The final publication is available at Springer via http://dx.doi.org/10.1007/s11032-015-0258-9

Identification and validation of a QTL influencing bitter pit symptoms in apple (*Malus* × *domestica*).

Buti M[1], Poles L[1], Caset D[1], Magnago P[1], Fernandez Fernandez F[2], Colgan RJ[3], Velasco R[1], Sargent DJ[1]*

[1]Centre for Research and Innovation, Fondazione Edmund Mach, Via E. Mach 1, 38010 San Michele all'Adige, Italy

[2]East Malling Research, New Road, East Malling, Kent ME19 6BJ, UK

[3]University of Greenwich, Natural Resources Institute, Chatham ME4 4TB, Kent, UK

*Author for correspondence

The final publication is available at Springer via http://dx.doi.org/10.1007/s11032-015-0258-9

Tel: +39 0461 615 625

Fax: +39 0461 650 872

E-mail: dan.sargent@fmach.it

The final publication is available at Springer via http://dx.doi.org/10.1007/s11032-015-0258-9

Abstract

Bitter pit is one of the most economically-important physiological disorders affecting apple fruit production, causing soft discrete pitting of the cortical flesh of the apple fruits, which renders them unmarketable. The disorder is heritable, however the environment and cultural practices play a major role in expression of symptoms. Bitter pit has been shown to be controllable to a certain extent using calcium sprays and dips, however, their use does not entirely prevent the incidence of the disorder. Previously, bitter pit has been shown to be controlled by two dominant genes, and markers on linkage group 16 of the apple genome were identified that were significantly associated with the expression of bitter pit symptoms in a genome wide association study. In this investigation, we identified a major QTL for bitter pit defined by two microsatellite (SSR) markers. The association of the SSRs with the bitter pit locus, and their ability to predict severe symptom expression, was confirmed through screening of individuals with stable phenotypic expression from an additional mapping progeny. The data generated in this current study suggest a two gene model could account for the control of bitter pit symptom expression, however, only one of the loci was detectable, most likely due to dominance of alleles carried by both parents of the mapping progeny used. The SSR markers identified are cost-effective, robust and multi-allelic and thus should prove useful for the identification of seedlings with resistance to bitter pit using marker assisted selection in apple breeding programs.

Key words: Marker-assisted breeding; Genomics; Mapping; Storage disorders; Calcium; Fruit

Introduction

Bitter pit is one of the most economically important physiological disorders affecting apple fruit production (Zupan et al. 2013). Despite its initiation in the pre-harvest period, symptoms are often expressed during post-harvest fruit storage and shipping, meaning that seemingly marketable fruits leaving the orchard, arrive at their destination in an unsaleable condition if the disorder is not controlled (Ferguson and Watkins 1989). Bitter pit is characterized by soft, discrete pitting of the cortical flesh of the apple fruits caused by the breakdown of the cell plasma membranes; the pitted areas turning brown and becoming desiccated over time (Faust and Shear 1968). The incidence of the disorder has been shown to be cultivar specific; however, the environment and cultural practices have been demonstrated to play a major role in expression of symptoms, with the same varieties displaying markedly different degrees of symptom severity in different geographical locations, or following over-thinning, hard pruning, dry summers or early fruit harvesting (Ferguson and Watkins 1989).

A number of works have identified a link between the incidence and severity of bitter pit symptoms and the relative concentrations of calcium in apple fruit tissues (Perring and Pearson 1986, Fallahi et al. 1997; Delong 1936) and leaves (Korban and Swiader 1984). Treatment of fruits using calcium sprays and dips has been shown to be help control symptom expression (Lotze and Theron 2006; Yuri et al. 2002) and thus their application is now a widely used practice in commercial-scale orchards, however, their use does not entirely prevent the incidence of the disorder (Lotze et al. 2008; Hewett and Watkins 1991).

Smock and Neubert (1950) detailed a comparison of the differential susceptibilities of 25 apple varieties to bitter pit, and Lewis (1980) suggested that bitter pit susceptibility is expressed when fruit calcium content was low. More recently, Volz et al. (2006) investigated the genetic variation in bitter pit incidence and calcium concentrations in a diverse germplasm collection containing 25 seedling families over two growing sites and concluded that, whilst there was a genetic component associated with both internal and external pit incidences, it was not correlated with the mean calcium

concentration of fruits from each family determined by atomic absorption spectrophotometry. These results contradicted a previous report investigating the inheritance of bitter pit (Korban and Swiader 1984) in three segregating populations, which demonstrated that the disorder was genetically inherited and correlated with dry-weight leaf, and peeled-fruit calcium content. The authors of the latter study suggested that the disorder was controlled by two dominant genes, which they denoted *Bp-1* and *Bp-2*, however, they did not elaborate on the likely genetic positions of the genes in relation to genetic markers.

Recently, Kumar et al. (2013), using a factorial (4×2) female×male mating design, raised a set of full-sib progenies with sizes ranging from 40 – 350 seedlings (total n=1200) in which bitter pit was scored as a binomial trait (i.e. present or absent in the seedlings). Using these progenies, the authors identified a set of genome-wide significant SNP markers that clustered on linkage group 16 of the apple genome (Velasco et al. 2010) and were significantly associated with the expression of bitter pit symptoms in the progeny. However, it was unclear from their study how many progeny segregated for the trait, and the SNP marker with the most significant association explained a total of just 11% of the trait variance in the study.

In this investigation, we performed phenotyping for bitter pit on a large segregating apple mapping progeny over three successive growing seasons. We identified a major QTL for bitter pit that was defined by two microsatellite (SSR) markers. The SSR markers were screened in a subset of susceptible and resistant seedlings from an additional mapping progeny that segregated for the trait, and for which stable phenotypes were observed over two successive growing seasons, and combinations of alleles were identified that were present in the seedlings most susceptible to the disorder. These markers will be useful when selecting parental genotypes and for screening progenies in apple breeding programs employing marker-assisted selection.

Materials and Methods

Plant material and DNA extraction

A cross was performed at the Research and Innovation Centre of the Fondazione Edmund Mach (FEM) between the moderately susceptible 'Redchief' and the resistant 'X6688' (a selection from the INRA apple breeding program) (R×X) in 2003. A total of 364 seedlings were raised, and a single replicate of each genotype was grafted onto 'M.9' rootstocks and maintained following standard technical management procedures in a field environment at Spini di Gardolo in Trentino, Italy. Newly emerging, unexpanded leaves were collected in the spring, freeze-dried within 2 h and ground to a powder using a MM 300 Mixer Mill (Retsch Inc). DNA was extracted using the CTAB extraction method described by Chen and Ronald (1999). The resultant DNA was quantified using a NanoDrop 8000 spectrophotometer (Thermo Scientific) and diluted to 10 ng/ul for use in PCR. A subset of seedlings from an additional progeny, raised from the cross between the moderately susceptible 'Braeburn', and 'Cameo', which displayed low susceptibility (symptoms were sometimes expressed at a low level only in storage) (B×C) at FEM in 2004 (DNA extracted as described above) was used for marker validation.

Phenotypic evaluation

Plants of the R×X population were phenotyped over three successive growing seasons between 2011 and 2013. Bitter pit susceptibility of each seedling carrying fruits was scored by eye based on the symptom expression on the fruits at full maturity (Figure 1). Symptoms were scored on a five point scale: no symptoms (0) – low symptoms (1) – medium symptoms (2) – high symptoms (3) – very high symptoms (4). Phenotyping was performed on the B×C population over the 2012 and 2013 growing seasons.

Microsatellite marker amplification and product visualization R×X

A total of 95 microsatellites distributed throughout the 17 linkage groups of published *Malus* linkage maps (Velasco et al. 2010; Silfverberg-Dilworth et al. 2006; Fernandez-Fernandez et al. 2008) were

selected for testing in the parental genotypes of the R×X mapping population. Fluorescent PCR was performed following the procedures described by Sargent et al. (2012), initially in simplex in the parental genotypes to determine marker amplicon sizes and heterozygosity, and then subsequently in multiplex for marker scoring in 94 seedlings of the R×X population. Products were electrophoresed through an ABI 3730x Genetic Analyzer (Life Technologies), following which, data were analyzed and scored using the GeneMapper (Life Technologies) software application. The resultant segregation data were scrutinized by eye to identify scoring errors.

Linkage map construction R×X

An initial framework linkage map was constructed from 94 individuals of the R×X mapping population using all SSR markers screened that were heterozygous in at least one parental genotype. Segregating markers were analyzed using `JOINMAP 4.1` (Van Ooijen 2006), using regression mapping. Marker placement was determined using a minimum LOD score threshold of 3.0, a recombination fraction threshold of 0.35, ripple value of 1.0, jump threshold of 3.0 and a triplet threshold of 5.0, and mapping distances were calculated applying the Kosambi mapping function. Linkage maps were plotted using `MAPCHART 2.2` for Windows (Voorrips 2002) with linkage group nomenclature following that of previous apple linkage maps (i.e. Fernandez-Fernandez et al. 2012).

Quantitative trait loci analysis

Quantitative trait loci analyses were performed using data from the individual years' evaluations, in addition to the average values obtained across the three years. Marker scores were used as input and data were analyzed by interval mapping, using `MapQTL 6.0` (Van Ooijen 2009). A genome-wide LOD threshold of 2.7 was determined following a permutation test over 20,000 permutations for each year. The restricted multiple QTL method (rMQM) was performed using markers with significant association with incidence of bitter-pit as co-factors to attempt uncover the presence of minor QTL throughout the linkage groups of the genetic map. The step size for both IM and rMQM was 1.0 cM.

Following initial QTL analyses using SSRs distributed throughout the 17 *Malus* linkage groups, a major QTL was identified on linkage group 16 with a maximum LOD score mapped between SSRs CH02a03 and Hi04e04. Five additional SSR markers located between these two SSRs were chosen from published apple genetic maps (Velasco et al. 2010; Silfverberg-Dilworth et al. 2006; Fernandez-Fernandez et al. 2008), scored and mapped on the same 94 progeny individuals following the methods described above. Interval mapping was again performed as described above to increase the resolution of the QTL detected on LG16. To confirm the QTL, using the methods described above, the four SSRs that defined the QTL interval in 94 individuals were scored in the full R×X mapping progeny (*n*=364) and in a subset of the mapping progeny for which the phenotype scored was consistent across the three evaluation years (*n*=106).

Comparison of QTL position with previously reported QTL position

To determine if the QTL on LG16 in the present investigation was the same QTL identified in a previous study (Kumar et al 2013), primer pairs were designed to amplify the region of LG16 containing the SNP ss475883359 (a SNP contained in a gene encoding a *leukoanthocyanidin reductase; LAR-1* MDP0000279135) most significantly associated with bitter pit incidence in the report of Kumar et al (2013). The primer pairs were designed using PRIMER3 to amplify a product of 335 bp with a T_m of 55–65°C (optimum 60°C), a primer length of 20–24 bp (optimum 22 bp) and a 2 bp GC-clamp at the 5' end. The primer sequences used were as follows: ss475883359F GAATAGGTCGGTGTCGTTGG and ss475883359R ACAACTACGATGGCGAATCC. Amplicons generated from the initial 94 seedlings and the parents of the R×X mapping population were sequenced directly and scored following the protocol reported in Padmarasu et al. (2014) and incorporated into the R×X linkage map as described above.

Marker validation in individuals from additional segregating mapping progenies

The B×C progeny (*n*=94) was phenotyped over two successive seasons for bitter pit susceptibility. From the progeny, the seven seedlings consistently displaying severe bitter pit symptoms (a score of 4 in both years) and nine consistently displaying resistance (a score of 0 in both years) were genotyped with markers linked to the bitter pit locus to validate the findings of mapping in the R×X mapping progeny.

Results

Phenotypic distribution in the R×X mapping population

Plants of the R×X mapping population were phenotyped over the course of three successive growing seasons (2011, 2012 and 2013). Depending on the year, between 14.3 and 16.5% of trees did not bear fruit. The remaining trees were classified on a 0 – 4 scale depending on the severity of the symptoms observed. The number of trees grouped into each of the five classes in each year of evaluation is shown in Figure 2. The parental line 'Redchief' displayed moderate bitter pit symptoms (1-2 on the above scale dependent on the year) in the field whilst 'INRA6688' did not present symptoms in the field in any of the three years of observation (0 on the above scale).

Linkage map construction and QTL analysis

The initial linkage map of a subset of *n*=94 individuals of the R×X mapping population contained a total of 86 heterozygous SSR markers spanning 828.26 cM across the expected 17 linkage groups associated with the *Malus* genome (Supplementary Figure 1). Following QTL analysis using interval mapping, a single stable QTL mapping to LG16 of the R×X linkage map was revealed in all three years' data as well as in the average dataset (Supplementary Figure 1). An additional five heterozygous SSR markers spanning the interval of the QTL identified on LG16 were mapped in the R×X (Figure 3). Following QTL analysis using all nine SSR markers mapping to LG16 in the R×X progeny, a QTL with the peak of the LOD (5.45 – 14.01) score associated with markers CH05c06 and NZmsCO905522 and explaining 25.5 – 50.9% of the trait variance observed was revealed (Figure

3; Supplementary Table 1). Following restricted MQM mapping using the three markers most closely associated with the LG16 QTL as co-factors, additional QTLs just above the level of significance and explaining below 10% of the observed trait variance were revealed on LG8 and LG17 (data not shown), however, these QTL were not stable across the three years' evaluation and were not investigated further in this study.

Subsequently, markers CH05a09, Hi22f06, CH05c06 and NZmsCO905522 were scored in the full mapping progeny of *n*=364 individuals. The markers spanned a total genetic distance of 6.6 cM and following QTL analysis, the locus observed in the subset of *n*=94 individuals was revealed, with the peak of the LOD (19.64 – 28.01) mapping between markers Hi22f06 and CH05c06, explaining 25.2 – 28.9% of the observed phenotypic variance (Supplementary Table 2). To minimize environmental effects on bitter pit symptoms severity, a QTL analysis was then performed on a subset of the mapping population (*n*=106) for which the phenotype scored was consistent across the three years' evaluation. In this analysis the peak of the LOD score (LOD = 12.84) was associated with SSR marker Hi22f06 (Table 1), and the QTL explained 42.8% of the observed phenotypic variance.

Mapping of marker ss475883359

Sequencing of the amplicons designed for marker ss475883359 most significantly associated with bitterpit susceptibility in the study of Kumar et al (2013) revealed the expected SNP in both parental genotypes of the R×X mapping population and thus the marker segregated approximating to a Mendelian 1:2:1 ratio in the mapping population. When the segregation data for the marker was incorporated into the outline SSR map developed for the population, the marker co-segregated with SSR marker Hi22f06 with no recombination events observed between the segregation patterns of the two markers.

Identification of alleles in coupling with severe bitter pit symptom expression

Scrutiny of the genotypes for the two SSR markers most closely associated with bitter pit symptom expression (Hi22f06 and CH05c06) in the subset of progeny for which symptoms were consistent over three years of evaluation (*n*=106; plants with 0 phenotype, plants with 1-2 phenotype, plants with 3-4 phenotype) revealed that severe bitter pit symptoms (phenotype class 3-4) were associated in 100% of cases with the homozygous genotype 236/236 for marker Hi22f06, and genotype 115/123 for marker CH05c06 (Figure 4).

Validation of alleles in coupling with severe bitter pit symptom expression

Markers Hi22f06 and CH05c06 were screened in seedlings from an additional population (B×C) for which bitter pit symptoms had been scored over successive growing seasons. The seven seedlings displaying a consistent phenotype of 4 (for two of which only one year's data were available due to biennial bearing) all carried the allelic combinations 236/236 and 115/123 genotypes for Hi22f06 and CH05c06 respectively, whilst none of the nine individuals displaying a resistant phenotype 0 carried either of these genotype combinations at the two loci (Supplementary Table 3).

Discussion

Bitter pit is one of the most economically important disorders affecting modern apple cultivation worldwide (Zupan et al. 2013). Despite chemical treatments being available, there is no effective method for the complete control of symptoms, and many commercially important cultivars such as Honeycrisp (DeLong et al. 2004), Granny Smith (Pesis et al. 2010) and Catarina (Miqueloto et al. 2014) which are widely cultivated and frequently used as parents in apple breeding programs, display at least moderately susceptibility to the disorder, and thus carry unfavorable alleles of genes that control symptom expression.

Apple breeding is a costly endeavor due in part to the long juvenility period of the species, with plants taking a minimum of three years to bear fruit, even when grafted to precocious rootstocks (Fernandez-Fernandez et al. 2011). For the evaluation of fruit characteristics, selection using traditional

approaches cannot be performed until seedlings have reached full maturity, meaning large numbers of inferior genotypes must be transplanted, grafted, and maintained in a field environment for many years before selection of those seedlings with acceptable fruit quality can be made. Employing selection using cost-effective molecular markers at an early stage of seedling growth enables seedlings with unfavorable characteristics to be eliminated whilst they are still in the seed tray, allowing significant savings to be made from early on in the breeding pipeline. This strategy of marker assisted selection (MAS) is already being employed for the selection of key disease resistance and fruit texture loci in the apple breeding program at Washington State University (Evans 2013) amongst others (http://ashsmedia.org/?p=454), but the success of such programs relies heavily on the reliability of the molecular markers employed for any given trait.

The purpose of the current investigation was to identify genetic loci controlling bitter pit symptom expression and to identify predictive, user-friendly, reliable markers to permit screening for bitter pit in seedling populations. To this end, a single major QTL for bitter pit susceptibility was identified which mapped to LG16 of the map developed from the cross 'Redchief' × 'X6688' associated with two SSR markers; Hi22f06 and CH05c06. The QTL identified is in a similar position to markers strongly associated with bitter pit reported by Kumar et al. (2013), who used SNPs from the IRSC whole genome genotyping array developed for apple by Chagne et al. (2012) to identify trait-genotype associations using a total of 1,200 seedlings where bitter pit was scored as a binomial trait, and through mapping of the SNP marker most strongly associated with bitterpit incidence in that investigation, we have demonstrated that the QTL identified in the progenies mapped here is the same QTL identified by Kumar et al (2013). Bitter pit severity has been shown to be influenced by the environment (Ferguson and Watkins 1989), and the severity of bitter pit symptoms expressed by a genotype can vary widely depending on the environmental conditions and the stage of maturity of the plant. Whilst the same locus was detected using phenotypic data collected in all three years' field evaluations in this investigation, symptom expression in many seedlings varied across the three years

and the percentage variance explained by the QTL and its associated LOD score ranged from 25.2-49.7% and 5.45-28.01 respectively. Since the present study lacked biological replication, a QTL analysis was performed using only those seedlings with a stable phenotype over the three years of evaluation to minimize the effect of environmental variability on symptom expression. This led to the identification of the same QTL found using data from the full mapping progeny with the peak of the QTL explaining 42.8% of the observed variance and an associated LOD score of 12.8.

Whilst SNP markers, such as those of the IRSC array employed by Kumar et al. (2013), have the advantage of being abundant and widely distributed throughout the genomes of eukaryotic organisms (Schlotterer 2000; Toth et al. 2000) they are binary in nature, and thus contain less information than multi-allelic marker types, with reports estimating that three or more SNPs are required in a haplotype block to contain the same information as a single SSR marker at any given locus (Rafalski 2002). Additionally, they are difficult to evaluate cost-effectively in a high-throughput but low-density fashion, as is often required for breeding programs incorporating MAS. In contrast, the LG16 QTL identified in this investigation was defined by two SSR markers, Hi22f06 and CH05c06. Scrutiny of the genotypes of these two markers associated with bitter pit symptoms revealed allelic combinations (236/236 and 115/123 respectively) that were associated with severe bitter pit symptoms in all seedlings scored from an additional segregating progeny (B×C) that displayed a stable phenotype over two years' evaluation, demonstrating the utility of these markers both for pre-selection of unfavorable parental genotype combinations and for eliminating seedlings with severe bitter pit symptoms in breeding progenies through the use of MAS.

Earlier genetic studies of bitter pit susceptibility provided compelling evidence that, in the progenies investigated, the trait was likely controlled by two genetic loci Korban and Swiader (1984). The relatively simple model of genetic control of bitter pit suggested by those authors was determined from the study of bitter pit susceptibility in crosses involving 'Prima', 'Coop 11' and three other numbered selections ('D1R99T188', '654102319' and 'PCF10-46'). In the report of their

investigation, the authors suggested that bitter pit was controlled by two major genes, *Bp-1* and *Bp-2*, for which the dominant allele of both loci must be present to confer at least partial resistance, since in the three progenies studied, they observed bitter pit segregation ratios (presence or absence of the trait) of 3:1 and 1:1. The R×X population studied here was derived from a cross between a mildly susceptible (R) and a resistant (X) cultivar. If two genes were controlling bitter pit symptom expression in the R×X progeny, the genotypes of the parental cultivars would have to be 'AABb' (*Bp-1,Bp-1,Bp-2,bp-2*) and 'AaBb' (*Bp-1,bp-1,Bp-2,bp-2*) to account for the observed resistant (X) and mildly susceptible (R) parental phenotypes respectively. Using this model, the segregation of alleles within the progeny and their associated expected phenotypes, is given in the schematic presented in Supplementary Figure 2. The allelic configuration proposed for a two gene model would imply that ~50% of the progeny would confer a resistant 0 phenotype, ~25% would display mild symptoms (1-2 phenotype), and the remaining ~25% would display a severely susceptible (3-4) phenotype. The observed ratio of resistant:susceptible seedlings in 2011 was 141:170, equating to a 1:1 Mendelian segregation ratio (χ^2=2.7), however, the distribution of phenotypes in the susceptible class was distorted, with the ratio of mildly susceptible (1-2): severely susceptible (3-4) of 139:31 (χ^2=68.61).

Since the environmental conditions have been shown to have a strong influence on the expression of bitter pit symptoms (Ferguson and Watkins 1989), the differences in observed and expected phenotypic ratios observed could be accounted for by fluctuations in the environmental field conditions between seasons and genotypes within the field, with a proportion of the seedlings scored as expressing mild (1-2) symptoms conferring a genotype associated with severe (3-4) symptoms. Thus given the available data collected in this investigation, it was not possible to conclusively confirm the two gene hypothesis of bitter pit control proposed by Korban and Swiader (1984), however, it is possible that two genetic loci control bitter pit symptom expression in the R×X progeny, and that the major QTL identified on LG16 corresponds with the B (*Bp-2*) locus in the model

elaborated above, with the A locus (*Bp-1*) not being detectable since all seedlings carry the dominant allele at least in heterozygous form.

Whilst rMQM mapping here identified additional putative loci influencing bitter pit symptom expression, they were not stable across the three years in which phenotyping was performed and thus were likely an artefact of the phenotyping process rather than true additional loci controlling bitter pit symptom expression. In order to characterize the possible A locus (*Bp-1*) further, additional progeny derived from a cross between two mildly susceptible genotypes (i.e in the configuration AaBb × AaBb) would need to be studied.

The genetic location of the bitter pit QTL on LG16 is a region of the apple genome that contains a number of other traits of interest to breeders closely linked to the bitter pit *Bp-2* locus. As well as presenting data on the genetics of bitter pit incidence, Kumar et al (2013) also reported significant associations between markers in this region and fruit splitting, and to a lesser extent, internal browning and weighted cortical intensity. Maliepaard et al (1998) first reported the mapping of the *Ma* locus (controlling fruit pH) to the top of LG16 and the characterization of this locus was performed in greater detail by Xu et al (2012), who performed fine mapping of the region and located the locus in a narrow genetic interval between SSR markers Hi22f06 and Hi02h08, equating to the 4.7 cM of genome immediately flanking the QTL interval identified as containing the *Bp-2* locus in this investigation. Recently, the genetic analysis of 17 fruit polyphenolic compounds identified QTLs in seven stable clusters, and identified a major locus controlling the content of flavanols catechin, epicatechin and procyanin B2, and five unknown procyanadim oligomers in the fruit that was located in the region of the bitter pit locus (Chagne et al 2012). In that investigation, the authors demonstrated co-segregation of the QTL with a SNP in the *Leucoanthocyanidin reductase* (*LAR-1*) gene, shown by Kumar et al (2013) and in this investigation to be closely associated with the major QTL identified on LG16.

Clearly, the bitter pit region of LG16 is a genomic 'hot-spot' for traits desirable in novel varieties, but the close proximity of numerous desirable and deleterious traits poses challenges for MAB. The data reported by Xu et al (2012) on desirable and undesirable alleles for the selection of genotypes heterozygous at the *Ma* locus could be used in conjunction with the data presented here to enable the ideal haplotypes to be selected from a progeny segregating for both levels of fruit titratable acidity and bitter pit incidence. Selection of seedlings associated with elevated levels of certain polyphenolic compounds in progenies also segregating for bitter pit incidence could prove more problematic, since the two loci are tightly linked. Further work using appropriate progenies or cultivar collections would need to be done before it could be determined if the traits are linked in coupling or resistance and thus if the breaking of linkage between these traits would be possible or even necessary.

The link between bitter pit and low cellular concentrations of calcium in the apple cortical tissue is well documented (Amarante et al 2006, Pavicic et al 2004, Ferguson and Watkins 1989), and lower levels of calcium in the fruits has been suggested to be caused by insufficient supply of calcium to the tissues following rapid increases in the nutrient requirement during fruit development (Greene 1991), along with possible changes in xylem functionality during fruit development (Amarante et al 2013). The uptake of macronutrients, including calcium, by plants and its allocation to various sub-cellular compartments and tissues is performed by transport proteins (Blatt 2004). Calcium remains in ionic form throughout plant growth and development and influx into the cytoplasm is performed through ion channels, whilst its removal is regulated by calcium pumps (Amtmann and Blatt 2009). Previous research has shown that abscisic acid (ABA) signaling is essential for the activation of calcium channels that permit entry of calcium ions into the cells (Hamilton et al 2000), whilst a relatively complex interaction between calcium ions, Ca^{++}binding proteins known as calmodulins and Ca^{++}-ATPases regulate the removal of calcium ions from the cells through a negative feedback loop that increases the rate of removal as the cytoplasmic calcium concentration increases (Allen et al. 2001). Of the 137 genes located between SSR markers Hi22f06 and CH05c06 on the 'Golden

Delicious' genome sequence, none were found to encode calcium transporters or calmodulins or to be clearly associated with ABA (data not shown), however, a number of transcription factors that could play a role in the regulation of cytoplasmic calcium concentrations were identified, the expression of which will be tested in future investigations.

Concluding remarks

The identification of robust, multi-allelic SSR markers in this investigation for which specific alleles can be used to identify seedlings conferring a severe bitter pit phenotype will permit savings to be made in the breeding of novel apple cultivars resistant to the disorder, and is a significant step towards understanding the genetic mechanisms controlling bitter pit in apple. Further work delimiting the QTL region with more precise phenotyping, and the development of additional markers will permit the identification of candidate genes and ultimately the functional characterization of the genetic control of the disorder at this major QTL.

Acknowledgements

This work was supported by a grant from the Autonomous Province of Trento to the Fondazione Edmund Mach, Trentino, Italy.

References

Allen G J, Chu SP, Harrington CL et al. (2001) A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature 411:1053-1057

Amarante CVT, Chaves DV, Ernani PR (2006) Mineral composition and bitter pit severity in 'Catarina' apples. Rev. Bras. Frutic. 28:51-54

Amarante CVT, Miqueloto A, Steffens CA, Santos A, Argenta LC (2013) Changes in xylem functionality during apple fruit development: implications on calcium concentration and incidence of bitter pit. Acta Hortic. 1012:135-140

Amtmann A, Blatt MR (2009) Regulation of macronutrient transport. New Phytol. 181:35-52

Blatt MR (2004) Membrane transport in plants. Oxford, UK: Blackwell Publishing

Chagne D, Crowhurst RN, Troggio M et al. (2012) Genome-wide SNP detection, validation, and development of an 8K SNP array for apple. Plos One 7:12

Chen DH, Ronald PC (1999) A rapid DNA minipreparation method suitable for AFLP and other PCR applications. Plant Mol. Biol. Rep. 17:53-57

DeLong JM, Prange RK, Harrison PA (2004) The influence of pre-storage delayed cooling on quality and disorder incidence in 'Honeycrisp' apple fruit. Postharvest Biol. Tec. 33:175-180

Delong WA (1936) Variations in the chief ash constituents of apples affected with blotchy cork. Plant Physiol. 11:453-456

Evans K (2013) Apple breeding in the Pacific Northwest. Acta Hortic. 976:75-78

Fallahi E, Conway WS, Hickey KD, Sams CE (1997) The role of calcium and nitrogen in postharvest quality and disease resistance of apples. Hortscience 32:831-835

Faust M, Shear CB (1968) Corking disorders of apples: a physiological and biochemical review Vol 34. Bot. Rev. 34:441-469

Ferguson IB, Watkins CB (1989) Bitter pit in apple fruit. Hortic. Rev. 11:289-355

Fernandez-Fernandez F, Evans KM, Clarke JB, Govan CL, James CM, Maric S, Tobutt KR (2008) Development of an STS map of an interspecific progeny of *Malus*. Tree Genet. Genom. 4:469-479

Fernandez-Fernandez F, Antanaviciute L, Govan CL, Sargent DJ (2011) Development of a multiplexed microsatellite set for fingerprinting red raspberry (*Rubus idaeus*) germplasm and its transferability to other *Rubus* species. J. Berry Res. 1:177-187

Fernandez-Fernandez F, Antanaviciute L, van Dyk MM, Tobutt KR, Evans KM, Rees DJG, Dunwell JM, Sargent DJ (2012) A genetic linkage map of an apple rootstock progeny anchored to the *Malus* genome sequence. Tree Genet. Genom. 8:991-1002

Greene DW (1991) Reduced rates and multiple sprays of paclobutrazol control growth and improve fruit quality of delicious apples. J. Am. Soc. Hort. Sci. 116:807-812

Hamilton DWA, Hills A, Kohler B, Blatt MR (2000) Ca^{2+} channels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and abscisic acid. Proc. Natl. Acad. Sci. USA 97: 4967–4972

Hewett EW, Watkins CB (1991) Bitter pit control by sprays and vacuum infiltration of calcium in 'Cox's Orange Pippin' apples. Hortscience 26:284-286

Korban SS, Swiader JM (1984) Genetic and nutritional status in bitter pit-resistant and pit-susceptible apple seedlings. J. Am. Soc. Hort. Sci. 109:428-432

Kumar S, Garrick DJ, Bink M, Whitworth C, Chagne D, Volz RK (2013) Novel genomic approaches unravel genetic architecture of complex traits in apple. BMC Genomics 14:13

Lewis TL (1980) Rate of uptake and longitudinal distribution of potassium, calcium and magnesium in the flesh of developing apple fruits of 9 cultivars. J. Hortic. Sci. 55:57-63

Lotze E, Joubert J, Theron KI (2008) Evaluating pre-harvest foliar calcium applications to increase fruit calcium and reduce bitter pit in 'Golden Delicious' apples. Sci. Hort. 116:299-304

Lotze E, Theron KI (2006) Dynamics of calcium uptake with pre-harvest sprays to reduce bitter pit in 'Golden Delicious'. Acta Hortic. 721:313-319

Maliepaard C, Alston FH, van Arkel G et al. (1998) Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers. Theor. Appl. Genet. 97:60-73

Miqueloto A, do Amarante CVT, Steffens CA, dos Santos A, Mitcham E (2014) Relationship between xylem functionality, calcium content and the incidence of bitter pit in apple fruit. Sci. Hort. 165:319-323

Padmarasu S, Sargent DJ, Jaensch M, Kellerhals M, Tartarini S, Velasco R, Troggio M, Patocchi A (2014) Fine-mapping of the apple scab resistance locus *Rvi12* (*Vb*) derived from 'Hansen's baccata #2'. Mol Breed DOI 10.1007/s11032-014-0167-3.

Perring MA, Pearson K (1986) Incidence of bitter pit in relation to the calcium content of apples - calcium distribution in the fruit. J. Sci. Food Agr. 37:709-718

Pesis E, Ebeler SE, de Freitas ST, Padda M, Mitcham EJ (2010) Short anaerobiosis period prior to cold storage alleviates bitter pit and superficial scald in Granny Smith apples. J. Sci.Food Agr. 90:2114-2123

Pavicic N, Jemric T, Kurtanjek Z, Cosic T, Pavlovic I, Blaskovic D (2004) Relationship between water-soluble Ca and other elements and bitter pit occurrence in 'Idared' apples: a multivariate approach. Ann. Appl. Biol. 145:193-196

Rafalski A (2002) Applications of single nucleotide polymorphisms in crop genetics. Curr. Opin. Plant Biol. 5:94-100

Sargent DJ, Passey T, Surbanovski N, Girona EL, Kuchta P, Davik J, Harrison R, Passey A, Whitehouse AB, Simpson DW (2012) A microsatellite linkage map for the cultivated strawberry (*Fragaria* × *ananassa*) suggests extensive regions of homozygosity in the genome that may have resulted from breeding and selection. Theor. Appl. Genet. 124:1229-1240

Schlotterer C (2000) Evolutionary dynamics of microsatellite DNA. Chromosoma 109:365-371

Silfverberg-Dilworth E, Matasci CL, Van de Weg WE, Van Kaauwen MPW, Walser M, Kodde LP, Soglio V, Gianfranceschi L, Durel CE, Costa F, Yamamoto T, Koller B, Gessler C, Patocchi

A (2006) Microsatellite markers spanning the apple (*Malus* × *domestica* Borkh.) genome. Tree Genet. Genom. 2:202-224

Smock RM, Neubert AM (1950) Apples and apple products. Interscience Pub., New York, USA

Toth G, Gaspari Z, Jurka J (2000) Microsatellites in different eukaryotic genomes: Survey and analysis. Genome Res. 10:967-981

Van Ooijen JW (2006) JoinMap® 4, Software for the calculation of genetic linkage maps in experimental populations. Kyazma B.V., Wageningen, Netherlands

Van Ooijen JW (2009) MapQTL® 6, Software for the mapping of quantitative trait loci in experimental populations of diploid species. Kyazma B.V., Wageningen, NL

Velasco R, Zharkikh A, Affourtit J et al. (2010) The genome of the domesticated apple (*Malus* × *domestica* Borkh.). Nat. Gen. 42:833-841

Volz RK, Alspach PA, Fletcher DJ, Ferguson IB (2006) Genetic variation in bitter pit and fruit calcium concentrations within a diverse apple germplasm collection. Euphytica 149:1-10

Voorrips RE (2002) MapChart: Software for the graphical presentation of linkage maps and QTLs. J. Hered. 93:77-78

Xu K, Wang A, Brown S (2012) Genetic characterization of the Ma locus with pH and titratable acidity in apple. Mol. Breed. 30:899-912

Yuri JA, Retamales JB, Moggia C, Vasquez JL (2002) Bitter pit control in apples cv. Braeburn through foliar sprays of different calcium sources. Acta Hort. 594:453-460

Zupan A, Mikulic-Petkovsek M, Cunja V, Stampar F, Veberic R (2013) Comparison of phenolic composition of healthy apple tissues and tissues affected by bitter pit. J. Agr. Food Chem. 61:12066-12071

Figures and Tables

Figure 1 – Apple fruits at full maturity displaying different levels of bitter pit symptoms. Symptoms were scored on a five point scale: no symptoms (0) – low symptoms (1) – medium symptoms (2) – high symptoms (3) – very high symptoms (4).

Figure 2 – Phenotypic distribution of bitter pit symptoms in the R×X mapping population over the course of three successive growing seasons (2011, 2012 and 2013). Fruiting trees were classified on a 0 – 4 scale depending on the severity of the symptoms observed, with class 5 representing trees that did not bear fruit. The number of plants grouped into each of the five classes is shown for each year of evaluation.

Figure 3 – Linkage group 16 of a subset (*n*=94) of the R×X mapping population showing the positions of the nine mapped markers along the linkage group in cM. Results of the QTL analysis using interval mapping of bitter pit symptom expression, detailing the LOD and percentage variance explained for each year and an average value across years is given in the accompanying graph.

Figure 4 – Haplotype distribution of CH05c06 and NZmsCO905522 SSRs in the 106 R×X mapping population plants that displayed a consistent phenotype across all three years of evaluation. Plants were classified by their phenotype as resistant (0), mildly susceptible (1-2) or highly susceptible (3-4).

Table 1 – Results of mapping and QTL analysis using interval mapping on a subset (*n*=106) of the R×X mapping population that displayed a consistent phenotype across all three years of evaluation. The position of each marker across the linkage group is given, along with the LOD score and associated percentage variance explained for each marker.

Supplementary Figure 1 – Initial linkage map and QTL analysis using interval mapping for 94 individuals of R×X mapping population. Marker positions are given in cM and accompanying graphs give LOD scores associated with bitter pit symptom expression.

Supplementary Figure 2 – Schematic representation of the segregation of alleles within the R×X progeny and their associated expected phenotypes, according to the two gene model for bitter pit susceptibility of Korban and Swiader (1984).

Supplementary Table 1 – Results of mapping and QTL analysis using interval mapping on a subset (*n*=94) of the R×X mapping population that displayed a consistent phenotype across all three years of evaluation. The position of each of the nine markers across the linkage group is given, along with the LOD score and associated percentage variance explained for each marker.

Supplementary Table 2 – Results of mapping and QTL analysis using interval mapping the full mapping progeny of *n*=364 individuals of the R×X mapping population across all three years of

evaluation. The position of each marker across the linkage group is given, along with the LOD score and associated percentage variance explained for each of the four markers scored.

Supplementary Table 3 – Validation of alleles in coupling with severe bitter pit symptom expression in nine susceptible (phenotype 4) and seven resistant (phenotype 0) plants belonging to B×C progeny that gave a consistent phenotype over two years evaluation.

Table 1.

Locus	Position	LOD	Percentage variance explained
CH05A09	0.000	9.25	33.1
Hi22f06	1.652	12.84	42.8
CH05c06	4.142	12.58	42.1
NZmsCO905522	6.621	11.81	40.1