping progeny using most closely linked SSR markers. Presence of the markers are indicated by a number 1 (firmness QTL). Alleles from 'Redgauntlet' indicated by R, from 'Hapil' indicated by H

Pheno-type	Cultivar	Marker-09		Marker-13		Marker-06		Marker-02		Marker-10		Sum
		111	H	351	H	194	R	232	R	213	R	
Soft	EM1792											0
Soft	Earliglow									1		1
Soft	RH006			1								1
Soft	RH051							1				1
Soft	RH122							1				1
Soft	RH130									1		1
Soft	RH158							1				1
Soft	RH164									1		1
Soft	RH179	1										1
Soft	Gorrela			1						1		2
Soft	Osmanli					1				1		2
Soft	RH105			1		1						2
Soft	RH115			1		1						2
Soft	RH153			1		1						2
Soft	P85	1				1		1				3
Soft	RH093	1		1						1		3
Firm	Holiday									1		1
Firm	Selva			1								1
Firm	SDBL122			1								1
Firm	Buddy							1		1		2
Firm	Flamenco							1		1		2
Firm	Florence			1		1						2
Firm	RH050			1						1		2
Firm	Argentera			1		1		1				3
Firm	Seascape	1				1		1				3
Firm	Albion			1		1		1		1		4
Firm	Diamante			1		1		1		1		4
Firm	Elegance			1		1		1		1		4
Firm	RH135			1		1		1		1		4
Firm	RH137	1		1		1				1		4
Firm	RH168			1		1		1		1		4
Firm	RH061			1		1		1		1		4
Firm	RH163	1		1		1		1		1		5

Two markers out of 16 identified were AFLPs and were excluded from further analysis. The majority of markers (9) identified explained incorrect phase group

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association with fruit firmness and were excluded. Only 5 SSR markers, which showed observed alleles in coupling with the QTL, were screened in plant material because only these markers potentially could be suitable for use in marker-assisted breeding.

The results obtained in this study showed that more than 58% of the firm plant material tested, consistently had higher number of molecular markers amplified (Table 4.). For example, three strawberry cultivars (‘Earliglow’, ‘Gorrela’ and ‘Osmanli’) producing soft fruits had lower number (1 or 2) of SSR markers amplified, whereas the majority of strawberry cultivars producing firm fruits had higher number (2 to 4) of molecular markers amplified. Individuals from the ‘Redgauntlet’ × ‘Hapil’ mapping progeny (names starting with RH) exhibited the most reliable results as expected. Seven out of ten individuals, producing soft fruits, amplified PCR products only for one SSR marker, while all five individuals producing firm fruits had enriched allele frequency (PCR products were amplified for 4 to 5 SSR markers).

Despite the expected trends observed for the majority of the plant material tested, there were some inconsistencies among the lines. For example, some lines producing soft fruits (e.g., P85 and RH093) had high number (3 markers) of SSR markers amplified, while lines producing firm fruits (e.g., ‘Holiday’, ‘Selva’ and ‘SDBL122’) had low (1 marker) SSR marker amplification (Table 4.). However, these results were based on 5 out of 16 markers, thus inconsistencies detected were expected.

Out of 5 SSRs tested, 4 markers (Marker-02, Marker-06, Marker-10 and Marker-13) expressed better amplification pattern among the lines tested, therefore marker Marker 9 is not suitable for use in MAB. However, fruit firmness is associated with SSR markers amplifying alleles from ‘Hapil’, thus only Marker-13 is the most reliable marker linked to firmness QTL identified in this study. Further marker validation analysis in a larger set of plant material, especially strawberry cultivars, is essential in order to confirm the significance level of these markers associated with firmness QTLs.

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Physical positions of SNPs and comparison to the strawberry expansin gene locations aligned to *Fragaria vesca* genome sequence

In order to investigate if QTL positions associated with fruit firmness were overlapping the expansin gene locations, mRNA sequences of the three expansin genes (*FaEXP1*, *FaEXP2* and *FaEXP5*) were aligned to the *Fragaria vesca* genome sequence v1.1. The identified physical positions of the expansin genes were compared to the physical positions of the closest SNPs linked to fruit firmness QTLs identified using SNP-based linkage map (SNP-based linkage map is not presented in this report). The list of SNP markers selected and the comparison of their physical positions against expansin gene physical positions are summarised in Table 5.

When physical locations of a total of 19 SNP markers where compared to the actual locations, one marker (SNP-05) mapped on LG1D had incorrect physical linkage group (Table 5, highlighted in red). This marker was excluded from further analysis. This suggests that potentially the *F. vesca* genome assembly has errors. Physical linkage groups were in agreement with the actual mapped linkage groups for the remaining 18 SNPs.

The comparison of the physical positions between the SNP markers and expansin genes showed that 3 SNPs were located close to the expansin gene locations (Table 5). The physical position of a single SNP (SNP-06) mapped to LG2C was 20.2 Mb, while the position of the *FaEXP2* expansin was 19.2 Mb. Moreover, this SNP marker (SNP-06) was closest linked to firmness QTL on the same linkage group (LG2C) for two years (2014 and 2015). Moreover, the *FaEXP2* expansin located on LG2C had only 66.2% identity, and therefore this gene is likely to be a homologous to the *FaEXP2* expansin (Table 5, in bold). Similarly, physical position of SNP-18 and SNP-19 SNPs mapped on LG7 were 16.9 Mb and 16 Mb respectively, whereas three expansin genes were located approximately at 18.2 Mb (Table 5). Interestingly, the results showed that *FaEXP1*, *FaEXP2* and *FaEXP5* were located at the identical position of the diploid *Fragaria* genome sequence (v1.1) on the linkage group 7.

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Table 5. List of SNP markers closest linked to firmness QTLs, their physical and actual linkage groups, position on the SNP-based linkage map and QTL locations on linkage groups. SNP marker physical positions were compared to the physical positions (aligned to the *Fragaria vesca* genome sequence v1.1) of the three proteins coding expansin genes. One SNP (in red) had incorrect physical location.

	Linkage group						Expansin genes			
SNP	Physical	Mapped	Position (cM)	QTL region (cM)	Parent	SNP physical position	Name	LG	Physical position	Identity
SNP-01	LG1	1A	43.527	43 - 44	Rg	8091967				
SNP-02	LG1	1A	43.527	43 - 44	Rg	8134908				
SNP-03	LG1	1A	43.887	43 - 44	Rg	8231059				
SNP-04	LG1	1A	42.448	42 - 43	Rg	7867898				
SNP-05	LG6	1D	64.548	63 - 64	H	16171921				
SNP-06	LG2	2C	78.788	66 - 80	Rg	20241367	***FaEXP2***	**LG2**	**19165616..19166515**	**66.2%**
SNP-07	LG2	2D	2.942	2 - 4	H	15946662				
SNP-08	LG2	2D	2.942	2 - 4	H	15933049				
SNP-09	LG2	2D	3.661	2 - 4	H	15757720				
SNP-10	LG2	2D	3.661	2 - 4	H	15756774				
SNP-11	LG3	3A	36.984	36 - 37	H	6986698	*FaEXP1*	LG3	20228990..20229307	80.0%
SNP-12	LG3	3A	13.398	13 - 17	Rg	2179493	*FaEXP2*	LG3	20228821..20229735	87.9%
SNP-13	LG3	3A	15.207	13 - 17	Rg	1952281	*FaEXP5*	LG3	30613024..30613750	76.5%
SNP-14	LG3	3A	17.066	13 - 17	Rg	1919703				
SNP-15	LG4	4A	11.418	9 - 11	H	6588568				
SNP-16	LG4	4B	38.17	36 - 39	Rg	21021764				
SNP-17	LG4	4C	31.532	20 - 31	H	18898079				
SNP-18	LG7	7A	20.126	19 - 21	Rg	16938915	*FaEXP1*	LG7	18154043..18154793	82.2%
SNP-19	LG7	7A	53.426	53 - 54	Rg	16004624	*FaEXP2*	LG7	18154043..18154793	100%
							FaEXP5	LG7	18154043..18154793	78.7%

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The same three expansin genes were identified on LG3 with the identity ranging from 76.5% to 87.9%. Four SNPs were also identified linked to firmness loci on LG3, although the physical locations between expansins and SNPs were distant and far away from each other (difference of more than 18 Mb were observed; Table 5).

In general, BLAST analysis yielded repetitive locations of the expansin genes. For example, all expansins showed a match on LG5, and of those, three expansins (*FaEXP4*, *FaEXP5* and *FaEXP6*) had the highest percentage of the identity (82.5% - 98.4%). Similarly, all expansins had a match on LG3, LG6 and LG7. In contrast, four expansins (*FaEXP2*, *FaEXP3*, *FaEXP4* and *FaEXP7*) were located on LG2 and only one gene (*FaEXP3*) or most likely to be a homologous gene of the *FaEXP3* gene was located on LG4 (the percentage of identity was 69.8%).

Discussion

In this report, the validation of QTLs linked to fruit firmness is reported as an example study. In addition, physical positions between closest linked SSRs and expansin genes were compared in order to investigate if SSR markers are overlapping the positions of the expansin genes reported to control fruit firmness.

A total of 16 SSRs closest linked to firmness QTLs were identified across 8 loci on the SSR-based genetic linkage map previously described in Year 2 report. The validation analysis of the strongest associated SSRs was performed using a wider strawberry germplasm representing soft and firm strawberry material. The results showed that 4 markers produced the most reliable pattern in the allele frequency among soft and firm lines tested, therefore potential 4 SSRs associated with firmness were identified.

The study also reports new findings in the discrepancies of the linkage group names between SNP-based and SSR-based linkage maps. The majority of linkage groups (20) of the SSR-based linkage map were incorrectly named previously and were re-named according to the SNP-based linkage groups. The correct linkage group naming is very important, especially for the comparison analysis as discussed in this report.

Furthermore, physical locations between 19 SNP markers linked to firmness QTLs and three expansin genes were compared. The results showed that three SNPs were closely located to the expansins and these findings provides supporting evidence that

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locations of several QTLs associated with firmness identified on the SNP-based map are correct. However, expansin genes exhibiting highest percentage of the identity were mostly located on linkage groups 5 and 6, but no QTLs linked to firmness were detected on these linkage groups on either SNP or SSR-based maps in this study. This could be explained by the large number of regions with low marker density observed on these linkage groups on both genetic linkage maps. It is very likely that high degree of homozygosity was observed in the mapping population ('Redgauntlet' × 'Hapil'), therefore the saturation of some regions of the genome, in this case linkage group 5 and 6, is difficult.

The comparison of QTL positions linked to fruit firmness between SSR and SNP maps

Nine QTLs associated with fruit firmness were identified on the SNP-based linkage map (see previous annual reports for more details), while eight QTLs linked to fruit firmness were identified on SSR-based linkage map, reported in this report. However, the results showed that only five QTL locations were mapped to the same linkage groups and the remaining 6 loci were mapped to the homoeologous linkage groups when QTL locations were compared between the SSR and the SNP maps (Table 2, a).

Two possible hypotheses may explain these inconsistencies. First of all, the quality between the two linkage maps used for QTL mapping is significantly different. More regions with low marker density were present and lower number of molecular markers was mapped on the SSR linkage map, and therefore the quality of the map was poorer than that of SNP map. The low density genetic linkage map is not suitable for QTL analysis and potentially important QTLs might have been missed due to the low QTL detection power. Secondly, annotation of some linkage group names, either of SSR map or SNP map, is incorrect. It is more likely that linkage group naming is incorrect for the SSR map because linkage groups of the SNP map were named according to a different *Fragaria* × *ananassa* high density linkage map derived from the cross 'Holiday' × 'Korona' (van Dijk et al. 2014). Indeed, the previously reported SSR-based linkage map, constructed for the 'Redaguntlet' × 'Hapil' mapping population, was compared to a diploid *Fragaria* reference map (*Fragaria vesca* × *Fragaria bucharica*). This suggests that linkage group naming was based on common molecular markers detected between diploid and octoploid strawberry populations (Sargent et al. 2009).

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After the re-naming of the linkage groups of the SSR-based map according to the SNP-based map, 7 out of 8 QTLs linked to fruit firmness were detected on the same linkage groups between the two maps, and only one QTL was mapped to the homoeologous linkage group (Table 2, c). These results suggest that incorrect linkage group names were the main reason why QTL locations between the SNP and SSR maps were not in agreement.

The results observed in this study demonstrated the importance of the consistent linkage group naming, especially for comparison analysis.

Transferability of SSRs linked to fruit firmness

The marker validation study showed, in general, consistent pattern of allele amplification among soft and firm lines tested. However, some discrepancies were observed among plant material. In some cases, lines producing soft fruits had more markers amplified instead of less, and vice versa. One of the reasons behind this could be that firmness is controlled by multiple loci and that markers linked to firmness were selected based on the biallelic cross between ‘Redgauntlet’ and ‘Hapil’, thus only a portion of fruit firmness was detected in a wider germplasm.

Similar findings were previously reported by Antanaviciute et al. (2015). The transferability of the identified molecular markers associated with *Veriticillium* wilt resistance was partial among the strawberry germplasm tested. The study also reports several other hypotheses to explain transgressive-like behaviour among the lines tested.

In addition, it is difficult to speculate the reasons behind the discrepancies observed in the marker transferability study however, relatively small number of individuals selected for the validation analysis may contribute to the false positive results. A further validation analysis would need to be performed in order to confirm the significance of the 4 SSRs identified showing consistent allele frequencies among plant material tested.

The results observed in this study also demonstrated that SSR markers associated with fruit firmness, are often on a wrong phase. Nine out of 16 SSRs identified were on a wrong phase, and therefore were excluded from the validation analysis and are not suitable for marker-assisted breeding (MAB), because these markers will have an inverse sign. This is a significant issue in the development of molecular markers for

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use in MAB. To overcome this issue, genotyping array containing thousands of SNPs may provide more reliable molecular markers associated with traits of interest for use in MAB. As a result, SNPs are likely to become the most preferable molecular markers for a wide range of studies.

An expansin genes associated with fruit firmness in strawberry

The analysis conducted here, based on three expansin genes (*FaEXP1*, *FaEXP2* and *FaEXP5*) and 19 SNP markers, demonstrated that two QTLs identified on linkage groups 2C and 7A are closely located to the expansin genes and/or homologous of the expansin genes. These genes were previously reported to show correlation between their mRNA level and fruit firmness.

The additional analysis of all 7 expansins showed that the most significant regions associated with these genes were located on LG5 and LG6. However, no QTLs linked to fruit firmness were identified on these linkage groups using SNP-based and SSR-based linkage maps in this study.

Interestingly, a large number of regions with low marker density were observed on linkage groups 5 and 6 on the SSR-based linkage map (Year 2 report), and therefore the power to detect QTL is low. Similar situation was observed on the SNP-based linkage map. Two gaps greater than 10 cM were observed on LG5B and LG6B on the SNP-based map, in addition to the 31 cM gap on LG6C (results are not reported here). This could be because of a high degree of homozygosity observed in 'Redgauntlet' × 'Hapil' progeny, resulting in difficulty in saturation of the specific regions of the genome. The low marker density observed on these linkage groups might be the reason why no QTLs linked to fruit firmness were detected.

Conclusions

- This research project unbaled the correlation, clustering and heritability analysis of a large number of different plant characteristics and fruit quality traits in cultivated strawberry. The analysis of phenotypic data collected in this study showed a number of highly significant differences between parental genotypes ('Redgauntlet' and 'Hapil'). The results provided new information on the correlations between physiological and fruit quality traits at the phenotypic

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level among the octoploid strawberry mapping population. Correlation coefficients were very low for a large number of traits analysed suggesting that many traits were not correlated to each other. The least and the most correlated traits were identified.

- The heritability analysis provided a better understanding between the genotypic and phenotypic associations among the progeny for the traits analysed. The high heritability coefficients observed for the majority of the traits revealed that phenotypic variations were affected by substantially large genotypic variations.
- The existing SSR-based linkage map with low marker density has been saturated with previously published SSR markers. In addition, the 90 K Affymetrix Axiom genotyping array was tested in an octoploid strawberry ('Redgauntlet' × 'Hapil') mapping progeny with the purpose of developing a high density novel SNP-based linkage map. This study demonstrated that mapping of the novel previously reported SSR markers within the targeted regions was ineffective and represents a relatively costly strategy because only three markers mapped to the targeted regions. Furthermore, the process is relatively slow and labour intensive.
- A total of 3,933 binned SNP markers were successfully mapped to the consensus linkage map resulting in the development of a high density linkage map for octoploid strawberry mapping progeny. The ongoing SNP marker data analysis will likely increase the number of loci could be mapped, thus increasing the quality of the linkage map even further. The development of the SNP-based linkage map will facilitate further studies, such as QTL associated with disease resistance and fruit quality traits identification. This application of the Affymetrix IStraw90® Axiom genotyping array is the first high-throughput genotyping platform for rapid, reliable and cost-effective method for linkage map development in the cultivated strawberry (*Fragaria* × *ananassa*).
- At least two potential QTL associated with plant characteristics and fruit quality traits were successfully identified for all 30 traits analysed in this 3-year research project. The results obtained here demonstrated that 30.2% of loci detected were present in 2 or 3 years and were stable over the years. However, the majority of loci were present in one year only and were year-dependable.

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Five QTL clusters (hotspots) overlapping chromosomal regions were detected. These findings are significant and could be considered as targeted regions for further analysis, such as candidate gene(s) identification. The results also suggest that the number of QTLs identified for two traits (truss length and skin strength) might be erroneous based on heritability analysis. This demonstrates the importance of heritability analysis of the traits prior to QTL mapping. Although a large number of potential QTLs was identified for all traits studied, further analysis, such as interval mapping using individual linkage maps for female and male, as well as permutation tests are essential to build on these results.

- An example study focusing on validation of the fruit firmness QTLs successfully provided novel information on SSR markers linked to firmness for use in marker-assisted breeding programmes. Four potential SSR markers associated with fruit firmness QTLs have been identified in this study, although further validation analysis is necessary to confirm the significance of the markers identified.
- The study also reported the importance of the consistent linkage group naming of the genetic linkage maps, especially those developed for the same mapping progeny. Twenty linkage groups of the SSR-based linkage map were named inconsistently when compared with SNP-based map, and were reported here for the first time for ‘Redgauntlet’ × ‘Hapil’ mapping progeny. The re-named linkage groups of the SSR-based map showed that 7 out of 8 QTLs identified on SSR map were located on the same linkage groups as on SNP map.
- The comparison of the physical positions between expansin genes aligned to the diploid *Fragaria* genome sequence (v1.1) and QTLs associated with firmness provided supporting evidence that two QTLs are located to close locations of the expansin genes. These findings suggest that expansin gene family, coding proteins are linked to fruit firmness and that two QTLs identified on the SNP-based genetic linkage map are significant.

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Knowledge and Technology Transfer

16th September 2015 - AHDB Studentship conference. Oral presentation titled ‘Genetic mapping and phenotyping plant characteristics and fruit quality traits in octoploid strawberry (*Fragaria* × *ananassa*)’.

14-18th June 2015 - XIV Eucarpia International conference. Oral presentation titled ‘Genetic mapping and phenotyping plant characteristics and fruit quality traits in octoploid strawberry (*Fragaria* × *ananassa*)’.

16-17th September 2014 – HDC Studentship conference. A poster was presented titled ‘QTL identification and phenotyping of fruit quality and disease resistance traits in octoploid strawberry (*Fragaria* × *ananassa*)’.

24-26th June 2014 – 7th International Rosaceae Genomics conference. A poster was presented titled ‘QTL identification and phenotyping of fruit quality and disease resistance traits in octoploid strawberry (*Fragaria* × *ananassa*)’.

9-10th September 2013 – HDC annual conference. A poster was presented titled ‘Phenotyping fruit quality disease resistance traits in octoploid strawberry (*Fragaria* × *ananassa*)’.

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