

## Review

# Genes and quality trait loci (QTLs) associated with firmness in *Malus x domestica*

Claudius Maronedze<sup>1,2\*</sup> and Ludivine Thomas<sup>2</sup>

<sup>1</sup>Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa.

<sup>2</sup>Division of Biological and Environmental Sciences and Engineering, 4700 King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia.

Accepted 11 December, 2012

**Fruit firmness, a quality quantitative trait, has long been established as a key textural property and one of the essential parameters for estimating ripening and shelf life of apples. Loss of firmness, also referred to as fruit softening, is undesirable in apples and represents a serious problem for growers in many countries. This results in the reduction of apple shelf life and in turn influences its commercialization. Low firmness impacts negatively on the sensory values of juiciness, crunchiness and crispness. Fruit firmness is affected by the inheritance of alleles at multiple loci and their possible interactions with the environment. Identification of these loci is key for the determination of genetic candidate markers that can be implemented in marker assisted selection and breeding for trees and/or cultivars that can yield firmer fruits with economic value. In turn, this technique can help reduce the time needed to evaluate plants and new cultivars could become available faster. This review provides an overview of quantitative trait loci (QTL), including additional putative QTLs that we have identified, and genes associated with firmness and their importance to biotechnology, the breeding industry and eventually the consumers.**

**Key words:** *Malus x domestica*, ethylene, fruit quality, multigenic traits, texture.

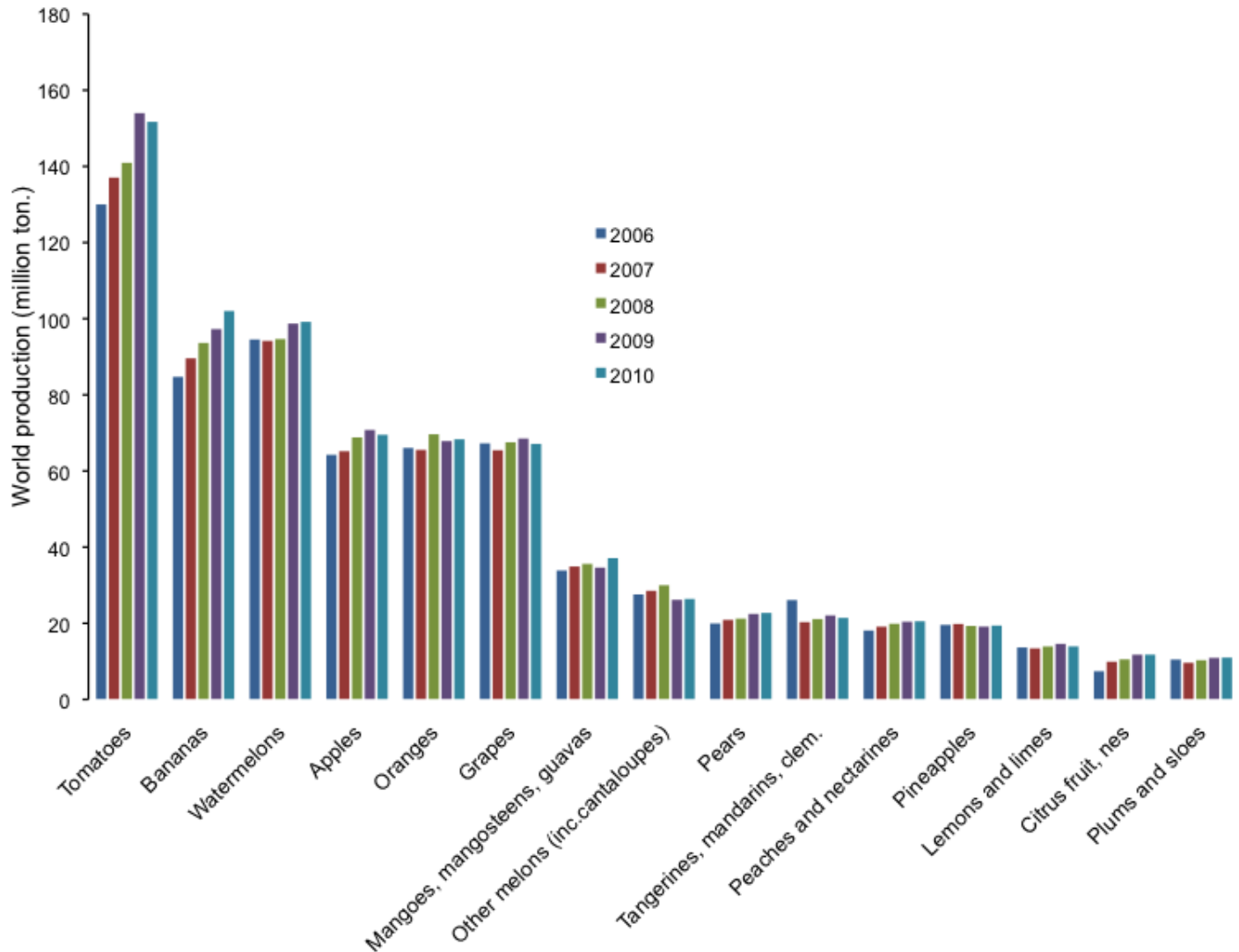
## INTRODUCTION

The climacteric apple (*Malus x domestica* Borkh.) is economically, one of the most important fruit crops worldwide, with a production of about 70 million tons in 2010 (FAOSTAT, 2012; Figure 1). Over 7,500 cultivars of apples have been identified (Bapat et al., 2010), depicting the genetic diversity of the species. Having said that, only a handful is of commercial interest. Fruit quality and disease resistance are crucial breeding targets in the pome industry as these characteristics ultimately determine the consumer perception about a specific cultivar. Generally, consumer's impression is portrayed directly by the fruit appearance, mainly characterized by its size and color.

Thus, apples showing signs of russetting or softening are regarded as flawed. Therefore, producing apple cultivars of high fruit quality, in addition to disease resistance, is the major goal of apple breeders all over the world (Eigenmann and Kellerhals, 2007).

Fruit quality is a complex commercial characteristic, which is evaluated based on a number of attributes or quantitative traits including size, color, sweetness, acidity and texture-based characteristics (Konopacka et al., 2003). It is also used as a critical determinant factor for estimating the shelf life of apples and pears (*Pyrus communis*), their close relatives. Texture-based attributes comprise crispness, juiciness, and firmness (sometimes referred to as hardness). Just like other quantitative traits, fruit texture characteristics are assumed to be under multigenic control. In horticulture, the firmness level is used to determine the maturity and ripeness of fruits (Abbott et al., 1992) and being a component of texture, it

\*Corresponding author. E-mail: Claudius.maronedze@kaust.edu.sa. Tel: +966 2 808 2526.



**Figure 1.** World total production quantity, expressed in tons, of top 15 fruit crops from 2005 to 2009 based on FAOSTAT 2009 updated data (<http://faostat.fao.org/>). This may include official, semi-official or estimated data.

influences the sensory perception of fruits by consumers (Harker et al., 2002; Surmacka-Szczesniak, 2002). Altogether, fruit firmness and ripening, contribute significantly towards perishability of climacteric fruits.

Fruit firmness is a developmentally programmed ripening process (Seymour et al., 2002) and its assessment, also known as fruit pressure test or penetrometer test, is commonly performed using a puncture test (Magness and Taylor, 1925). Additionally, firmness can be assessed using the HyperSpectral Imaging system, a more reliable and non-destructive technique based on the diffraction of infrared light (Lu et al., 2000; Lu, 2007). Noteworthy, the level of firmness differs from one cultivar to another and is also dependent upon the environmental conditions (Konopacka and Plocharski, 2004). This difference in firmness levels

indicate that the rate at which cellular structural modifications occur, involving solubilization and depolymerization of pectins as well as hemicelluloses, differs among cultivars and more importantly, that the nature, timing and extent of the modification of the cell wall polysaccharides also varies (Rose et al., 1998; Brummell and Harpster, 2001).

In tomato (*Solanum lycopersicum*), a model system for fruit ripening, the role of the cell wall modifying enzymes such as  $\beta$ -galactosidase, polygalacturonase, pectin methylesterase, expansin and xyloglucan endotransglycosylase in fruit firmness or softening has been extensively studied using genetically engineered mutant lines (Goulao and Oliveira, 2008). For example, studies using mutant lines lacking  $\beta$ -galactosidase and expansin showed fruit softening reduction in early

ripening (Brummell and Harpster, 2001).

### CONSUMER PERCEPTION ON FRUIT FIRMNESS

Major biochemical and sensory changes in texture, color and flavor occur during apple fruit growth and development, ripening and senescence, and even after harvest during storage just like in pear, peach (*Prunus persica*) and apricot (*Prunus armeniaca*). Genetic dissection of these complex processes has fundamental applications in crop improvement and will aid the exploitation and preservation of pome fruit quality from production and processing through to storage and consumption.

Fruits of the Rosaceae family are considered inedible when over-ripe, which is characterized by excessive softening and changes in taste, aroma and skin color (Bapat et al., 2010). Excessive softening is an undesirable characteristic particularly in pomes as it lowers the sensory values for firmness, juiciness, crispness and crunchiness (Abbot et al., 1984; Jaeger et al., 1998), which ultimately reduces consumer acceptability (Liu and King, 1978). Generally, consumers consider texture as a positive quality attribute symbolizing freshness of apples and in turn this contributes to gratification of consumption (Surmacka-Szczesniak, 2002).

For example, the exceptional crispness and juiciness of the 'Honeycrisp' apple greatly accounted for its commercial success (Kilcast and Fillion, 2001). Besides, numerous markets routinely use fruit firmness as a guide to ensure that apples reach the required textural characteristics for customers all year-round (Johnston et al., 2002).

It is also important to note that the physiology and the (bio)-chemistry involved in fruit ripening are irreversible processes and cannot be paused once started. Nevertheless, these processes could be delayed by controlling atmospheric and/or other environmental conditions (Martinez-Romero et al., 2007; Prasanna et al., 2007).

### INFLUENCE OF ETHYLENE ON FRUIT FIRMNESS

Apples, like other climacteric fruits, display a sharp increase in endogenous ethylene production during ripening, which contributes to variation in firmness as well as color (Abeles and Biles, 1991). The onset of ethylene production is widely believed to drive key cellular structural modifications leading to the loss of firmness. These changes include modifications of the cell wall structure and composition (Seymour et al., 2002). Other factors influencing firmness include cell to cell cohesion, cell size, cell packing, intercellular spaces and cell turgor pressure (Volz et al., 2003).

Ethylene has been identified as the major hormone that initiates and controls ripening in fleshy fruits (Ross et al., 1992; DeEll et al., 2002; Stella et al., 2007). Slowing down the biosynthesis of ethylene during ripening in fleshy fruits has been the foremost attempt to arrest post harvest deterioration. It is widely believed that the onset of ethylene production during fruit ripening drives key cellular structural changes leading to softening. Further, early and late ripening events in apples were shown to be regulated by differential ethylene concentrations (Johnston et al., 2009). In this study, antisense suppression of apple Md-ACO1 led to a very low ethylene production in fruits and exposure of these fruits to different exogenous ethylene concentrations showed differential ethylene sensitivities by various ripening factors, such as hypanthium softening, ester volatile production and starch degradation. Biochemically, the levels of ethylene production increase proportionally from early harvested fruits to later harvests (Wiersma et al., 2007). It is important to note that just like any other biological process occurring *in vivo*, once ethylene is synthesized, it is almost impossible to delay fruit ripening and softening.

Novel insights into fruit firmness and shelf life control in apple have been reported via a candidate-gene approach investigating the effects of genes involved in ethylene biosynthesis, Md-ACS1, Md-ACO1 and Md-ACS3 (Oraguzie et al., 2004; Costa et al., 2005, 2008; Wang et al., 2009). This approach positioned the Md-ACO1 on the linkage group (LG) 10, genetically connecting ethylene production and apple softening (Costa et al., 2005). Additionally, an allelic variation of a simple sequence repeat within the Md-Exp7 (an expansin apple homolog) gene that mapped on LG01 was associated with fruit softening (Costa et al., 2008).

Furthermore, cultivars homozygous for Md-ACS1-2, coding for 1-aminocyclopropane-1-carboxylate synthase (ACS), an enzyme involved in ethylene biosynthesis, showed an explicit reduction in ethylene production, which correlated with increased firmness and extended shelf life (Sunako et al., 1999). In addition, DNA sequencing showed that a null mutation in Md-ACS3a, Mdacs3a, another ripening-specific ACS gene in the Md-ACS3 family, could not be transcribed (Wang et al., 2009). In this study, it was further observed that apple cultivars either homozygous or heterozygous for both null alleles revealed no or very low expression of ripening-related genes leading to longer shelf life on apples. This suggested that Md-ACS3a plays an essential role in the regulation of fruit ripening process in apple, and is possibly a determinant of ethylene synthesis and maintenance of fruit firmness. In the same study, the other two genes in the Md-ACS3 family, Md-ACS3b and Md-ACS3c, were found to possess a transposon-like insertion in their 5' flanking region that is thought to prevent transcription of these genes during ripening. In relation to the latter, a comparative proteomic

study identified a transposase expressed only in the high firmness fruits (Maroneddze and Thomas, 2012). This may imply a link between the transposon-like insertion in the 5' flanking region and the transposase in altering the expression of some of the ethylene biosynthesis genes, thus possibly preventing or reducing their expression.

Further attempts towards understanding fruit firmness has been accelerated via studies aimed at identifying variations in gene expression during ripening and storage of apples (Dandekar et al., 2004; Gessler and Patocchi, 2007). A direct association has been reported between ethylene and aroma production during apple ripening (Wang et al., 2007). However, fruit firmness seems not to be entirely under the immediate control of ethylene as a number of metabolic pathways and processes have been shown to, directly or indirectly, be involved in controlling this trait through comparative proteomics studies (Maroneddze and Thomas, 2012). In this study, up regulation of the cytoskeleton related proteins was observed in high firmness fruits suggesting a putative involvement in the maintenance of cell structure and integrity. Such investigations provide a significant contribution towards a better understanding of the regulatory networks involved in fruit firmness, which could help in targeting future studies of apple breeding.

### QTLs FOR FIRMNESS

The wide scale application of marker-assisted selection (MAS) has permitted hastening of traditional breeding and selection of high-quality apple cultivars. The availability of genetic linkage maps, constructed by positioning molecular markers throughout the apple genome, has enabled the detection and analysis of quantitative trait loci (QTLs) and major genes associated with valuable fruit quality traits (Maliepaard et al., 1998; Liebhard et al., 2002, 2003a). For multigenic controlled traits, QTL mapping approach is usually applied, although pedigree genotyping approach can also be used. The latter has been shown to be more flexible and focuses rather on small genetically related progenies and varieties than on a single progeny (Van de Weg et al., 2004). Detection of putative QTLs is vital for the identification of potential candidate genes, making MAS implementable and this could lead to the creation of new cultivars with specific desired characteristics. This technique has the advantage of being cost and time effective, by reducing the expenses involved in the maintenance of the trees that may carry the 'undesirable' characteristics rather than the traditional way of breeding involving lifelong and costly field maintenance. In fruits like apples, it is well recognized that most of the economically valuable agronomic traits are quantitatively inherited, implying that traits are under the control of several genes. Attempts to position physical locations of these quantitative traits and candidate genes on genetic

maps have been carried out with the long-term goal of implementing MAS and marker assisted breeding (MAB) to create new cultivars. Notably, detection of these loci and genes would provide the key to improve fruit quality and disease resistance.

The construction of more elaborate reference genetic linkage maps stimulated genome wide association studies towards the detection of QTLs associated with fruit quality (Maliepaard et al., 1998). These linkage maps have since been used as reference for the construction of different genetic maps for diverse apple populations and led to an avalanche of data for implementing MAS in the breeding system. Some QTLs linked to fruit quality traits were identified using the genetic map derived from 'Prima' × 'Fiesta' mapping population (Maliepaard et al., 2001), 'Fiesta' × 'Discovery' population (Liebhard et al., 2003b) and 'Telamon' × 'Braeburn' population (Kenis et al., 2008). A considerable body of literature was reported on the assessment of apple fruit development and quality using a wide range of mechanical and instrumental measurements and sensory parameters, and also on the identification of QTLs and major genes controlling firmness and other sensory attributes (King et al., 2000, 2001; Maliepaard et al., 2001; Liebhard et al., 2003b; Costa et al., 2005; Gao et al., 2005a; Gao et al., 2005b, c; Chagné et al., 2007; Kenis et al., 2008; Cevik et al., 2010).

Quantitative trait loci associated with firmness and flesh texture were identified on 10 of the 17 linkage groups in apple (Table 1). In our study, a total of eight putative QTLs associated with firmness were identified located on LG02, LG06, LG10, LG12, LG15 and LG16 using a partially saturated genetic linkage map derived from a progeny of 'Golden Delicious' × 'Dietrich'. Most of the QTLs identified in this study corroborated the findings from previous reports, with the exception of a QTL located on LG02, which could be an additional QTL for firmness. However and in contrast to other studies, no QTLs were identified on LG01, LG08, LG11 and LG14, possibly because the map was not completely saturated or the occurrence of variations in QTL positions amongst cultivars. The latter suggests that QTL detection may be cultivar dependent and/or that these QTLs are under the influence of environmental conditions since the populations used in these studies were grown in different locations (Liebhard et al., 2003b). In addition, five studies (King et al., 2000; Maliepaard et al., 2001; Seymour et al., 2002; Liebhard et al., 2003b; Cevik et al., 2010) identified distinct QTLs controlling firmness using apple populations with 'Fiesta' as one of the parents, suggesting that the expression of alleles contributing to fruit firmness could be influenced by genetic backgrounds, and/or that certain alleles may only be expressed in specific environments (Liebhard et al., 2003a; Gardiner et al., 2007). Nevertheless, these studies were comparable since the linkage maps used for QTL detection were constructed in part using co-dominant

**Table 1.** Quantitative trait loci controlling fruit firmness and related quality traits in *Malus*.

Trait	Linkage group													
	1	2	4	5	6	8	9	10	11	12	13	14	15	16
Fruit flesh firmness	X <sup>a, d</sup>	X <sup>e</sup>			X <sup>c</sup>	X <sup>a, d</sup>		X <sup>a, d</sup>	X <sup>c</sup>	X <sup>c</sup>		X <sup>c</sup>	X <sup>e</sup>	X <sup>e</sup>
Hardness								X <sup>a, d</sup>						
Crispness	X <sup>a, d</sup>	X <sup>e</sup>	X <sup>e</sup>	X <sup>a, d</sup>				X <sup>a, d</sup>		X <sup>a, d</sup>	X <sup>a, d</sup>			X <sup>a, d</sup>
Juiciness	X <sup>a, d</sup>		X <sup>e</sup>				X <sup>e</sup>			X <sup>a, d</sup>		X <sup>f</sup>	X <sup>e</sup>	X <sup>a</sup>
Sponginess	X <sup>a, d</sup>			X <sup>a, d</sup>	X <sup>a, d</sup>									X <sup>a, d</sup>
Compression	X <sup>b</sup>				X <sup>b</sup>	X <sup>b</sup>				X <sup>b</sup>			X <sup>b</sup>	
Slow breakdown	X <sup>a, d</sup>													

<sup>a</sup>King et al., 2000; <sup>b</sup>King et al., 2001; <sup>c</sup>Liebhard et al., 2003b; <sup>d</sup>Maliepaard et al., 2001 and <sup>e</sup>Marondedze, 2011.

and transferable simple sequence repeat (SSR) markers (King et al., 2000; Maliepaard et al., 2001; Liebhard et al., 2003b; Kenis and Keulemans, 2005). It is worth noting that a linkage map with a high marker density is of great importance for the precise detection of potential QTLs. This may be one of the reasons why some studies successfully detected QTLs on some linkage groups while others failed. For example, we could not detect any QTLs for firmness and acidity on LG01 and LG08, respectively, while Costa et al. (2008) and Liebhard et al. (2003b) managed to identify the respective QTLs.

In addition, Kenis et al. (2008) assessed the inheritance of fruit quality traits derived from 'Telamon' × 'Braeburn' mapping population. In this study, a total of 74 putative QTLs were identified as controlling some major fruit physiological traits that include fruit stiffness, flesh firmness, rate of flesh browning and harvest date. Only 26 of these putative QTLs were detected in both of the two years of investigation, demonstrating that QTLs are indeed influenced by the environmental conditions making them rather unstable. Although direct comparisons between studies are difficult

as they use different markers and trait evaluation protocols, they still however provide insights about QTLs or genes controlling fruit quality traits.

Recently, two orthologous candidates of Fruitfull-like genes, *MdMADS2.1* and *MdMADS2.2*, were identified in the *Malus* genome using genetic association studies and were demonstrated to genetically link with QTLs of fruit flesh texture (Cevik et al., 2010). The two paralogous genes showed 99% identity in their predicted coding sequences but illustrated divergence in their non-coding regions. Further, the genetic association between the cortex texture and *MdMADS2.1*, *MdMADS2.2* and *MdMADS14*, a Shatterproof orthologue, was evaluated using progeny obtained from 'Prima' × 'Fiesta' cross (Maliepaard et al., 1998) and allowed positioning of *MdMADS2.1*, *MdMADS2.2* and *MdMADS14* on LG14, LG06 and LG09, respectively. In another study, one SSR marker mapped corresponding to *MdMADS2* (U78948) on LG14 at 45.6 cM using a 'Fiesta' × 'Discovery' cross (Silfverberg-Dilworth et al., 2006). This marker showed close proximity to a firmness QTL at position 47 cM (Liebhard et al., 2003b). Conversely, Cevik et al. (2010) showed that conventional QTL analyses could not detect

any association between fruit flesh firmness and *MdMADS2.1*, *MdMADS2.2* and *MdMADS14* genes in the 'Prima' × 'Fiesta' population. This suggested either a genetic interaction between loci poised by the sizes of the individual locus (Carlborg and Haley, 2004) or differences in alleles inheritance from the two parents (Cevik et al., 2010). Nevertheless, evidence for the association between *MdMADS2.1* and flesh fruit firmness in apple was obtained using population-based genetic association studies, an approach successfully used to identify genes or alleles underlying multifaceted plant traits (Rafalski, 2002; Neale and Savolainen, 2004; Andersen et al., 2005; Gupta et al., 2005; Brescghello and Sorrells, 2006). Although this study linked the *MdMADS2.1* locus with aspects of texture in apple, further important functionalisation could be obtained from analogous genes in other species. For example, in *Arabidopsis thaliana*, the *Fruitfull-like* gene is known to be involved in the negative regulation of genes controlling for valve-margin development (Ferrandiz et al., 2000; Liljegen et al., 2004). The *Fruitfull-like* orthologues in other fleshy fruit species have been suggested to play an analogous role to the *Arabidopsis Fruitfull-like*

gene in repressing cell separation and preventing a total loss of cell-cell adhesion that in turn could lead to firmer fruit phenotypes (Cevik et al., 2010).

## OTHER TRAITS INFLUENCING FRUIT TEXTURE

Firmness together with crispness and juiciness are the main attributes of fruit texture. These attributes have been shown as closely related (Tu et al., 1997; Konopacka and Plochanski, 2001; Kingston, 2010). While firmness can be viewed as the force required to bite into an apple, crispness can be defined as the sound intensity or pitch produced when an apple is first bitten with the front teeth (Fillion and Kilcast, 2002; Harker et al., 2002).

An in-depth analysis of the relationship amongst texture attributes was first attempted by assessing the contributions of mechanical properties of fruit flesh to the observed genetic variations (Maliepaard et al., 1998). Further, quantitative trait analysis was employed to account for the variation of traits like crispness and juiciness using mechanical techniques derived from the compression and wedge fracture tests (King et al., 2000). Significant QTLs, associated with both juiciness and crispness, have been located on LG01 and LG16 (Table 1). An additional QTL for juiciness has been identified in 'Fiesta' × 'Prima' population, located on LG12 (King et al., 2000). Further, we identified putative QTLs for this trait on LG04, LG09 and LG15 using 'Golden Delicious' × 'Dietrich' population. Crispness associated QTLs were detected on LG01, LG05, LG10, LG12, LG13 and LG16 (King et al., 2000, 2001; Maliepaard et al., 2001), and an additional QTL has been detected on LG04. Of interest, is that LG16, in particular, shows a cluster of QTLs associated with firmness, crispness and juiciness, as well as other quality traits compared to other linkage groups (Table 1). Taken together, these studies revealed that specific regions of the apple genome might account for some substantial genetic variation associated with fruit quality.

Recent advanced techniques such as diversity arrays technology (DArT), a technique that offers enhanced multiplexing and generate whole-genome fingerprints by scoring the presence versus absence of DNA fragments through the process of complexity reduction, can be applied to improve accuracy in QTL detection. This technology has successfully been used in rice (*Oryza sativa*), wheat (*Triticum aestivum*), cassava (*Manihot esculenta*), barley (*Hordeum vulgare*) (Wenzl et al., 2004, 2006) and sorghum (*Sorghum bicolor*) (Mace et al., 2008). Its application in apple has also been initiated (Soriano et al., 2008). Owing to the algorithms of the MapQTL software, the identification of QTLs associated with traits of interest is critical during the implementation of MAS since it paves the way to fish out candidate markers for use during MAB. In essence, these candidate markers could be used to screen gene inheritance in

individuals of a given population as early as the seedling stage, thus greatly hastening the selection of individuals displaying quality traits of interest (Mohan et al., 1997; Liebhard et al., 2002; Graham et al., 2009).

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Fruit quality traits can be affected by inheritance of alleles at multiple loci and their possible interactions in addition to environmental influence. This factorial combination poses a key challenge to modern fruit genetic research. As such, further functional genetic studies are needed to elaborate on the biological basis that links traits to genes. For example, linking the relation between *MdMADS2.1* and fruit flesh firmness. While genetic mapping allows for the identification of putative QTLs associated with agronomic traits, transcriptomics and proteomics on the other hand, are complements by providing insight into the functome influencing the variability observed at the phenotypic level between cultivars and/or plants of a same cultivar. These approaches in turn promote targeted candidate gene mapping. Further, they provide connections between datasets from proteomics, genomics and phenotypic analyses, thus integrating these modern 'omics' tools of crop biotechnology with traditional fruit breeding approaches to transform selection of cultivars and/or individuals with desired characteristics. This integration could be the ultimate breeding strategy in order to produce cultivars with increased shelf life and firmness conservation after storage. However, this is still a challenging operation for breeders and plant scientists and despite plant breeders' achievements, using classical approaches, tremendous opportunities do still exist to accelerate and expedite new cultivar(s) development and deployment through MAB. This could help increasing profitability and sustainability faced by industries due to poor fruit quality and therefore, meeting the dynamic industry, market and consumer preferences. Of importance to note is that several groups have since taken initiatives contributing towards developing sustainable agricultural practices to meet consumer demands, like the National Apple Breeding Association in China, the USDA-ARS/Cornell University Apple Rootstock Breeding in the US and numerous EU-projects such as the European Apple Genome Mapping Project (EAGMAP), the Durable Apple Resistance in Europe (DARE) and the High Quality Disease Resistant Apples for a Sustainable agriculture (HIDRAS, QLK5-CT-2002-01-01492) and have significantly contributed to molecular apple breeding (King, 1996; Lespinasse et al., 2000; Gianfranceschi and Soglio, 2004).

In précis, a number of factors contribute to disparities in the outcome of QTL analysis, for example, selection of cultivars, population size and structure, environmental conditions, or number and density of markers per linkage

group (Liebhard et al., 2003b; Pillen et al., 2003; Gardiner et al., 2007). Despite all this, an increasing number of QTLs and candidate genes have been identified as associated with agronomical and economical valuable traits. Introduction of the DArT markers, and the recent release of the apple genome (Velasco et al., 2010) will beyond no doubt accelerate saturation of linkage maps hence, making the detection of QTLs and candidate genes more accurate and valuable. This may assist in developing a common reference genetic map that would provide efficient means of locating genes of interest within a target genome.

## ACKNOWLEDGEMENTS

The authors wish to thank Prof. Christoph Gehring and Dr. Fouad Lemtiri-Chlieh for critically revising the review.

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