Genetical metabolomics in apples

(*Malus* x *domestica* Borkh)

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Thesis

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Dedicated to my beloved parents

Chapter 1

General Introduction

1.1 Introduction

Cultivated apple (*Malus x domestica* Borkh) is one of the most diverse and ubiquitously cultivated fruit species. It belongs to the order of Rosids, the family of Rosaceae and the sub-family of Maloideae which includes many commercial fruit species such as pear, strawberry, cherry, peach, apricot, almond, black cherry, and crab apple (Park *et al.*, 2006; Harris *et al.*, 2002). It is the main fruit crop of temperate regions of the world. Apple is produced commercially in 91 countries with a total production of 64 million tons (FAO statistics, 2009).

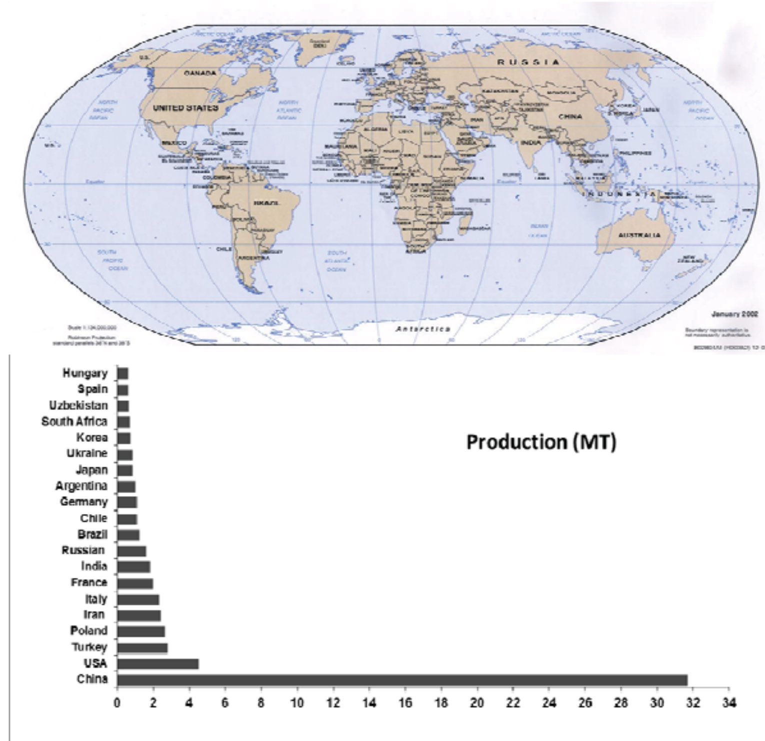

Fig. 1.1 Global apple production in million tons for selected countries (FAO statistics, 2009). The globe map is shown on the top.

China is at the top with 31.7 million tons followed by USA with 4.5 and Turkey with 2.8 million tons production. Other major producers include Poland, Iran, Italy, France, India, and Russia, etc (Fig. 1.1). It is the fourth most widely produced fruit crop in the world after banana, orange and grape (FAO statistics, 2009).

1.2 Apple crop breeding and the potential challenges

Apple was improved through selection within natural variation over a period of thousands of years starting by early farmers with domestication. The haploid (x) chromosome number for most Rosaceae crops is 7, 8 or 9, however, Pyreae has a distinctive number of $x = 17$. The reason is that Pyreae has long been considered to be allopolyploid between species related to extant Spiraeoideae ($x = 9$) and Amygdaleoideae ($x = 8$) (Evans and Campbell, 2002). It has been reported that an auto-polyploidization also occurred in apple (Evans and Campbell, 2002) which is recently proven with whole genome sequence information of apple cv. 'Golden Delicious' (Velasco *et al.*, 2010). The transition from 9 to 17 chromosomes in the Pyreae is the result of a relatively recent genome wide duplication (Velasco *et al.*, 2010), followed by partial reduction at chromosome and gene level. There is no clear agreement among the taxonomists on how many species the genus *Malus* consists of and as a result of this, the number of species belonging to the genus *Malus* varies from 25 to 47 according to Robinson et al. (2001) while Harris et al. (2002) mentioned that *Malus* comprises of 55 species.

The cultivation of apple is supposed to be started long time ago by domestication (Juniper *et al.*, 1999). Sweetness of the fruit appeared to be one of the first domestication traits (Juniper *et al.*, 1999). There is evidence of apple fruit gathering having started as early as the Neolithic times (Juniper *et al.*, 1999). Later cultivation of apple geared up with vegetative propagation of selected phenotypes through cuttings and also with the discovery of grafting techniques (Morgan and Richards, 1993). Nowadays, because of grafting, it is treated as a vegetatively propagated crop in breeding schemes. About 200 years ago, the traditional method of apple improvement by selecting the best individual phenotypes from seedlings grown from open-pollinated seeds was replaced by deliberate hybridization by artificial hand pollination (Gardener *et al.*, 2007).The scientific breeding started with the first controlled cross-pollination carried out by Thomas Knight early in the nineteenth century (Brown, 1975).

Apple is, however, a self-incompatible and highly heterozygous crop, which results in highly diverse progeny plants with only a few of them combining a sufficient number of traits being a major improvement on both the parents (Gardener *et al.*, 2007). Moreover, most of the characters are under polygenic control which results in low efficiency in genetic improvement of breeding clones. This situation is further hampered by the long juvenile period which makes breeding in this crop a very slow and expensive process (Gardener *et al.*, 2007). Another problem is the linkage drag i.e. the introgression of other (undesired) trait(s) along with the trait of interest. This problem is more prominent if introgression of traits from wild species is needed. In that case several backcrosses are needed to remove undesired traits

from the donor species which take at least seven years per single backcross cycle. Development of new apple varieties by means of traditional (introgression) breeding is very slow and takes 25-50 years in developing a superior variety to be available at the market and generally during this long period the new variety becomes deteriorated for its newly acquired traits. This demands the use of new breeding techniques in apple such as marker assisted breeding (Kellerhals *et al.*, 2000; Markussen *et al.*, 1995; Khan *et al.*, 2007; Moriya *et al.*, 2009; Durel *et al.*, 2009; Zhu *et al.*, 2011; Bus *et al.*, 2000) which allows indirect selection of desired traits at a much earlier stage and genetic modification for improvement of existing varieties (James *et al.*, 2003; Puite and Schaart, 1996; Telias *et al.*, 2011; Chagne *et al.*, 2007). Such genetic modification could be done in many ways e.g. by cisgenesis. Cisgenesis is defined as 'the genetic modification of a recipient plant with natural gene(s) from a sexually compatible plant' (Schouten *et al.*, 2006a, b).

1.3 The metabolic diversity in apple crop

Plants are a rich source of secondary metabolites. It has been estimated that over 100,000 metabolites can be found in plants (Keurentjes *et al.*, 2006). Moreover, substantial qualitative and quantitative variation in metabolite composition is often observed within different individuals of the same plant species (Keurentjes *et al.*, 2006). The currently available metabolic profiling technologies have shown to be effective in detecting differences and similarities in the composition of plant breeding germplasm and species (Harrigan *et al.*, 2007a). Metabolic changes are associated with plant development and responses to applied stresses (Harrigan *et al.*, 2007a). Though, variation in the levels of metabolites within a species or population is quantitative and moderately heritable (Schauer *et al.*, 2006; Rowe *et al.*, 2008), and shows polygenic inheritance (Beló *et al.*, 2008) controlled by the interaction of environmental and genetic factors (Harrigan *et al.*, 2007b; Skogerson *et al.*, 2010). However, it could also be monogenic and could be mapped accurately. Quality traits in many important crop plants are dependent on metabolic composition (Harrigan *et al.*, 2007a). The metabolome represents a critical aspect of a plant's physiology, growth characteristics, and ultimately its economic value (Harrigan *et al.*, 2007a).

Many cultivated apple genotypes trace their ancestry to a small number of progenitor lineages and, therefore, share a relatively high degree of genetic identity (Richards *et al.*, 2009). Therefore, these relatively few progenitors caused sharp decrease in the genetic biodiversity within the cultivated apples. The narrowness of this genetic base has been further increased, as the number of different varieties used in commercial production has decreased over the last century (Richards *et al.*, 2009). In contrast, wild species within this genus are known to contain substantial genetic diversity such as resistance to biotic stress and quality traits (Richards *et al.*, 2009).

1.4 Potential role of apple in human health

Fruits are well known for their high value in human health. Epidemiological studies indicate that consumption of fruits, vegetables and their (processed) products are inversely related to carcinogenesis, coronary heart disease, mortality, and aging processes (Sanchez *et al.*, 2004). On one hand, it has been suggested that this reduction is not only due to increased levels of vitamins and fibers, but that other compounds, such as phenolic compounds, also appear to play an important role in the overall antioxidant capacity of fruits and vegetables (Sanchez *et al.*, 2004). On the other hand, it is clear from the fact that there has been recorded a substantial increase in heart and vascular diseases, obesity, cancer and also diabetes due to less fruit intake in food (Hollman and Katan, 1999; Kaur and Kapoor, 2001). The antioxidant properties of phenolic compounds present in plant food may contribute to these health beneficial effects (Hollman and Katan, 1999; Kaur and Kapoor, 2001). In other studies, during the past few years, these secondary metabolites, which occur abundantly in plant foods, have been discovered to be beneficial components of functional food (Treutter, 2001). Their positive effects on human health were first proposed by Bentsáth (1936) and are now widely accepted. Plant phenolic compounds are well recognized for their antioxidative activities and apple is well known for its high levels of these compounds. They scavenge free radicals, thus breaking the free radical chain reaction of lipid peroxidation (Hertog *et al.*, 1993; Hertog, 1992).

"An apple a day keeps the doctor away". This saying has encouraged many researchers to search for the "magic" ingredients of apple. The beneficial role of apple phenolics prompted Ridgway et al. (1997) to call the apple a "new agrochemical crop". Apple possesses many health beneficial properties for human being as a rich source of phenolic compounds. It has been associated with lowering the risks of cancers, particularly prostate, liver, colon, and lung cancers (Xing *et al.*, 2001) cardiovascular diseases (Hertog *et al.*, 1993; Hertog, 1992; Hyson *et al.*, 2000), coronary heart diseases, asthma, type-2 diabetes, thrombotic stroke, and ischemic heart disease (Mcghie *et al.*, 2005).

Phenolic compounds are an important part of the human diet and have great importance in the nutritional and commercial properties of agricultural foodstuffs. Phenolic compounds comprise one of the largest and most ubiquitous group of plant metabolites. The most commonly occurring ones in foods are flavonoids and phenolic acids. The diversity of the chemical structures and variability in foods make calculation of the phenolic content difficult. One study in The Netherlands on flavonols and flavones estimated the average intake at 23 mg/day (Hertog *et al.*, 1993). Phenolic content of plant food is currently considered a measure of product quality (Hertog *et al.*, 1993). Some of the major phenolic compounds isolated and

identified from apple are chlorogenic acid, hydroxycinnamic acid, epicatechin, catechin, gallaocatechin, procyanidins, phloretin-glucosides, quercetin glycosides, and dihydrochalcone glycosides (Treutter, 2001; Lu and Foo, 1997; Mazza and Velioglu, 1992; Lancaster, 1992; Nicolas *et al.*, 1994; Awad *et al.*, 2000).

1.5 Importance of taste in fruit consumption

The consumption quality of a fruit is one of the important criteria which determine its value for cultivation and breeding (Visser *et al.*, 1968). The organoleptic appreciation of apple depends on both the acid and sugar contents, or rather on the ratio between them, though this ratio alone did not entirely explain the sensation of sweetness. It was concluded that at optimal acidity, the content of aromatic compounds becomes of primary importance for the appreciation of apple. On the other hand, in breeding it should be taken into account that sugar content and acidity inherit independently. A proper balance between sugar content and acidity - the former high, the latter medium - is an essential prerequisite of a dessert apple (Visser *et al.*, 1968; Van der Sluis *et al.*, 2001). The low acidity behaves as a recessive character and because most cultivars appear to be heterozygous, a quarter of the seedlings in progenies may be of the low acid kind (Van der Sluis *et al.*, 2001). The so-called `sweet' fruits constitute a loss to the breeder. However, high-acid cum `sour' fruits are equally undesirable in progenies because their sourness remains unpleasant, irrespective of their sugar content. Composition of apple fruit for selected quality traits as average for 15 different cultivars is given in Fig. 1.2 (Nour *et al.*, 2010).

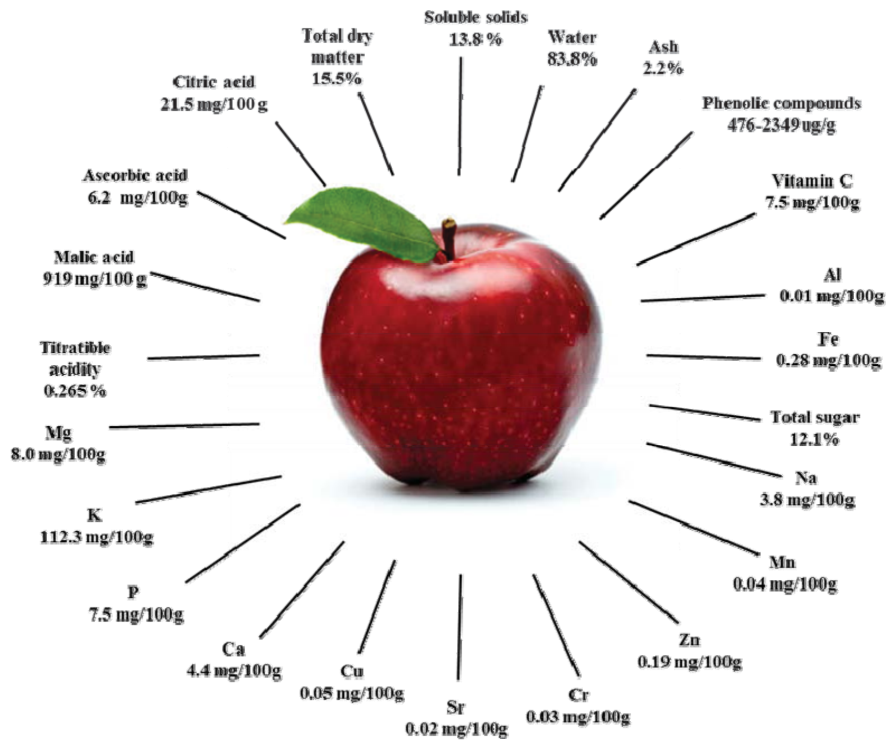

Fig. 1.2 Composition of apple fruit for selected quality traits. Source: Nour et al. (2010), Campeanu et al. (2009), and Lata (2005).

1.6 Phenolic compounds found in apple fruit

The content of phenolic compounds varies in different plant species. It also varies from tissue to tissue within the same plant species. For instance, Lata (2005) measured the average phenolic content in 53 different apple cultivars in two successive growing years 2003 and 2004. The average phenolic content for different cultivars was 777-1842 ug/g in year 2003 and 476-2349 ug/g in 2004. This shows some seasonal variation in phenolic content and more over there was also variation among the different cultivars for the content of the phenolic compounds. The amount of phenolics for cutivars 'Prima' and 'Fiesta' falls within the range for different cultivars, for 'Prima' being more than 'Fiesta'. They also found flavonols and anthocyanins with some variation between the two years. The amount of these compounds in both 'Prima' and 'Fiesta' was in the range of different cultivars.

'Prima' showed a bit higher amount than 'Fiesta' (Lata *et al.*, 2005). This also shows that both 'Prima' and 'Fiesta' contain significant amounts of the important phenolic compounds. We used a segregating F1 population during our study derived from the cross of both these parents. Apple cultivars with increased levels of beneficial phenolic compounds could incline the consumer for more fruit consumption because of improved health values.

In another study by Lata (2007) phenolics for whole fruit was found to be 1.6-4.6 mg/g where 'Fiesta' contained 2.0 mg/g and 'Prima' 2.4 mg/g. They found that the total phenolics in peel were three times higher than in flesh by HPLC and folin-ciocalteu method (Lata, 2007). Van der Sluis et al. (2001) also found that flavonoids are not equally distributed throughout the apple. For instance, quercetin glycosides are almost exclusively found in peel and have very low concentration in flesh. Phloridzin was found both in peel and flesh. Mcghie et al. (2005) found on average 46% of the phenolic compounds of apple in the peel and determined that flavanols contents were higher in flesh than in peel. Like Van der Sluis et al. (2001), Mcghie et al. (2005) also observed that phloridzin was present in both peel and flesh as well as procyanidins. Chlorogenic acid was more in flesh (80-85%) whereas anthocyanins only were determined (100%) in peel (Mcghie *et al.*, 2005).

1.7 Improvement of apple through advanced technologies

In spite of its importance to agriculture and its pervasive role in human health, relatively little is known about apple fruit and its development, physiology, biochemistry, and metabolomics, including secondary metabolites. This lack of knowledge has contributed to perpetual difficulties in breeding, production, and storage of apple (Park *et al.*, 2006).

There is a direct need for the fast development of apple varieties with increased health promoting compounds. There is a need to identify genes responsible for these health beneficial compounds. The developments in plant biotechnology offer a very promising solution for that, especially for direct improvement of existing varieties of vegetatively propagated crops like apple. Extensive research on genetic engineering of crop plants is in progress. Literature is also available regarding the genetic modification of apple (Puite and Schaart, 1996; James *et al.*, 1989; Bolar *et al.*, 2000; Faize *et al.*, 2003; Faize *et al.*, 2004; Belfanti *et al.*, 2004; Malnoy *et al.*, 2008). A good collection of apple expressed sequence tags (ESTs) is also available (Park *et al.*, 2006). This can be further accelerated when the whole genome sequence for apple will become publically available in near future.

1.7.1 QTL mapping

It has been known for nearly a century that the approximate genetic position of loci controlling the quantitative traits can be identified through association of markers with phenotypic variation in a structured population (Flint and Mott, 2001; Mackay, 2001). This association is the foundation for quantitative trait locus (QTL) mapping that attempt to identify number, phenotypic impact and interaction of the loci controlling a quantitative trait (Flint and Mott, 2001; Mackay, 2001; Asins, 2002). The diversity between individuals is partitioned into both environmental and genetic variation. Frequently genetic variation studied to date tends to be qualitative so that there are one or more distinct and non-overlapping phenotypic states. However, most phenotypic differences are quantitative such that there are numerous overlapping phenotypic states (Flint and Mott, 2001; Mackay, 2001). The availability of genetic linkage maps enable the detection, analysis and use of QTL contributing to quantitative traits of the genotype (Zini *et al.*, 2005). The combination of molecular markers and trait data to explore individual genes concerned with quantitative traits has become an important tool for biologists to dissect the genetics of complex traits (Kearsey, 1998).

In crop plants, quantitative variation is a feature of many important traits, such as those related with yield, quality or disease resistance (Asins, 2002). Although most of the advancements in genetics over the last century have been concerned with structural variation in single ‘major genes’, much of the natural variation observed in our species and crops, domestic animals and other populations that are studied are due to minor genetic changes in many genes, called polygenes (Kearsey, 1998). Over the last 15 years, QTL mapping has become a popular method for understanding the genetic basis of continuous variation in a variety of

complex traits. For example, the technique of QTL mapping is now an integral tool in medical genetics, livestock production, plant breeding and population genetics of model organisms (Slate, 2005). Plant breeding is a dynamic area of applied science. It relies on genetic variation and uses selection methods to improve plants for traits and characteristics that are of interest for the growers and the consumers (Asins, 2002). This allows the QTL mapping to unravel the genetic base of many important complex traits such as disease resistance (Calenge *et al.*, 2004; Calenge and Durel, 2006; Khan *et al.*, 2006), fruit quality (Liebhard *et al.*, 2003; King *et al.*, 2001; Davey *et al.*, 2006) or tree morphology (Kenis and Keulemans, 2007).

1.7.2 Marker assisted selection

Conventional apple breeding is a slow process and many of the traits nowadays can be selected by the use of molecular markers technology. In apple this technology is also more and more available because of the presence of molecular markers maps and the availability of markers linked with important traits such as resistance to diseases (Durel *et al.*, 2009) and even to select indirectly for skin colour of apple fruit (Zhu *et al.*, 2011). The knowledge of positions of markers on chromosomes can be used and could help to identify genes responsible for the production of health beneficial phenolic compounds. This can be done easily by extracting the DNA and developing molecular markers for marker assisted selection (MAS) as already practised for several biotic stress resistances (Kellerhals *et al.*, 2000; Markussen *et al.*, 1995; Khan *et al.*, 2007; Moriya *et al.*, 2009; Durel *et al.*, 2009; Zhu *et al.*, 2011; Bus *et al.*, 2000). The molecular markers developed for localization of different phenolic compounds in the genome may be used to obtain the presence of genes responsible for synthesis of potential health beneficial compounds. This approach can also be used to assess the genetic diversity of crops (Schulman, 2007). Marker assisted selection is also employed for columnar type growth in apple (Moriya *et al.*, 2009). Khan et al. (2007) found two markers (SCAR, SSR) linked to a fire blight resistance QTL, explaining nearly 50% of the phenotypic variation for this trait in apple. These markers were fixed to the resistant progenies and were stable across different backgrounds and thus are useful for marker assisted selection. In another study, Bus et al. (2000) performed marker assisted selection for pest and disease resistance in an apple breeding programme.

1.7.3 Genetic modification

The most important advantage of genetic modification is the use of existing varieties of crops with a long term of safe use in the market. A major disadvantage of this approach is the lack of acceptance with transgenes. This is especially the case in fruit crops because of their consumption as fresh products. Two main developments have changed the world of genetic modification: 1. the possibility of marker free transformation or making GMO plant afterwards marker free by an additional step of active marker removal by site specific recombination using *cre-lox* system (Dale and Ow, 1991), *FLP/FRT* system (Lyznik *et al.*, 1993), or*R/Rs* system (Schaart *et al.*, 2004), and 2. the growing availability of useful cisgenes, coding for example for disease resistance, in addition to transgenes. Marker free transformation with cisgenes could be an acceptable solution for the consumer, as shown in the Eurobarometer (2010) at one hand and extent conventional breeding at the other hand after it is exempted from GMO regulation in Europe (2001/18/EC) and/or USA.

Marker free transformation of apple, at this moment, means the use of marker assisted transformation with kanamycin resistance (*nptII*) gene. This can be followed after selection of back bone free plants with the induced excision of the selection marker coding for *nptII* and other helper genes. This process is resulting in an apple plant with only the desired cisgene. Another possibility is the development of a cisgenic selection marker for example, based on the dominant *MdMYB10* transcription factor, which was mutated in the variety 'Red Field'. This mutated transcription factor is visibly accumulating anthocyanin in many tissues of apple.

There are more and more cloned cisgenes available coding for important traits such as disease resistance. In apple, for example, the *Vf2* gene has been cloned, coding for resistance to apple scab. In the future more genes will be found in apple that are directly or indirectly influencing other traits coding, for example, for the synthesis of health promoting compounds and abiotic stress resistance. Through the knowledge of cloned key genes and their alleles involved, development of superior apple cultivars which provide desired health beneficial compounds is possible. These genes could be transferred into high quality apple cultivars with a long safe use by means of transgenesis and cisgenesis.

Transgenesis is defined as 'the genetic modification of a recipient plant with one or more genes from any non-plant organism, or from a donor plant that is sexually incompatible with the recipient plant' (Schouten *et al.*, 2006a). Cisgenesis is defined as 'the marker free genetic modification of a recipient plant with natural gene(s) from the plant itself or from a sexually compatible plant' (Schouten *et al.*, 2006a, b). This is in contrast to transgenesis where transgenes, originating from non-crossable species or consisting of functional parts of different genes, are used. It has been clearly shown in the Eurobarometer (2010) that the application of cisgenes is much more acceptable for the European consumer than transgenes. There are strong discussions going on within the USA to exempt cisgenesis from the GMO regulations of that country (Waltz, 2011).

1.8 Outline of the thesis

In this thesis, variability for synthesis of secondary metabolites in apple has been investigated as well as genetic factors involved. In **Chapter 2,** genetic linkage maps were constructed and extended with the inclusion of a great number of DArT (Diversity Array Technology) markers. An integrated map of 1286 cM length containing 820 molecular markers was obtained. Availability of a high density genetic linkage map is a prerequisite for mapping of many genetic traits. In **Chapter 3,** we studied the metabolic variation in wild germplasm and advanced selections of apple as well as the metabolic variation in a segregating F1 population in comparison with the crossing parents involved. It was found that many different metabolites could be found in wild species but to a lesser extent in advanced breeding selections and the described F1 population of apple. It was also found that certain metabolites could be lost during inbreeding while certain other metabolites could be gain. The genetic factors causing this loss and gain of metabolites are discussed in this chapter.

In **Chapter 4**, we investigated the genetic basis of the quantitative variation of the earlier described potentially health beneficial compounds. We mapped these secondary metabolites in the segregating F1 population from the cross between the cultivars ‘Prima’ and ‘Fiesta’. Mapping of genetic factors influencing synthesis of metabolites is the next step in developing apple cultivars with increased levels of specific health beneficial metabolites. Two approaches were adopted for mQTL (metabolite QTL) mapping, a wide non-targeted approach using MetaNetwork (Fu *et al.*, 2007) and a targeted approach using MapQTL® (Van Ooijn, 2009). Untargeted metabolic profiling of peel and flesh tissues of ripe fruits was applied by liquid chromatography-mass spectrometry (LC-MS), resulting in the detection of 418 metabolites in the peel and 254 in the flesh. An mQTL hotspot was found on LG16. We located structural genes involved in the phenolic biosynthetic pathway, using the recently published whole apple genome sequence of cv. Golden Delicious (Velasco *et al.*, 2010). The structural gene coding for *leucoanthocyanidin reductase* (*MdLAR1*) was found within the mQTL hotspot window on LG16, as were in addition seven transcription factor genes. **Chapter 5** deals with the expression profile of the genes in the mQTL on LG16 and also all the structural genes of the phenylpropanoid pathway in apple. Structural gene (*MdLAR1*) showed differential expression. We believe that this *MdLAR1* has caused the mQTL hotspot which is reported in chapter 4 above. The mQTLs located upstream in the pathway could be explained by the negative feedback mechanism. **Chapter 6** deals with the finding of genes influencing acidity in apple fruits. Acidity is an important determinant for fruit quality. An optimum acidity together with optimum sugar content is required

for consumers. We located *MdALMT2*, coding for a malic acid transporter, as candidate gene which causes changes in acidity and is ultimately influencing the pH in apple.

In **Chapter 7** *MdMYB10*, a dominantly mutated transcription factor gene, involved in the up-regulation of anthocyanin in many tissues of apple, was inserted into apple cultivar 'Gala' and also in potato cv. Desiree and strawberry cv. Calypso by antibiotic marker free selection. It can be used as a cisgenic selectable reporter gene for cisgenic apple plant transformation. All the experimental chapters are combined and discussed in **Chapter 8**. The different steps from genotyping and phenotyping of a segregating F1 population, studying the metabolic diversity of the apple germplasm, mapping of the phenolic compounds, the expression profile of the candidate genes and all other phenylpropanoid pathway genes, finding candidate genes for pH determination in apple fruits, and transformation of the *MdMYB10* gene are discussed in more detail in chapter 8.

Chapter 2

Diversity Arrays Technology (DArT) markers in apple for genetic linkage maps

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Abstract

Diversity Arrays Technology (DArT) provides a high-throughput whole genome genotyping platform for the detection and scoring of hundreds of polymorphic loci without any need for prior sequence information. The work presented here details the development and performance of a DArT genotyping array for apple. This is the first paper on DArT in horticultural trees. Genetic mapping of DArT markers in two mapping populations and their integration with other marker types showed that DArT is a powerful high-throughput method for obtaining accurate and reproducible marker data, despite the low cost per data point. This method appears to be suitable for aligning the genetic maps of different segregating populations. The standard complexity reduction method, based on the methylation-sensitive *Pst*I restriction enzyme, resulted in a high frequency of markers, although there was 52 to 54% redundancy due to the repeated sampling of highly similar sequences. Sequencing the marker clones showed that they are significantly enriched for low copy, genic regions. The genome coverage using the standard method was 55 to 76%. For improved genome coverage, an alternative complexity reduction method was examined, that resulted in less redundancy and additional segregating markers. The DArT markers proved to be of high quality and were very suitable for genetic mapping at low cost for the apple, providing moderate genome coverage.

Keywords: apple, DArT, genetic mapping, molecular markers, diversity

1 Introduction

The genus *Malus* has been a focus of molecular studies since the mid 1980s, when Weeden and Lamb (1985) used isozymes as a means of discriminating between different apple cultivars. As new marker types have been developed, scientists have readily adopted them into their studies of apple genetics, progressing from RAPDs (Koller et al. 1993), through RFLPs and isozymes (Maliepaard et al. 1998), to AFLPs and SSRs (Guilford et al. 1997, Hokanson et al. 1998) and on to targeted markers (e.g., Broothaerts 2003; Calenge et al. 2005; Chagné et al. 2007) and SNP arrays (Micheletti et al. 2011). The progress in the molecular characterisation of the *Malus* genome has recently gained further momentum with whole-genome sequencing (Velasco et al. 2010).

Molecular markers have been applied widely to evolutionary and pedigree studies in apple, including both wild *Malus* species (Richards et al. 2009) and domestic cultivars (e.g., Cabe et al. 2005, Evans et al. 2010). Additionally, markers developed for the apple have been applied fairly widely to other pome species, and vice versa, notably pear (*Pyrus* spp., Yamamoto et al. 2001; Hemmat et al. 2003; Dondini et al. 2004), quince (*Cydonia oblonga*; Yamamoto et al. 2004) and loquat (*Eriobotrya japonica*; Gisbert et al. 2009; He et al. 2010). Simultaneously, linkage maps have been constructed for a number of domestic cultivars, and several map alignments have been reported (Maliepaard et al. 1998; N’Diaye et al. 2008; Patocchi et al. 2009; Van Dyk et al. 2010). The construction of linkage maps has facilitated the identification of molecular markers associated with numerous phenotypic traits. Among the traits examined to date are resistance to apple scab caused by the fungus *Venturia inaequalis* (Koller et al. 1994; Calenge et al. 2004; Bus et al. 2005a, b; Soriano et al. 2009), fire blight caused by the bacterium *Erwinia amylovora* (Peil et al. 2007), columnar growth habit (Moriya et al. 2009), several fruit quality traits (e.g., King et al. 2001; Liebhard et al. 2003a; Costa et al. 2005, 2008, 2010; Kenis et al. 2008) and chilling requirement (Van Dyk et al. 2010). The identification of such molecular markers is essential for marker-assisted selection in apple breeding programs (Gianfranceschi et al. 1996; Liebhard et al. 2003b; Gardiner et al. 2007; Zhu and Barrett 2008). In recent years, genomic methods have been embraced by apple researchers. The enhanced ability to study gene expression has resulted in new understandings of developmental processes (Ban et al. 2007; Espley et al. 2009). Transcription analyses of apple fruit development using cDNA microarrays (Soglio et al. 2009) and plant physiological responses to pathogens (Norelli et al. 2009) has facilitated the development of new molecular markers (e.g., Chagne et al. 2008; Igarashi et al. 2008). The further development of high-throughput genetic technologies will continue to expand the ability of scientists to

investigate the details of the genetics of apple and its relatives (Shulaev et al. 2008). Since the proof-of-concept paper (Jaccoud et al. 2001), DArT has been developed as an inexpensive whole-genome profiling technique for many organisms, especially plants. The website www.diversityarrays.com has a current list of organisms for which arrays are available (>50). DArT, in its current implementation, is a hybridisation-based genome profiling technology that does not require sequence information and uses microarrays to identify and type DNA polymorphisms. As the DArT markers are typed in parallel, it is possible to identify hundreds or even thousands of polymorphic markers in a single experiment (Wittenberg et al. 2009). This highly parallel assay results in a reduction of the per data point price to around US$ 0.01 in organisms with well-developed arrays. The DArT assay primarily detects dominant markers, mostly resulting from single nucleotide polymorphisms and indels in restriction sites and differences in methylation of restriction sites. When methylation-sensitive restriction enzymes (like *Pst*I; see Gruenbaum et al. 1981) were used in the large genomes of cereals (Wenzl et al. 2004, 2006; Akbari et al. 2006), DArT markers were located preferentially at the gene-rich, subtelomeric regions of the chromosomes. The use of methylation-sensitive enzymes may provide also an insight into epigenetic variation (Wenzl et al. 2004).

Interestingly, while DArT has performed well in over 50 crops, there are no reported applications of DArT in horticultural trees. As DArT has been applied successfully to complex amphidiploid genomes like wheat (Akbari et al. 2006), oat (Tinker et al. 2009) and sugarcane (Heller-Uszynska et al. 2010), application to the duplicated genomes of the pome fruits such as apple (Velasco et al. 2010), pear and loquat should have good perspectives too. Here, we present the development and validation of Diversity Arrays Technology (DArT) for apple using a complexity reduction method similar to the one used for the cereal genomes. We compare this with a second complexity reduction method that is similar to the method used for the fungus *Mycosphaerella graminicola* (Wittenberg et al. 2009), and we discuss the characteristics of the detected markers. We combined DArT markers with other marker types in genetic linkage maps, providing insight into the coverage of the DArT markers in the apple genome. We used as a starting point the progeny and genetic linkage map of ‘Prima’ x ‘Fiesta,’ which was the first genetic linkage map for apple covering all 17 chromosomes (Maliepaard 1998). In addition, we used a more recent progeny of other parents for genetic mapping. Furthermore, we evaluated the performance of DArT in a genetic diversity analysis of 44 diverse apple accessions and a set of Australian breeding lines.

2. Materials and methods

2.1 Plant Material

For making of the DArT libraries, care was taken to represent a wide genetic diversity, including several major founders in apple breeding worldwide, founders of more local breeding programs, modern cultivars and some very recent selections from ongoing breeding programs. Forty-four accessions of *Malus* were used for the library development (Online Resource 1a).

For mapping, two populations were used. The first, 'Prima' x 'Fiesta,' was used to examine the reliability of the DArT data by evaluating the ease by which the DArT markers were integrated in an existing, well-established linkage map. This population consists of 156 individuals (Maliepaard et al. 2008), of which a subset of 121 individuals were DArT genotyped. The second population, 2000-012 (Soriano et al. 2009), was used to demonstrate the ease by which DArT markers allow for the alignment between mapping populations. Further, this population was used to compare two DArT complexity reduction methods with respect to the number of markers and genome coverage. It comprises 894 individuals, 399 of which were used in the current study. The parentages of both populations are presented in Fig. 2.1.

2.2 DNA extraction

For the development of the DArT libraries and genetic diversity studies, leaves were collected from grafted trees with a preference for younger, actively expanding leaf material. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) / chloroform / isoamylalcohol protocol based on the method of Doyle and Doyle (1987). The addition of 2% W/V polyvinylpyrrolidone (PVP-40, Sigma, K value: 29-32) (Aljanabi et al. 1999; Kim et al. 1997) appeared to be essential for preventing the inhibition of restriction endonuclease digestion in many of the apple leaf samples tested. For the mapping populations, DNA was extracted according to Maliepaard et al. (1998) and Soriano et al. (2009).

2.3 Construction of DArT Arrays

A crucial step in the Diversity Arrays Technology is the complexity reduction of genomic representations. In this manuscript, complexity reduction refers to the reproducible selection of a subset of DNA fragments from a whole genome. These fragments, after being cloned into *E. coli* vectors (TOPO) and amplified with M13 primers, were printed onto slides as probes for microarray hybridisations. The complexity reduction method used most often in DArT involves digestion with the methylation-sensitive restriction enzyme, *Pst*I. In conjunction with digestion using

this relatively rarely-cutting restriction enzyme (six bp recognition site plus methylation sensitivity; Gruenbaum et al. 1981), an enzyme with frequent cutting capabilities is used (Wenzl et al. 2004). In this study, the frequently-cutting enzymes *Alu*I, *BstN*I, *Taq*I or *Mse*I were used. PCR adapters were ligated to the *Pst*I fragment ends, and the PCR-amplification was performed using primers complementary to the *Pst*I adapters, according to Wenzl et al. (2004). Only those fragments with *Pst*I adapters at both ends were amplified.

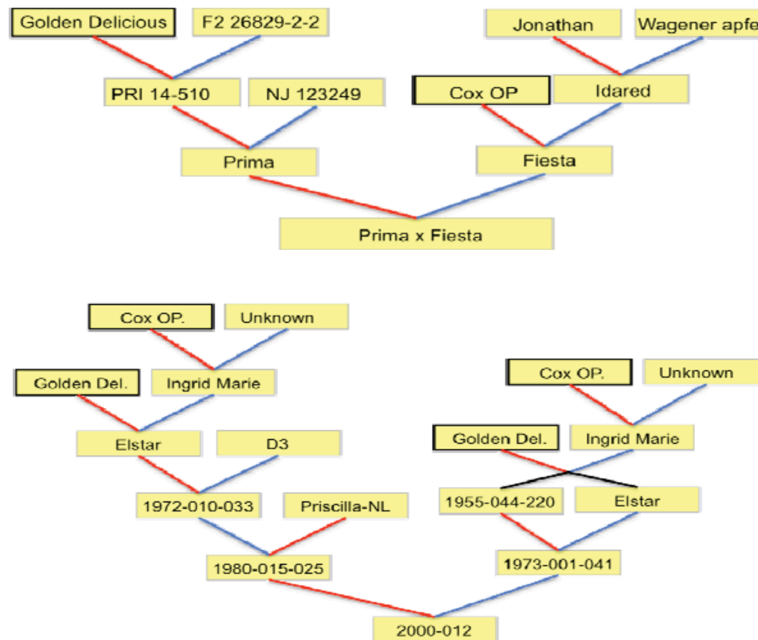


Fig. 2.1. Pedigree of the two examined mapping populations. Common parents are high-lighted.

Initial assessment of the enzyme combinations was performed by agarose gel electrophoresis, as described by Jaccoud et al. (2001). On this basis, the genomic representations produced by the digestion with *Pst*I, in combination with either *Alu*I or *BstN*I (*Pst*I/*Alu*I and *Pst*I/*BstN*I), were considered to be the most suitable due to the absence of visible bands in the gel smear (Kilian et al. 2005). An initial library of 768 clones was prepared for *Pst*I/*Alu*I and a second initial library of the same size for *Pst*I/*BstN*I, using DNA from 15 diverse heritage apple varieties (Online Resource 1a). The inserts of the 2 x 768 clones were amplified and printed on glass slides to provide small arrays for testing. The 15 cultivars listed in Table 1 were

hybridised in duplicate to the arrays, according to Wenzl et al. (2004). The *Pst*I/*Alu*I complexity reduction method was found to give a higher number of candidate polymorphic markers (16% of clones) as compared to the *Pst*I/*Bst*NI method (10% of clones). Therefore the *Pst*I/*Alu*I library was expanded by an additional 3,840 clones derived from the 15 heritage cultivars, and 9,984 clones from modern apple cultivars and breeding lines (Online Resource 1a). The total size of the expanded library for the *Pst*I/*Alu*I complexity reduction was 14,592 clones.

2.4 Hybridisation to the expanded array

To gain insight into the applicability of DArT in mapping, two segregating populations were hybridised to the expanded array. All genotypes were hybridised with two to four replicate arrays per genotype, using both Cy-3 and Cy-5 fluorescent labelling. Hybridisation, subsequent processing and data analysis were performed according to Wenzl et al. (2004).

2.5 Integration of existing genetic markers into genetic linkage maps

The genetic linkage map of ‘Prima’ x ‘Fiesta’ was the first for apple covering all 17 chromosomes (Maliepaard 1998). It consisted of 138 dominant markers (mainly RAPDs and some AFLPs) and 152 essentially co-dominant markers (mainly RFLP, some isozymes and SSRs). Since then, 313 new markers have been added through successive European projects, of which 180 are dominant (mainly AFLP) and 133 are co-dominant, mainly consisting of SSR markers from the gDNA based CH and Hi series (Liebhard et al. 2003b, Silfverberg-Dilworth et al. 2006) as gathered from EST sequences (Soglio 2009). Some of the new markers were specifically designed for fruit quality genes (Costa et al. 2005, 2008, 2010) and allergy genes (Gao et al. 2005a,b,c). The quality of the map was thoroughly validated and improved, using JoinMap (Van Ooijen and Voorrips 2008). This newly enriched, evaluated and extended map of ‘Prima’ x ‘Fiesta’, covers approximately 90% of the apple genome, and was used as a starting point for the mapping of DArT markers.

2.6 An alternative method for complexity reduction

We evaluated an alternative complexity reduction method that was applied by Wittenberg et al. (2005) to microbial genomes. This approach involved digestion with two six-base cutters, *Pst*I and *Eco*RI. A standard adapter was ligated to the *Pst*I ends of the restriction fragments and a long, asymmetric adapter with a 3'-amino (NH_2) group on the short strand was ligated to the *Eco*RI ends. The amino group, combined with PCR suppression (Siebert et al. 1995; Broude et al. 2000), was used to prevent amplification of the *Eco*RI - *Eco*RI fragments. Only the *Pst*I - *Pst*I and *Pst*I - *Eco*RI fragments were amplified. To further reduce the complexity of the genomic representations, a third endonuclease, the four basepair cutter *Mbo*I, was used. No adapters were ligated to the *Mbo*I sites. Consequently, the fragments cut by *Mbo*I were not amplified (Wittenberg et al. 2005).

For this alternative complexity reduction method, the DNA of the apple selection 1980-015-025, a parent of population 2000-012 (Fig. 2.1), was used to construct a library of 6144 fragments, which were printed onto slides (Wittenberg et al. 2005). Target DNA from this selection's progeny, population 2000-012, was assayed with this array, using the same alternative complexity reduction method. The adapters ligated to the target DNA of this progeny differed from those of the parental fragments printed on the slides, in order to prevent hybridisation of adapters to one another (Wittenberg et al. 2005).

The alternative array was used for the genotyping of 244 progenies of population 2000-012, all of which had also been genotyped with the standard method too. The maternal map of the heterozygous parent 1980-015-025 was constructed using DArT markers from both complexity reduction methods. In addition, several SSR markers were used as references on the linkage map. They were generated according to Patocchi et al. (2008).

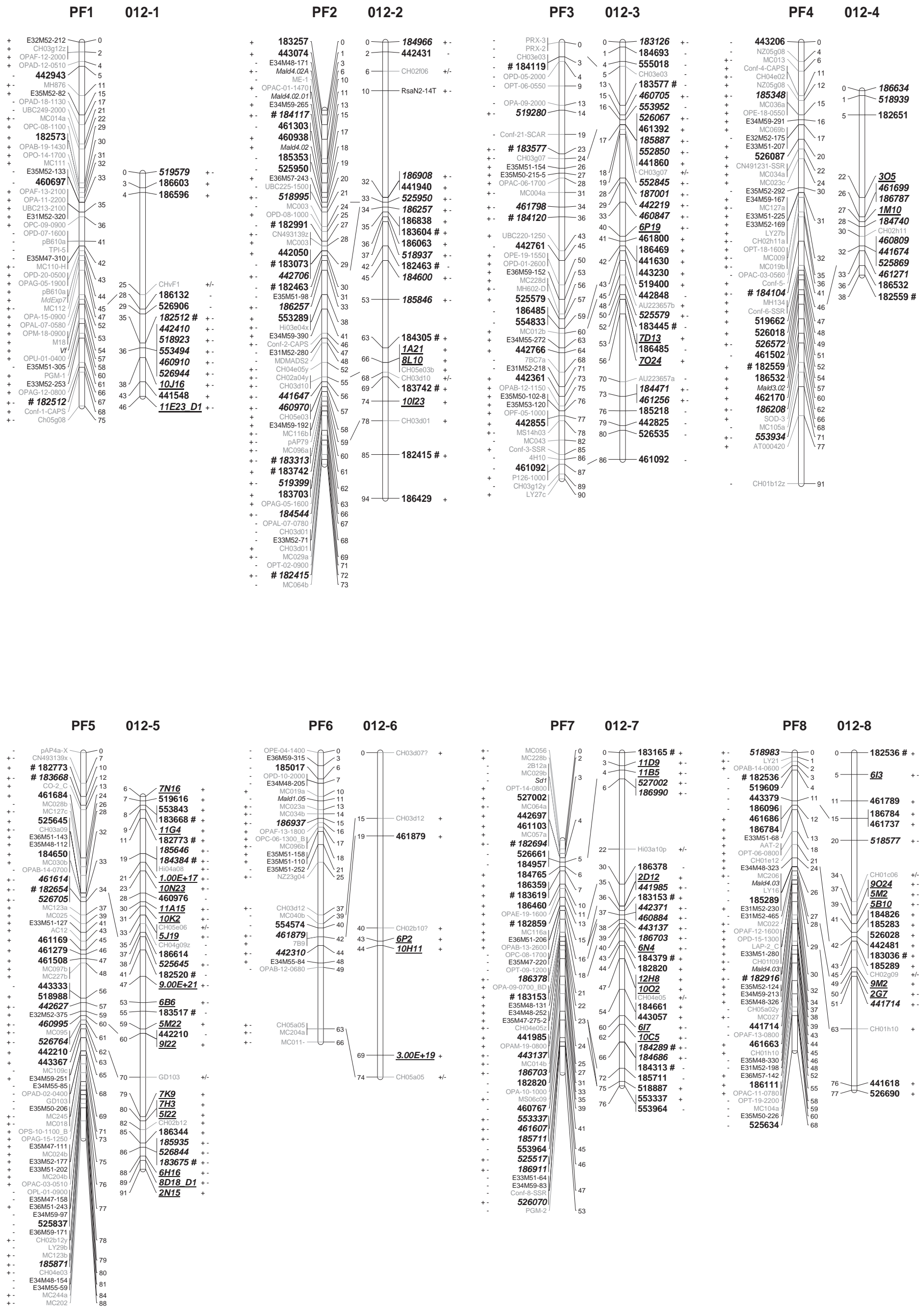
3. Results

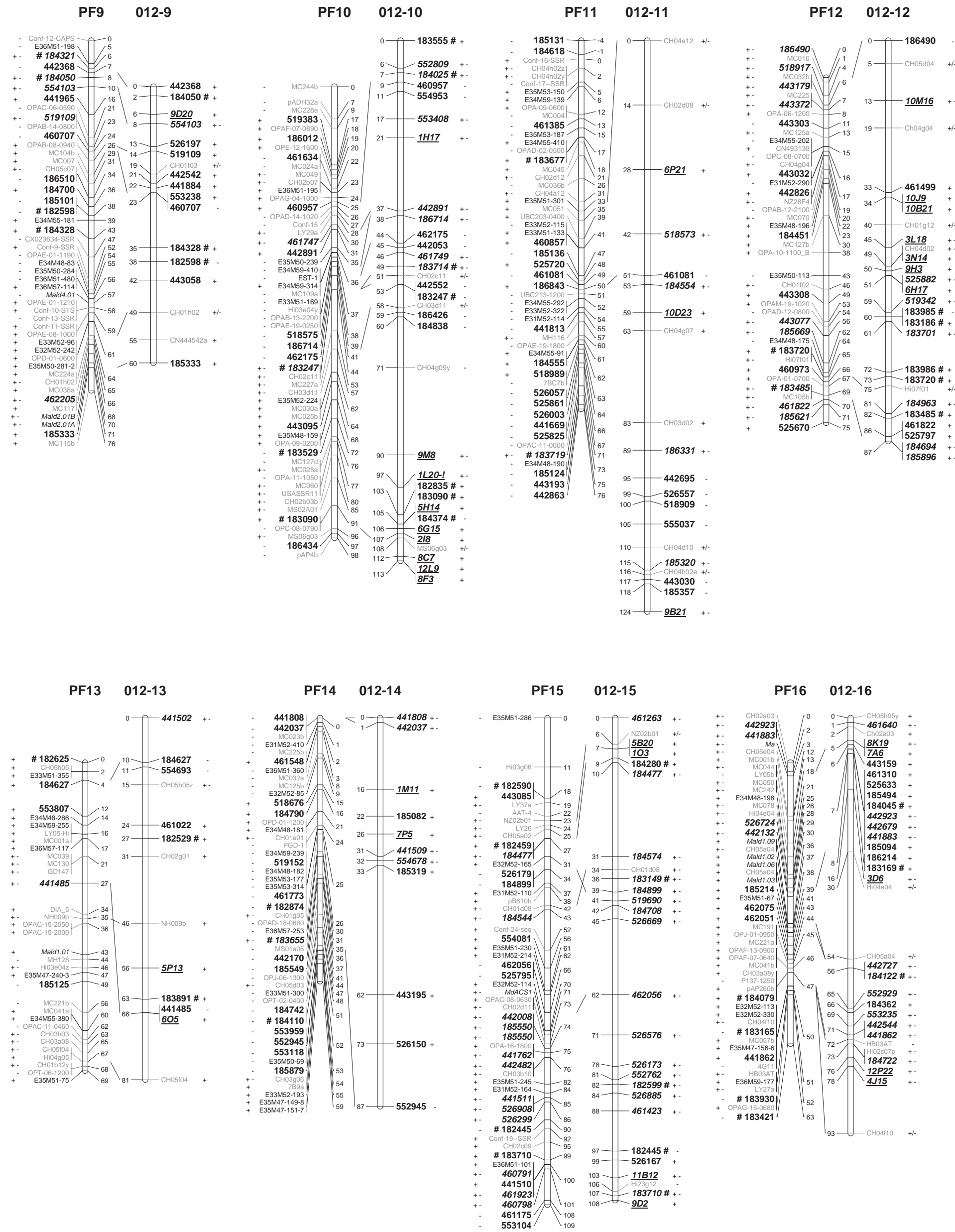
3.1 Mapping of DArT markers in ‘Prima’ x ‘Fiesta’

The ‘Prima’ x ‘Fiesta’ progeny were hybridised to the expanded DArT array for the standard complexity reduction method, which provided 776 polymorphic markers. The call rate for the parental genotypes was 99.2%. The call rate is the percentage of targets that could reliably be assigned a score of ‘0’ or ‘1’ for a given candidate marker. The average call rate for the entire ‘Prima’ x ‘Fiesta’ mapping population was 96.7%.

Of the aforementioned 776 ‘Prima’ x ‘Fiesta’ DArT markers, 247 (32%) were mapped to a unique position (Fig. 2.2). The other 68% were eliminated during the mapping process for several reasons (Table 2.1). Only 4.6% of the markers were discarded due to possible scoring problems. Of these, 3.5% were due to incomplete data on the mapping parents, leaving only 1.1% of the markers being possibly discarded for inadequate scoring, remaining ungrouped or showing irregular segregation patterns. The two latter phenomena could also be due to reasons other than scoring difficulties, such as a lack of marker coverage of the genomic regions or the presence of duplicated loci. Online Resource 1b documents that adding DArT markers did not affect the previous high overall map quality, as measured by the average χ^2 value.

A considerable number of clones exhibited identical segregation patterns in ‘Prima’ x ‘Fiesta’ compared to other clones, and therefore did not provide a higher genetic resolution in the map. As a result, a total of 364 (54%) of the 677 clones (=776-99; Table 2.1) were classified as redundant.

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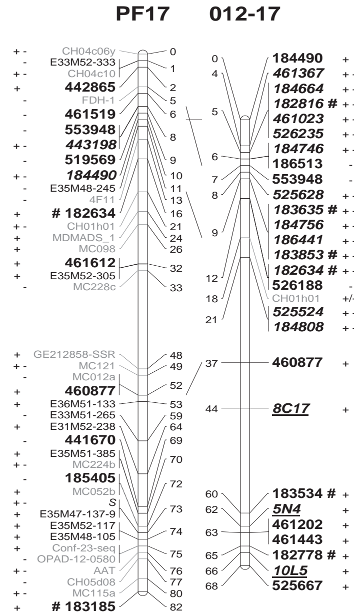

Fig. 2.2: Alignment of the 17 linkage groups of the mapping populations ‘Prima’ x ‘Fiesta’ (PF) and 2000-012 (012). DArT markers are displayed in bold, those segregating in both parents are in italics and those generated with the alternative complexity reduction method are underlined. The + and – symbols next to a marker name indicate that the DArT marker was polymorphic in the mother and father, respectively. The # symbols indicate the DArT markers that have been sequenced.

Table 2.1. Number of DArT markers from the standard complexity reduction method, during successive mapping stages in the 'Prima' x 'Fiesta' population.

Mapping stages	'Prima'		'Fiesta'		Both parents		Total		
	No. of events	No. of cumu lative	No. of events	No. of cumu lative	No. of events	No. of cumu lative	No. of events	% events	No. of cumu lative
Preparation data									
Polymorphic		257		234		285			776
Unclear parentage	11	246	14	220	0	285	25	3.2%	751
Polymorphic Information Content < 0.10	47	199	49	171	3	282	99	12.8%	652
Mapping									
Redundant, completely identical	1	198	40	131	73	209	114	14.7%	538
Remaining ungrouped	0	198	4	127	0	209	4	0.5%	534
Insufficient linkage for phase determination	1	197	4	123	32	177	37	4.8%	497
Irregular segregation pattern	2	196	4	119	1	176	7	0.8%	490
Redundant, differences only for missing values	112	83	42	77	96	80	250	32.2%	240
Double recombinant	0	83	1	76	0	80	1	0.1%	239
Unique mapped		**83**		**76**		**80**			**239**
Markers with solved parentages	3	86	3	79	2	82	8	1.0%	247
Total uniquely mapped	**86**		**79**		**82**		**247**		

3.2 Genome coverage

The current DArT array provided moderate genome coverage. Fig. 2.2 illustrates this. Many markers clustered, producing several short genomic segments containing multiple markers, such as a segment of 4 cM at the top of LG7 that contained eight unique 'Prima'-specific DArT markers and one marker for both parental cultivars. However, several extended regions had no or very few markers, such as the entire LG1 of 'Prima,' which contained only one 'Prima'-specific and one common marker. Genome coverage was estimated using the integrated map of Fig. 2.2 as a reference and the following thresholds: i) only parent-specific markers were considered, as markers common to both parents carry little genetic information, and ii) a single marker covers 10 cM surrounding its position. In these calculations, the current DArT array offered sufficient coverage for performing classical QTL mapping studies on around 55% of the 'Prima' and 60% of the 'Fiesta' genome. If a single marker was to sufficiently cover a larger window of 30 cM, then the genome coverage for 'Prima' and 'Fiesta' would be 76% and 74%, respectively.

3.3 Suitability of DArT markers for map alignment

To examine the power of DArT markers for aligning maps, the second mapping population, 2000-012, was hybridised to the same DArT array used for 'Prima' x 'Fiesta'. Additionally, several previously mapped SSR markers were included to confirm the DArT marker alignment. A similar number of polymorphic DArT markers was obtained (774 in 2000-012 versus 776 in 'Prima' x 'Fiesta'), in addition to a similar call rate (97.3%) and a similar redundancy level, leading to a comparable number of unique single locus markers (260 versus 247).

The two mapping populations were shown to have 70 common polymorphic DArT markers that consistently aligned homologous linkage groups with regard to their identity and orientation for all 17 linkage groups of apple (Fig. 2.3). Several cases of minor differences in marker order were observed, depicted as the crossing lines in Fig. 2.2. The overall consistency in the identity and orientation of the linkage groups and the marker order show that the DArT markers support the alignment of the mapping populations.

3.4 DNA sequencing of DArT markers and redundancy estimation

In the mapping process, DArT markers were classified as redundant when they showed identical scores among the progeny, leading to clustering at one genetic position. Such clustering may be caused by high DNA sequence similarity or tight genetic linkage of the markers. To obtain an estimate of sequence-based redundancy, we sequenced 384 clones from the *Pst*I/*Alu*I DArT array. We performed a local pairwise blast of all of these sequences and then clustered them into bins of highly similar sequences (e < 1.0E-50), using an in-house developed Perl script (DArT PL unpublished). As Fig. 2.3 shows, the number of clones per bin varied from one (278 bins) to four (1 bin only). There were 324 sequence bins identified; therefore, the percent of redundant clones in the initial sampling of the 384 clones was 15.6% when based on the sequences. Since these 384 sequenced clones represent only 2.6% of the 14,592 clones used for printing on the slides, the real redundancy was higher. Fitting the observed data from Fig. 2.3 to a Poisson distribution, we deduced that sequence similarity led to approximately 50% redundancy within the total set of clones.

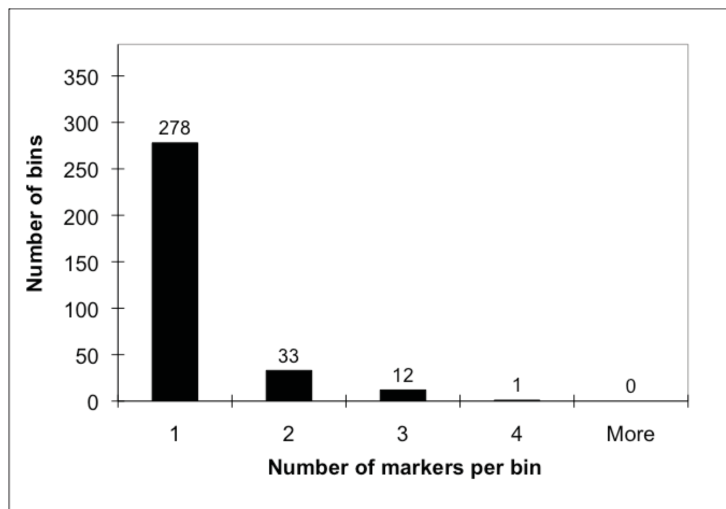

Fig. 2.3 Frequency distribution of 384 DNA-sequenced DArT markers from the standard *PstI/AluI* method. If DNA sequences of one or more clones were highly homologous to each other, then these clones were clustered into a common bin. The 384 markers provided 324 bins.

Comparison of these DNA-sequences to sequences in NCBI GenBank databases showed that close to 90% of BlastN and TblastX searches returned highly significant similarities to EST sequences (Online Resource 2). This indicates that the *Pst*I/*Alu*I DArT clones were derived mainly from genes or gene-like sequences.

3.5 Alternative complexity reduction method

The high proportion of redundant markers hampers the statistical and genetic analyses of the data. Therefore, we wondered whether marker redundancy could be decreased by a less stringent complexity reduction method and whether such an alternative approach could also be useful in increasing genome coverage. The standard complexity reduction method was based on *Pst*I/*Alu*I digestion. The alternative method that we examined here was based on *Pst*I/*Eco*RI/*Mbo*I digestion, which resulted in a larger number of fragments, so the chance that a clone was sampled multiple times diminished. The alternative array was used for the genotyping of 244 progenies of population 2000-012, all of which had also been genotyped with the standard method. The maternal map of the heterozygous parent 1980-015-025 was constructed using DArT markers from both complexity reduction methods.

Table 2.2 Number of DArT clones and resulting markers from two complexity reduction methods for the maternal map of population 2000-012.

Complexity reduction method	Number of spotted clones	Number (and percentage[1]) of segregating markers	Number of mapped markers	Number (and percentage[2]) of redundant markers	Number (and percentage[1]) of mapped markers after removal of the redundant ones
Standard	14592	547 (3.7%)	542	282 (52%)	260 (1.8%)
Alternative	6144	105 (1.7%)	104	24 (23%)	80 (1.3%)

[1] as % of the number of spotted clones
[2] as % of the number of mapped markers

Table 2.2 shows the number of spotted clones per complexity reduction method, the number of obtained polymorphic maternal markers, the number of maternal markers that could be mapped and the number of ‘unique’ markers. Fig. 2.2 shows the resulting maps. Table 2.2 and Fig. 2.2 led to the following conclusions:

1. The degree of redundancy was lower for the alternative method;
2. The two methods gave a similar genome coverage;

3. There are no clear indications that the two methods differ in the genomic regions for which they raise polymorphic markers;
4. Performance of both methods increased genome coverage compared to application of one method only.

4. Discussion

4.1 Comparison of DArT with other marker technologies

After being initially developed for rice, DArT markers have been developed for many additional plant species (Jaccoud et al. 2001; Xie et al. 2006). These include barley (Wenzl et al. 2004), wheat (Akbari et al. 2006; White et al. 2008), cassava (Xia et al. 2005), *Arabidopsis* (Wittenberg et al. 2005), pigeon pea (Yang et al. (2006), oat (Tinker et al. 2009), sorghum (Mace et al. 2008) and many others (collated at www.diversityarrays.com/publications.html). The present study demonstrates the performance of DArT technology for low-cost, high-throughput genotyping in apple (*Malus*).

Several lines of evidence support the utility of DArT for apple genomics studies. First, the call rate and reproducibility appear to be high. Non-DArT marker data required repeated examinations for identification of erroneous data, consuming many months of labour. DArT markers, however, did not require this laborious scrutinising; DArT genotyping was fully automated and more accurate than other marker systems. The non-DArT markers were gel-based systems, i.e. RFLP, RAPD, AFLP, SSR, and were scored manually. Second, the DArT markers integrated smoothly into the existing 'Prima' x 'Fiesta' map. Only one marker could not be placed (0.4%). This percentage is very low compared to that found in other marker systems, like RFLP, RAPD, AFLP and SSRs. Also, DArT markers were robust among different mapping populations, allowing for map alignment.

The standard DArT array gave similar numbers of non-redundant markers in the two mapping populations, indicating that it is robust over populations. Moreover, the majority of these markers were population-specific, indicating that the extensive pool of clones that are not polymorphic in one population are a vast reservoir of possible new markers in other populations. Thus, the DArT array is applicable over a wide range of mapping populations. The number of unique markers is therefore expected to increase further in more extensive studies.

DArT lends its general applicability for wide germplasm coverage to the hybridisation of PCR fragments that are hundreds of base pairs long. Polymorphisms are based on the presence or absence of restriction sites and are insensitive to SNPs and short indels in the hundreds of basepairs between the restriction sites (Wittenberg et al. 2005). This is reflected by the sequence data. Map-redundant DArT markers were often based on clones that differed in size and sequence but nevertheless showed identical segregation patterns in mapping. For instance, the three markers 183247, 183997 and 184057 showed identical segregation patterns and therefore mapped to the same position (LG10, 'Prima' x 'Fiesta', linkage group 4, 44cM; Fig. 2.2, only 183247 is shown). The three DArT fragments varied in size (479, 469 and 502 bp, respectively) and exhibited some sequence polymorphism as occurs for different alleles of a single gene. Their map-redundancy was confirmed at the sequence level as "blasting" matched them to the same gene (Resource Online 2). This insensitivity to SNPs and short indels in the probe makes DArT robust for applications on genetically diverse germplasm. This contrasts with the sensitivity of SNP arrays to similar polymorphisms. The proportion of informative markers from SNP arrays drops quickly when applied to germplasm that is genetically distinct from accessions that were used in the design of the SNP array. DArT appears to be less prone to this limitation.

DArT and SNP platforms are both suitable for high-throughput genotyping, benefiting from automated scoring and data quality checks. The first small-scale SNP arrays for *Malus* were developed recently for 'Golden Delicious' (Micheletti et al. 2011). Recently, initiatives have been undertaken for worldwide collaboration on the development of large-scale, multiple accession-based arrays. We expect that DArT will also play a longer term role because of its own specific advantages, including wide applicability, as discussed above, and low cost even when small numbers of accessions have to be genotyped.

4.2 Impact of pedigree structure

Although the standard DArT array gave similar numbers of non-redundant markers for both mapping populations (247 for 'Prima' x 'Fiesta' versus 260 for 2000-012), differences were observed in the distribution of these markers among the parents. Whereas the proportion of maternal, paternal and bi-parental markers were similar in 'Prima' x 'Fiesta' (35%, 32%, 33%, respectively), unbalanced proportions were observed in 2000-012, with relative under-representation of paternal markers and over-representation of bi-parental markers (35%, 20%, 45%). These differences in representation reflect differences in the pedigree structures of these populations (Fig. 2.1). The father of population 2000-012 arose from a cross between two sibs, resulting in an expected level of homozygosity of approximately 25%. This parent would yield no segregating markers in these homozygous regions. In addition, the parents of population 2000-012 have common ancestors, 'Golden Delicious' and 'Ingrid Marie', reducing levels of allelic diversity. This increases the number of bi-parental markers that segregate for both the father and the mother. 'Prima' and 'Fiesta' lack such recent common ancestors. The distribution of segregating DArT markers among the parents thus reflects the pedigree structures, further supporting the suitability of DArT for genetic studies.

4.3 Redundancy

Markers that perfectly co-segregate do not provide additional genetic information, but slow down the mapping process and lead to the statistical overweighting of that specific position. Therefore, redundant markers were removed. This reduced the number of polymorphic markers used in the mapping process by about half. Markers that segregated in both populations needed to be preferentially retained to facilitate linkage map alignment.

Clustering of DArT markers can be caused by: (i) an absence of recombination between the markers due to tight genetic linkage; (ii) sequence identity; or (iii) high sequence similarity derived from different alleles from different apple accessions. The effective population size of 'Prima' x 'Fiesta' was 121, which can only partially explain the current high degree of redundancy in the DArT markers as a result of a lack of recombination between markers. Consequently, sequence identity and high sequence similarity were likely to be significant sources of redundancy. This is consistent with our estimation from Fig. 2.3 that sequence identity indeed led to approximately 50% redundancy. Based on the sequence information, we conclude that the clustering of *Pst*I-*Alu*I markers is mainly due to sequence similarity among the markers, in addition to genetic linkage.

In classifying markers as "redundant" using a mapping process, more than just similar mapping positions were taken into account. For a proper classification, the parental origin and linkage phase have to be identical too. For example, JoinMap® mapped the bi-parental DArT markers 183313 and 184063 within 0.1 cM from each other. Considering the size of the 'Prima' x 'Fiesta' population (n=121 for the DArT markers) and the type of marker (bi-parental), both markers belonged to the same genetic bin: there was no evidence that they mapped to different positions. Their scores did not indicate any recombination. Although this suggests that they are identical, their linkage phase was different: They were in repulsion phase, indicating that these markers come from different genomic positions. Indeed, DNA sequencing confirmed a substantial sequence difference of the two clones (Online Resource 2).

The positive side of redundancy is that the underlying markers can be regarded as repeats when present on the same slide and thus confirm validity of marker scores. The co-segregation of markers with highly similar sequences highlights the high reliability of the DArT markers.

4.4 DArT markers mainly derived from gene rich regions

Our analysis of the sequences shows that 90% of the sequenced markers are highly homologous to mRNA sequences that are deposited in EST databases (Resource Online 2). This indicates that the set of DArT markers is highly enriched for genic regions. This phenomenon has been observed previously in other species like barley (Wenzl et al. 2006), wheat (Akbari et al. 2006) and sorghum (Mace et al. 2008). DArT markers are predominantly located in gene-rich islands in the subtelomeric regions of chromosomes. This is not surprising, as the *Pst*I enzyme is methylation sensitive (Gruenbaum et al. 1981), and targets hypomethylated, low-copy sequences, which occur primarily in the gene-rich regions (Feng et al. 2010).

4.5 Comparison of complexity reduction methods

The standard DArT array developed here provided moderate coverage of the apple genome (Fig. 2.2), allowing for a quick start in QTL mapping experiments. Coverage, however, was not uniform across the entire genome. Enhancing the array with more clones could increase genome coverage, but using the standard complexity reduction method to do so would not be efficient, due to sequence redundancy. Therefore, we tested an alternative complexity reduction method in order to develop more DArT markers and improve the genome coverage.

Whereas the standard method only amplified *Pst*I–*Pst*I fragments, the alternative method amplified *Pst*I–*EcoR*I fragments as well as *Pst*I–*Pst*I fragments, yielding higher complexity with the alternative method than with the standard method. We simulated the number of fragments that would be amplified using the published whole genome sequences of *Arabidopsis thaliana*, and found that the alternative would result in approximately 4.5 times more amplicons in *Arabidopsis* than did the standard method (Mark Fiers, unpublished). The higher complexity of the alternative method and the printing of fewer of these amplicons resulted in less redundancy for population 2000-012 (23%), compared to the standard method (52%).

Despite the lower redundancy, the percentage of clones leading to unique single locus markers was low for the alternative method (1.3%) compared to the standard method (1.8%; Table 2). The most likely explanation is an unfavourable signal-to-noise ratio due to the higher degree of complexity. The analysis of raw image data, looking at the noise level, supports this explanation (data not presented). Another possible explanation is a reduction in polymorphism due to variation in methylation. Whereas the standard procedure only amplifies *Pst*I-*Pst*I segments, the alternative method also amplifies *Pst*I–*EcoR*I segments. The *EcoR*I enzyme is methylation insensitive, thus not using possible polymorphism in methylation. Wittenberg et al. (2005) ascribed up to 8 % of the polymorphisms of the alternative method to differences in methylation in *Arabidopsis thaliana.*

Importantly, a single standard DArT assay was clearly capable of providing reasonable genome coverage, as exemplified by the 17 linkage groups of the ‘Prima’ x ‘Fiesta’ map, with non-redundant markers distributed across all of the chromosomes, albeit unevenly (Fig. 2.2).

4.6 Linked research

The genetic maps shown here are the basis for several QTL studies that are currently underway, examining metabolomics (Khan et al., submitted), disease resistance, fruit quality traits, and low allergenicity (Van de Weg et al., in prep.). Additionally, the standard DArT assay is being applied worldwide to a number of mapping populations and to the construction of consensus genetic maps (Van Dyk et al., in prep.). All of the markers in this study are being sequenced and will be aligned with the apple genome sequence (Velasco et al. 2010). This will allow for the integration of the genetic positions of phenotypic traits with the whole genome sequence of apple, thereby aiding in the search for underlying genes.

Acknowledgements

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Chapter 3

Loss and gain of metabolites during apple breeding

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Abstract

The domesticated apple (*Malus* x *domestica* Borkh) is a rich source of secondary metabolites. In this study, fruits from 23 wild species, eight advanced selections and a segregating population from the cross between 'Prima' and 'Fiesta' were analysed for metabolic profiling, using liquid chromatography-mass spectrometry (LC-MS). The data were subjected to Principle Components Analysis (PCA). Variance analysis of the first principle component (PC) showed that genetic variation accounted for 96.6 % in peel and 97.4 % in flesh of the total metabolic variation. Technical variation accounted for 1.4 % and 0.8%, while environmental variation accounted for 2.0% and 1.8% in peel and flesh respectively. The genetic variation between wild genotypes was very large, compared to the advanced selections and the F1 progeny. Only 8 % of the genetic variation of the first principle component was captured by the advanced selections. This indicates a strong genetic erosion. This genetic erosion was mainly caused by reduction of the contents of several flavonoids including catechin, epicatechin and procyanidins. PCA of the F1 progeny of 'Prima' x 'Fiesta' cross showed a clear 3:1 Mendelian segregation of metabolites. These metabolites were 4.2 fold less in both peel and flesh in progeny that had inherited the recessive alleles of a gene at the top of Linkage Group 16 (LG16) from the heterozygous parents. We found a separate group of 11 metabolites in peel and 12 in flesh. These metabolites were putatively identified as glycosylated forms of β-glycols: R-octane-1,3-diol and its unsaturated form R-5-(Z)-octene-1,3-diol. The contents of these metabolites were up to 50 fold more abundant in some progeny compared to both parents. Genetic mapping showed that this strong increase was caused by one locus at the top of LG8, in progeny that had inherited only the recessive alleles of that locus from the heterozygous parents. These metabolites belong to β-glycols group of secondary metabolites which have a potential role in controlling infecting microorganisms and influence the aroma of some ciders. This research illustrates the strong genetic erosion in apple breeding regarding metabolic diversity, but also shows that inbreeding can lead to a strong increase of metabolites that were present at much lower contents in the parents and advanced selections, because of accumulation of recessive alleles during inbreeding.

1. Introduction

The domesticated apple (*Malus* x *domestica* Borkh) belongs to family Rosaceae, tribe Pyreae and is the main fruit crop of temperate regions (Faize *et al.*, 2004; Velasco *et al.*, 2010). Apple is produced commercially in 91 countries with a total production of 64 million tons (FAO, 2009). China is at the top with 31.7 million tons followed by USA with 4.5 and Turkey with 2.8 million tons production. Other major producers include Poland, Iran, Italy, France, India, and Russia (FAO, 2009). It is the fourth most widely produced fruit crop in the world after banana, orange and grape (FAO, 2009).

1.1 Genetics of *Malus*

The haploid chromosome number for most Rosaceae crops are 7, 8 or 9, but Pyreae have 17 pairs of chromosomes (Evans and Campbell, 2002). Pyreae have long been considered to be allopolyploid between species related to extant Spiraeoideae and Amygdaleoideae (Evans and Campbell, 2002). However, based on the whole genome sequence information of apple cultivar 'Golden Delicious', Velasco et al. (2010) have shown that in ancestors of apple, the genome had been duplicated twice. The transition from 9 to 17 chromosomes in the Pyreae is the result of relatively recent genome wide duplication (Velasco *et al.*, 2010). There is no clear agreement among the taxonomists on the number of species in the genus *Malus,* and as a result the number of species in genus *Malus* varies from 25 to 47 according to Robinson et al. (2001) while Harris et al. (2002) mentioned that *Malus* comprises of 55 species.

1.2 Genetic diversity in apple

Apple is a highly heterozygous and self-incompatible crop. Apple breeders have developed more than 1,000 apple cultivars worldwide in the last 50 years (Chen *et al.*, 2007). Many cultivated apple genotypes trace their ancestry to a small number of progenitor lineages and, therefore, share a high degree of genetic identity (Richards *et al.*, 2009). These few progenitors caused sharp decrease in the genetic diversity within the cultivated apples. The genetic diversity has been further narrowed down as the number of genotypes used in commercial production has decreased over the last century (Richards *et al.*, 2009; Harrigan *et al.*, 2007). In contrast, wild species within this genus are known to contain substantial genetic diversity (Richards *et al.*, 2009). In worldwide apple breeding programmes, about 70%-80% of the parents of over 1,000 cultivars are such "Founder parents" as 'Ralls', 'Fuji', 'Delicious', and 'Golden Delicious' (Chen *et al.*, 2007). Inbreeding between these founder parents leads to narrow genetic background, lower environmental adaptability, and loss of resistance to diseases and insects of current apple cultivars (Chen *et al.*, 2007).

Many modern varieties resulted from domestication of wild species to produce plants with more desirable traits, thereby reducing the original diversity among resources (Sasaki, 2009).

1.3 Functions of plant secondary metabolites

Plants are a rich source of secondary metabolites. It has been estimated that over 100,000 metabolites can be found in plants (Keurentjes *et al.*, 2006). Dixon and Strack, (2003) reported ~ 200, 000 metabolites in plant kingdom and the number is even more as reported by Trethewey (2004). Moreover, substantial qualitative and quantitative variations in metabolites composition is often observed within the plant species (Keurentjes *et al.*, 2006). Many of these secondary metabolites e.g. quercetin, procyanidins and chlorogenic acid have potential health benefits for humans (Eberhardt *et al.*, 2000; Mcghie *et al.*, 2005).

The current metabolic profiling technologies have shown to be effective in detecting the similarities and differences in the composition of different plant breeding germplasm and wild species (Harrigan *et al.*, 2007). Metabolic changes are associated with plant development and responses to stresses (Harrigan *et al.*, 2007). Quality traits such as flavour, aroma, colour and texture, their storage properties and performance in the field in many important crops and ornamental plants are dependent on metabolic composition (Harrigan *et al.*, 2007; Memelink, 2005). The metabolome represents a critical aspect of a plant's physiology, growth characteristics and ultimately economic value (Harrigan *et al.*, 2007). Secondary metabolites in plants are also involved in the interaction between host and parasite. For example, the resistance of apple against fungus *Venturia inaequalis* is associated with a rapid and localized accumulation of phenylpropanoids which is induced by the infection (Leser and Treutter, 2007).

Metabolomics has proven to be of increasing popularity in assessing genotypic and phenotypic diversity in plants and in defining biochemical changes associated with developmental changes during plants' growth (Harrigan *et al.*, 2007). The metabolic profiling technologies can be applied to investigate metabolic differences between different plant species and cultivars (Harrigan *et al.*, 2007). Further, the plant metabolome has been suggested as bridging the genotype-phenotype gap (Fiehn, 2002; Hall, 2006).

1.4 Present study

In this study, the metabolic diversity in wild germplasm and advanced selections of apple was evaluated. Also metabolic diversity in the F1 progeny of a segregating population from 'Prima' x 'Fiesta' cross was compared to the metabolic diversity in the parents of that population. A large number of metabolites were found in the wild species and advanced selections of apple. The variation in metabolites was mainly because of the genetical differences between genotypes and to a little extent because of the environmental and technical differences. Moreover, metabolites were detected that were significantly less or more abundant in some of the progeny compared to both the parents. The loss of metabolites was caused by an mQTL hotspot on LG16. These metabolites appeared to be phenolic compounds of the phenylpropanoid pathway, as described in Chapter 4. The gain of metabolites in some of the progeny was caused by an mQTL on LG 8. The annotation revealed that these metabolites were glycosylated forms of β-glycols: R-octane-1,3-diol and its unsaturated form R-5-(Z)-octene-1,3-diol.

2. Materials and methods

2.1 Plant material and samples collection

Fruits from 23 wild *Malus* species were harvested in the botanical garden of Wageningen, the Netherlands in 2008. For each genotype, fruits from one to four trees (trees as biological replicates), depending on the availability of replicates, were harvested. Additionally, fruits from eight advanced selections of apple were harvested, preferably from four trees per genotype (Table 3.1). These fruits were harvested at the experimental station, Randwijk, the Netherlands. A segregating F1 population from the cross between 'Prima' and 'Fiesta', containing 118 progeny genotypes, located in the experimental orchard in Elst, the Netherlands, was also included. For this population we had two trees per genotype and five trees per parent. More than ten fruits from each individual tree were picked, in order to compensate for the injuries and other damages of the fruits during transportation.

Table 3.1 *Malus* wild germplasm and advanced selections evaluated for the metabolic diversity

Wild germplasm	Replicates	Advanced selections	Replicates
D3	4	cv. 'Braeburn'	3
Dre 324	3	cv. 'Golden Delicious'	4
Dre 708	2	cv. 'Fuji Kiku'	4
M. baccata	3	cv. 'Fiesta'	5
M. brevipes	1	cv. 'Jonathan'	4
M. coronaria	1	cv. 'Prima'	5
M. 'David'	3	1973-001-041	2
M. evereste	4	1980-015-025	4
M. floribunda	4		
M. fusca	1		
M. gorgeous	3		
M. halliana	3		
M. hupehensis	4		
M. Neville Copeman	2		
M. x purpurea	2		
M. Red Silver	1		
M. sargentii	1		
M. sikkimensis	2		
M. spectabilis	2		
M. strathmore	1		
M. sylvestris	4		
M. toringo	4		
Malus (unspecified)	2		

2.2 Storage conditions for harvested apples

The harvested fruits were immediately stored at 0°C and later before grinding, the fruits were shifted to 20 °C for one week to mimic the storage conditions at consumers.

2.3 Samples preparation

The best apples were selected based on their appearance and size. For wild germplasm, advanced selections, and parents of the segregating population, eight apples were pooled per tree. In case of some very small wild species, more than eight apples were taken to get sufficient amount of material after grinding. For the F1 progeny of the segregating population, four fruits from each tree of the genotype were taken. The fruits from both trees of the segregating population were pooled to make one representative sample of eight apples per genotype. All the eight apples of

each sample were peeled off and a representative part of the flesh was taken. Peel and flesh were pooled separately for each sample and ground to powder according to the method described in Chapter 4.

2.4 Metabolite extraction and LC-QTOF-MS analysis

A total of 2x212 samples were extracted separately for peel and flesh with methanol and the semi-polar metabolite extracts derived were analyzed using LC-QTOF-MS in two continuous runs as described in Chapter 4. A quality control sample, for both peel and flesh, was prepared separately by pooling fruit material of the two randomly chosen wild species *Malus hupehensis* and *Malus Nivelle copman*, the two randomly selected advanced selections cv. 'Braeburn' and cv. 'Golden Delicious' and five genotypes from the segregating population which were also selected randomly. This pooled sample was analyzed 12 times during the two runs in order to i) train the analytical settings ii) to control the analytical performance during the measurements and iii) to determine true metabolite signals during data analyses. The pooled sample is named here 'quality control sample' (QC) for either peel or flesh.

2.5 Data pre-processing

The resulting chromatograms were subjected to the total mass peak alignment using MetAlign software package (www.metalign.nl). Flesh and peel chromatograms were aligned separately. This resulted into two data matrices consisting of 18,582 and 11,817 mass peaks in peel and flesh, respectively, aligned throughout the 212 samples. To distinguish the true mass peak signals from the noise, a noise average (N_{QC}) and a noise standard deviation ($SD_{N.QC}$) were calculated in the quality control (QC) sample (from a corresponding MetAlign output file), and their standard deviation. Then true signal of a metabolite mass was determined as a value that exceeds a sum of N_{QC} and 1x $SD_{N.QC}$. The Multivariate Mass Spectra Reconstruction (MMSR) approach was used to reduce the data amount and to detect putative metabolites by clustering mass peaks based on their quantitative pattern similarity and retention time distance (Tikunov *et al.*, 2005). Clusters containing two or more peaks were considered as putative metabolites. This resulted in 317 and 149 putative metabolites in peel and flesh respectively. The most abundant mass peak was selected from each cluster (putative metabolite mass spectrum) for further multivariate analyses.

2.6 Evaluating the metabolic diversity in apple fruits

Principal Components Analysis (PCA) was performed to assess the metabolic diversity. For this purpose the experimental materials were divided into wild germplasm, advanced selections, 'Prima', 'Fiesta', F1 progeny of the segregating population from 'Prima' x 'Fiesta', and a 'quality control sample'. PCA was

performed in GenMaths XT version (www.applied-maths.com). The analyses were performed on two data sets separately, i.e. peel and flesh.

2.7 Annotation of metabolites

Semi-polar metabolites were putatively annotated by comparing their accurate mass data and fragmentation patterns to the following mass spectral databases: the moto database (http://appliedbioinformatics.wur.nl/moto/), the Komics database (http://webs2.kazusa.or.jp/komics), the Human Metabolite database (www.hmdb.ca), MassBank (www.massbank.jp), and the Dictionary of Natural Products (www.dnp.chemnetbase.com).

3. Results

3.1 Sources of variation

The results of the PCA for the putative metabolites in the peel and flesh are shown in the Figs. 3.1 and 3.2. This provides insight into the various sources of variation that contributed to the total metabolic diversity in the evaluated *Malus* germplasm, i.e. i) technical variation, caused by noise during LC-MS measurement, ii) environmental variation when comparing different trees from the same genotype, and iii) genetic variation when comparing the different *Malus* genotypes such as wild versus advanced selections.

i) Technical variation

The technical variation can be estimated from the QC sample, which was measured as 12 replicates. These replicate measurements are represented by the 12 grey dots at the centre of the graph in Figs. 3.1B and 3.2B for peel and flesh respectively. These dots are tightly clustered, indicating a very low content of technical variation. The 12 replicates are also tightly linked in the dendrograms in Figs. 3.1A and 3.2A. The technical variation, estimated from the 12 measurements of the QC sample, appeared to account for 1.4 % and 0.8 % of the total variation for the first component (PC1) in peel and flesh respectively (Figs. 3.1B, 3.2B).

ii) Environmental variation

The environmental variation was estimated by comparing the metabolic profiles of five trees per parental genotype. The trees of the parental ‘Prima’ and ‘Fiesta’ genotypes are represented by green and red dots respectively. Fig. 3.1B provides the results for peel, and Fig. 3.2B for flesh. The dots appeared to be clustered per parent. This indicates the presence of small differences between trees of the same genotype. The dispersal of the dots within a parent in the PCA is the result of both technical and tree-to-tree variations. By means of multiple variance analysis, the content of

these two sources of variation could be distinguished. Environmental variation, estimated as tree-to-tree variation, accounted for 2.0 % and 1.8 % of the total variation for the PC1 for peel and flesh respectively.

It must be noted that the environmental variation caused by e.g. sunlight was reduced by the experimental setup, as the samples were not from individual apples, but from eight apples per tree. This lowered the impact of differences between apples of same tree.

iii) Genetic variation in wild genotypes and advanced selections

The far majority of the metabolic diversity was caused by genetic variation, mainly between wild genotypes in both peel and flesh samples. Figs. 3.1B and 3.2B show that the genetic variation between wild genotypes was very large compared to the advanced selections and the F1 progeny. Analysis of the variance components showed that 96.6 % and 97.4 % of the variation of the PC1 was caused by genetic differences between the genotypes for peel and flesh respectively. Only 8 % of this genetic variation was captured by the advanced selections.

A separate group of metabolites was found both in peel and flesh (Figs. 3.1C, 3.2C). These metabolites were far more abundant in a group of wild germplasm compared to the advanced selections and the segregating population (Figs. 3.1B, 3.2B). The average pattern for abundance of these metabolites is shown in Figs. 3.1D and Fig. 3.2D. These metabolites were subjected to putative annotation based on their accurate mass and fragmentation pattern. The identification showed that these metabolites represent β-glycols: glycosylated forms of β-glycols: R-octane-1, 3-diol and its unsaturated form R-5-(Z)-octene-1, 3-diol, R-octane-1, 3-diol (Tables 3.2, 3.3).

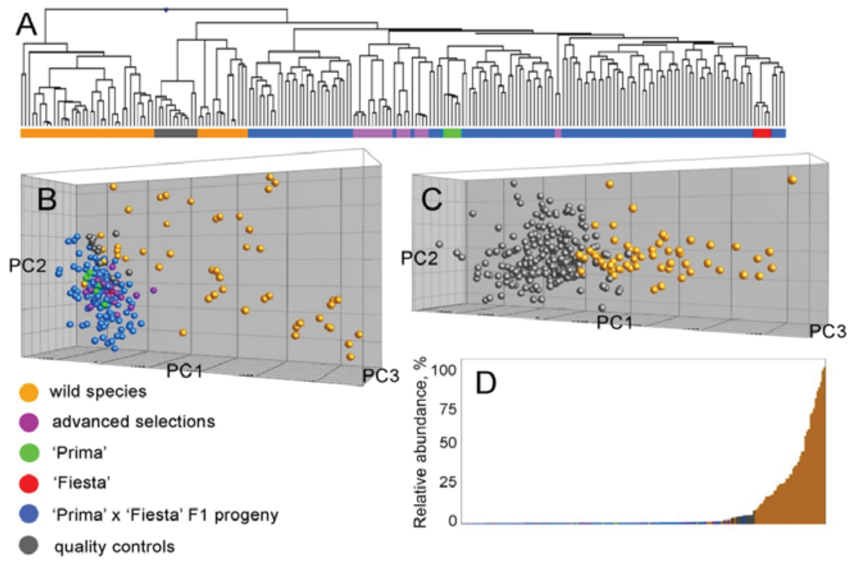

Fig. 3.1

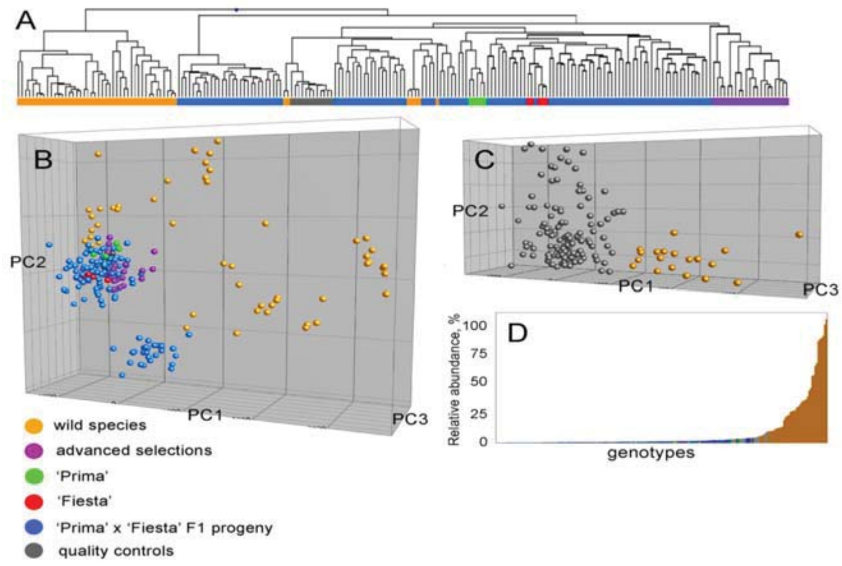

Fig. 3.2

Fig. 3.1. Hierarchal Cluster Analysis (HCA) and Principle Components Analysis (PCA) of apple diversity based on 317 metabolites detected in peel. Plot A represents HCA of apple genotypes. Plot B shows genotype diversity based on metabolic variation which is shown in plot C. PC1 describes 23.6%, PC2 11.2% and PC3 5.5% of the variation. PC1 describes a difference between a group of wild species and the rest of the genotypes. This group of wild species is more abundant in metabolites depicted in orange in plot C and an average pattern of these metabolites is shown in chart D.

Fig. 3.2. HCA and PCA of apple diversity based on 149 metabolites detected in flesh. PC1 describes 24.8%, PC2 21.7% and PC3 7% of the variation. The detail of each plot is the same as given in the legend of Fig. 3.1.

3.2 Loss of metabolites in F1 progeny compared to both parents

The PCA for peel of the F1 progeny of the 'Prima' x 'Fiesta' cross showed segregation into two groups in Fig. 3.3A. One group of metabolites could be recognized that was largely lost in some progeny (Fig. 3.3B). The group of genotypes that largely lost these metabolites is denoted as 'g1' in the Fig. 3.3A. This segregation into two groups was caused by the metabolites represented by red dots in Fig. 3.3B.

This segregation and loss of metabolites was observed even clearer for flesh (Fig. 3.4). The g1-group comprised approximately one quarter of the progeny of the F1 population (Figs. 3.4A, 3.4C), which resembles a 3:1 Mendelian segregation. The average pattern of abundance for the metabolites group 'm1' found in 'g1' is shown in Fig. 3.4C for flesh.

3.3 Gain of metabolites in F1 progeny compared to both parents

Figs. 3.3B, 3.4B show a separate group of 11 and 12 metabolites, i.e. group 'm3' in peel and 'm2' in flesh respectively. These metabolites are strongly enhanced in some F1 progeny compared to both parents and advanced selections (Figs. 3.3G, 3.4D). Histograms of these metabolites (Figs. 3.5, 3.6 and Figs. S3.1, S3.2) show that the metabolite contents were up to 1.7 fold higher in some progenies on a ^{10}log scale, which resembles an up to 50 fold increase in metabolite contents on linear scale compared to the parents. Genetic mapping showed that this strong increase was caused by one locus at the top of Linkage Group 8 (Tables 3.2, 3.3). Comparison of the molecular masses of the enhanced metabolites to known metabolites indicated that these metabolites belong to the β-glycols: glycosylated forms of β-glycols: R-octane-1, 3-diol and its unsaturated form R-5-(Z)-octene-1, 3-diol, R-octane-1, 3-diol (Tables 3.2, 3.3).

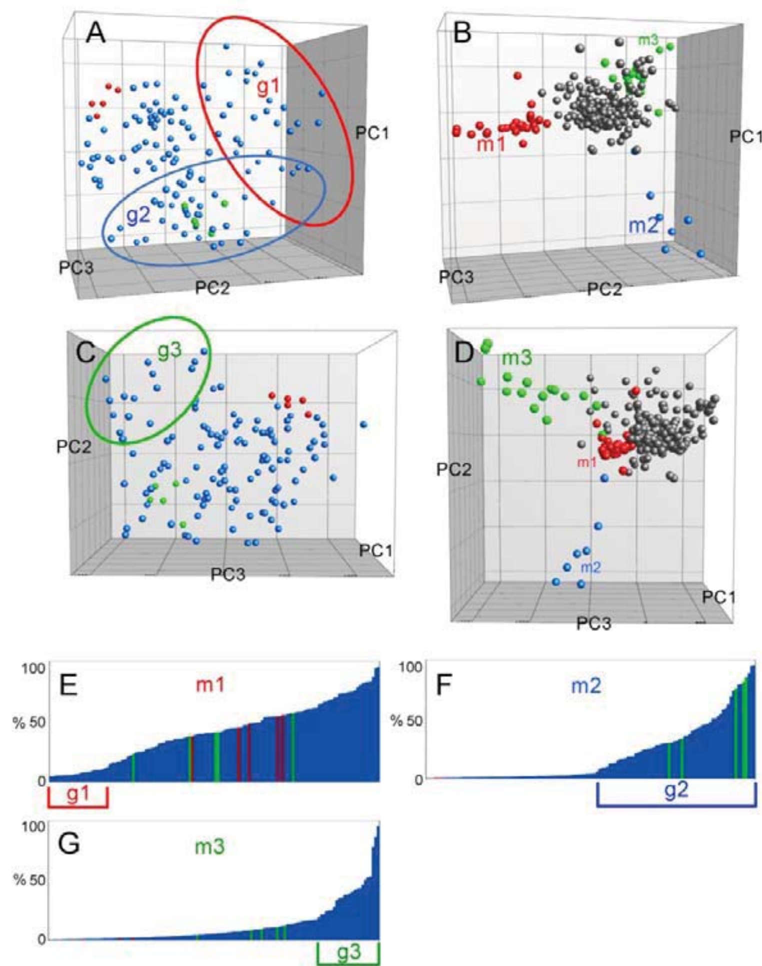

Fig. 3.3. Peel: PCA of diversity of F1 progeny derived from a cross between 'Prima' and 'Fiesta' based on metabolite composition of peel. Plot A shows the distribution of genotypes along the first two principal components: PC1 = 13.4% of variation, PC2 = 11.2%. 'Prima'-green dots, 'Fiesta'-red dots, F1 genotypes-blue dots. Plot B shows metabolic variation that determines the genotype variation in plot A. A group of genotypes (g1) that diverges along the first principal component shows much lower contents of metabolites of (m1) depicted in red in plot B. Another group of genotypes (g2) that diverges along the second principal component shows higher accumulation of metabolites of group (m2) depicted in blue in plot B. Plot C shows an additional variation of genotypes along PC3 (8.4% of variation) that is determined by metabolites of a group (m3) depicted in green in plot D. Charts E, F and G show average patterns of metabolites of (m1, m2 and m3) groups, respectively, across F1 progeny (blue bars) and the two parents-Prima (green bars) and Fiesta (red bars).

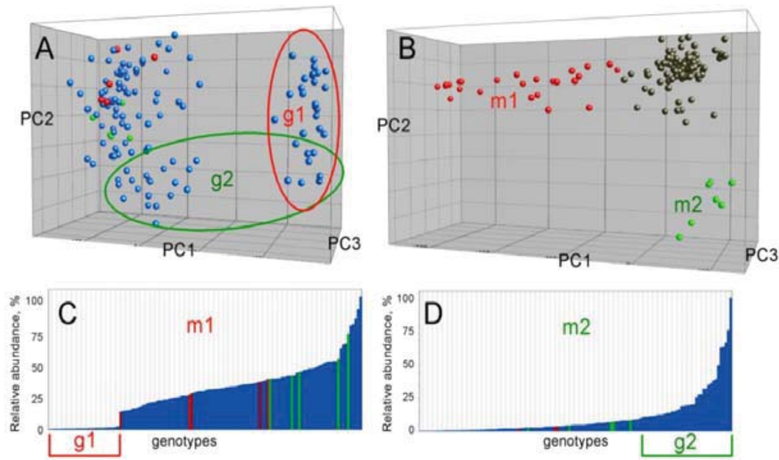

Fig. 3.4. Flesh: A Principal Components Analysis of diversity of F1 progeny derived from a cross between 'Prima' and 'Fiesta'. A group of genotypes (g1) that diverges along the first principal component (PC1= 27.2%) shows much lower contents of metabolites (m1) depicted in red in plot B. Another group of genotypes (g2) that diverges along the second principal component (PC2 = 11.5%) shows higher accumulation of metabolites of group (m2) depicted in green in plot B. The details of each plot are same as mentioned in Fig. 3.3.

Table 3.2 Putative metabolites in peel having significantly higher contents in some of the progeny than both the parents of the segregating population. These metabolites belong to group ‘m3’ in Fig. 3.3, and are more abundant in genotypes that belong to group ‘g3’.

Putative metabolite number	Annotation	Genetic position	LOD value	2-LOD support interval (cM)
1442	Unknown	LG8	18.8	0.8-5.5
2556	Unknown	LG15	6.0	6.0-85.4
3428	Unknown	LG8	3.7	0-39.1
3810	Unknown	LG8	20.0	0-5.5
3937	Unknown	LG8	29.3	1.0-5.5
4123	R-5(Z)-octane-1,3-diol; (R)-form, 6-O-(Î²-D-Xylopyranosyl-(1->6)-Î²-D-glucopyranoside)	LG8	22.9	1.0-4.5
5045	Unknown	LG8	39.6	1.5-4.5
5738	Unknown	LG8	38.9	1.5-4.5
6050	Unknown	LG3	10.1	44.8-56.3
6511	Unknown	LG8	19.9	1.0-6.0
4588	Unknown	----	----	---

Table 3.3 Putative metabolites in flesh having significantly higher contents in some of the progeny than both the parents of the segregating population

Putative metabolite number	Annotation	Genetic position	LOD value	2-LOD support interval (cM)
2477	Unknown	LG8	33.6	1.0-4.5
2224	R-5(Z)-octane-1,3-diol; (R)-form, 6-O-(Î²-D-Xylopyranosyl-(1->6)-Î²-D-glucopyranoside)	LG8	22.0	1.0-4.5
2489	Unknown	LG8	7.3	0-14.4
2564	1,3-Octanediol; (R)-form, 1-O-(Î²-D-Apiofuranosyl-(1->2)-Î²-D-glucopyranoside)	LG8	15.6	0-7.0
2670	Unknown	LG8	7.9	0-7.0
2585	1,3-Octanediol; (R)-form, 1-O-(Î²-D-Apiofuranosyl-(1->2)-Î²-D-glucopyranoside	LG8	13.0	0.0-7.2
2022	R-5(Z)-octane-1,3-diol; (R)-form, 6-O-(Î²-D-glucopyranosyl-(1->6)-Î²-D-glucopyranoside)	LG8	10.6	0.0-8.2
2407	1,3-Octanediol; (R)-form, 1-O-(Î²-D-Glucopyranosyl-(1->6)-Î²-D-glucopyranoside)	LG8	9.1	0.0-13.4
2143	Unknown	LG8	15.3	0.0-7.2
2650	1,3-Octanediol; (R)-form, 1-O-Î²-D-Glucopyranoside	LG8	10.0	0.0-7.2
2370	Unknown	LG10	6.8	91.6-99.1
1578	Unknown	LG16	16.6	2.1-11.1

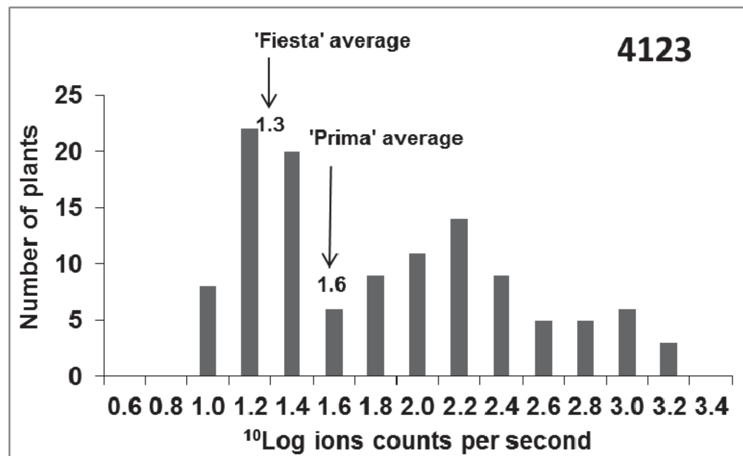

Fig. 3.5. Example of putative metabolite (4123) showing higher content in some progeny than the parents in apple peel. This metabolite shows segregation. Both parents i.e. 'Prima' and 'Fiesta' belong to the low content group of metabolite. The metabolite 4123 is annotated in Table 3.2.

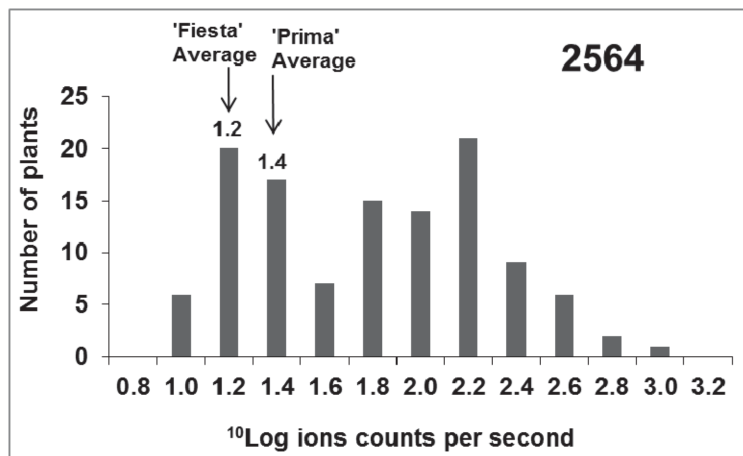

Fig. 3.6. Example of putative metabolite (2564) showing higher content in some progeny than the parents in apple flesh. This metabolite shows segregation. The parents 'Prima' and 'Fiesta' both belong to the low content group of metabolite. The metabolite 2564 is annotated in Table 3.3.

3.4 Metabolites inherited from only one parent

Additionally a separate group of metabolites was found in peel. These metabolites led to a separate group, shown as ‘g2’ in Fig. 3.3A. The average pattern of abundance of metabolites in ‘m2’ is shown in Fig. 3.3F. The contents of these metabolites were high in the parent ‘Prima’, but low in the other parent ‘Fiesta’. These metabolites showed a 1:1 Mendelian segregation (Fig. 3.3F). The annotation revealed that these metabolites were isorhamnetin glycosides. These metabolites were previously mapped on LG13 (Chapter 4). This metabolite group is a clear example of a 1:1 Mendelian segregation of metabolites in the progeny. This is caused by heterozygosity of one parent (*Mm*), and a homozygous recessive state (*mm*) of the other parent, leading to a segregation of 1*Mm* : 1*mm*.

4. Discussion

4.1 Loss of metabolic diversity

When metabolic variation in wild germplasm of *Malus* was compared with metabolic variation in advanced selections in apple, it was obvious that only 8 % of the variation was caused by the advanced selections. This was the case for both apple peel and apple flesh. We have to admit, however, that only a small set of genotypes both of wild and advanced selections were sampled in this study but in spite of that the conclusion was very clear that wild *Malus* germplasm represents most of the metabolic diversity in apple fruits. Further, it should be realize that this analysis was performed for the first principle component of the PCA, and not for the overall variation.

This wild germplasm covered about 89 % of the genetically determined metabolic variation in our research. The advanced selections clustered closely (Figs. 3.1 and 3.2). This indicates that the far majority of the metabolic variation was lost during breeding and selection process.

Previous studies have shown that genetic diversity can be performed either through studying the morphological traits or through molecular markers (Khlestkina *et al.*, 2004). Here we have shown that this could also be done with metabolic traits. There is a general concern in scientific community that modern plant breeding could narrow down the diversity of currently released varieties compared to older ones (Hoisington *et al.*, 1999; Lee, 1998; Reif *et al.*, 2005). Hammer and Laghetti (2005) reported genetic erosion in wheat in Italy. They observed genetic erosion not only in crop plants but also in the garden plants and wild species. They reported no significant difference in the genetic erosion of both crop plants and wild species. The genetic erosion had occurred almost at the same rate in both garden and crop

plants.

There are several reports where studies were conducted on wheat varieties showing that modern plant breeding causes this genetic erosion (Gregova *et al.*, 1997; Donini *et al.*, 1998). Comparable studies performed in barley have confirmed the differences in genetic diversity between landraces and cultivars, however, it appeared not to be as dramatic as has often been assumed (Backes *et al.*, 2003; Petersen *et al.*, 1994; Struss and Plieske, 1998).

4.2 Loss of metabolites during breeding

Comparison of metabolite contents in the F1 progeny to the contents in the parents revealed that the contents of metabolites could drop strongly during one generation, both in peel and flesh. This is illustrated by Figs. 3.3 and 3.4. Analysis of mass fragmentation and retention time revealed that these metabolites belong to the phenylpropanoid pathway as explained in Chapter 4. The gene that caused this segregation is located at the top of linkage group 16 (Chapter 4). Both parental varieties, 'Prima' and 'Fiesta' harbour one dominant and one recessive allele of this gene (*Mm*), leading to a 3:1 segregation in the F1 progeny (1*MM*:2*Mm*:1*mm*). One quarter of the population inherited the two recessive alleles from the parents (*mm*), and lacked therefore the dominant allele that provided high contents of the phenolic compounds (Chapter 4). The 3:1 segregation can also be recognized in Figs. 3.3 and 3.4. This example illustrates that due to loss of a dominant allele, metabolic contents can drop strongly within one generation. In this case, the contents dropped by 0.62 on ^{10}log scale, which is 4.2 fold decrease on the linear scale (Chapter 4).

As both parents contained one dominant allele, the contents of the phenolic compounds were similar in both parents. As a result, the parents grouped rather closely to one another in the PCA (Figs. 3.3, 3.4). The segregation in the F1 was apparent in the second PC in Fig. 3.4, but not in the first PC. This indicated that the metabolic diversity in the F1 population was not caused by the metabolites that segregated in the wild germplasm, but by other metabolites.

4.3 Gain of metabolites during breeding

Remarkably, not only some metabolites were largely lost during one generation in some progeny, but other metabolites were up to 50 fold more abundant in some progeny genotypes compared to both parents. This was the case for 11 metabolites in the peel and 12 metabolites in the flesh (Tables 3.2, 3.3). The majority of these metabolites could be mapped genetically, and appeared to segregate as a monogenic trait, leading to sometimes very high LOD scores of up to 40. The causal locus was positioned at the top of LG8. The southern flanking marker of the mQTL region was

a DArT marker 519609, located 4.5 cM from the top of LG8 (Schouten *et al.*, 2011). The DNA sequence of this marker aligned well with a physical position on chromosome 8 of the DNA sequence of 'Golden Delicious' (Velasco *et al.*, 2010).

Analysis of the masses and retention times indicated that the compounds that mapped at the top of LG8 were putatively identified as glycosylated forms of β-glycols: R-octane-1, 3-diol and its unsaturated form R-5-(Z)-octene-1, 3-diol. R-octane-1,3-diol and R-5-(Z)-octene-1,3-diol have been previously identified and play an important role in some cider apple varieties as aroma precursors (Beuerle and Schwab, 1999; Dietrich *et al.*, 1997). These compounds are produced from linoleic acid via β-oxidation or lipoxygenase pathway both of which lead to biosynthesis of R-3-hydroxyoctanoyl-SCoA. These compounds can be further metabolized via two pathways: (i) to its S-enantiomer through 2(E)-octenoyl-SCoA or (ii) to 3-hydroxyoctanoic acid which is subsequently reduced to R-octane-1,3-diol (Beuerle and Schwab, 1999). The segregation of these metabolites in the F1 population analysed suggests that their accumulation was the result of inheritance of two recessive alleles of one of the genes involved in the first pathway, which subsequently redirects the flux to the second pathway leading to R-octane-1, 3-diol and R-5-(Z)-octene-1, 3-diol.

This research illustrates not only the strong genetic erosion in apple breeding regarding metabolic diversity, but also shows that inbreeding can lead to a strong increase of metabolites that were present at much lower contents in the parents and advanced selections, because of accumulation of recessive alleles during inbreeding.

Chapter 4

Genetical metabolomics in apple indicates an mQTL hotspot for phenolic compounds on Linkage Group 16

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Abstract

Apple (*Malus x domestica* Borkh) is among the main sources of phenolic compounds in the human diet. We investigated the genetic basis of the quantitative variations of these potentially beneficial compounds. A segregating population was used to map metabolite quantitative trait loci (mQTLs). Untargeted metabolic profiling of peel and flesh tissues of ripe fruits was applied by liquid chromatography-mass spectrometry (LC-MS), resulting in the detection of 418 metabolites in the peel and 254 in the flesh. In mQTL mapping using MetaNetwork, 669 significant mQTLs were detected: 488 in the peel and 181 in the flesh. Four linkage groups (LGs) i.e. LG1, LG8, LG13 and LG16 were found to contain mQTL hotspots, mainly regulating metabolites that belong to the phenylpropanoid-flavonoid pathway. The genetics of annotated metabolites was studied in more detail using MapQTL®. The quercetin conjugates had mQTLs on LG1 and LG13. The most important mQTL hotspot with the largest number of metabolites was detected on LG16: mQTLs for 33 peel-related and 17 flesh-related phenolic compounds. We located structural genes involved in the phenolic biosynthetic pathway, using the apple genome sequence. The structural gene *leucoanthocyanidin reductase* (*MdLAR1*) was in the mQTL hotspot on LG16, as were seven transcription factor genes. We believe this is the first time that a QTL analysis was performed on such a high number of metabolites in an outbreeding plant species.

Key words: *Malus x domestica* Borkh, untargeted and targeted mQTL mapping, genetical metabolomics, LC-MS, MetaNetwork, MapQTL.

1. Introduction

The fruit of apple (*Malus* x *domestica* Borkh) is a rich source of phytochemicals including phenolic compounds (Gerhauser, 2008). There is increasing evidence that apple is an important source for various compounds that are beneficial for human health (Han and Korban, 2009). Its consumption has been associated with a risk reduction of many human diseases, such as asthma, type-2 diabetes, thrombotic stroke, ischemic heart disease, and various cancers (Eberhardt *et al.*, 2000; Mcghie *et al.*, 2005). Some of the major phenolic compounds isolated and identified from apple are procyanidins, anthocyanins, chlorogenic acid, hydroxycinnamic acid, flavan-3-ols such as; (-)-epicatechin, (+)-catechin, and gallaocatechin; phloridzin and quercetin glycosides (Lu and Foo, 1997; Mazza and Velioglu, 1992; Lancaster, 1992; Nicolas *et al.*, 1994; Treutter, 2001; Awad *et al.*, 2000).

The current study aims at elucidation of the genetic basis of metabolic variability in apple fruits. We initiated this study without any a priori with regard to the specific metabolites group. For that reasons we chose for large-scale LC-MS based metabolic profiling.

Metabolomics is defined as the large scale analysis of metabolites in an organism, and it concerns the simultaneous measurement of these metabolites in a given biological system (Dixon and Strack, 2003). Metabolomics is developing as an important functional genomics tool in crop plants, including fruit trees (Moco *et al.*, 2006; Carrari and Fernie, 2006). Although QTLs have been mapped in cultivated apples for different traits such as disease resistance (Calenge *et al.*, 2004; Calenge and Durel, 2006; Khan *et al.*, 2006), fruit quality (Liebhard *et al.*, 2003; King *et al.*, 2001; Davey *et al.*, 2006) and tree morphology (Kenis and Keulemans, 2007), there is only one report on the genetic mapping of a large number of metabolites in apple fruits, and, in that case, on volatiles (Dunemann *et al.*, 2009).

In our study, the LC-MS metabolomics showed numerous metabolic compounds in the segregating F1 population, both in peel and in flesh of the fruits, allowing mQTL mapping. Standard QTL mapping software is designed to map individual traits, one by one, and is not suited to map hundreds of metabolites simultaneously. Therefore we decided to use the software MetaNetwork (Fu *et al.*, 2007). MetaNetwork enables simultaneous genome-wide screening of numerous traits. MetaNetwork has been designed for self-pollinating species, and not for highly heterozygous, cross-pollinating parents that segregate in the F1, such as apple. Therefore we developed an approach to make MetaNetwork also suitable for a segregating F1 population.

MetaNetwork revealed clusters of mQTLs on the apple genome. We annotated the underlying LC-MS mass peaks of mQTL clusters, and aimed at studying the genetics of these metabolites in more detail, such as revealing the

allelic contributions to traits, and perform co-factors analysis to filter out the effect of strong mQTLs. MetaNetwork appeared to be less suitable for these deeper analyses of individual metabolites. Therefore, we decided to use MapQTL® (Van Ooijn, 2009a) for this.

2. Materials and methods

2.1 Plant Materials

For mQTL mapping, a segregating F1 population from the cross 'Prima' x 'Fiesta' (PF) was used. This population was also used for the first international reference linkage map of apple covering all chromosomes (Maliepaard *et al.*, 1998). In this study we used a subset of 113 progenies and both parents. For the F1 population, two trees per genotype were present.

2.2 Harvesting and storage of the apples

Mature fruits of all genotypes were harvested in September and October, 2008 in a trial orchard in Elst, The Netherlands. The maturity of the fruits was assessed by checking the colour of the peel, the taste, and the browning of the seeds. For each progeny, more than ten fruits from each of the two trees were harvested separately, while for the two parents 'Prima' and 'Fiesta' fruits from five trees (five replicates) were harvested. The fruits were harvested randomly from different sides of each individual tree to level out possible differences due to environmental factors such as light. Fruits were over sampled in the field to forestall the possible damage or decay during transit. After harvesting, fruits were immediately stored at 0 °C in a cold storage room to minimize enzymatic activities. Once fruits for all of the genotypes were harvested, these were shifted to a storage room at 20 °C for seven days. This was done to mimic the storage conditions in a consumer's household.

2.3 Selection and grinding of apples

Samples of eight apples per genotype were selected. For the progeny genotypes, four apples from each of the two trees of one genotype were combined as one sample. For each sample, the individual fruit was cut transverse wise to obtain a 1-cm thick round slice, and the round slice was peeled. The peel (1.4 mm thick) was chopped into small pieces and snap-frozen in a separate beaker with liquid nitrogen. The core was removed from the flesh and a slice (3.2 mm thick) of the fresh flesh was also chopped into small pieces and snap-frozen. This was repeated for all of the eight apples of one genotype, and the samples from a tissue were pooled per genotype. The samples were then ground using an IKA coffee grinder (model A11 basic). The powder for the flesh and peel was collected separately in 50-ml falcon tubes and

stored at -80 °C. For the parents 'Prima' and 'Fiesta', the samples were treated separately in five replicates each and treated in the same way as described for the progenies.

2.4 Extract preparation

The aqueous-methanol extracts were prepared as described by De Vos et al. (2007), with minor modifications (Keurentjes *et al.*, 2006a). Ice-cold 99.9% methanol (1.5 ml) acidified with 0.133% (vol/vol) formic acid, was added to each plant sample (final methanol concentration of 75%, assuming 90% water in the 500 ± 5 mg tissues). The ensuing steps, from sonication to the injection of the samples and separation using the Alliance 2795 HT system, were performed as described by De Vos et al. (2007). The separation was performed at 40 °C, by applying a 45 min gradient of 5-35% acetonitrile in water (acidified with 0.1% formic acid) at a flow rate of 0.19 ml/min. The compounds eluting from the column were detected online, first by a Waters 996 photodiode array detector at 200-700 nm and then by a Q-TOF Ultima MS (Waters) with an electron spray ionisation (ESI) source. Ions were detected in negative mode in the range of m/z 80 to 1,500 at a resolution of 10,000, using a scan time of 900 ms and an interscan delay of 100 ms. The desolvation temperature was 250 °C, with a nitrogen gas flow of 500 l/h, the capillary spray was 2.75 kV, the source temperature was 120 °C, the cone voltage was 35 V with 50 l/h nitrogen gas flow and the collision energy was 10 eV.

The mass spectrometer was calibrated as described by De Vos *et al.* (2007). MassLynx software version 4.0 (Waters) was used to control all instrumentation and for the calculation of accurate masses.

2.5 Pre-processing and quality improvement of data set

Pre-processing of the LC-MS data was performed according to Keurentjes et al. (2006b). As, a single metabolite may resolve into a cluster of mass peaks, the data were clustered, taking one representative centrotype per cluster. A centrotype was intended to represent one metabolite. This clustering was performed with the Metabolite Mass Spectrum Reconstruction (MMSR) script (Tikunov *et al.*, 2005). The LC-MS data were ^{10}log transformed to normalise the variances among the signals.

2.6 Molecular genetic linkage maps

Molecular genetic linkage maps were available for both 'Prima' and 'Fiesta', representing the 17 linkage groups of apple. The maternal map consists of 562 markers and the paternal map consists of 452 markers, including DArT, AFLP,

RFLP, NBS-LRR, SSRs, RAPD markers and some isozymes (Schouten *et al.*, 2011).

In the untargeted mQTL mapping of metabolites in apple, the individual maps of 'Prima' and 'Fiesta' were used, as the MetaNetwork could not incorporate the integrated map of cross-pollinating crops such as apple. In the targeted mQTL mapping using MapQTL® 6.0, an integrated map of both parents was constructed and used for the analysis of the annotated metabolites. The integrated map contained 801 markers, spanning 1,348 cM.

2.7 Untargeted mQTL mapping of metabolites with MetaNetwork

MetaNetwork was designed for the mQTL analysis of homozygous recombinant inbred line (RIL) populations of inbreeding plants such as *A. thaliana* (Fu *et al.*, 2007), and was therefore not applicable for the analysis of a segregating F1 population of an out-crossing species. Therefore, we transformed the data, using single parental maps, giving 2x17=34 linkage groups. Hereby each parent was considered to be derived from a cross between two inbred lines, and the F1 progeny was considered to be the result of a backcross. The linkage phase information from the linkage map was used to assign F1 marker alleles to the respective parental inbred lines, thus giving the dichotomous marker scores as required by MetaNetwork. Missing marker data were imputed using information of flanking markers if they were within a 20-cM distance and in a non-recombinant segment.

MetaNetwork uses a user-defined spike value to distinguish qualitative segregation from quantitative differences. The value chosen as spike was 37, because this value was the noise level in the LC-MS analysis. MetaNetwork also allows setting a threshold for the significance of mQTL by performing permutation tests on samples. A bootstrap procedure was performed, and the obtained threshold of 3.8 $-^{10}\log(p)$ value was used for all analyses.

2.8 Annotation of metabolites

Metabolites known to be present in apple fruit were identified by their accurate mass and retention time compared with standards and with the Moto-database (Moco *et al.*, 2006).

Further, centrotypes from the untargeted approach that appeared to be controlled by a major mQTL were annotated by selecting the molecular ion mass within the selected centrotype and by comparing this ion mass with the metabolite databases KNapSack, Metabolome Japan and the Dictionary of Natural Products. A subset of known metabolites given in supplementary Tables S4.1 and S4.2 was detected, both in apple peel and flesh.

2.9 Targeted mQTL mapping of annotated metabolites with MapQTL® 6.0

MapQTL® 6.0 (Van Ooijn, 2009a) was used to study in more detail the mQTLs of the annotated metabolites in apple peel and flesh. Interval mapping, followed by rMQM mapping with regression algorithm, Haldane's mapping function, with a mapping step size of 1, and independent LOD (logarithm of odds) test statistics was used. The threshold for mQTL significance was determined using a genome-wide permutation test with 1,000 iterations, which gave $\alpha = 0.005$ for the 17 chromosomes of apple, to obtain a 95% confidence interval. Two LOD support intervals were used to estimate the range in cM where the mQTLs reside. Markers near mQTL peaks or at mQTL peaks were used as co-factors for rMQM mapping of a certain metabolite. This was followed by another round of co-factor selection by using markers from the newly found minor mQTLs from the rMQM. The results from this second round were recorded as the final result. An mQTL was named as minor QTL if its LOD score was close or just at threshold level.

2.10 Mapping of the metabolites that segregated as a monogenetic trait

The metabolites procyanidin dimer I, procyanidin dimer II, procyanidin trimer I, procyanidin trimer II, (+)-catechin and (-)-epicatechin had only one mQTL, segregating in a clear 3:1 ratio. This single locus explained a major part of the variation in the metabolite content (up to 81 %, supplementary Table S4.3). These metabolites were treated as monogenic traits and were integrated into the genetic linkage map by JoinMap 4.0 (Van Ooijn, 2009b). This was performed with the aim of locating the positions of the underlying genes more precisely.

2.11 Testing additional simple sequence repeats (SSR) loci for LG16

To map the monogenetically segregating metabolites more precisely, 17 additional SSR loci at the upper part of LG16 were tested for the 42 progenies that showed recombination in this genetic area. These SSRs along with their primers, have been previously published in other apple molecular marker linkage maps (Celton *et al.*, 2009; Liebhard *et al.*, 2002; Silfverberg-Dilworth *et al.*, 2006; Kenis and Keulemans, 2005). The 17 SSR loci along with their primer sequences are listed in supplementary Table S 4.7.

3. Results

3.1 Liquid chromatography-mass spectrometry (LC-MS) profiling of apple fruits revealed hundreds of metabolites in the peel and the flesh

From the cross of 'Prima' x 'Fiesta', 113 progeny individuals were analysed by accurate LC-MS. A total of 18,582 and 11,817 mass signals were detected in the peel and flesh tissues respectively. Clustering of the mass signals based on their corresponding retention time and abundance profile across samples resulted in 672 centrotypes: 418 and 254 for the peel and flesh, respectively. In the following sections these centrotypes are named metabolites.

3.2 Untargeted mQTL mapping of metabolites showed 669 mQTLs in the peel and the flesh

In the untargeted mQTL mapping using MetaNetwork, a total of 669 mQTLs were detected (Table 4.1), spread over all of the 17 linkage groups of the apple genome (Fig. 4.1). Not all of the metabolites showed mQTLs; 50% of the metabolites in the peel and 44% in flesh exhibited statistically significant mQTLs. Fig. 4.2 shows that several mQTLs had very high $-^{10}\log(p)$ values.

Table 4.1. MetaNetwork results of mQTL mapping in peel and flesh of apple from F1 segregating population from 'Prima' x 'Fiesta'cross

	'Prima' Peel	'Prima' Flesh	'Fiesta' Peel	'Fiesta' Flesh	Total Peel	Total Flesh	Total
Number of metabolites with at least one mQTL	184	77	169	67			
Number of mQTLs	288	101	200	80	488	181	669
Number of markers with at least one mQTL	133 (28%)	62 (13%)	81 (21%)	50 (13%)	214	112	326

3.3 LG16 has a strong hotspot of mQTLs in both parental genotypes and in both peel and flesh of the fruit

The striking thing in Fig. 4.1 and 4.2 is the strong hotspot of mQTLs on LG16. On other linkage groups such as LG1 and LG13, also many mQTLs were detected, but these were not as strongly clustered as on LG16. The mQTLs on LG16 clustered mainly around a single locus. Notable also is that the hotspot of mQTLs on LG16 was present in both parents and in both tissues, in contrast to the mQTL hotspot on LG8, which was explicitly present in 'Prima' but absent in 'Fiesta' (Fig. 4.1).

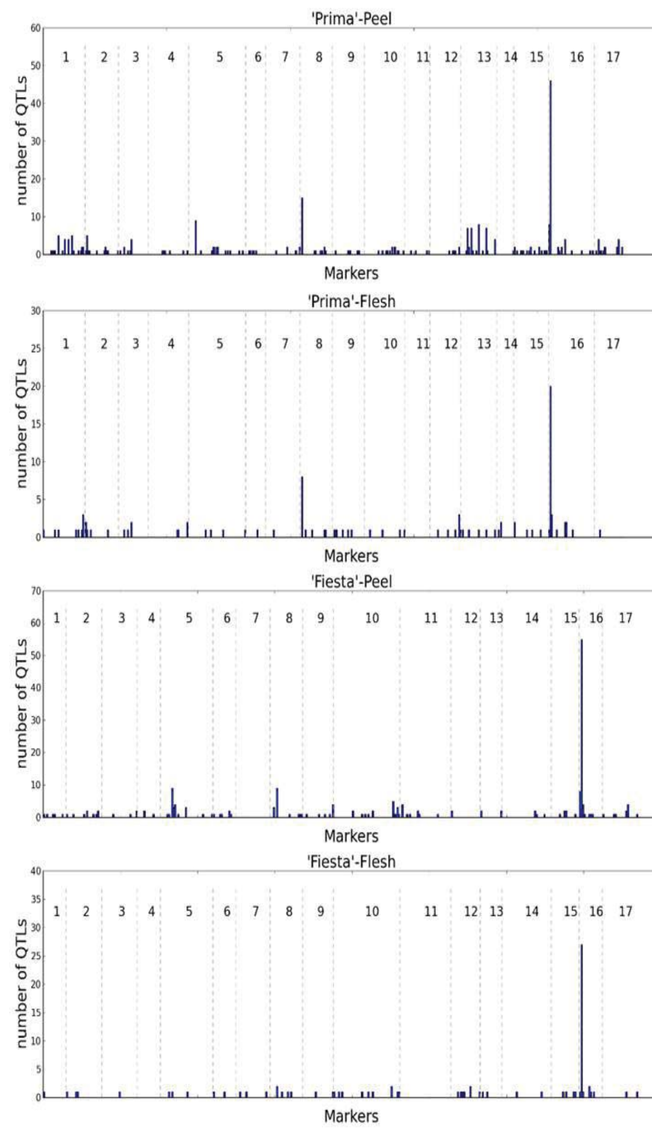


Fig. 4.1 Number of mQTLs over the apple genome. The linkage groups are separated by vertical dotted lines. In this Figure, markers are ordered and positioned equidistantly, thus ignoring their genetic distances.

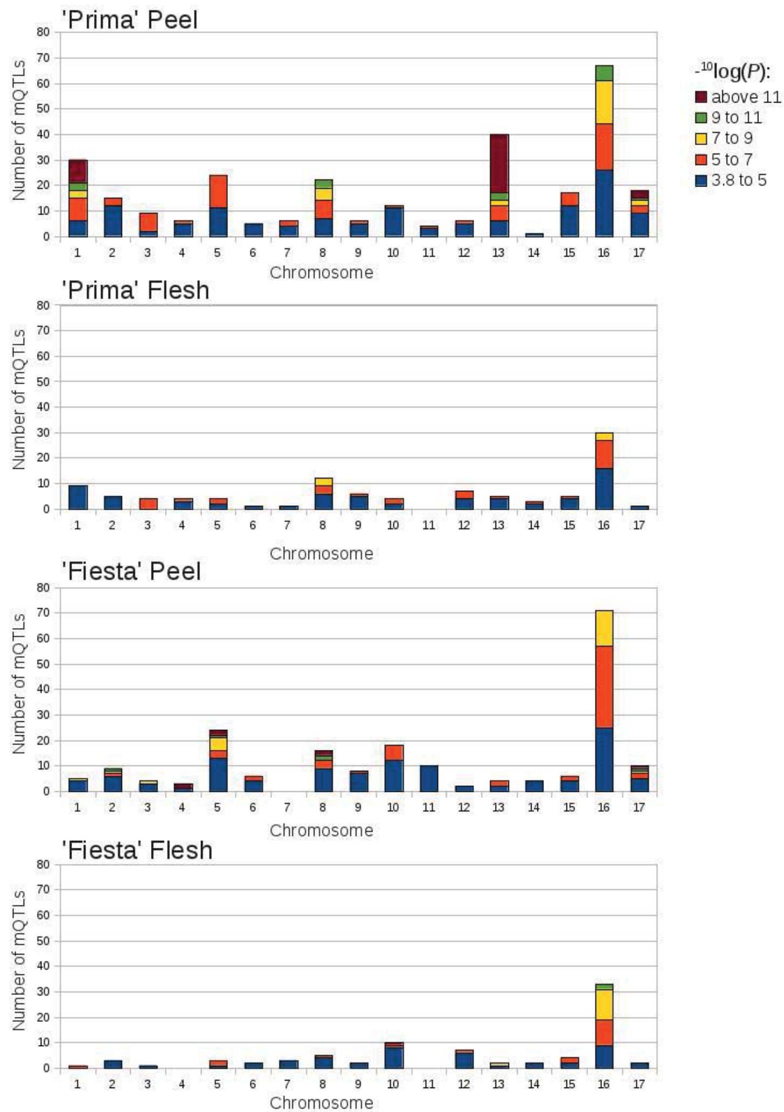


Fig. 4.2 Significant mQTLs with range of $-^{10}\log(p)$ values over the apple genome. An mQTL was considered as significant if its $-^{10}\log(p)$ value was higher than 3.8.

3.4 The mQTL hotspot on LG16 is not caused by the co-localizing major locus for pH

Maliepaard *et al*. (1998) mapped the pH of apple fruits on LG16 in the same segregating population. They observed monogenic inheritance for low versus high acidity and mapped pH to our current mQTL hotspot on LG16 (Fig. 4.3). Remarkably, both parents had one dominant allele for low pH at the LG16 mQTL hotspot. They denoted the locus as *Ma* for malic acid, being the major acid in apple, although they measured pH rather than malic acid itself (Maliepaard *et al.*, 1998). As the pH might influence different enzymatic processes and biochemical reactions in plant cells, differences in the pH may possibly have caused the mQTL hotspot. We evaluated this hypothesis. Both at low and high pH, high levels of the metabolites were found (Fig. 4.4). Apparently, the dominant allele for high acidity was in repulsion to the allele for high level of metabolite content in both parents. Consequently, the occurrence of the hotspot was not a side effect of major differences in the pH.

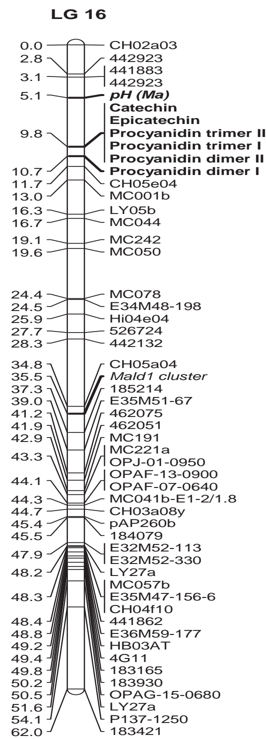


Fig. 4.3 Mapping of (+)-catechin, (-)-epicatechin, several procyanidins and pH (*Ma*) on linkage group 16.

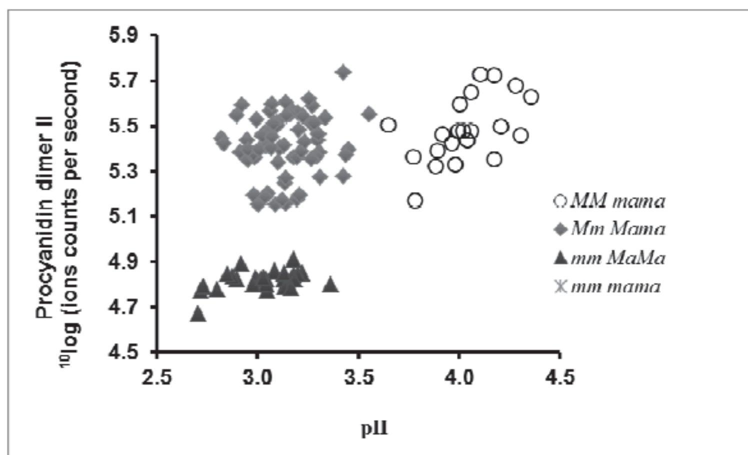


Fig. 4.4. A scatter plot showing the distribution of F1 progenies of 'Prima' x 'Fiesta' over the four genotype classes for low/high pH and low/high procyanidin dimer II content, whereby procyanidin dimer II represents the metabolites that share the strong mQTL on LG16. The trait pH co-localizes to this hotspot. The dominant allele for high metabolite level is denoted as *M*, and for low pH (high acidity; presumably high level of malic acid) as *Ma*. As the dominant alleles *M* and Ma are in repulsion phase in both parents, giving as alleles in the gametes *Mma* and *mMa*, the progeny segregates into three genotypes, lacking the genotype *mm mama*.

3.5 All annotated metabolites that mapped to the hotspot on LG16 belong to the phenylpropanoid pathway of phenolic compounds

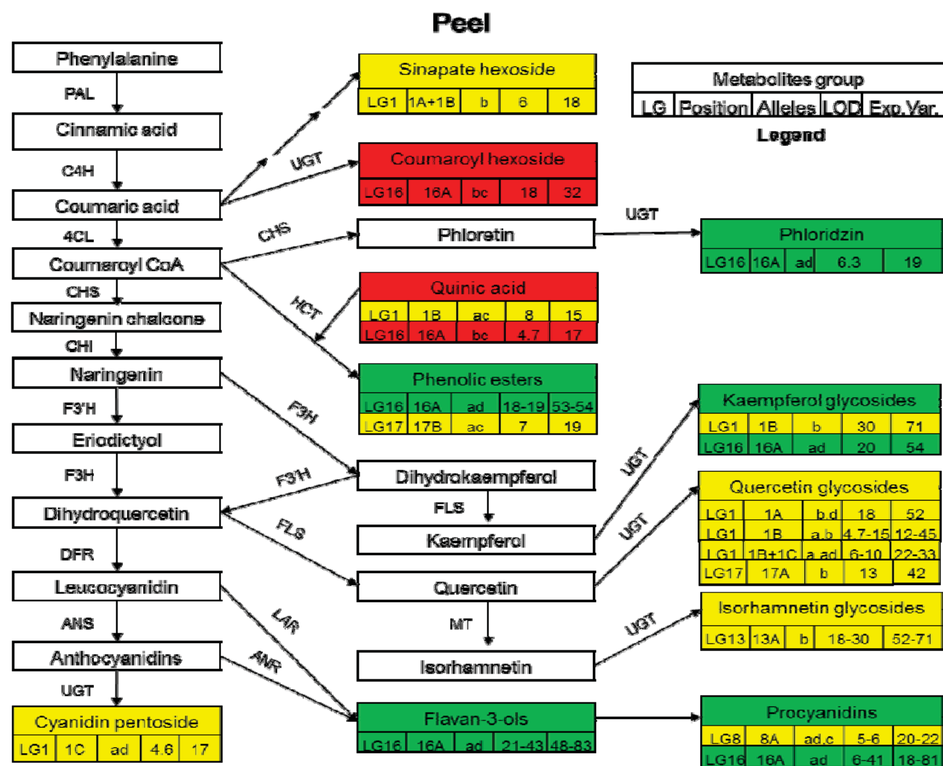
For the peel 69 and for the flesh 30 metabolites were annotated (supplementary Tables S4.1, S4.2). Of the annotated metabolites, 81 out of 99 were phenolic compounds belonging to the two groups of phenylpropanoids and polyphenols (Tables S4.1, S4.2).

3.6 Targeted mQTL mapping

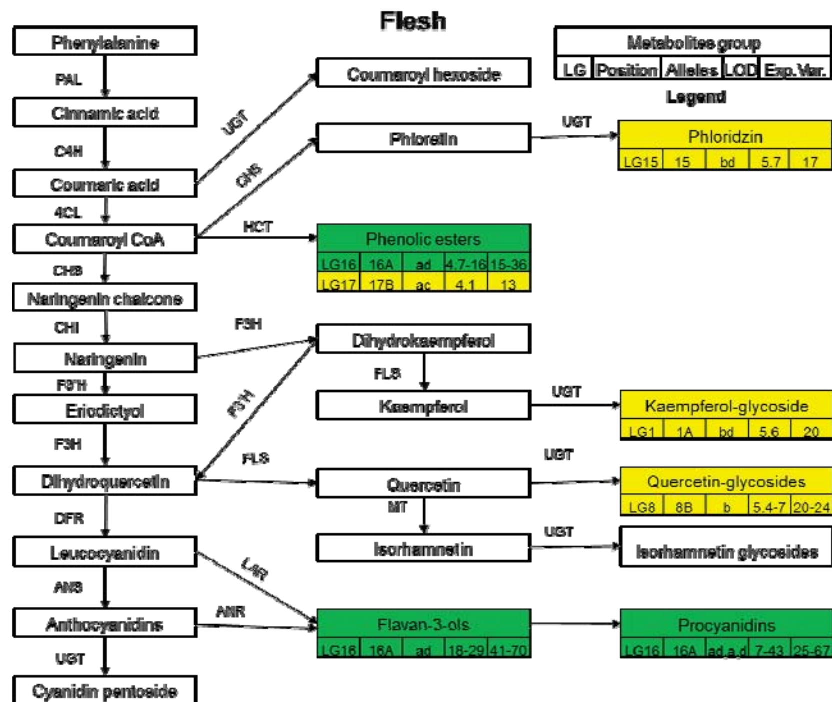
The genetics of these annotated metabolites was studied in more detail using MapQTL® 6.0. mQTL hotspots were confirmed on LG1, LG8, LG13, and LG16. Two mQTLs were also found on LG17 (Fig. 4.5). Similar to the MetaNetwork analysis, the hotspot with the highest number of mQTLs was detected on LG16. Among the 69 annotated metabolites in the peel, 33 had an mQTL on LG16 (Fig. 4.5A, Table S4.3). Majority of these metabolites represented procyanidins of various chain lengths, including the monomeric building blocks of procyanidins, the flavan-

3-ols (+)-catechin and (-)-epicatechin. The same region of LG16 showed mQTLs for quinic acid, phenolic esters, coumaroyl hexoside, kaempferol glycoside, and phloridzin. Interestingly, all these metabolites originate from the phenylpropanoid pathway (Fig. 4.5A,B). Most of the metabolites had only a single mQTL; however, a few metabolites were found to have some additional minor mQTLs on other LGs (Fiure. 4.5A,B). A restricted multiple QTL mapping (rMQM) analysis confirmed the major mQTLs and revealed in addition several minor mQTLs (Tables S4.3, S4.4).

For the 69 annotated metabolites in the peel tissue, mQTLs were located on five different linkage groups (Fig. 4.5A, Table S4.3). LG1 contained specific mQTLs for quercetin glycosides. There is also an mQTL for Kaempferol-rhamnoside on LG1. Glycosides of isorhamnetin had an mQTL on LG13. Chlorogenic acid showed an mQTL on LG17. A distinguished group of mQTLs, mapped on LG8, was formed by alcohol glycosides such as octane-di-ol hexoside and phenylethanol glycoside (Table S4.3).



4.5A



4.5B

Fig. 4.5 The phenylpropanoid pathway of phenolic compounds in two apple fruit tissues, peel (A) and flesh (B). The metabolites for which mQTLs were found are presented in coloured boxes. Colourless boxes show the metabolites that were not detected in our analysis or have no mQTL. Boxes with green colour indicate mQTLs of which the + alleles are in coupling phase. Boxes with yellow colour show mQTLs for metabolites other than on LG16A. The metabolites in the red box show a negative correlation with the metabolites in the green boxes, having an mQTL on LG16A. The linkage group (LG) where an mQTL was located is given. If different mQTLs were present on different regions of a LG, these regions are distinguished with the letters A, B, C etc. The alleles 'a' and 'b' originate from the parent 'Prima', and the alleles 'c' and 'd' originate from the parent 'Fiesta', thus following JoinMap codes for outcrossers. As many metabolites in the phenylpropanoid pathway were mapped, for the purpose of simplicity, metabolites that belong to a similar group of compounds are shown as a group (e.g. phenolic esters is a group of several metabolites). Gene names are abbreviated as: *phenylalanine ammonia-lyase* (*PAL*), *cinnamate*-4-*hydroxylase* (*C4H*), 4-*coumaroyl*:*CoA-ligase* (*4CL*), *chalcone isomerase* (*CHI*), *chalcone synthase* (*CHS*), *flavonone* 3' *hydroxylase* (*F3'H*), *dihydroflavonol* 4-*reductase* (*DFR*), *hydroxycinnamoyl-CoA quinate*/*shikimate hydroxycinnamoyl transferase* (*HCT*), *leucoanthocyanidin* 4-*reductase* (*LAR*), *UDP-glycosyltransferase* (*UGT*), *flavonol synthase* (*FLS*) and *anthocyanidins synthase* (*ANS*).

3.7 The genetic loci controlling metabolite levels in the peel also appear to control these in the flesh although less significantly

Like those in peel, mQTLs in flesh were detected on the same five linkage groups. Most of the metabolites showing mQTLs in the peel also showed mQTLs in the flesh; however, the number of mQTLs in the flesh was lower than in the peel (Figs. 4.1, 4.2). Like in peel, in flesh also kaempferol glycosides had an mQTL on LG1 and LG16. In contrast to peel, quercetin rhamnoside is the only quercetin glycoside which had a clear mQTL on LG8. As in the peel, several octane-di-ol glycosides also had mQTLs on LG8 (Table S4.4). LG16 contained mQTLs for procyanidins at the same genetic region as in the peel. In this genetic area on LG16, mQTLs for phenolic esters, (+)-catechin and (-)-epicatechin were also found (Fig. 4.5B). Chlorogenic acid, which had an mQTL in the peel on LG17, had a minor mQTL in the same genetic region in the flesh (Table S4.4). Glucuronic acid which is not part of the phenypropanoid pathway also has an mQTL on LG16 (Table S4.4).

3.8 The levels of metabolites in the LG16 mQTL hotspot were controlled by a single, dominant locus present in both 'Prima' and 'Fiesta', and showed a clear 3:1 segregation in the progeny

The individual parental alleles and different allele-pairs gave higher or lower level of metabolites in the progeny. Tables S4.3 and S4.4 show the effects of different parental allele pairs at the LG16 hotspot on metabolite levels in the progeny, allowing the detection of dominant, recessive or additive genetic effects. For the hotspot of mQTLs on LG16, both 'Prima' and 'Fiesta' had one dominant and one recessive allele each (*Mm*). The combination of the two dominant alleles in the progeny (*MM*) occasionally showed a further increase in the metabolites level, indicating an additive effect or incomplete dominance in these cases.

As LG16 showed a cluster of many mQTLs, we analysed this particular hotspot in more detail. The metabolites (+)-catechin, (-)-epicatechin, two of the procyanidin dimers, and two procyanidin trimers gave one major mQTL per parent, showing two contrasting groups representing monogenic segregation. Fig. 4.6 shows a typical example based on procyanidin dimer II, indicating a Mendelian 3:1 segregation of *Mm* x *Mm* (χ3:1= 0.87; P>0.05), whereby the amount of this metabolite was apparently predominantly controlled by a single locus. Both parents were heterozygous and the *Mm* and *MM* offspring genotypes showed a similar average content for this metabolite, indicating full dominance both in peel and flesh (Fig. 4.6). The effect of a single dominant allele was on average an increase of 0.62

compared to the recessive allele on the ^{10}log scale (Fig. 4.6). This resembles a 4.2-fold increase on a linear scale.

Procyanidins, coumaroyl quinic acid, (+)-catechin, (-)-epicatechin, and kaempferol hexose rhamnose showed similar segregation patterns, apparently being controlled by the same dominant and recessive alleles of LG16 from both parents. Coumaroyl hexoside and quinic acid appeared to be controlled by the same locus, but in contrast to phenolic esters and other phenolic compounds, their level was negatively correlated to the other phenolic compounds that mapped to this hotspot (Tables S4.5, S4.6).

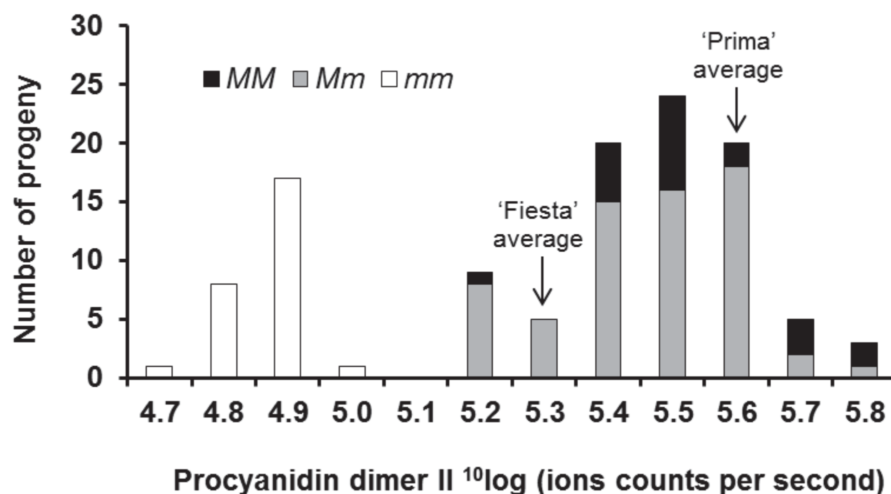


Fig. 4.6 Frequency distribution for the amount of the metabolite procyanidin dimer II *MM*, *Mm* and *mm* in the F1 population of 'Prima' x 'Fiesta', showing a 3:1 segregation pattern. The two classes (i.e. *mm* and *Mm* + *MM*) show the full dominance.

The metabolites that segregated according to a 3:1 ratio and had only one mQTL behaved as monogenic traits, and could be mapped as genetic markers, which was true for (+)-catechin, (-)-epicatechin, two procyanidin dimers, and two procyanidin trimers. In case where the relative metabolite level of a progeny was high, it was not clear whether that genotype had inherited the dominant allele from the mother, the father, or from both. Only in case of a low metabolite level, it was evident that both the mother and the father provided the recessive allele. Therefore, as for all dominant markers that segregate in a 3:1 fashion, the marker information could only be used for 25% of the progeny. In spite of this limitation, it was still worthwhile and helpful to locate the genetic window of the locus (Fig. 4.7).

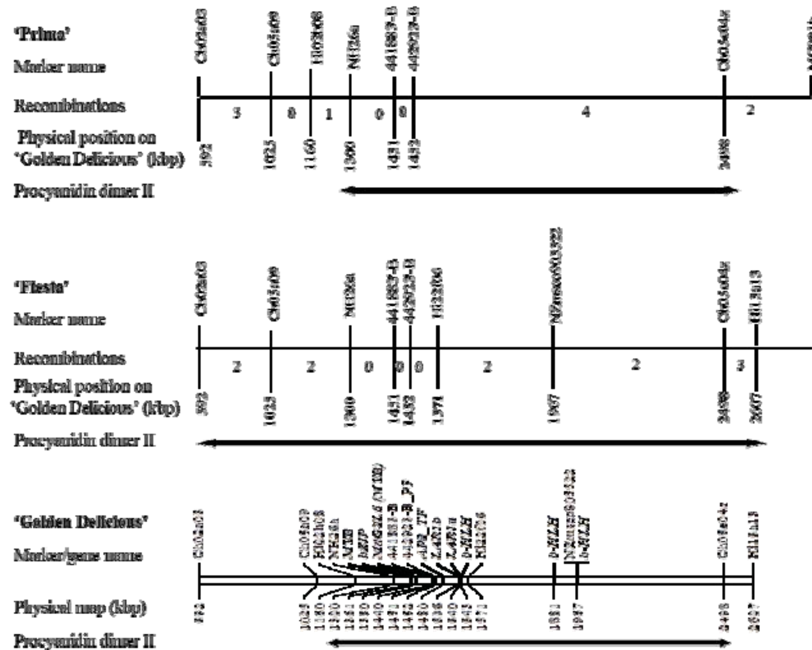


Fig. 4.7 Genetic linkage maps of 'Prima' and 'Fiesta' and a physical map of the apple cultivar 'Golden Delicious' for the mQTL hotspot region on LG16. Procyanidin dimer II was used as representative for the metabolites that mapped to the LG16 mQTL hotspot. The mQTL regions were genetically mapped as monogenic traits by means of graphical genotyping in both parents and are indicated as horizontal black arrows. The structural gene *leucoanthocyanidin reductase* (*MdLAR1*) of the phenylpropanoid pathway appeared to be present in this region, according to the putative genes in the whole genome sequence of cv. 'Golden Delicious'. Seven putative transcription factor genes including *MYB* and *bHLH* were also detected in this region. The structural gene *MdLAR1* and transcription factor genes are presented in bold text.

For more-detailed mapping of this locus on LG16, additional SSR markers in the LG16 mQTL hotspot were mapped. Several of the new SSR loci mapped in the genetic window (Fig. 4.7). The names and allelic sizes of these markers are given in Table S4.7. Graphical genotyping of the metabolite ‘procyanidin dimer II’ revealed that the gene causing the mQTL hotspot on LG16 is located between the locus NH26a and the locus Ch05e04 in ‘Prima’, and between the locus Ch02a03 and locus Hi15a13 in ‘Fiesta’ (Fig. 4.7).

3.9 The structural gene *MdLAR1* and seven transcription factors are at the mQTL hotspot

To find the position of the orthologous genes on the 17 chromosomes of apple, the DNA sequences of the structural genes of *A. thaliana* (Table 4.2) were aligned to the entire genome sequence of the apple cv. ‘Golden Delicious’ (Velasco *et al.*, 2010). The results of these alignments are shown in Table 4.2 and also in Figs. 4.5 and 4.7. Among the fifteen different structural genes, only the structural gene *MdLAR1* was found in the mQTL hotspot on LG16 (Figs. 4.5, 4.7). A closer look revealed that the published ‘Golden Delicious’ genome sequence had at this locus at least five *LAR*-like sequences in overlapping contigs. However, a sequence homology search using the EMBOSS software package revealed that these sequences were identical or highly homologous, and actually only two different genomic sequences were found. These two sequences probably represent the two alleles for the *MdLAR1* gene. Apart from *MdLAR1*, seven putative transcription factor genes were also identified in the genetic window of the mQTL hotspot on LG16 (Table 4.3). Two of these belong to *MYB* class, four to basic *helix-loop-helix* (*bHLH*) class, one to *bZIP* and one to *AP2* class of transcription factor genes.

Table 4.2. Structural genes of the phenylpropanoid pathway in *Arabidopsis* and apple

Gene	Full name	*Arabidopsis* locus	Known apple sequence	Homologues in *Arabidopsis*	Genetic positions in apple	At mQTL hotspot on LG16
4CL	*4-coumarate-CoA ligase*	At1g65060	GO565912, GR882782, GO577298, GO568847, etc	12	3 (LG1,3,7)	No
ANR	*Anthocyanin reductase*	At1g61720	AY830130	1	3 (LG5)	No
ANS	*Anthocyanin synthase*	At4g22870	AF117269	5	4 (LG6)	No
C3H	*p-coumarate 3-hydroxylase*	At2g40890	TC28151 (http://compbio.dfci.harvard.edu/)	1	6 (LG8,15)	No
C4H	*Trans-cinnamate 4-monooxygenase*	At2g30490	GO549874	1	2 (LG3,11)	No
CHI	*Chalcone isomerase*	At3g55120	X68978	3	9 (LG1,11,14)	No
CHS	*Chalcone synthase*	At4g34850	X68977	4	3 (LG2,5,7)	No
DFR	*Dihydroflavonol 4-reductase*	At5g42800	AF117268	2	2 (LG8,12)	No
F3'H	*Flavonoid 3'-monooxygenase*	At5g07990	Apple_0223.261.C2.Contig645, Apple_0223.261.C1.Contig644 (http://titan.biotec.uiuc.edu/cgi-bin/ESTWebsite/estima_start?seqSet=apple)	1	2 (LG6,14)	No
F3H	*Flavanone 3-hydroxylase*	At3g51240	AF117270	2	2 (LG2,5)	No
FLS	*Flavonol synthase*	At5g08640	AF119095	2	2 (LG0)	No
HCT	*Shikimate O-hydroxycinnamoyltransferase*	At5g48930	Apple_0223.2950.C2.Contig4990, Apple_0223.850.C1.Contig1757	1	3 (LG9,17)	No
LAR	*Leucoanthocyanidin reductase*	*AJ550154	AY830131, AY830132	0	5 (LG13,16)	Yes
PAL	*Phenyalanine ammonium lyase*	At2g37040	Apple_0223.263.C1.Contig648, Apple_0223.215.C2.Contig537, Apple_0223.263.C2.Contig649	4	6 (LG1,4,8,12)	No
UGT	*UDP-dependent glycosyltransferase*	At5g17050	AF117267	3	6 (LG0,1,7,9)	No

* Not found in *Arabidopsis* but in *Desmodium uncinatum*.

Table 4.3. Transcription factor genes at the mQTL hotspot on LG16

Putative gene	Apple sequence	Position on chromosome (bp)	Size (kbp)
MYB	MDP0000375685	1361220-1362093	1.3
MYB	MDP0000703817	1440436-1442198	1.7
bHLH	MDP0000319726	1543934-1555640	11.7
bHLH	MDP0000154272	1881558-1884164	2.6
bHLH	MDP0000261293	1967365-1970040	2.6
AP2	MDP0000939633	1475660-1476865	1.2
bZIP	MDP0000250967	1376596-1386527	9.9

4. Discussion

Phenolic compounds are not equally distributed throughout the apple fruit and are more abundant in the peel (Van der Sluis *et al.*, 2001). Quercetin glycosides are found almost exclusively in the peel tissue (Van der Sluis *et al.*, 2001). Anthocyanins, the red pigments, are predominantly present in the peel, except for the red-fleshed genotypes of apple. In contrast, chlorogenic acid and flavanols are more abundant in the flesh (Mcghie *et al.*, 2005; Van der Sluis *et al.*, 2001).

Many genes involved in the biosynthesis of phenylpropanoids and flavonoids in different plant species have been identified and appear to occur throughout the plant kingdom (Dixon and Steele, 1999). In apple, most of the structural genes and several regulatory genes have been isolated as cDNA sequences (Takos *et al.*, 2006; Szankowski *et al.*, 2009; Kim *et al.*, 2003). However, it is not yet clear which of these genes are critical for the variation in metabolite levels between tissues and genotypes. Genetic mapping of metabolites is a useful step to elucidate which genes are critical for this variation in metabolite levels.

By combining the metabolomic data with genetic linkage maps, we detected 488 mQTLs in peel and 254 mQTLs in flesh, using the software MetaNetwork. MetaNetwork is designed for the simultaneous mapping of a large series of traits in homozygous recombinant inbred line (RIL) populations of self-fertilising crops or in F2 populations from crosses between homozygous inbred lines. However, the parents of the apple population are heterozygous, and the markers and traits do segregate in the F1. Therefore, the input files had to be transformed, as indicated in the Materials and methods, to make MetaNetwork compatible with the apple genetic model. To our knowledge, the present study is the first report of this large-scale mapping software being applied to a cross-fertilising crop. This opens wider applications for MetaNetwork.

To gain more insight into the biochemical pathways regulated by the detected mQTLs, the centrotypes that showed highly probable mQTLs were manually annotated using accurate mass, in-source MS/MS and UV/Vis-absorbance information. The annotation revealed several groups of metabolites in both peel and flesh, including phenylpropanoid esters and glycosides (such as coumaroyl-hexoside, chlorogenic acid and coumaroyl-quinic acid) and flavonoids (such as (+)-catechin, (-)-epicatechin, procyanidins, quercetin, and kaempferol glycosides) (Tables S4.1, S4.2). Except for the alcohol glycosides and glucuronic acid all of these metabolites originate from the phenylpropanoid and flavonoid biosynthetic pathway. This bias towards the phenylpropanoid pathway was at least partially caused by the sample preparation (aqueous-methanol extracts) and the LC-MS system used (C18-reversed phase LC and ESI ionisation in negative mode; Moco *et al*. (2006). Other apple fruit metabolites, such as apolar steroids and volatiles could not be detected with these parameter settings and thus did not show up in our approach.

4.1 A flavonoid-mQTL hotspot is located on LG16

From MetaNetwork analysis, an mQTL hotspot on LG16 was found for both the parents and both the peel and flesh tissues (Figs. 4.1, 4.2). To find the putative underlying gene(s) causing this mQTL hotspot, we positioned the structural genes of the phenylpropanoid pathway on the chromosomes of apple, as shown in Fig. 4.5 and Table 4.2. In this case, the position of the structural genes was not determined by means of genetic mapping in a segregating population, but via the alignment of the known sequences of these structural genes in *A. thaliana* with the first draft whole genome sequence of the apple cv. ‘Golden Delicious’ (Velasco *et al.*, 2010). Only the structural gene *MdLAR1* was found in the mQTL hotspot on LG16. *MdLAR 1* catalyzes the conversion of leucocyanidins into the flavan-3-ols (+)-catechin and (-)-epicatechin, which are the building blocks of procyanidins (Fig. 4.5). Both the flavan-3-ols and the procyanidins showed an mQTL in this region. The *MdLAR1* gene may explain the mQTL hotspot on LG16, as 23 procyanidins in peel and 13 procyanidins in flesh were mapped to this locus, as well as the two flavan-3-ols (+)-catechin and (-)-epicatechin in both tissues. This was also observed with the alleles involved: the level of flavan-3-ols was increased by the ‘a’ allele from ‘Prima’ and/or the ‘d’ allele from ‘Fiesta’ (Fig. 4.5). These same alleles also increased the level of procyanidins (Fig. 4.5, Tables S4.3, S4.4).

A contra-indication that *MdLAR1* being the responsible gene for the hotspot is the presence of mQTLs at this locus for the chlorogenic acid and coumaroyl quinic acid (phenolic esters), phloridzin, and kaempferol glycosides. These metabolites are upstream of the substrate for *MdLAR1* (Fig. 4.5). Furthermore, their

levels were simultaneously increased with flavan-3-ols and procyanidins (Fig. 4.5). It is unlikely that *MdLAR1* causes all these mQTLs. A likely explanation for this result is the presence of a transcription factor gene that regulates the structural genes for these metabolites. In view of this, we searched for transcription factor genes at the mQTL hotspot and detected seven transcription factor genes here. Some of these transcription factor genes belong to the *MYB* and *bHLH* types of transcriptional regulators. One of these transcription factor genes may be responsible for the mQTL hotspot for the phenolic esters and kaempferol glycosides and possibly also for the mQTL hotspot for flavan-3-ols, procyanidins and coumaroyl hexoside.

The level of coumaroyl hexoside and quinic acid (Fig. 4.5A) were negatively correlated with the level of flavan-3-ols and procyanidins (Tables S4.5, S4.6) in the progeny, as indicated by a red colour in Fig. 4.5A. This was also indicated by the alleles that increase the levels of coumaroyl hexoside: whereas the levels of flavan-3-ols and procyanidins were elevated by presence of the marker alleles ‘a’ and ‘d’, the coumaroyl hexoside and quinic acid were elevated when these alleles were absent (Fig. 4.5A, Tables S4.3, S4.4). This may be explained by a strong sink effect for the production of flavan-3-ols and procyanidins, thus, competing with coumaroyl hexoside for the substrates cinnamic acid and coumaric acid. This sink effect may have been enhanced by the subsequent side branches from the main route from the cinnamic acids to the flavan-3-ols and the procyanidins (Fig. 4.5).

4.2 Quercetin glycosides are not controlled by the flavonoid-mQTL hotspot on LG16

Quercetin glycosides are commonly found in apple fruits (Van der Sluis *et al.*, 2001). Although quercetin glycosides are part of the phenylpropanoid and flavonoid biosynthetic pathways, they did not exhibit any significant mQTL on LG16 (Fig. 4.5). Instead, these compounds had mQTLs on LG1 in peel. On LG1, the structural gene *UGT* is located, which is responsible for the glycosidation of quercetins. This *UGT* gene may be responsible for the mQTL on LG1 (Fig. 4.5). Takos et al. (2006) identified and characterised an *UGT* gene in apple, using a functional genomics approach. As *UGT* genes consist of a large gene family, further studies would be needed to verify which *UGT* gene would be responsible for the glycosidation of quercetin. In flesh, quercetin glycosides showed an mQTL on LG8 (Fig. 4.5).

Another quercetin derivative, i.e. isorhamnetin (a methoxylated form of quercetin) had a strong mQTL on LG13. Possibly, a gene for methoxylation is located on LG13 (Fig. 4.5). We observed that both quercetin metabolites (quercetin glycosides and isorhamnetin glycosides) were not depending on LG16, and we did not detect any free, unmodified quercetin in apple. Together, these observations

suggest that the rate-limiting step in the formation of quercetin derivatives in apple is determined by the modifying enzymes (*UGT*, *OMT*), and that the flux of phenylpropanoid towards quercetin is adapted to the availability of modification opportunities.

4.3 Structural and transcription factor genes of the phenylpropanoid pathway in other studies

Several structural genes for flavonoid biosynthesis have been described in apple. Takos et al. (2006) described two *LAR* genes and an *Anthocyanidin reductase* (*ANR*) gene, detected in cDNA from the peel of the red apple cv. 'Cripps Red'. They named the two *LAR* genes *MdLAR1* and *MdLAR2*. BLAST results of the sequences of these genes from apple revealed that only *MdLAR1* is present in the LG16 mQTL hotspot. The *MdLAR2* is located on LG13. A major part of LG16 contains homoeologous sequences of LG13 due to whole genome duplication (Maliepaard *et al.*, 1998; Velasco *et al.*, 2010). Park et al. (2006) also identified *LAR* and *ANR* in apple fruits by statistically analyzing the expressed sequence tags (ESTs). *ANR* utilizes anthocyanidin and *LAR* use leucocyanidin as substrate. Both *ANR* and *LAR* participate in synthesis of flavan-3-ol monomers, whereas these monomers are the building blocks of procyanidin polymers (Xie *et al.*, 2003). In grape, *ANR* and *LAR* genes strongly influence procyanidin accumulation and composition during berry development (Bogs *et al.*, 2005).

In pear (*Pyrus communis L.*), a closely related species to apple, cDNAs for the prominent genes in flavonoids biosynthesis mentioned in Fig. 4.5 have been isolated via homology with the apple sequences in order to elucidate the functions of these genes (Fischer *et al.*, 2007). They found high homology to apple in the DNA and cDNA. Substrate specificities of the recombinant enzymes expressed in yeast were determined for physiological and non-physiological substrates and found to be in general agreement with the characteristic pear flavonoid metabolite pattern (Fischer *et al.*, 2007). In strawberry, another member of the rosaceous family, genes in the flavonoid pathway could be clearly classified into two groups according to their expression pattern; one having two transcription peaks at early and late stages (i.e., *FaANR*, *FaANS*, *FaCHI*, *FaFHT* and *FaLAR*), and the other showing an up-regulation trend with a single peak at the turning and/or ripening stage (i.e., *FaDFR*, *FaFGT*, *FaFLS* and *FaMYB* (Almeida *et al.*, 2007). This shows that expression pattern for flavonoid genes can be different and fruit stage for the expression of certain flavonoid genes can be very critical.

Bogs *et al*. (2007) characterized a grapevine *MYB* transcription factor gene, *VvMYBPA1*. This regulatory gene was shown to be able to activate the *LAR* and *ANR* genes, and several other flavonoid pathway genes in grapevines (Bogs *et al.*,

2007). Two other *MYB* genes, *VvMYBPA2* (Terrier *et al.*, 2009) and *VvMYB5b* (Deluc *et al.*, 2008) were also found to promote procyanidin biosynthesis in grapes. We compared *VvMYBPA1, VvMYBPA2* and *VvMYB5b* with the *MYB* gene in the hotspot on LG16, but found no convincing homology.

Three transcription factor genes from *Arabidopsis* that regulate procyanidin accumulation have been identified. *TRANSPARENT TESTA 2* (*TT2*), encoding *AtMYB123* transcription factor (Nesi *et al.*, 2001), is required for the normal expression of *BANYUL* (*BAN*) and therewith procyanidins accumulation (Nesi *et al.*, 2001). *BAN* genes encode *ANR,* a crucial gene in procyanidins biosynthesis (Xie *et al.*, 2003). *BAN* is actually a mutant of *ANR* and that is why we used *ANR* instead of *BAN* in our later discussion. *TRANSPARENT TESTA* 8 (*TT8*) (Nesi *et al.*, 2000), is a *AtbHLH42* and is required for the normal expression of *dihydroflavonol 4-reductase* (*DFR*) and *ANR* (Nesi *et al.*, 2000). *AtTTG1* (*Arabidopsis TRANSPARENT TESTA GLABRA* 1) is a *WD40* protein, regulating the expression of *ANR* (Nesi *et al.*, 2000). Recently, (Brueggemann *et al.*, 2010) reported *MdTTG1* from apple and proved it to be a functional homologue of *AtTTG1*. Li *et al.* (2007) reported the upregulatation of mRNA for several structural enzymes of the flavonoid pathway in apple by overexpressing the maize leaf colour (*Lc*) transcription factor gene. A BLAST analysis was performed to identify putative homologues of these transcription factor genes on the apple genome, particularly in the mQTL hotspot on LG16. None of the above transcription factor genes showed close homology and thus these cannot explain the mQTL hotspot on LG16.

4.4 Consequences of tight genetic linkage of the dominant alleles for high metabolites to the recessive alleles for pH

Figs. 4.3 and 4.4 illustrate that in both parents the dominant alleles for high levels of metabolites are genetically tightly linked to the recessive alleles for high pH. This has consequences for apple breeding. In Northern Europe, apples with a low pH are usually preferred to high pH, and therefore should contain the dominant allele for low pH. As the dominant allele for low pH is in repulsion phase to the dominant allele for high metabolites, at least in the genotypes we investigated, the selection for the dominant low pH allele implies the selection for the recessive allele for low levels of the phenolic compounds. Therefore, progeny that are more acidic, have higher chances of having lower levels of procyanidins and other phenolic compounds. This can be solved by the selection of progeny that have one dominant allele for low pH from one parent and one dominant allele for high metabolite levels from the other parent. This is the *MmMama* group in Fig. 4.4. In the southern countries of Europe and in Asia, consumers usually prefer a higher pH. In that case, the desired absence of the dominant allele for pH is automatically combined with the presence of the dominant allele for high levels of metabolites. This is the *MMmama* group in Fig. 4.4. For apple, it takes usually six to eight years after sowing to have fruits that can be evaluated for pH and metabolites. Selection of the desired progeny for these fruit traits is feasible already at a very young stage, using DNA from leaves of seedlings and DNA markers (Fig. 4.3).

4.5 Other fruit quality traits mapped in ‘Prima’ x ‘Fiesta’ segregating population

King et al. (2000) mapped several other traits in the same population. They detected a significant QTL for different sensory traits like crispness, juiciness, sponginess, and overall liking on LG16 (King et al., 2000). This QTL mapped in the region of Ma (pH), which is actually mapped in our mQTL hotspot. A QTL for hardness and granularity was also suggested in the same region (King et al., 2000). These traits are different than the metabolites used in our studies and it shows the importance of this region on LG16 having QTLs for different important traits. King et al. (2000) also found that Ma (pH) locus was in coupling phase to the sensory traits, but in our studies the Ma (pH) locus was found to be in repulsion phase to the mQTLs for different metabolites. This means that the important traits detected by King et al. (2000) are in repulsion to high levels of phenolic compounds. This has practical implications. When the breeder selects in ‘Prima’ or ‘Fiesta’ progeny for low pH, he will also select for the different sensory traits. When he would like to have high levels of phenolic compounds too, he should select for heterozygous plants on LG16

hotspot, as discussed above. Marker assisted breeding can be helpful.

4.6 Phenotypic buffering

Fu *et al*. (2009) analyzed a segregating *A. thaliana* population for variation in transcript, protein and metabolite abundance. They mapped QTLs for 40,580 molecular and 139 phenotypic traits, and found six QTL hotspots with major, system-wide effects. For the far majority of the 500,000 SNPs between the two parental lines, no or minor impact on the phenotype was detected. The authors interpreted this lack of dramatic changes by genetic variation as robustness of the system. The six hotspots are exceptions. These hotspots seem to correspond to a few molecular fragilities of an otherwise robust regulatory system (Fu *et al.*, 2009). In another study, Keurentjes *et al*. (2006b) have described these hotspots in more detail for metabolites. Their results show striking similarities with our results in apple, although the *Arabidopsis* population contained only up to two alleles per gene because of the homozygous parents, whereas in the apple population up to four alleles were present per gene. Although a series of genes are involved in the pathway of phenolic compounds, a large extent of quantitative variation in these compounds is explained by one locus only, i.e. the hotspot on LG16.

4.7 Follow-up studies

In subsequent studies, the expression profiles of the *MdLAR1* candidate gene, other structural genes of the phenylpropanoid pathway, and the seven transcription factor genes found in the mQTL hotspot will be studied in progeny that have either low or high procyanidins levels. Final proof of their involvement needs complementation studies. Next, to increase the level of these beneficial metabolite(s), the most promising alleles may be inserted into existing, highly popular apple cultivars with low procyanidin levels, e.g. by means of a cisgenesis approach. Cisgenesis is defined as ‘the genetic modification of a recipient plant with natural gene(s) from a sexually compatible plant (Schouten *et al.*, 2006a, b). Whatever the outcome of these follow-up studies, the knowledge obtained from the current study of the mQTL hotspot genes is already of use for the breeding of new cultivars with increased levels of these putatively beneficial metabolites through application in marker-assisted breeding.

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Chapter 5

The *leucoanthocyanidin reductase* gene causes an mQTL hotspot on LG16 for phenolic compounds in apple fruits

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Abstract

Apple (*Malus* × *domestica* Borkh) is an important source of phenolic compounds in our food. In the previous study on ripe apples from a progeny of a cross between 'Prima' and 'Fiesta', it was shown that at the top of LG16 there was a hotspot of mQTLs for phenolic compounds in peel and flesh. In the present study, we investigated the expression profiles of genes that are related to the metabolism of phenolic compounds during fruit development. This was done for structural genes of the phenylpropanoid pathway, putative transcription factor genes on the LG16 mQTL hotspot and putative transcription factor genes elsewhere in the apple genome that may regulate the phenylpropanoid pathway. The gene expression profiles were studied for peel and flesh of apples from 19 progeny genotypes. Only the structural gene *leucoanthocyanidin reductase* (*MdLAR1*) showed a significant correlation between transcript level of different alleles and content of metabolites that mapped on the mQTL hotspot. Progeny that had inherited one or two copies of the dominant alleles (*Mm, MM*) showed on the average a 4.4- and 11.8-fold higher expression level of *MdLAR1,* respectively*,* compared to the progeny that had inherited the recessive alleles (*mm*). This led to a 4.0-fold increase of procyanidin dimer II content at the ripe stage. Upstream metabolites such as phenolic esters and kaempferol glycosides also showed a positive correlation with transcript levels of *MdLAR1*, suggesting feedback mechanism within the pathway. We also found in literature that the *4-coumarate-CoA ligase* (*4CL*) step is regulated in the phenylpropanoid pathway. This could also explain the mQTLs upstream in the pathway. The consequences for indirect breeding for increased phenolic compounds content in apple fruits are discussed.

1. Introduction

Apple (*Malus* × *domestica* Borkh) is a member of the Rosaceae family, sub family Pomoideae, which includes crop species such as pear, cotoneaster, and quince (Janssen *et al.*, 2008). It is an important fruit crop of the temperate regions of the world. Apple is an important source of many secondary metabolites known as phenolic compounds. These phenolic compounds have various functions in the plant such as protection against ultra violet (UV) light and defence against pathogens. One of the important benefits of these compounds to consumers is their potential role against various human diseases such as cancer, coronary heart diseases, cardiovascular diseases, and diabetes (Eberhardt *et al.*, 2000; Mcghie *et al.*, 2005).

The phenylpropanoid pathway is the biochemical pathway of flavonoids in plants. This is, however, a complex metabolic pathway and involves several enzymes. Many of these enzymes have been functionally characterized (Han and Korban, 2009; Jugde *et al.*, 2008; Takos *et al.*, 2006; Kim *et al.*, 2003).

To date, variations in gene expression have been extensively studied in different populations, species and tissues of plants (Ince *et al.*, 2010). For example, Ince et al. (2010) studied the expression of genes containing SSRs in different tissues and at different developmental stages of pepper plant. Janssen et al. (2008) made gene expression studies for apple fruit development at eight time points using microarray. Costa et al. (2010) also studied gene expression in apple during development, maturation and ripening with specific focus on ethylene. They found that gene expression in apples is coordinated with specific developmental stages (Janssen *et al.*, 2008; Costa *et al.*, 2010).

Expression of candidate genes can be correlated with the end-products of the pathway, from which the genes are part of, by correlating the transcript levels to the compound content such as phenolic compounds. The expression studies can be performed through various techniques such as microarray hybridization and quantitative reverse transcriptase PCR (q-RT-PCR). q RT-PCR is a very efficient technique for measuring the expression profiles of candidate genes. It is a more targeted and reliable approach for studying the expression of target genes.

Apple fruit develops over a period of 150 days from fertilization to fully ripe fruit (Janssen *et al.*, 2008). During this period it passes through different developmental stages. A pictorial representation of the different stages of apple can be found on the Michigan University website as http://web1.msue.msu.edu/fruit/applgrw.htm.

In Chapter 4, phenolic compounds were genetically mapped that were detected in peel and flesh of ripe apple fruits, and a hotspot of mQTLs was detected on LG16. By looking closer at the draft sequence of the whole genome of the apple cultivar 'Golden Delicious' (Velasco *et al.*, 2010), the structural gene

leucoanthocyanidin reductase (*MdLAR1*) and eight transcription factor genes were detected in the genetic window of the mQTL hotspot.

The aim of the present study was to know whether one or more of the candidate genes are responsible for this mQTL hotspot. The approach was the analysis of expression of structural and transcription factor genes of the phenylpropanoid pathway. Therefore the expression of the structural gene *MdLAR1* and seven transcription factor genes in the mQTL hotspot was investigated. In addition, expression profiles of transcription factor genes outside of the mQTL hotspot and the structural genes of the phenylpropanoid pathway were also studied. A strong positive correlation between the expression level of the *MdLAR1* gene and the level of metabolites that mapped on LG16 was observed. This was not found for any other gene among the genes that were studied. This indicates that different alleles of the *MdLAR1* gene cause the mQTL hotspot on LG16.

2. Materials and methods

In this study fruits from the F1 segregating population derived from the cross between the cultivars 'Prima' and 'Fiesta' were used. This population was used in our previous study too in which the mQTL hotspot was detected (For detail see Chapter 4).

2.1 Selection of genotypes and harvesting of fruits for gene expression studies

We selected genotypes based on the clear segregation of metabolite 'procyanidin dimer II'. This metabolite belongs to the phenylpropanoid pathway and its metabolic content showed a clear segregation and was mapped at the mQTL hotspot on LG16 as described in Chapter 4. This metabolite was used as representative metabolite for all metabolites that mapped at the mQTL hotspot. The progeny genotypes from the cross 'Prima' x 'Fiesta' were divided into two groups based on 'procyanidin dimer II' clear segregation, i.e. one group having high content and one having low content of 'procyanidin dimer II'. The trees were at full bloom from 26-30th April, 2010. The different developmental stages of fruits were observed. Fruits from trees in the trial orchard located in Randwijk, the Netherlands were harvested as described in Chapter 6. Ten genotypes were selected from 'Group A' and nine genotypes were selected from 'Group B' with two trees per genotype (two biological replicates). For reference, the parents of the segregating population were included in the analysis with two trees for each parent. Both parents belong to the heterozygous dominant class. The genotypes number and genotypes classes used in this study are given in Table 5.1. The sizes (diameter) of individual fruits was measured at each developmental stage using an electronic digital caliper, model VWRI819-0012 of

Control Company. The average size at 34 DAFB was 24 mm, at 60 DAFB 40 mm, and at 95 DAFB was 62 mm (Fig. 5.1; Table S5.1).

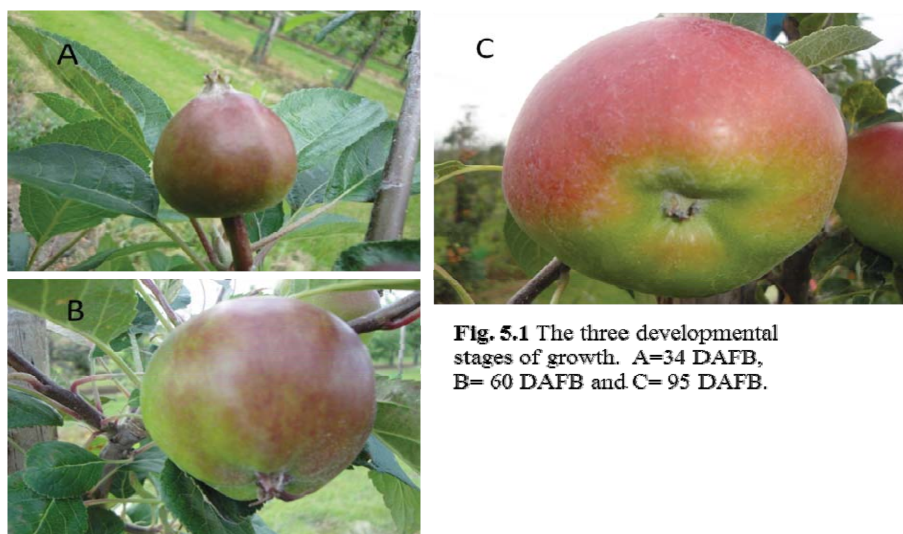


Fig. 5.1 The three developmental stages of growth. A=34 DAFB, B= 60 DAFB and C= 95 DAFB.

There were three classes of genotypes based on co-segregating genetic markers: *MM* is a homozygous dominant class which has both dominant alleles for increased content of the procyanidin dimer II. These progenies inherited from each parent one dominant allele. *Mm* is the heterozygous class, which has one dominant allele from one parent and one recessive allele from the other parent. The heterozygous progeny has high content of the metabolite. The homozygous recessive class *mm*, has received both recessive alleles from the two parents, and showed a low content of the metabolite.

2.2 RNA isolation from apple fruits

Total RNA was isolated from peel and flesh of apple fruits separately according to the CTAB method described by Asif et al. (2000). The RNA quantity was measured on NanoDrop® spectrophotometer model ND-1000 from isogen lifescience scientific company as explained in Chapter 6 and the RNA quality and quantity was measured by running two µls of the RNA sample on a 1.5% agarose gel.

Table 5.1 Genotypes used for measuring relative gene expression. 'Group A' had low and 'Group B' had high content of 'Procyanidin dimer II'. The content is given as [10]log transformed values

Group A	Geno type class	Procyanidin dimer II content (peel)	Procyanidin dimer II content (flesh)	Group B	Genotype class	Procyanidin dimer II content (peel)	Procyanidin dimer II content (flesh)
1988-001-014	*mm*	3.25	4.82	1988-001-002	*MM*	3.76	5.36
1988-001-015	*mm*	3.44	4.78	1988-001-003	*MM*	3.96	5.33
1988-001-019	*mm*	3.29	4.82	1988-001-006	*MM*	3.97	5.43
1988-001-030	*mm*	3.31	4.78	1988-001-008	*Mm*	3.82	5.52
1988-001-037	*mm*	3.37	4.84	1988-001-009	*Mm*	3.89	5.57
1988-001-044	*mm*	3.35	4.89	1988-001-010	*MM*	3.91	5.48
1988-001-088	*mm*	3.28	4.80	1988-001-013	*Mm*	3.78	5.59
1988-001-099	*mm*	3.31	4.79	1988-001-021	*MM*	3.94	5.48
1988-001-127	*mm*	3.34	4.83	1988-001-023	*Mm*	3.61	5.44
1988-001-138	*mm*	3.35	4.83	--	--	--	--

2.3 Complementary DNA (cDNA) synthesis

First single-strand complementary DNA (cDNA) was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's manual.

2.4 Selection of genes for q RT-PCR studies

i) Structural genes of the phenylpropanoid pathway

The *MdLAR1* gene was detected in the middle of the genetic window for mQTL hotspot at the top of the LG16 using sequence information of cv. 'Golden Delicious' (Velasco *et al.*, 2010). Therefore this structural gene was included in the gene expression study. For this gene, two primer pairs were designed at different regions of the gene. In addition, other structural genes that are involved in the phenylpropanoid pathway were included. The details of these genes are given in Table 5.2.

ii) Transcription factor genes in the mQTL hotspot on LG16

Seven transcription factor genes that were found in the mQTL hotspot window on LG16 were included in the q RT-PCR studies (Table 5.2).

iii) Transcription factor genes located outside the mQTL hotspot on LG16

Further, 11 other transcription factor genes located outside the mQTL hotspot but are known as candidates for regulation of the phenylpropanoid pathway such as *TTG1* and *bHLH3* were also added (Table 5.2).

Table 5.2 Description of 35 structural and transcription factor genes of the phenylpropanoid pathway including the seven candidate transcription factor genes found in the genetic window of the mQTL hotspot on LG16

	Gene name	Gene ID	Forward Primer (5'--→ 3')	Reverse Primer (5'--→ 3')	Gene position (bp)	LG on "Golden Delicious"
Structural genes	*MdLAR1pair1*	MDP0000171928	GTGGTTAACGGAGGCACAGT	CCGAGGAGAAAGGACTACCC	1536040-1540362	LG16
	MdLAR1pair2	AY830131	GTGCTTCGATGGCTTTCTTC	TAACAAGCTCACCCCCAAAC	--	LG16
	MdLAR2	AY830132	ATGCCACAATCGTGTCAAAA	GGCTGGCTTCAGCTACAAAC	--	LG13
	MdPAL	ES790093	CGAGGAGTGTGACAAGGTGTTCCA	AGGAATGCAGCATGTAAACCGTGAC	--	*LG4*
	MdC4H	EB135197	GGACGTTTAGTCCAGAACTTCGAGCT	ACTTCATCACAATGGTGGAATGCTTC	--	*LG11*
	Md4CL	EB122629	CATAAACAGTGTCCCCAAGTCAGCAT	AGTGTTCCTACAAGCCTTCCCGATAA	--	*LG11*
	MdCHS	CN944824	GGAGACAACTGGAGAAGGACTGGAA	CGACATTGATACTGGTGTCTTCA	--	*LG9*
	MdCHI	CN946541	GGGATAACCTCGCGGCCAAA	GCATCCATGCCGGAAGCTACAA	--	*LG1*
	MdF3H	CN491664	TGGAAGCTTGTGAGGACTGGGGT	CTCCTCCGATGGCAAATCAAAGA	--	*LG5*
	MdDFR	AF117268	GATAGGGTTTGAGTTCAAGTA	TCTCCTCAGCAGCCTCAGTTTTCT	--	*LG12*
	MdANS	AF117269	GATGAAGGGAGGCTGGAGAAAG	GTGGAGGATGAAGGTGAGTGC	--	*LG6*
	MdFLS	EB137300	TCAGATGGAGATAATGAGCAATGGAAA	ATTAACGGGGTTCACAAGCTGTGG	--	*LG8*
	MdANR	EB125405	TCGCTGGCTTATGATCCTCCTGTT	CCGTTTGCCAAACTCAGCAAATTA	--	*LG5*
	MdHCTchr9	MDP0000851389	CGATGCTGTTTTCAGAACCA	GCAGCAGACGAGGATGATTA	24590557-24590820	*LG9*
	MdC3Hchr8	MDP0000466557	CAAAGGAGGTGCTCAAGGAG	TGGACTCGACCATAGCAGTG	29024267-29030089	*LG8*
	MdF3'Hchr6	MDP0000539956	ACTCTCTTCATGCGCTTGGT	TGCCTATCCTCACCCAAAAG	22805618-22810170	*LG6*
	MdF3'Hchr14	MDP0000370951	ACCATTAAACCCCAACAACG	ATCACGGTTTGGAGCTCTTG	27562625-27562933	*LG14*
	MdUFGT	AF117267	AAGGTCTCTCCAATGTACGAAT	AGGAGTTTGTTGACTTTGGACT	--	LG1, LG7

Table 5.2 continued-----

Transcription factor genes at mQTL hotspot	*MYB1361*	MDP0000375685	CTGGGGGTTCAAGTAGTCCA	CTCCGTGGTGGCTTGATAAT	1361220-1362614	LG16
	b-HLH1967	MDP0000261293	GATACGGCATCATTCCTGCT	GCCTGAGGATTTCCAACAAA	1967365-1970040	LG16
	b-HLH1881	MDP0000154272	CTCAACCGGGACTTATCCAA	GCTCATCCTCCCACACATTT	1881558-1884164	LG16
	b-HLH1543	MDP0000319726	GAGCTGAAACGCCAAACTTC	CGGTGATGAACAACACGTTC	1543934-1555640	LG16
	AP2_1480K	MDP0000939633	GCACCTTCAACGAAGAGGAC	GACTTGGAGTGGGAGCTCAG	1475660-1476865	LG16
	MdG2L61440K	MDP0000202657	AGACCGACTCCAACAATTCG	GGACTGGTGGTGAGACCTGT	2702256-2703966	LG13
	bZIP1380K	MDP0000250967	CTGTTTCTGGCAAAGGCTTC	CCATCAACATTGCAGTGGAC	1376596-1386527	LG16
Transcription factor genes out side the mQTL hotspot	*COL1220K*	MDP0000185616	TGATTTTATGGGGTGCCAAT	TAATCACCGCCTCGTAATCC	1224811-1226412	LG16
	b-HLH1080K	MDP0000725991	GGCCAATGACACCTCCTTTA	TGAGCTGTGGAATGAGCAAC	1084386-1086076	LG16
	MYB1070K	MDP0000659260	ACTCCGCAAGAACAGCTCAT	GCTGTTCGACTCGATGTTCA	1058455-1059577	LG16
	C2H2_1020K	MDP0000183099	CCTCCTCACCTCCTCTCTCC	CCCGGCTCTGTTGTAGTACC	1021788-1022492	LG16
	C2H2_1000K	MDP0000283750	ATTCAGCAAGTTGGGTGTCC	TTTGCTTTGTGCAGTTGAGG	1003724-1013102	LG16
	MdMyb5a.A	MDP0000791870	GGGGAGGAGGAAATGAAGAG	CAGAGTCCCAGCCAAATGTT	967439-968759	LG3
	MdbHLH33	MDP0000309179	GGAGACATCAAAACCCGAAA	TGAAGGACATGCAAAGCAAG	37144212-37148163	LG15
	MdTTG1	MDP0000906307	GACCCGGATACCCTTTCAAT	AAACTCGCTGGTCTTGCTGT	28763873-28764901	LG1
	MdbHLH3	MDP0000225680	GTCGCCATTGGTAAGGCTAA	CCACCGTGGTCTCAATTCTT	32877432-32884492	LG11
	MdMyb9	MDP0000210851	GGCCACTAGGTTGACCAAAA	ATCATCGCAGCCAAAGTTCT	9430777-9431972	LG8
	MdMyb11.A	MDP0000437717	TGAAGGTCGCTCATGTTCTG	ATTCACCGCCTGGTTTAGTG	30373863-30379290	LG13
	mGAPDH	CN494000	GCTGCCAAGGCTGTTGGAA	ACAGTCAGGTCAACAACGGAAAC	--	--

2.5 Designing and testing of primers for q RT-PCR

Primer pairs for candidate genes *MdLAR1,* transcription factor genes in the mQTL hotspot and outside the mQTL hotspot were designed with online available program 'Primer3Plus' (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primer pairs for structural genes of phenylpropanoid pathway were kindly provided by HortResearch New Zealand. The primer names and their forward and reverse sequences are given in Table 5.2. The primers were tested using q RT-PCR in the same way as explained in the chapter 6. The q RT-PCR products were checked for quality by checking their clear single peak in the melting curve and a clear band of the expected amplicon size on 1.5% agarose gel.

2.6 Sequencing of *MdLAR1* gene

The amplicons of the two primer pairs of the *MdLAR1* gene were sequenced. For this purpose several genotypes from each *MM* (homozygous dominant), *Mm* (heterozygous dominant) and *mm* (homozygous recessive) genotype classes were sequenced. This was done for verification of the specificity of the primers, as *MdLAR1* and *MdLAR2* have very similar sequences. Moreover, as the primers were designed on cv. 'Golden Delicious' sequence, it was checked for the correct primer annealing sites in the 'Prima' x 'Fiesta' segregating population.

2.7 Performing q RT-PCR

Gene expression in 216 cDNA samples was measured using Fluidigm Dynamic Array integrated fluidic circuits. The Fluidigm q RT-PCR for quantification of relative expression of the candidate genes was used. Fluidigm used the BioMark™ System and Evagreen DNA binding dye (http://www.fluidigm.com). Three 96x96 Dynamic Arrays of Integrated Fluidic Circuits**,** comprising 48 primer pairs in two replicates were used for this purpose. The q RT-PCR set up for reference gene and other control samples was same as explained in Chapter 6.

2.8 Analysis of q RT-PCR data

The q RT-PCR data were analysed using the Fluidigm® Real-Time PCR Analysis Software version 3 (http://www.fluidigm.com) as explained in the Chapter 6.

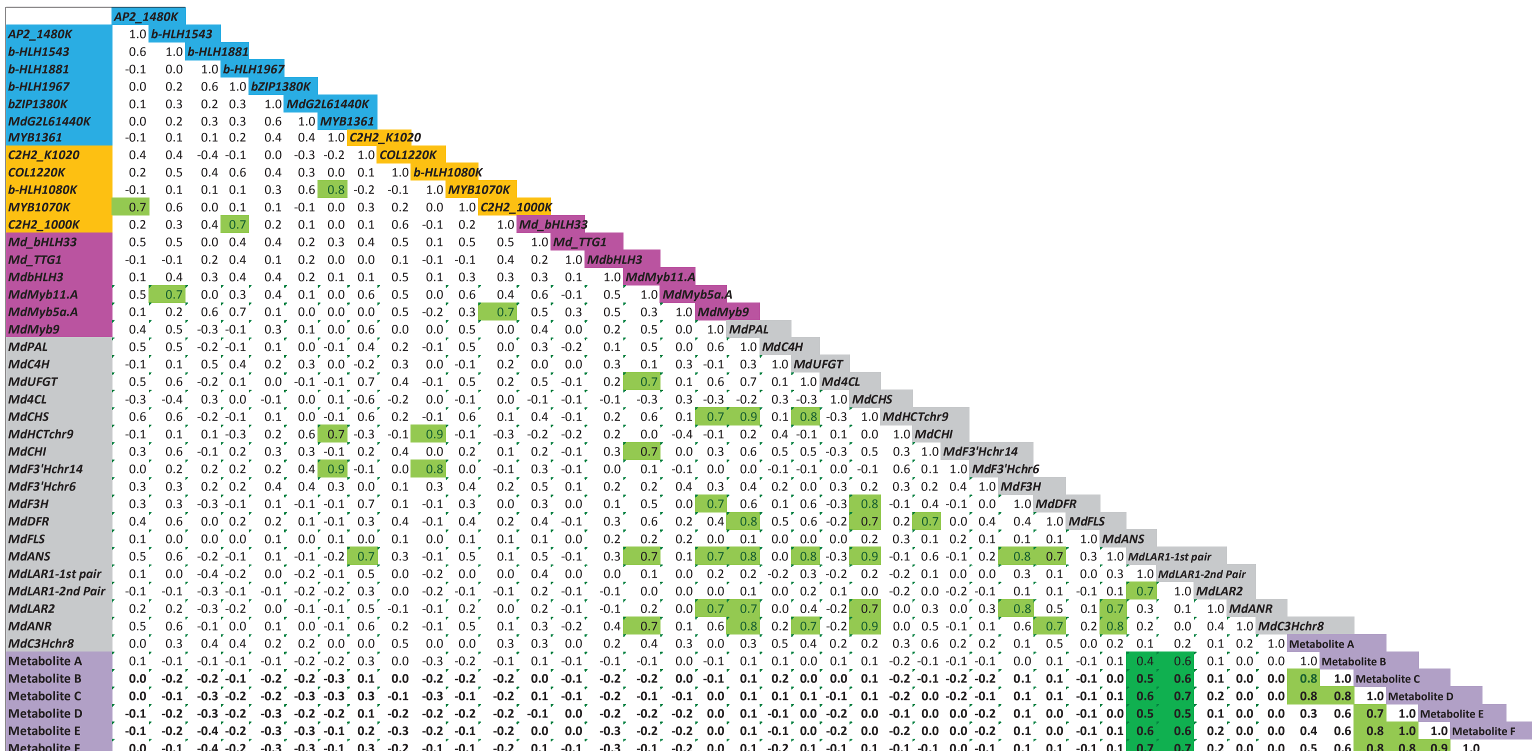
3. Results

3.1 Expression profiles of structural genes of the phenylpropanoid pathway

None of the 17 structural genes of phenylpropanoid pathway which were evaluated for gene expression, showed a significant correlation with the content of procyanidin dimer II, except the *MdLAR1* gene (Table 5.3 and Table S5.1). This was observed both in peel and flesh tissues and at the three different developmental stages. For *MdLAR1* we evaluated the expression using two different primer pairs, annealing at different places of the *MdLAR1* gene. Expression using both primer pairs showed a positive correlation between the expression level and the procyanidin dimer II and other metabolites content which mapped in the mQTL hotspot.

The progeny that had inherited the recessive alleles for low procyanidin dimer II content (*mm*), showed a low expression of *MdLAR1* throughout the fruit development, both in peel and flesh (Fig. 5.1). However, the heterozygous group (*Mm*) showed a higher expression, compared to the homozygous recessive (*mm*) group, whereas the homozygous dominant progeny (*MM*) with high content of procyanidin dimer II showed the highest expression of *MdLAR1* (Fig. 5.2). For all other structural genes we could not find any differential expression for the three genotype classes (data not shown). This strongly indicates that *MdLAR1* gene is the causal gene for the mQTL of procyanidin dimer II and all other phenolic compounds that mapped at the mQTL hotspot on LG16 and are highly correlated with procyanidin dimer II content in peel and flesh.

Table 5.3 (next page) Correlations between the expression levels of the phenylpropanoid pathway genes in flesh. The blue colour represents the transcription factor genes that were detected in the mQTL hotspot. Yellow colour shows the transcription factor genes that were not in the mQTL hotspot but were located on the LG16. Pink colour represents transcription factor genes of the phenylpropanoid pathway that are not located in the mQTL hotspot nor on the LG16 but are at other regions of the genome. In grey colour are the structural genes. The metabolites are highlighted in purple and the correlations with the expression level of all the genes is given in bold text. The metabolites contents were ^{10}log transformed. The significant correlation between expression level of genes and metabolite content is given in dark green colour. Metabolite A=Procyanidin dimer I , metabolite B=Procyanidin dimer II , Metabolite C=Procyanidin trimer I, Metabolite D=Procyanidin trimer II, Metabolite E=Epicatechin and Metabolite F=Catechin. Light green colour shows other significant correlations.



	AP2_1480K	b-HLH1543	b-HLH1881	b-HLH1967	bZIP1380K	MdG2L61440K	MYB1361	C2H2_K1020	COL1220K	b-HLH1080K	MYB1070K	C2H2_1000K	Md_bHLH33	Md_TTG1	MdbHLH3	MdMyb11.A	MdMyb5a.A	MdMyb9	MdPAL	MdC4H	MdUFGT	Md4CL	MdCHS	MdHCTchr9	MdCHI	MdF3'Hchr14	MdF3'Hchr6	MdF3H	MdDFR	MdFLS	MdANS	MdLAR1-1st pair	MdLAR1-2nd Pair	MdLAR2	MdANR	MdC3Hchr8	Metabolite A	Metabolite B	Metabolite C	Metabolite D	Metabolite E	Metabolite F
AP2_1480K	1.0																																									
b-HLH1543	0.6	1.0																																								
b-HLH1881	-0.1	0.0	1.0																																							
b-HLH1967	0.0	0.2	0.6	1.0																																						
bZIP1380K	0.1	0.3	0.2	0.3	1.0																																					
MdG2L61440K	0.0	0.2	0.3	0.3	0.6	1.0																																				
MYB1361	-0.1	0.1	0.1	0.2	0.4	0.4	1.0																																			
C2H2_K1020	0.4	0.4	-0.4	-0.1	0.0	-0.3	-0.2	1.0																																		
COL1220K	0.2	0.5	0.4	0.6	0.4	0.3	0.0	0.1	1.0																																	
b-HLH1080K	-0.1	0.1	0.1	0.1	0.3	0.6	0.8	-0.2	-0.1	1.0																																
MYB1070K	0.7	0.6	0.0	0.1	0.1	-0.1	0.0	0.3	0.2	0.0	1.0																															
C2H2_1000K	0.2	0.3	0.4	0.7	0.2	0.1	0.0	0.1	0.6	-0.1	0.2	1.0																														
Md_bHLH33	0.5	0.5	0.0	0.4	0.4	0.2	0.3	0.4	0.5	0.1	0.5	0.5	1.0																													
Md_TTG1	-0.1	-0.1	0.2	0.4	0.1	0.2	0.0	0.0	0.1	-0.1	-0.1	0.4	0.2	1.0																												
MdbHLH3	0.1	0.4	0.3	0.4	0.4	0.2	0.1	0.1	0.5	0.1	0.3	0.3	0.3	0.1	1.0																											
MdMyb11.A	0.5	0.7	0.0	0.3	0.4	0.1	0.0	0.6	0.5	0.0	0.6	0.4	0.6	-0.1	0.5	1.0																										
MdMyb5a.A	0.1	0.2	0.6	0.7	0.1	0.0	0.0	0.0	0.5	-0.2	0.3	0.7	0.5	0.3	0.5	0.3	1.0																									
MdMyb9	0.4	0.5	-0.3	-0.1	0.3	0.1	0.0	0.6	0.0	0.0	0.5	0.0	0.4	0.0	0.2	0.5	0.0	1.0																								
MdPAL	0.5	0.5	-0.2	-0.1	0.1	0.0	-0.1	0.4	0.2	-0.1	0.5	0.0	0.3	-0.2	0.1	0.5	0.0	0.6	1.0																							
MdC4H	-0.1	0.1	0.5	0.4	0.2	0.3	0.0	-0.2	0.3	0.0	-0.1	0.2	0.0	0.0	0.3	0.1	0.3	-0.1	0.3	1.0																						
MdUFGT	0.5	0.6	-0.2	0.1	0.0	-0.1	-0.1	0.7	0.4	-0.1	0.5	0.2	0.5	-0.1	0.2	0.7	0.1	0.6	0.7	0.1	1.0																					
Md4CL	-0.3	-0.4	0.3	0.0	-0.1	0.0	0.1	-0.6	-0.2	0.0	-0.1	0.0	-0.1	-0.1	-0.1	-0.3	0.3	-0.3	-0.2	0.3	-0.3	1.0																				
MdCHS	0.6	0.6	-0.2	-0.1	0.1	0.0	-0.1	0.6	0.2	-0.1	0.6	0.1	0.4	-0.1	0.2	0.6	0.1	0.7	0.9	0.1	0.8	-0.3	1.0																			
MdHCTchr9	-0.1	0.1	0.1	-0.3	0.2	0.6	0.7	-0.3	-0.1	0.9	-0.1	-0.3	-0.2	-0.2	0.2	0.0	-0.4	-0.1	0.2	0.4	-0.1	0.1	0.0	1.0																		
MdCHI	0.3	0.6	-0.1	0.2	0.3	0.3	-0.1	0.2	0.4	0.0	0.2	0.1	0.2	-0.1	0.3	0.7	0.0	0.3	0.6	0.5	0.5	-0.3	0.5	0.3	1.0																	
MdF3'Hchr14	0.0	0.2	0.2	0.2	0.2	0.4	0.9	-0.1	0.0	0.8	0.0	-0.1	0.3	-0.1	0.0	0.1	-0.1	0.0	0.0	-0.1	-0.1	0.0	-0.1	0.6	0.1	1.0																
MdF3'Hchr6	0.3	0.3	0.2	0.2	0.4	0.4	0.3	0.0	0.1	0.3	0.4	0.2	0.5	0.1	0.2	0.2	0.4	0.3	0.4	0.2	0.0	0.3	0.2	0.3	0.2	0.4	1.0															
MdF3H	0.3	0.3	-0.3	-0.1	0.1	-0.1	-0.1	0.7	0.1	-0.1	0.3	0.0	0.3	0.0	0.1	0.5	0.0	0.7	0.6	0.1	0.6	-0.3	0.8	-0.1	0.4	-0.1	0.0	1.0														
MdDFR	0.4	0.6	0.0	0.2	0.2	0.1	-0.1	0.3	0.4	-0.1	0.4	0.2	0.4	-0.1	0.3	0.6	0.2	0.4	0.8	0.5	0.6	-0.2	0.7	0.2	0.7	0.0	0.4	0.4	1.0													
MdFLS	0.1	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.1	0.0	0.2	0.2	0.2	0.0	0.1	0.0	0.0	0.0	0.2	0.3	0.1	0.2	0.1	0.1	0.1	1.0												
MdANS	0.5	0.6	-0.2	-0.1	0.1	-0.1	-0.2	0.7	0.3	-0.1	0.5	0.1	0.5	-0.1	0.3	0.7	0.1	0.7	0.8	0.0	0.8	-0.3	0.9	-0.1	0.6	-0.1	0.2	0.8	0.7	0.3	1.0											
MdLAR1-1st pair	0.1	0.0	-0.4	-0.2	0.0	-0.2	-0.1	0.5	0.0	-0.2	0.0	0.0	0.4	0.0	0.0	0.1	0.0	0.2	0.2	-0.2	0.3	-0.2	0.2	-0.2	0.1	0.0	0.0	0.3	0.1	0.0	0.3	1.0										
MdLAR1-2nd Pair	-0.1	-0.1	-0.3	-0.1	-0.1	-0.2	-0.2	0.3	0.0	-0.2	-0.1	-0.1	0.2	-0.1	-0.1	0.0	0.0	0.0	0.1	0.0	0.2	0.1	0.0	-0.2	0.0	-0.2	-0.1	0.1	0.1	-0.1	0.1	0.7	1.0									
MdLAR2	0.2	0.2	-0.3	-0.2	0.0	-0.1	-0.1	0.5	-0.1	-0.1	0.2	0.0	0.2	-0.1	-0.1	0.2	0.0	0.7	0.7	0.0	0.4	-0.2	0.7	0.0	0.3	0.0	0.3	0.8	0.5	0.1	0.7	0.3	0.1	1.0								
MdANR	0.5	0.6	-0.1	0.0	0.1	0.0	-0.1	0.6	0.2	-0.1	0.5	0.1	0.3	-0.2	0.4	0.7	0.1	0.6	0.8	0.2	0.7	-0.2	0.9	0.0	0.5	-0.1	0.1	0.6	0.7	0.2	0.8	0.2	0.0	0.4	1.0							
MdC3Hchr8	0.0	0.3	0.4	0.4	0.2	0.2	0.0	0.0	0.5	0.0	0.0	0.3	0.3	0.0	0.2	0.4	0.3	0.0	0.3	0.5	0.4	0.2	0.2	0.3	0.6	0.2	0.2	0.1	0.5	0.0	0.2	0.1	0.2	0.1	0.2	1.0						
Metabolite A	0.1	-0.1	-0.1	-0.1	-0.1	-0.2	-0.2	0.3	0.0	-0.3	-0.2	-0.1	0.1	-0.1	-0.1	-0.1	0.0	-0.1	0.1	0.1	0.0	0.1	0.1	-0.2	-0.1	-0.1	-0.1	0.0	0.1	-0.1	0.1	0.4	0.6	0.1	0.0	0.0	1.0					
Metabolite B	**0.0**	**-0.2**	**-0.2**	**-0.1**	**-0.2**	**-0.2**	**-0.3**	**0.1**	**0.0**	**-0.2**	**-0.2**	**-0.2**	**0.0**	**-0.1**	**-0.2**	**-0.2**	**0.0**	**-0.1**	**0.1**	**0.2**	**0.0**	**0.0**	**0.1**	**-0.2**	**-0.1**	**-0.2**	**-0.2**	**0.1**	**0.1**	**-0.1**	**0.0**	**0.5**	**0.6**	**0.1**	**0.0**	**0.0**	0.8	1.0				
Metabolite C	**0.0**	**-0.1**	**-0.3**	**-0.2**	**-0.2**	**-0.3**	**-0.3**	**0.3**	**-0.1**	**-0.3**	**-0.1**	**-0.2**	**0.1**	**-0.1**	**-0.2**	**-0.1**	**-0.1**	**0.0**	**0.1**	**0.1**	**0.1**	**-0.1**	**0.1**	**-0.2**	**0.0**	**-0.2**	**-0.1**	**0.1**	**0.1**	**-0.1**	**0.1**	**0.6**	**0.7**	**0.2**	**0.0**	**0.0**	**0.8**	**0.8**	1.0			
Metabolite D	**-0.1**	**-0.2**	**-0.3**	**-0.2**	**-0.3**	**-0.2**	**-0.2**	**0.1**	**-0.2**	**-0.2**	**-0.2**	**-0.2**	**-0.1**	**0.0**	**-0.2**	**-0.2**	**-0.2**	**0.0**	**0.1**	**-0.1**	**0.0**	**-0.2**	**0.0**	**-0.1**	**0.0**	**0.0**	**-0.2**	**0.1**	**0.0**	**-0.1**	**0.0**	**0.5**	**0.5**	**0.1**	**0.0**	**0.0**	**0.3**	**0.6**	**0.7**	1.0		
Metabolite E	**-0.1**	**-0.2**	**-0.4**	**-0.2**	**-0.3**	**-0.3**	**-0.1**	**0.2**	**-0.3**	**-0.2**	**-0.1**	**-0.2**	**0.0**	**0.0**	**-0.3**	**-0.2**	**-0.2**	**0.0**	**0.1**	**-0.1**	**0.0**	**-0.2**	**0.0**	**-0.2**	**0.0**	**0.0**	**-0.2**	**0.1**	**0.0**	**-0.1**	**0.1**	**0.6**	**0.6**	**0.2**	**0.0**	**0.0**	**0.4**	**0.6**	**0.8**	**1.0**	1.0	
Metabolite F	**0.0**	**-0.1**	**-0.4**	**-0.2**	**-0.3**	**-0.3**	**-0.1**	**0.3**	**-0.2**	**-0.1**	**-0.1**	**-0.2**	**0.1**	**-0.1**	**-0.3**	**-0.1**	**-0.2**	**0.0**	**0.1**	**-0.2**	**0.1**	**-0.1**	**0.1**	**-0.1**	**-0.1**	**0.0**	**-0.1**	**0.1**	**0.1**	**-0.1**	**0.1**	**0.7**	**0.7**	**0.2**	**0.0**	**0.0**	**0.5**	**0.6**	**0.8**	**0.8**	**0.9**	1.0

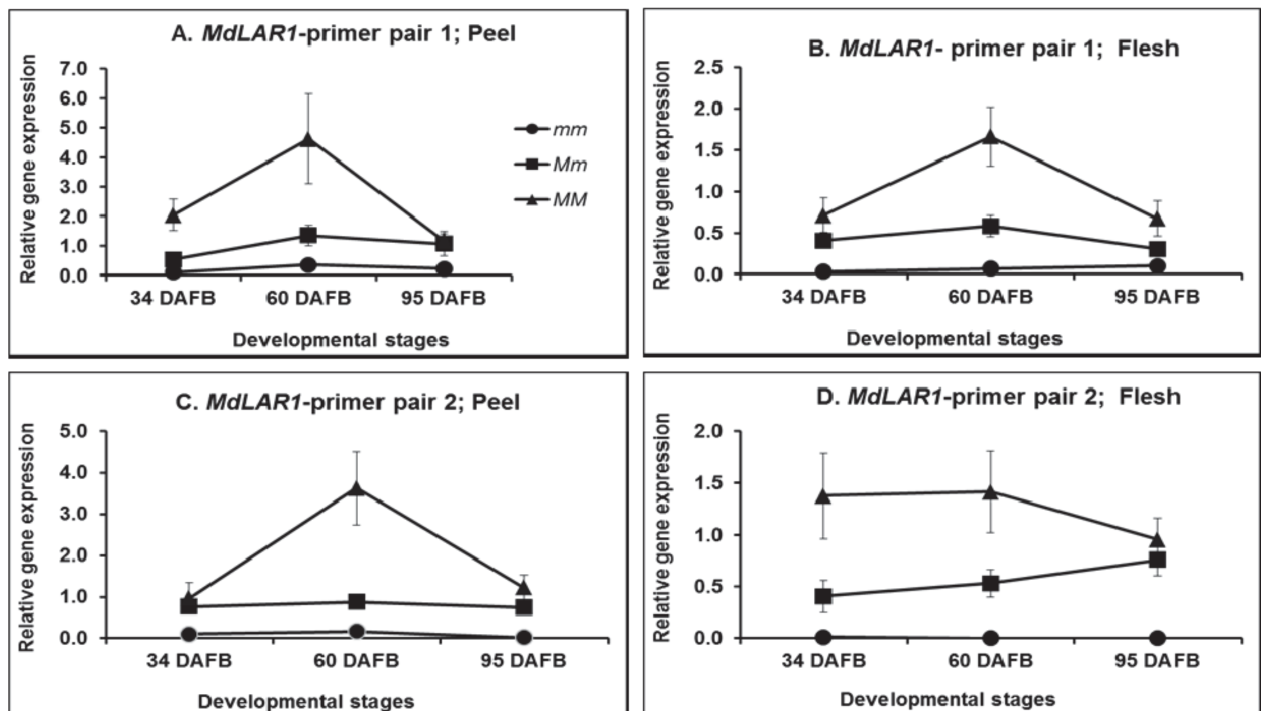


Fig. 5.2 Relative gene expression of the structural gene *MdLAR1*. Figs. 5.2A and 5.2B are the results with primer pair 1for *MdLAR1* in peel and flesh and Fig. 5.2C and 5.2D are the results for primer pair 2 of *MdLAR1* gene in peel and flesh respectively. The genotype classes are given in legend as *mm* (homozygous recessive), *Mm* (heterozygous dominant) and *MM* (homozygous dominant). The three different developmental stages are given on the horizontal axis as 34 Days After Full Bloom (DAFB), 60 DAFB and 95 DAFB.

3.2 Expression profiles of transcription factor genes at the mQTL hotspot on LG16

There was no clear correlation for any of the candidate transcription factor genes with the procyanidin dimer II content in flesh (Table 5.3). However, some transcription factor genes showed some correlation with other structural genes in the pathway (Table 5.3). When the expression level of these genes was compared with metabolite procyanidin dimer II content, we could not see any positive correlation. This means that the transcription factor genes found in the mQTL hotspot are not responsible for the mQTL hotspot, although they may regulate the expression levels of several structural genes of the phenylpropanoid pathway. This was observed in both peel and flesh. There was no differential expression pattern observed for these transcription factor genes for the low and high metabolite content in the different genotype classes (data not shown).

3.3 Expression profiles of transcription factor genes located outside the mQTL hotspot on LG16

No clear correlation was found between the expression profile of any of the putative transcription factor genes, which were not in the mQTL hotspot but were located on either the same LG16 or somewhere else in the genome, and the tested structural genes of the phenylpropanoid pathway (Table 5.3 and Table S5.1). However, some correlations were observed between some transcription factor genes (Table 5.3). For example *MYB9* and *MYB11A* showed strong correlations with several structural genes. None of these transcription factor genes showed correlation with *MdLAR1* gene. Moreover, the expression level of these genes was not correlated with the procyanidin dimer II content. This clearly indicated that none of these transcription factor genes could explain the mQTL hotspot. This was observed in both peel and flesh tissues.

This shows that neither the transcription factor genes at mQTL hotspot nor outside the mQTL hotspot were involved in the regulation of the structural genes for metabolites in the mQTL hotspot on LG16.

3.4 Expression level of *MdLAR1* and procyanidin dimer II content

As explained in table 5.3, the expression level of *MdLAR1* was positively correlated with procyanidin dimer II content. This increase was 0.6 ions counts per second from *mm* to *MM* on ^{10}log scale, which is equivalent to a 4.0-fold increase on linear scale (Fig. 5.3).

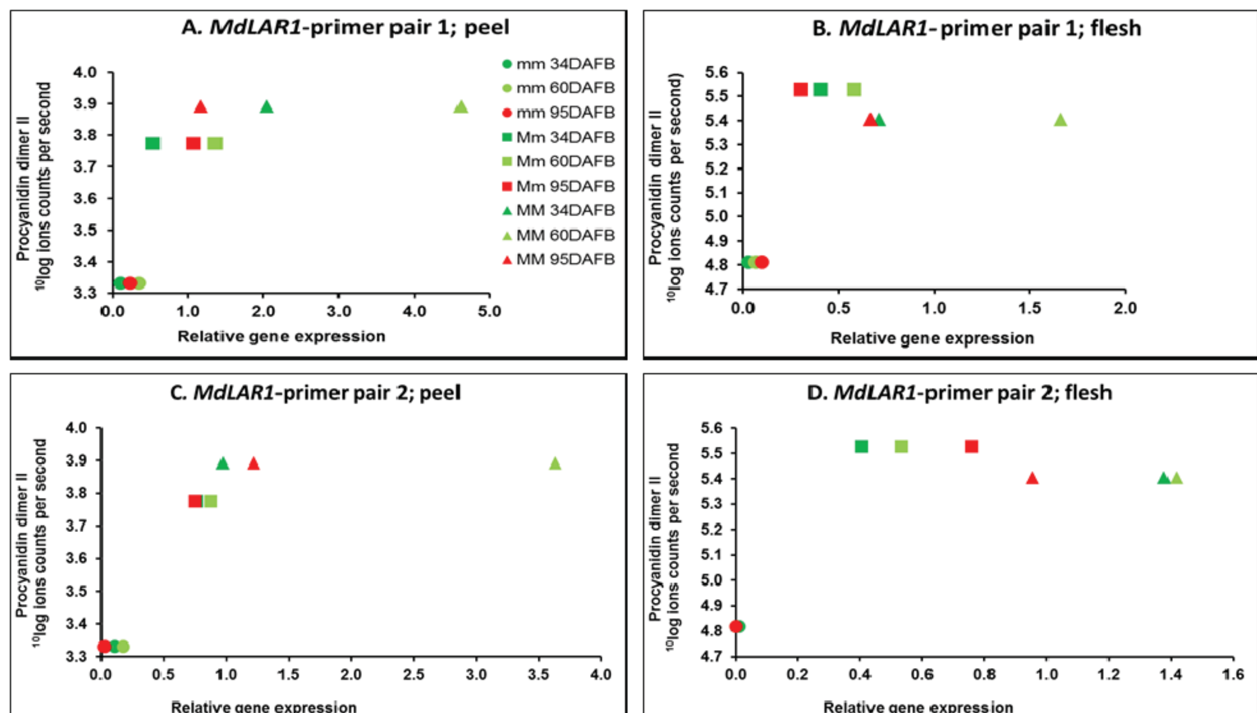


Fig. 5.3 Correlations of relative gene expression of *MdLAR1* and the content of procyanidin dimer II (ions counts per second) at three different developmental stages (34 DAFB, 60 DAFB, 95 DAFB) and in three genotype classes (*mm*, *Mm*, *MM*). These genotype classes are explained in Fig. 5.2. The procyanidin dimer II content was measured at only full mature stage of apple fruits. Figs. 5.3A and 5.3B are the results with primer pair 1 for *MdLAR1* in peel and flesh and Figs. 5.3C and 5.3D are the results for primer pair 2 of *MdLAR1* gene in peel and flesh respectively.

4. Discussion

In Chapter 4, we reported on mapping of phenolic compounds in ripe fruits of a segregating population derived from the cross between 'Prima' and 'Fiesta'. There appeared to be a strong hotspot of mQTLs at the top of LG16. Annotation of the metabolites showed that the compounds that mapped on the LG16 hotspot belong to the phenylpropanoid pathway of secondary metabolites (adapted Fig. 5.3 from chapter 4).

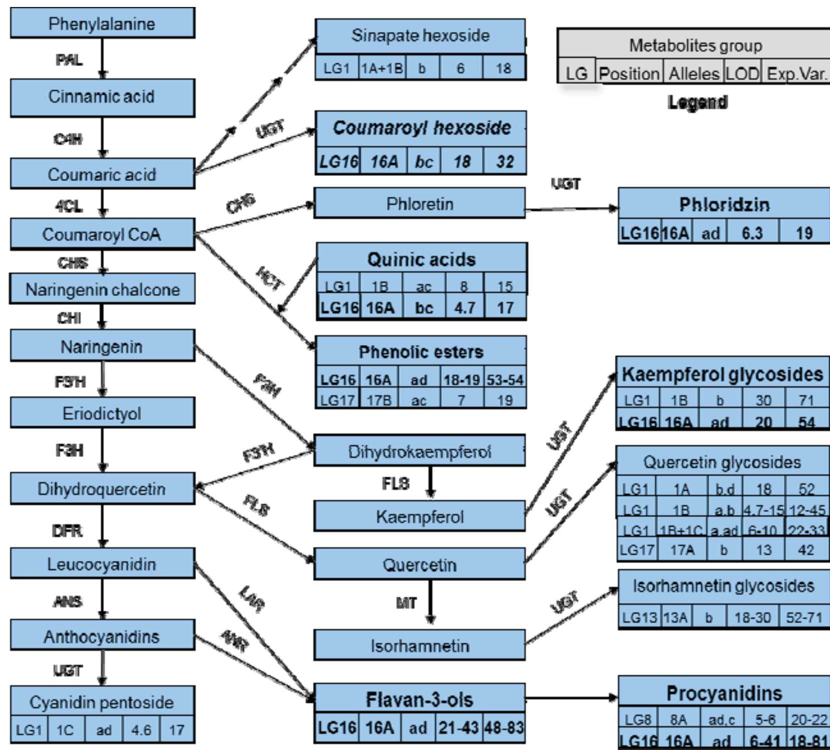


Fig. 5.4 Mapping results of phenolic compounds in the mature apple fruits. The metabolites that mapped at the mQTL hotspot on LG16 are shown in bold. The mQTL which was found negatively correlated with other mQTLs is in bold italic. The LOD value, % explained variance and favorable allele(s) for increased content of the metabolite are shown in the legend for each mQTL (Fig. adapted from Chapter 4). The alleles 'a, b' come from parent 'Prima' and 'c, d' are from parent 'Fiesta'.

We wanted to detect the underlying gene(s) in this mQTL hotspot. Therefore, in the present research, gene expression studies in selected genotypes for the candidate genes in the mQTL region were performed. In addition, structural genes of the phenylpropanoid pathway and putative transcription factor genes that are candidates for regulating the phenylpropanoid pathway and located elsewhere were also evaluated as mentioned in Materials and Methods section in detail.

4.1 *MdLAR1* seems to be the only gene which can explain the mQTL hotspot on LG16

As shown in Table 5.3, *MdLAR1* was the only gene for which the expression was clearly correlated with the metabolite content. None of the other genes showed a clear correlation with procyanidin dimer II content. Moreover, Fig. 5.2 shows clearly that the expression of *MdLAR1* was low for the genotypes that had inherited the recessive alleles (*mm*), and had low content of the representative metabolite procyanidin dimer II. The progeny that had inherited one or two dominant alleles (*Mm, MM*) had higher expression levels of *MdLAR1* and higher content of procyanidin dimer II (Fig. 5.3) and this pattern was observed both in peel and in flesh. This was not the case for any of the other studied genes, which indicates that *MdLAR1* was responsible for the hotspot of mQTLs on LG16. Further, it shows that *MdLAR1* exerted its influence by means of its expression level. Most likely the recessive alleles of *MdLAR1* did not lose their ability to increase the metabolite content via a mutation that affected the *MdLAR1* gene function, but lost their ability to increase the metabolite content because of a mutation that led to a low transcript level of the recessive *MdLAR1* alleles. This mutation may be in the promoter region of *MdLAR1*, or another region of the gene that affects the expression level of this gene. The sequences of *MdLAR1* in ‘Prima’ x ‘Fiesta’ also showed good alignment with sequences from cv. ‘Golden Delicious’ on which the primers for q RT-PCR were designed.

Based on the obtained results in this study, it is clear that *MdLAR1* is the probable responsible gene for controlling the mQTL hotspot for phenolic compounds on LG16 of apple.

4.2 More upstream mQTLs in the pathway might be explained by a feed-back mechanism

The *MdLAR1* gene is located rather downstream in the phenylpropanoid pathway (Fig. 5.4). However we found several mQTLs related to upstream metabolites in this pathway in the same mQTL hotspot on LG16. This may indicate that more upstream

genes could play a role in causing the mQTL hotspot but this was not indicated by the expression data. An alternative explanation is a feedback mechanism determined by the low expression of *MdLAR1* in the recessive plant.

In literature it has been reported that flavonoid pathway is often regulated at the *4-coumarate-CoA ligase* (4CL) step (Muir *et al.*, 2001; Verhoeyen *et al.*, 2002; Voo Kui *et al.*, 1995). This fits with our results and could explain the mQTLs found for metabolites upstream of the *MdLAR1* step such as for phloridzin and kaempferol glycoside. These compounds are upstream of the 4CL step and were also found in mQTL hotspot on LG16. This would suggest regulation at enzyme activity level and not at gene expression level (Table 5.3). The coumaroyl hexoside and quinic acid mQTL which has negative correlation with other mQTLs found on LG16 could be explained by the *MdLAR1* activity. The strong activity of *MdLAR1* could cause a strong flow towards the production of procyanidins causing a decrease in the coumaroyl hexoside content. There are also reports on feedback inhibition in tomato peel flavonoid pathway upstream of the *chalcone isomerase* step (Muir *et al.*, 2001; Verhoeyen *et al.*, 2002) as well as direct *in vitro* evidence for inhibition of 4CL by naringenin chalcone (Voo Kui *et al.*, 1995). So probably this negative feedback mechanism also plays a role in apple. However, this has been reported for only one compound so far i.e. naringenin and to prove this, further research is needed.

4.3 Metabolite content at the ripe stage may be a result of expression of genes at earlier stages

Gene expression can be studied at different fruit developmental stages. Metabolite genes may be expressed earlier in the fruit development and give their final product during later stages. Therefore, the fruit developmental stage is critical for the expression of such genes. Janssen et al. (2008) made gene expression studies for fruit development at eight time points using microarray. They found that gene expression in apple fruit is depending on developmental stages (Janssen *et al.*, 2008). In the present study gene expression was performed at three different developmental stages i.e. 34 DAFB, 60 DAFB and 95 DAFB (Fig. 5.1). Different expression levels were found at different stages. The mid stage i.e. 60 DAFB showed increased expression of the *MdLAR1* gene. This indicated that perhaps the metabolites were produced more at certain stage. However, in the present study metabolites were measured only at mature stage and therefore it is apparently difficult to prove about this hypothesis.

4.4 Metabolic engineering

Two classes of genes determine the flavonoid pathway of secondary metabolites, i.e. structural genes which encode enzymes that directly participate in the formation of flavonoids such as *PAL*, *DFR,* and regulatory genes that control the expression of these structural genes (Szankowski *et al.*, 2009). Harrigan et al (2007) also found that the genetic diversity in metabolites as intermediate and end products are controlled by both structural and transcription factor genes. Most known regulatory genes involved in the regulation of genes of the flavonoid pathway are belonging to *MYB* and basic helix-loop-helix (*bHLH*) transcription factor families (Szankowski *et al.*, 2009). In our case, we have shown that a structural gene is responsible for the mQTL hotspot, rather than a regulatory gene.

Metabolic engineering is an interesting and growing field and several groups in the world are performing research in this area in apple. Using metabolic engineering many genes responsible for regulating important metabolites have been cloned. For example, recently, three genes, designated as *MdF3'H1*, *MdF3'H2*-1, and *MdF3'H2*-2 were identified from cv. 'GoldRush' based on EST database assembly and BAC library screening (Han and Korban, 2009). In another study, using a functional genomics approach, a novel *UGT* from apple belonging to the *UGT88* family was identified and characterized. It was established that the *MdPGT1* enzyme utilizes the dihydrochalcone phloretin as its primary substrate (Jugde *et al.*, 2008). Relative expression of *MdPGT1* was assessed in apple root, leaf, flower and fruit by quantitative PCR experiments which were repeated twice (Jugde *et al.*, 2008). In another study, three genes encoding enzymes involved in condensed tannins synthesis from cDNA of peel of the red apple variety 'Cripps Red', two *LAR* and an *ANS* gene, were cloned. Steady-state transcript levels of the CT genes and the other structural genes of the flavonoids pathway were measured by Real Time PCR and correlated with flavonoid accumulation in the peel throughout early fruit growth and during ripening (Takos *et al.*, 2006).

Genes involved in anthocyanin biosynthetic pathway, which is a sub-branch in the flavonoid pathway were isolated from apple fruit and characterized to understand the regulation of the genes during fruit development (Kim *et al.*, 2003). Espley et al (2007) isolated an apple transcription factor gene, *MdMYB10,* responsible for skin colour of apple fruits. A mutant allele was found in the apple cv. Red Field that results in purple-stained skin and flesh of fruits. We reported the mutant *MdMYB10* allele from cv. Red Field as a selectable marker gene for plant transformation, Chapter 7 (Kortstee *et al.*, 2011). In pear, a close relative to apple, Fischer et al (2007) have cloned cDNAs of main enzymes such as *PAL*, *CHS*, *CHI*, *FLS*, *DFR*, *LAR1*, *LAR2*, *ANS*, *ANR*, and *flavonoid* 7-O-*glucosyltransferase* (*F7GT*),

which have been isolated via homology with apple sequences to elucidate gene functions, gene copy numbers, and gene relationships within the Maloideae.

Next step could be genetic engineering with these genes. All these findings indicate that in coming years more research will be focused on gene cloning and gene characterization of apple and other important fruits for potential health beneficial compounds in different biochemical pathways such as flavonoid pathway.

4.5 Marker assisted breeding and cisgenesis

The dominant allele of the *MdLAR1* gene causing increased content of metabolites which are potentially health beneficial could be used in marker assisted selection of current apple breeding programs. This selection could be made at seedling stage. This would reduce the production costs for the breeders by discarding the undesired seedlings at earlier stage of growth. Another possibility is to clone the dominant allele or alleles for engineering increased content of metabolite(s) into existing apple cultivars by different transformation technologies including cisgenesis. In this case cisgenesis is defined as 'the genetic modification of a recipient plant with the natural dominant allele of *MdLAR1* from the apple variety itself' (Schouten *et al.*, 2006a, b).

Chapter 6

Differences in acidity of mature apples are mainly caused by a malic acid transporter gene on LG16

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Submitted

Abstract

Apple (*Malus* x *domestica* Borkh) is an important fruit crop of temperate regions. Acidity has profound effects on the organoleptic quality of apples. Malic acid is the predominant organic acid associated with the pH in apples. Its concentration decreases during development and maturation of the fruit and varies strongly between cultivars. It is synthesized in the cytosol, and transported into the vacuoles, where it can be accumulated. In view of the vital role of malic acid in the citric acid cycle, it is unlikely that apples of low malic acid content, have a defect in a structural gene that would hamper the production of malic acid. Furthermore, the cytosol where most enzymatic activities occur should have a rather constant pH. In view of that, it is more likely that differences in malic acid content and pH in apple are caused by differences in accumulation of malic acid in the vacuole. The pH of mature apples was genetically mapped on LG16 in the segregating F1 population from the cross 'Prima' x 'Fiesta'. A strong negative correlation was found between malic acid content and pH of apples, which confirmed the strong impact of malic acid on pH. The genetic position of the pH gene on LG16 in the segregating population from 'Prima' x 'Fiesta' was determined more precisely by adding more markers and applying graphical genotyping. In the obtained genetic window, two candidate genes, i.e. *MdALMT1* and *MdALMT2* were detected, using the published genome sequence information of cv. 'Golden Delicious'. Putatively, these two candidate genes are malic acid transporters. The relative expression of the candidate gene *MdALMT1* showed no correlation to pH or malic acid content, whereas the expression of *MdALMT2* had a significant positive correlation with the malic acid content and a negative correlation with pH. The combination of the genetic position, annotation and the expression profile indicates that the malic acid transporter gene *MdALMT2* was responsible for the clear differences in malic acid content and pH in mature apple fruits of the segregating F1 population. This malic acid transporter gene is located on the top of LG16. The genetic inheritance of at least one dominant allele of this gene sufficed for an increased expression level that led to a three-fold increase of the malic acid concentration and a reduction of the pH from 4 to 3 in ripe apples, compared to the presence of the recessive, lowly expressed allele only. To our knowledge, this is the first time that the genetic segregation of the pH in apples is assigned to a specific gene. Further, this gene has not been reported yet in conjunction to pH of apple or other fruits.

1. Introduction

Apple (*Malus* x *domestica* Borkh) is an important fruit crop of temperate regions. Its consumption is global, available throughout the year, and represents a major source of dietary antioxidants (Wojdyło *et al.*, 2008). The major part of the production is consumed as fresh fruits, while a lesser part is processed into juices, concentrates, and purees (Wojdyło *et al.*, 2008).

Acidity has profound effects on the perception of apple fruit quality (Lobit *et al.*, 2006). In organoleptic evaluations of apple juice, sweet taste was found to be strongly correlated with low acid content. Sweetness appeared to be almost exclusively determined by the pH while the sugar content was found to have a minor role (Visser *et al.*, 1968). A proper balance between sugar and acid content is therefore important for a desired apple variety (Brown and Harvey, 1971). From a genetic point of view, however, the sweetness (sugar content) and acidity are inherited independently (Brown and Harvey, 1971). Sweetness is quantitatively inherited while acidity is mainly caused by a single gene (Brown and Harvey, 1971).

Malic acid is the predominant acid in many fruits, especially in apples (Yao *et al.*, 2009). It was first isolated in 1785 by Carl Wilhelm Scheele from apple juice (Jensen, 2007). Malic acid contributes to the sourness of fruits, and is the source of extreme tartness in confectionery. When added to food products, it is denoted by E number E296 (http://www.food-info.net/uk/e/e296.htm). Via acidity, malate content of apple is an important determinant for fruit quality, and has enormous effects on the taste and flavour (Maliepaard *et al.*, 1998). Among the eleven organic acids in apple pulp and five in the whole fruit, malic acid was the predominant one (Hulme *et al.*, 1971). Malic acid accumulated up to 100 mmol g^{-1} fresh weight in young fruit, and gradually decreased with fruit ripening (Ulrich R, 1970). Zhang et al. (2010) found that malic acid was the major organic acid in the flesh of mature 'Honeycrisp' apples**.** Yamaki (1984) isolated vacuoles from apple fruit flesh, and detected that more than 90% of the malic acid was located in the vacuole.

The salts and esters of malic acid are known as malates. Malate is the predominant form of malic acid in many fresh fruits (Yao *et al.*, 2011). Malate works together with other organic acids, soluble sugars and aromas to contribute to the overall organoleptic quality of fruits (Yao *et al.*, 2011). Malic acid is also known as carboxylic diacid. It has two stereoisomeric forms (L- and D-enantiomers), though only the L-isomer exists naturally (Yamamoto *et al.*, 2001).

Malic acid, in the form of its anion malate, occurs in all metabolizing cells, as a key intermediate in the major energy-producing biochemical pathway, known as the citric acid or Krebs cycle that occurs in the mitochondria. Also it is part of the cytosolic pyruvate metabolism as an intermediate between oxaloacetate and acetyl CoA. Both the citric acid cycle and the pyruvate metabolism have extensively been

studied, and the enzymes involved in metabolism of malate are well known. For example *PEPC* is considered to be an important enzyme involved in fruit malic acid synthesis, while *cyME* is indicated as the key enzyme in malic acid degradation (Yao *et al.*, 2009; Berüter, 2004). Yao et al. (2011) cloned the apple gene *MdcyMDH,* encoding the cyMDH enzyme that catalyzes the reaction from oxalacetic acid to malate. The expression level of this gene was positively correlated with malate dehydrogenase activity throughout fruit development, but not with malate content (Yao *et al.*, 2011). The activity of the citric acid cycle is regulated based on requirements of energy, rather than on requirements of fruit acidity. In fact, acidity of apples is considered to be determined by storage of malate in the vacuole (Ulrich R, 1970).

The vacuole is by far the largest organelle in fruits and can occupy more than 90 % of the cell volume (Shiratake and Martinoia, 2007). This is because it stores sugars, organic acids and secondary metabolites in high concentration which are required for the fruit quality (Shiratake and Martinoia, 2007). Transport of metabolites into the vacuole is often an active, energy-requiring process, in which many different transporters are involved (Shiratake and Martinoia, 2007).

Vacuolar transport of malic acid in *Arabidopsis* is controlled by a transporter (Emmerlich *et al.*, 2003) and by a channel (Kovermann *et al.*, 2007). The channel is more passive, and can be opened or closed, but there is no energy needed to transport the malic acid, while a transporter consumes energy when pumping malic acid into the vacuole. A detailed biochemical analysis of two genetic variants of cv. “Usterapfel” revealed that vacuolar transport of malic acid plays an important role in malic acid fruit content (Beruter, 2004). Therefore it is likely that malic acid transporters, homologous to those found in *Arabidopsis*, play a role in differences in apple acidity.

In previous work, a segregating F1 population from the cross of apple cultivars ‘Prima’ and ‘Fiesta’ was used to map acidity on a locus of linkage group 16. Recently, the genome sequence of the apple cv. ‘Golden Delicious’ has been published. Here information of both genetic and genomic resources has been used for detection of a novel putative vacuolar malic acid transporter *MdALMT2*, determining malic acid levels and acidity in apple fruit.

2. Materials and Methods

2.1 Genetic mapping of the pH locus

Maliepaard et al (1998) were the first to construct genetic linkage maps for apple, covering all 17 chromosomes. They used a segregating F1 population of 164 genotypes from the cross ‘Prima’ x ‘Fiesta’. They measured the pH of mature fruits

of these progenies using bromocresol and pH indicator paper (Maliepaard *et al.*, 1998). The pH appeared to segregate as a monogenic trait in a 3:1 fashion, and both parents appeared to be heterozygous for the dominant trait for high acidity (low pH). We improved the genetic linkage maps of this population by adding DArT and other markers as described by Schouten et al. (Schouten *et al.*, 2011), and combined the pH data from Maliepaard with the improved genetic linkage maps, and mapped the pH again on LG16, using JoinMap® 4.0 (Van Ooijn, 2009). For more-detailed mapping of the pH locus, additional simple sequence repeats (SSR) markers on LG16, reported previously, were run and mapped (Chapter 4).

pH was scored as a dominant marker. Both parents were heterozygous for the pH locus. Progeny that had a low pH had received one dominant allele from 'Prima' or one dominant allele from 'Fiesta', or the dominant alleles from both parents. From the pH data only, it could not be judges which dominant allele(s) were inherited. Due to this uncertainty, only progeny with high pH were used for mapping, as for these progeny plants it was clear that they inherited from both parents the recessive alleles. This reduced the number of progeny genotypes that could be used for mapping, and therewith this reduced the mapping accuracy. In spite of this, a genetic window for the pH locus could be drawn on LG16, using graphical genotyping. The genome sequence information of cv. 'Golden Delicious' (Velasco *et al.*, 2010) was used to find candidate genes for the pH in this genetic window.

2.2 Plant material for measurement of malic acid content and expression of candidate genes

Progeny genotypes of the F1 segregating population from the 'Prima' x 'Fiesta' cross were selected based on their genotype at the pH locus. The genotypes selected for sampling of fruits are shown in Table S6.1. Each genotype was represented by two trees. These trees were located in the experimental station, Randwijk, the Netherlands. In addition, both parents ('Prima', 'Fiesta') of the population were included.

The progeny and parents were at full bloom from 26th to 30th April, 2010. Fruits were sampled at three developmental stages, i.e. 34 days after full bloom (DAFB), 60 DAFB and 95 DAFB. Per tree, eight fruits were sampled, giving 16 fruits per genotype. Fruits from different sides of each individual tree were harvested to level out the effects of the environment such as sunlight during fruit development. Fruits were peeled off, cut into small pieces and frozen in liquid nitrogen just after harvesting according to method used before (Chapter 4). Care was taken to exclude the seeds of the fruits. The knife was cleaned with water and dried up with tissue paper before every next fruit. Peel and flesh were separately put into

polythene bags, then shock-frozen in liquid nitrogen and stored on dry ice. Once all fruits were harvested, they were transferred to the laboratory and stored at -80 °C.

The sizes (diameter) of the individual fruits were measured at each developmental stage using an electronic digital caliper, model VWRI819-0012 of Control Company. The average size at the 34 DAFB was 24 mm, at 60 DAFB 40 mm and at 95 DAFB 62 mm (Table S6.1). The harvested fruit material was ground in the same way as explained by Chapter 4. This led to one sample per tree per tissue per developmental stage, and to two replicates (two trees) per genotype. The powder was collected in falcon tubes of 50 ml volume and stored immediately at -80°C in freezer before any further processing.

In addition, mature fruits were included for malic acid measurement. These fruits were harvested and stored in autumn 2008, as described in Chapter 4. For this mature stage, fruits from the two individual trees per genotype (four apples from each tree) were pooled before grinding to make one representative sample of eight apples per genotype. We used for expression studies fruits from 34, 60 and 95 DAFB. Malic acid was measured in fruits at 60 and 95 DAFB and at mature stage, but not at 34 DAFB as fruits at this stage were too small for malic acid measurement.

2.3 Measurement of malic acid content

Extracts of flesh samples were prepared from the ground powder according to a modified protocol by Lisec et al. (2006). First, 250 mg frozen apple powder was transferred to 2 ml cryo-cooled eppendorf tubes where 750 µl of methanol including 25 µl internal standard (ribitol 0.5mg/ml) was added. Samples were briefly vortexed, sonified for 30 minutes, and centrifuged for 10 min at 21,000 rpm. A 400 µl of the supernatant was transferred to a new eppendorf tube and 600 µl dH_2O and 300 µl chloroform were added. Thereafter, samples were again vortexed for 20 seconds and were centrifuged for 10 min at 21,000 rpm. 40 µl of the upper polar phase was then transferred to a 1.5 ml crimp neck glass vial containing a 0.1 ml micro-insert. The samples were dried overnight in a SpeedVac® concentrator (SPD121P, Thermo Scientific Savant, Asheville, USA). Samples were then capped under an Argon atmosphere.

The dried samples were derivatized on-line as described by Lisec et al. (2006). First, 12.5µl methoxyamine hydrochloride (20 mg/ml pyridine) was added to the samples and incubated for 30 min at 40 ºC with agitation. Then the samples were derivatized with 17.5µl MSTFA (N-methyl-*N*-trimethylsilyltrifluoroacetamide) for 60 min. An alkane mixture was added to determine retention indices of metabolites.

The derivatized samples were analyzed by a gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) system consisting of a Combi PAL

autosampler (CTC Analytics AG), an Optic 3 high performance injector (ATAS GL Int.) and an Agilent 6890 gas chromatograph (Agilent Technologies) coupled to a Pegasus III time-of-flight mass spectrometer (Leco Instruments). 2 µl of each probe was introduced into the injector at a split flow of 20 ml min-1, a transfer column flow of 1 ml min-1 was used. The chromatographic separation was performed using a ZB-5 capillary column (30 m x 0.32 mm x 0.25 µm, Phenomenex) including a 10m guardian column with helium as carrier gas at a column flow rate of 1 ml min^{-1}. The GC program started at 70 °C for 2 min, then rose by 10 °C min^{-1} to 310 °C and was maintained at this temperature for 5 min. The GC transfer line temperature was set to 270 °C. The column effluent was ionised by electron impact at 70eV. Mass spectra were acquired at 20 scans/sec within a mass range of m/z 50 – 600, at a source temperature of 200°C.

Raw data were processed by ChromaTOF software 2.0 (Leco instruments) and further by using MetAlign software (Lommen, 2009) to extract and align the mass signals (s/n≥ 3). Mass signals that were below s/n of 3 were randomized between 2.4 and 3 times the calculated noise value. Mass signals that were present in less than four samples were discarded. Malic acid and the internal standard ribitol were identified by comparing the retention time and the mass spectra with those of authentic reference standards (Sigma-Aldrich). The abundance of malic acid was normalised to ribitol based on m/z 133.

2.4 Expression analysis of the pH candidate genes

i) RNA isolation and cDNA synthesis

The expression profiles of the two candidate genes in the genetic window were compared for low pH progenies on the one side and high pH progenies on the other side. In addition expression profile of a third apple gene *Cytosolic malate dehydrogenase* (*MdcyMDH* GenBank Accession No. DQ221207) was also studied. This gene is involved in the synthesis of malate in cytosol of the plant cell (Yao *et al.*, 2011) and is located on LG17 of apple. For the expression analysis, total RNA was isolated from flesh of apple according to CTAB method (Asif *et al.*, 2000) of the genotypes mentioned in Table S6.1 at developmental stages of 34, 60 and 95 DAFB. Each RNA sample represented one developmental stage for one tree. The RNA quantity was measured on NanoDrop® spectrophotometer model ND-1000 from isogen life science scientific company. Further the RNA quality and quantity were evaluated by running 2µl of the RNA sample on 1.5% agarose gel. Single-strand complementary DNA (cDNA) was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's manual.

ii) Designing and testing of primers for quantitative RT-PCR (q RT-PCR)

For q RT-PCR, primers were designed for each candidate gene using the online available primer designing programme Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primer names, sequences and amplicon sizes are given in Table 6.1. Primers were tested using q RT-PCR with iQ SYBR® green super mix (Bio-rad). Two µl cDNA, ten times diluted of the original cDNA obtained with iScript™ cDNA Synthesis Kit (Bio-Rad), was used in the reaction. The PCR programme was as 95°C for 3 min, 95°C for 15 sec, 60°C for 1 min, 95°C for 10 sec. The PCR products were checked for quality by checking their clear single peak in the melting curve and a clear band of the expected amplicon size on 1.5% agarose gel.

iii) Performing q RT-PCR

The gene expression was measured for 216 cDNA samples. As we studied simultaneously the expression profiles of 42 genes related to the phenylpropanoid pathway for the same cDNA samples, we used the BioMark™ System from Fluidigm (http://www.fluidigm.com). Three 96.96 Dynamic Arrays of Integrated Fluidic Circuits were used, comprising 48 primer pairs in two replicates. The expression profiles of the genes related to the phenylpropanoid pathway will be reported elsewhere.

Three reference genes were included for normalisation of the expression of target genes. These three genes were *Actin* (Genbank accession *DT002474*), *MdUBQ* (Genbank accession *U74358*) and *mGAPDH* (Genbank accession *CN494000*). Eventually, only *GAPDH* (Genbank accession *CN494000*) was used as reference gene, based on overall performance. The Ct values for the candidate genes were normalised using this reference gene, giving ΔCt values.

A pooled sample containing cDNA from all the samples was used as reference sample. The expression of a gene in a target sample was compared with the expression of that gene in the pooled sample, giving ΔΔCt values. We named this as relative expression of the target genes.

A dilution series of the reference sample was also included which allowed calibration for each individual primer combination. The serial dilutions were as 1, $1/4^{th}$, $1/16^{th}$, $1/64^{th}$ and $1/256^{th}$ of the original sample concentration used.

iv) Analysis of q RT-PCR data

The q RT-PCR data were analyzed using the fluidigm® Real-Time PCR Analysis Software version 3 (http://www.fluidigm.com). Two main analyses were made, i.e. melting curve analysis and ΔΔCt values determination. Subsequent analyses were performed using Microsoft Office Excel 2010 and IBM SPSS statistics.

3. Results

3.1 The apple genome has 27 putative malic acid transporters

Alignment of amino acid sequence of the malic acid transporter *AtALMT1* from *Arabidopsis* to the protein sequences of apple (http://genomics.research.iasma.it/blast/blast.html) indicated the presence of 27 putative malic acid transporters in apple. We made a phylogenetic tree for these proteins, using the multiple sequence alignment program ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Although *AtALMT1* has some homology to the putative malic acid transporters in apple, it is not highly similar to a specific protein in apple (Fig. 6.1).

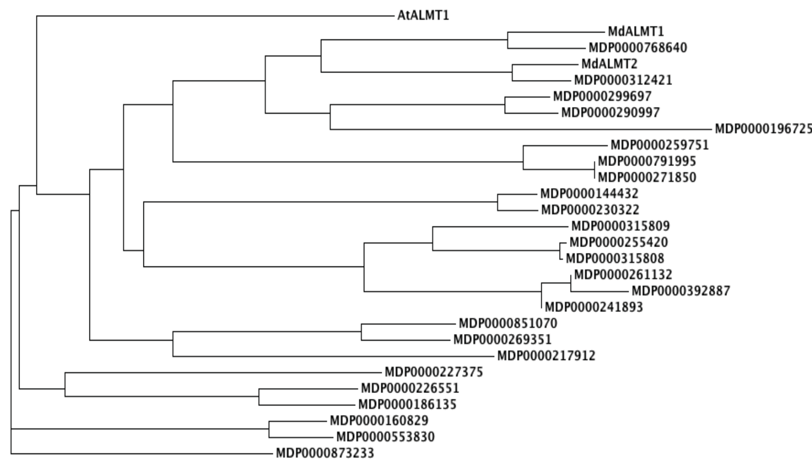

Fig. 6.1. Phylogenetic tree of the malic acid transporter protein *AtALMT1* in *Arabidopsis* (Hoekenga *et al.*, 2006) and 27 putative malic acid transporter proteins in apple. The two genes that reside at the genetic locus for pH of ripe apples were named as *MdALMT1* and *MdALMT2*.

3.2 pH locus on LG16

The pH of the mature apples of the F1 progeny from the ‘Prima’ x ‘Fiesta’ cross showed a clear 3: 1 segregation (Fig. 6.2).

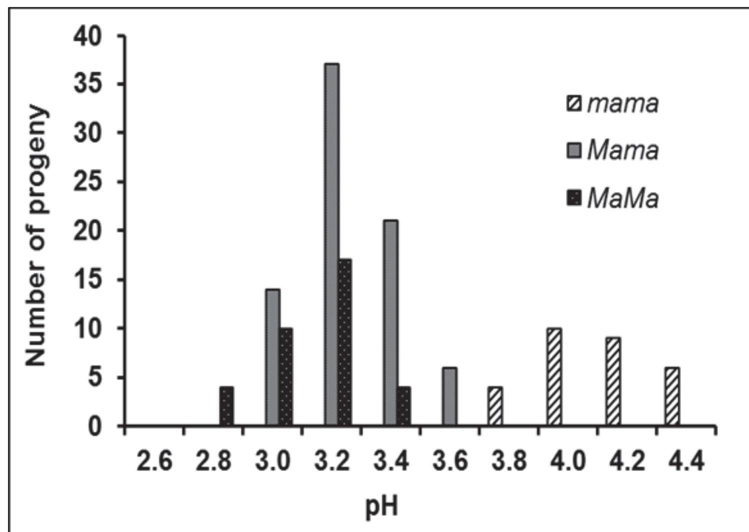

Fig. 6.2. Histogram of the F1 segregating population of 'Prima' x 'Fiesta' cross, sorted to pH of the ripe apple fruits. By means of genetic markers at the top of linkage group 16 (LG16), the progeny has been grouped into three classes, i.e. progeny that is homozygous for the dominant allele for low pH (*MaMa*), heterozygous (*Mama*), and homozygous recessive as it did not inherit the dominant allele from either of the two parents (*mama*). Both parents are heterozygous. The three genotype classes show a 3:1 Mendelian segregation.

The pH could be mapped as phenotypic trait genetic marker. Maliepaard and colleagues mapped this pH genetically on LG16 of apple (Maliepaard *et al.*, 1998). We mapped it more precisely with JoinMap® 4.0 (Van Ooijn, 2009) by including additional markers. The results of this mapping are shown in Fig. 6.3.

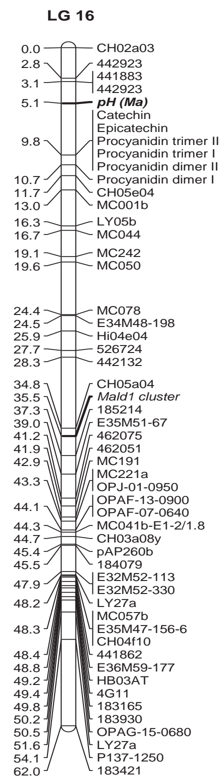

Fig. 6.3. Integrated genetic linkage map of LG16 of apple, based on segregating markers in the F1 progeny from the cross 'Prima' x 'Fiesta'. The pH locus is shown in bold. This Figure is adapted from Chapter 4.

Based on the genetic markers on LG16, the progeny could be divided into three genetic groups: One group of progeny that had inherited from each parent a copy of the dominant allele for malic acid, giving homozygous dominant genotypes (*MaMa*). The second group inherited one copy of the dominant allele from one parent, and one copy of the recessive allele from the other parent, leading to heterozygous progeny (*Mama*). The third group represents the homozygous recessive genotypes, lacking the dominant allele (*mama*). Fig. 6.2 shows clearly that the genotypes that had one or two copies of the dominant allele, had a lower pH, compared to the *mama* genotypes. Fig. 6.2 also shows that homozygous dominant progeny (*MaMa*) had on the average a slightly lower pH compared to the heterozygous progeny, but this difference was insufficient to distinguish the *MaMa*

genotypes from the *Mama* genotypes, using the pH values only. Using the graphical genotyping, the genetic window for the pH locus was constructed for both parents (Fig. 6.4).

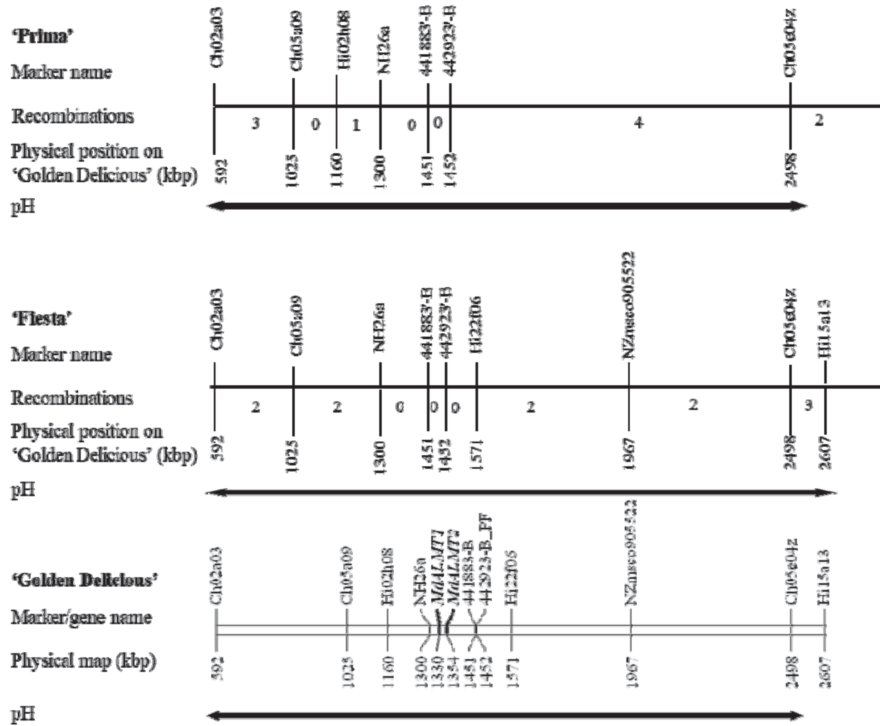

Fig. 6.4. Genetic window of the pH locus on LG16 for both heterozygous parents, and the physical distances between the markers on the sequenced genome of cv. 'Golden Delicious'. The two putative malic acid transporter genes are given in bold text. These genes reside in the middle of the genetic windows of both 'Prima' and 'Fiesta'.

3.3 Malic acid during apple fruit development

Malic acid was measured in apple flesh at three different developmental stages (60 and 95 DAFB, and at mature stage) using GC-TOF-MS. Malic acid was clearly detectable in the apple flesh samples. The level of malic acid at 60 and 95 DAFB was rather high as compared to mature stage (Fig. 6.5).

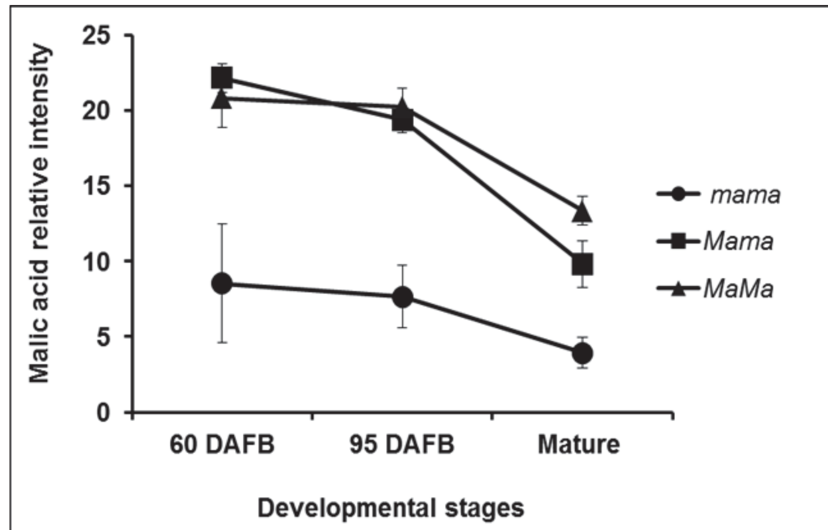

Fig. 6.5. Malic acid content of apples at three different developmental stages, i.e. 60 and 95 days after full bloom (DAFB) and at full maturity of the fruits. The three genotype classes (*MaMa, Mama, mama*) are explained at Fig. 6.2. The error bars refer to the standard deviations of the means, and are based on 8 to 20 measurements per genotype class per developmental stage.

This means that during maturation the acidity of the apples decreased. This is in line with the general observation that apples are sourer at immature stages as compared to the mature stage. The *mama* group, lacking the dominant allele for acidity, had lower levels of malic acid, as expected (Fig. 6.5). This agrees with results from others (Yao *et al.*, 2009; Zhang *et al.*, 2010; Sweetman *et al.*, 2009; Yao *et al.*, 2007) that malic acid is the main determinant for pH of apples. Apple genotypes that were sour at the ripe stage (*Mama* and *MaMa* genotypes) contained already more malic acid at the earlier developmental stages compared to the *mama* genotypes (Fig. 6.5). Apparently, the sourness of these sour genotypes was not the result of a slower breakdown of malic acid during ripening, but was a result of higher levels of malic acid during the whole period of fruit development.

3.4 Two putative malic acid transporters were detected in the genetic window of the pH locus on LG16

The genetic windows of the pH locus on LG16 were screened for candidate genes, using the sequence information of cv. 'Golden Delicious' (Velasco *et al.*, 2010).

Two candidate genes were detected that may be responsible for the segregation of the pH of the mature fruits. These two candidate genes are putatively annotated as

malic acid transporters in the cv. 'Golden Delicious' genome browse annotation (http://genomics.research.iasma.it/gb2/gbrowse/apple/), and are coded on this website as MDP0000244249 and MDP0000252114. In view of similarity of these malic acid transporters to the *Arabidopsis* malic acid transporter *AtALMT1*, we named these candidate genes as *MdALMT1* and *MdALMT2* (Fig. 6.4, Table 6.1).

Table 6.1 Names, putative functions and primer sequences of candidate genes used in q RT-PCR

Gene Name	Gene ID	Gene function	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)
MdAL MT1	MDP000 0244249*	Malic acid transporter	ACTCGCCAAGA AAGTGGAGA	GCATGTC CTCTGTT GAAGCA	185
MdAL MT2	MDP000 0252114*	Malic acid transporter	GACTTGGGCTTC AACAGCTC	TTTTCGA GGATCCG AATGAC	150
MdcyM DH	DQ2212 07#	Cytosolic malate dehydrogen ase	CTTAGATGCTCT TGGCCTTTCC	TCAGCTG CTTTTCT CACACAT TG	73

* Derived from cv. 'Golden Delicious' gbrowse
# Gene bank accession number

The *Arabidopsis* gene *AtALMT1* is involved in malate transport and aluminium tolerance (Kobayashi *et al.*, 2007). No structural genes for malic acid synthesis or degradation were detected within the genetic windows.

3.5 Only *MdALMT2* showed positive effects on malic acid content

Fig. 6.6 shows the relationships between the expression of the two putative malic acid transporter genes at the pH locus, i.e. *MdALMT1* and *MdALMT2* on one side, and malic acid content on the other side. No correlation could be found between the gene expression and malic acid content for *MdALMT1* (Fig. 6.6; $r = -0.01$; $P = 0.94$). However, the relative expression of *MdALMT2* showed a positive correlation with the malic acid content (Fig. 6.6; $r = 0.42$; $P = 0.004$). The *mama* genotypes that lack the dominant allele, showed a low expression of *MdALMT2*, and also a low level of malic acid (Fig. 6.6).

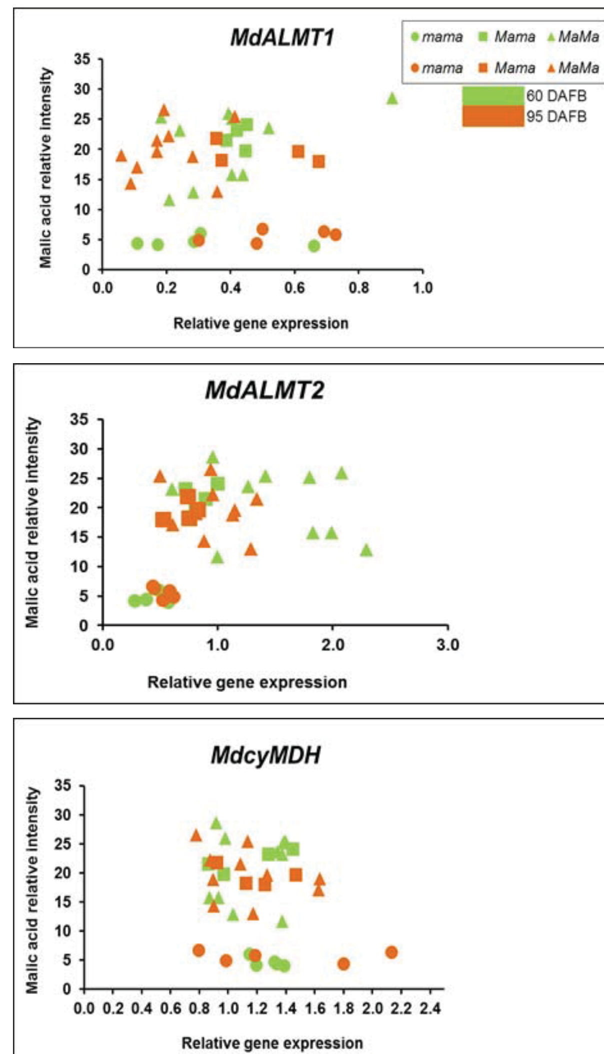

Fig. 6.6. Relationships between gene expression and malic acid content of three candidate genes at two different stages of apple fruit development for three genotype classes (*mama*, *Mama*, *MaMa*). The different colours show different developmental stages as shown in legend. The malic acid content is normalised to ribitol.

The genotypes with one or two copies of the dominant allele (*Mama, MaMa*) showed variable but higher expression levels, and also higher levels of malic acid. This suggests that *MdALMT2* was responsible for the increased level of malic acid in apple fruits. The gene indicated as *pH*/*Ma* in the genetic map in Fig. 6.3, is likely *MdALMT2*.

A third gene *MdcyMDH* (*cytosolic malate dehydrogenase*) was included in our expression analysis. This gene was not in the genetic window of pH locus and rather located on LG17, but has been reported as a gene that is critical for malic acid synthesis in apple (Yao *et al.*, 2011). This structural gene showed no significant correlation between expression level and malic acid content (Fig. 6.6; r = -0.25; P = 0.14).

3.6 Out of the three genes, the relative expression of only *MdALMT2* was negatively correlated with pH

The relative expression of only the *MdALMT2* gene showed a significant negative correlation with pH of mature fruits (Fig. 6.7; for the three consecutive stages: r = -0.49, -0.67, -0.62; P = 0.03, 0.003, 0.007). This confirmed that *MdALMT2* is involved in the regulation of the malic acid, which in turn contributes strongly to the pH. The relative expression of the genes *MdALMT1* and *MdcyMDH* did not correlate with pH (Fig. 6.7). This indicates that the expression patterns of these two genes were not involved in the segregation for pH in the progeny.

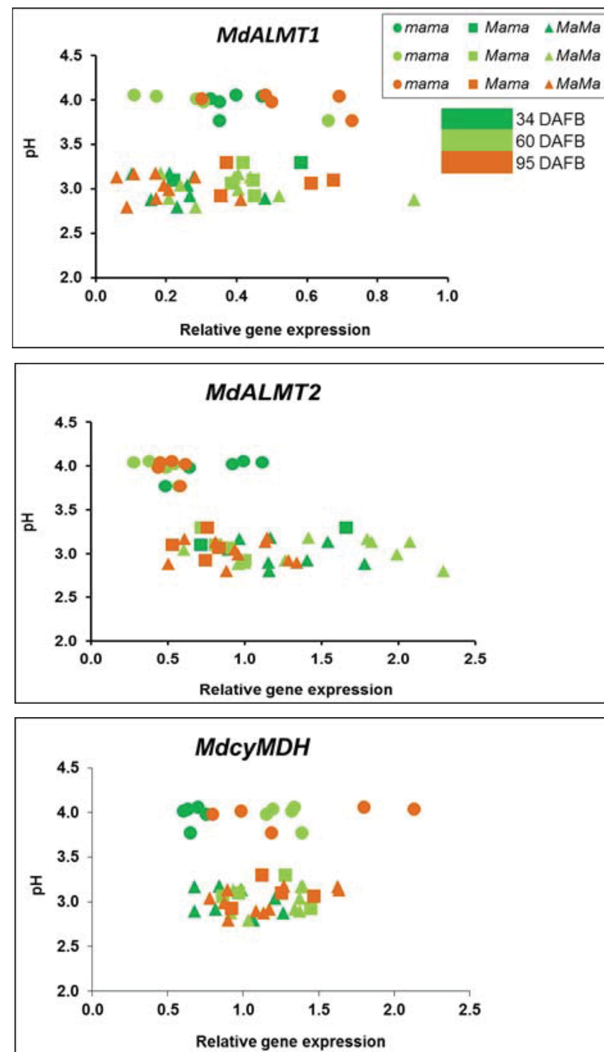

Fig. 6.7. Relationships between relative expression and pH of three candidate genes at three developmental stages for three genotype classes. The genotype classes are explained in Fig. 6.2. Different colours represent the different developmental stages as shown in legend for relative expression, however, pH was measured only at mature stage.

3.7 Relative gene expression of candidate genes *MdALMT1*, *MdALMT2* and *MdcyMDH* at three developmental stages

In Fig. 6.8, the expression level of *MdALMT2* is shown for the three genotype groups at three developmental stages of the apple fruits.

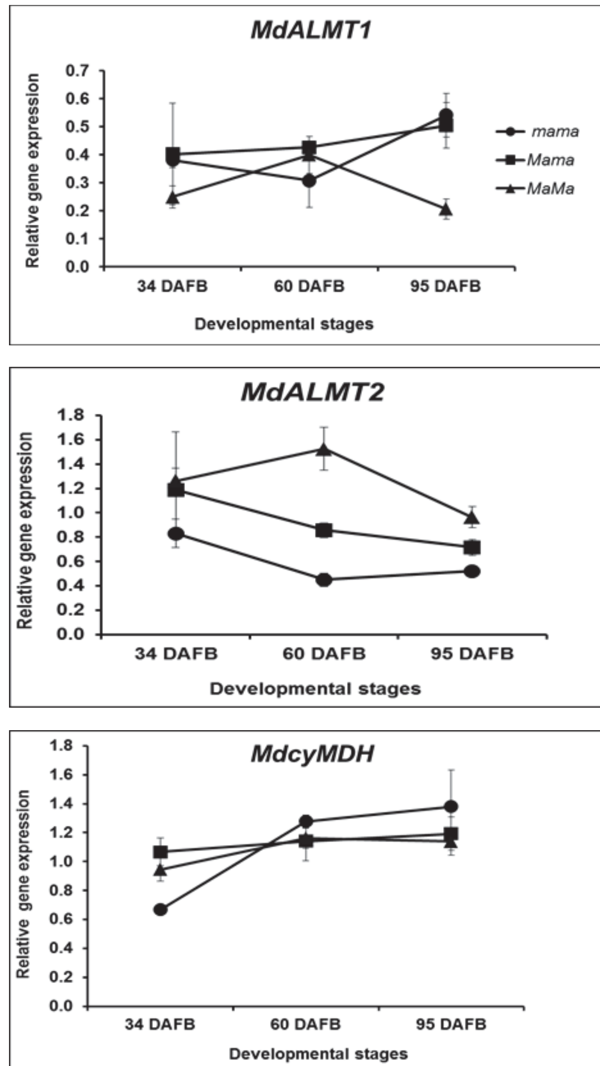

Fig. 6.8. Relative expression of three candidate genes for the three different pH genotype classes at three different stages of the fruits.

The *mama* class showed lower expression during all three stages, compared to both the *Mama* and *MaMa* group. The expression of the *MaMa* group was in general the highest. For the other two genes i.e. *MdALMT1* and *MdcyMDH,* no clear correlation was found between the relative expression and pH groups (Fig. 6.8). *MdALMT1* showed strange expression pattern for one data point. At 95 DAFB the *MaMa* class showed a sharp decrease in the relative expression (Fig. 6.8).

4. Discussion

4.1 Genetic position of the pH gene

Maliepaard et al. (1998) mapped genetically the pH of mature apples, using the segregating F1 population of ‘Prima’ x ‘Fiesta’. We used the same F1 population. Since the paper of Maliepaard and co-workers, many other DNA markers were added to the genetic map of this population (Schouten *et al.*, 2011). We used this denser genetic map, and added SSR-markers that are located near the pH locus on LG16 (Chapter 4). This allowed us to map the pH locus more precisely. We focused on the pH of mature fruits, as the mature stage is most relevant to eating quality. The pH appeared to segregate clearly (Fig. 6.2). The X^2 tests for 1:1 and 3:1 segregation gave as *P*-values 0.000 and 0.21, which indicates that the segregation deviated significantly from a 1:1 segregation, and resembled a 3:1 segregation. The pH locus could be mapped as a monogenic trait at the top of LG16 (Maliepaard *et al.*, 1998). Both parents appeared to be heterozygous for the dominant allele for high acidity and thus low pH.

Graphical genotyping was applied for setting the borders of the genetic interval in which the gene that is responsible for the segregation of the pH should reside (Fig. 6.4). As both parents are heterozygous for the dominant allele for high acidity, genetic windows could be made for both parents, using the pH values and genetic markers of the F1 population. The genetic windows appeared to overlap to a large extent (Fig. 6.4). As explained in the Materials and Methods section, only a part of the F1 population could be used for genetic mapping, due to the dominant inheritance in a 3:1 ratio.

4.2 The dominant allele for acidity increased the malic acid content by a factor of three

The F1 population could be grouped into three classes, i.e. genotypes that were homozygous for the dominant allele for low pH (*MaMa*), the heterozygous group (*Mama*), and genotypes that lacked the dominant allele (*mama*), and had therefore a low acidity and high pH. From each of these three groups, trees were selected for picking of apples at different developmental stages. The apples were peeled off, and the malic acid content and other organic acids in the apple flesh were measured. The data confirmed the supposition from Maliepaard et al. (1998) that malic acid was the important organic acid that was responsible for the segregation in pH. Maliepaard and co-workers assumed that malic acid was the organic compound that was responsible for the clear differences in pH of the mature fruits, and therefore named this locus as *Ma* (Maliepaard *et al.*, 1998). However, they did not measure the malic acid content of the progeny, but solely the pH.

The genotypes that had one or two copies of the dominant allele (*Mama, MaMa*), and a pH of approximately 3.0, had a three times higher malic acid content compared to the *mama* genotypes that had a pH of approximately 4.0. This is in agreement with several other investigations that indicate that malic acid is the major organic acid in apples (Yao *et al.*, 2009; Zhang *et al.*, 2010; Sweetman *et al.*, 2009; Yao *et al.*, 2007). This suggests that the gene that was responsible for the segregation in pH, was indeed a gene that raised the malic acid content strongly. The dominant allele of that gene, *Ma,* raised the malic acid content by a factor 3, compared to the recessive allele *ma*. The addition of a second copy of the dominant allele, slightly raised the malic acid content further, and therewith slightly lowered the pH, as can be seen in Fig. 6.5, when comparing the *Mama* group with the *MaMa* group.

4.3 Two putative malic acid transporter genes reside at the pH locus

Thanks to the published genome sequence of the apple cv. ‘Golden Delicious’ (Velasco *et al.*, 2010), we could align the genetic markers at the locus on LG16 to the genome sequence, and could scan the genetic windows for genes that could be responsible for the differences in malic acid content, and therewith pH.

No structural genes for malic acid production or degradation were detected in these genetic windows. However, two putative malic acid transporter genes appeared to be present at the centres of the genetic windows for both parents (Fig. 6.4). These genes show homology to the malic acid transporter gene *AtALMT1* in

Arabidopsis (Fig. 6.1). In view of the sequence homology to *AtALMT1*, we named the putative malic acid transporter genes at the pH locus on LG16 as *MdALMT1* and *MdALMT2*.

We looked in the cv. 'Golden Delicious' genome for genes similar to *MdALMT2,* and detected 27 other putative malic acid transporter genes, scattered among the chromosomes of apple. Fig. 6.1 shows that homologies to the *Arabidopsis* gene *AtALMT1* did not allow yet to select one of these apple genes as the most likely candidate. There was no apple protein that was far more homologous to the *Arabidopsis* protein compared to the other apple proteins represented in Fig. 6.1. This indicates that in our research, genetic mapping was an important step for the reduction of the number of candidate malic acid transporter genes.

4.4 The expression of one of these malic acid transporter genes was the limiting factor in the acidity of the fruits

The expression of both candidate genes was measured in the three groups of genotypes. *MdALMT1* did not show clear differential expression among the three groups, but *MdALMT2* appeared to have a low transcript level in the *mama* group, a moderate expression in the *Mama* group and a high expression in the *MaMa* group (Fig. 6.8). This provides evidence that *MdALMT2* is the gene responsible for the segregation for pH in the F1 progeny, shown in Fig. 6.1.

The recessive allele of *MdALMT2* is expressed at a low level (Fig. 6.8), and led to a low level of malic acid (Fig. 6.5). The dominant allele is expressed more strongly (Fig. 6.8), which increased the malic acid content by a factor 3 (Fig. 6.5). Addition of a second copy of the dominant allele, further increased the transcript level (Fig. 6.8), but raised the malic acid content only slightly, and therewith reduced the pH to a low extent (Figs. 6.2, 6.5). This indicates that the expression level of *MdALMT2* was the limiting factor in the acidity of the fruits, and that presence of one copy of the higher expressed allele *Ma* was sufficient to remove this limitation.

4.5 Genes for synthesis of malic acid are not associated with pH

Malic acid is an intermediate in the citric acid cycle, also known as the tricarboxylic acid cycle or the Krebs cycle, which is of central importance in all living cells. In eukaryotic cells, the citric acid cycle occurs in the matrix of the mitochondrion. Yao et al. (2011) isolated and functionally analyzed the *malate dehydrogenase* gene (*MdcyMDH*) from apple. The enzyme cyMDH is crucial for malate synthesis in the cytosol. They localized the protein in the cytoplasm and plasma membrane. The expression level of *MdcyMDH* was positively correlated with Malate dehydrogenase (MDH) activity throughout fruit development, but not with malate content. We also found that *MdcyMDH* expression was not associated with malic acid content (Fig. 6.6). Further, this gene is not located at the pH locus on LG16, but on LG17, as detected by aligning the DNA sequence to the published sequence of cv. 'Golden Delicious'.

Sweetman et al. (2009) have indicated that *PEPC* activity, while linked to the synthesis of malate in normal acid varieties, cannot explain the variation in malate levels seen in the low acid fruits, nor can *NAD*-MDH and NADP-ME activities. Berüter (2004) studied the carbohydrate metabolism in an apple variety and its mutant that differed in fruit acidity. The high-acid genotype had five times more malic acid compared to the low-acid genotype. However, Berüter (2004) detected no difference in the catalytic activity of enzymes involved in malate metabolism, such as *PEP carboxylase*, *Malate dehydrogenase*, and *NADP* malic enzyme. The rate of respiration and the rate of malate synthesis were similar in both genotypes. However, the uptake of ^{14}C malate was significantly lower in excised tissue of lower acid fruit. These findings suggest that low malate content in these fruits was not the result of reduced ability to synthesize malate, but of a restricted ability to accumulate it in the vacuoles (Berüter, 2004). This is consistent with our findings, as we did not detect any gene for the malate biosynthesis at our genetic locus for pH, but detected there only putative malic acid transporter genes.

It is the storage of malic acid in plant cell vacuoles that causes the change in pH in apple (Berüter, 2004). Malate storage within the vacuole allows the plant to accumulate this metabolite to a very high concentration (up to >300 mM) and thus maintain the cytosolic concentration constant (Emmerlich *et al.*, 2003). We have indicated here that the malic acid transporter *MdALMT2* could perform this job, by transporting the malic acid from the cytoplasm into the vacuole.

4.6 Marker assisted selection for pH locus may yield apple cultivars with optimum acidity

Acidity of the fruits has a clear impact on overall liking by consumers (Fig. 6.9). In conventional breeding of apples, due to the long juvenile period of apple seedlings, it takes six to eight years after making a cross to evaluate the progeny for fruit quality. This leads to high costs for maintenance of progeny over many years. Marker assisted breeding, using sequence information of the desired allele for acidity, allows selection of young seedlings within one year after making the cross, although these seedlings do not bear fruits yet.

Early selection avoids the costs of growing of many seedlings over a long period, and discovering their poor acidity not until the adult phase when they start to fruit. As *MdALMT2* is the main gene for genetic segregation of the pH, we recommend using markers in or very close to this gene.

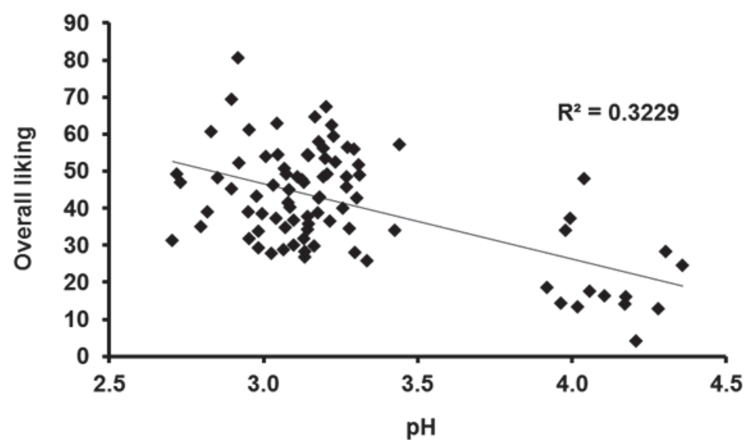

Fig. 6.9. The relationship between acidity of mature fruits and overall liking of these fruits in sensory evaluations (derived from Maliepaard et al (1998) and King et al (2001).

5. Conclusions

Evidence has been provided that in apple the expression level of a malic acid transporter gene is the limiting factor in malate accumulation in the vacuoles, and therewith the major determining factor for acidity of apple. This gene resides at the top of linkage group 16. The genetic inheritance of at least one dominant allele of this gene sufficed for an increased expression level that led to a three-fold increase of the malic acid concentration and a reduction of the pH from 4 to 3 in ripe apples, compared to inheritance of the recessive, lowly expressed allele only. To our knowledge, this gene has not been described before in association with pH of apples.

Acknowledgements

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Chapter 7

Anthocyanin production as a potential visual selection marker during plant transformation

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Abstract

A mutant allele of the transcription factor gene *MdMYB10* from apple induces anthocyanin production throughout the plant. This gene, including its upstream promoter, gene coding region and terminator sequence, was introduced into apple, strawberry and potato plants to determine whether it could be used as a visible selectable marker for plant transformation as an alternative to chemically selectable markers, such as kanamycin resistance. After transformation, red coloured calli, red shoots and red well-growing plants were scored. Red and green shoots were harvested from apple explants and examined for the presence of the *MdMYB10* gene by PCR analysis. Red shoots of apple explants always contained the *MdMYB10* gene but not all *MdMYB10* containing shoots were red. Strawberry plants transformed with the *MdMYB10* gene showed anthocyanin accumulation in leaves and roots. No visible accumulation of anthocyanin could be observed in potato plants grown *in vitro*, even the ones carrying the *MdMYB10* gene. However, acid methanol extracts of potato shoots or roots carrying the *MdMYB10* gene contained up to four times higher anthocyanin content than control plants. Therefore anthocyanin production as result of the apple *MdMYB10* gene can be used as a selectable marker for apple, strawberry and potato transformation, replacing kanamycin resistance.

Keywords: anthocyanin, alternative selection marker, *Malus x domestica*, *Solanum tuberosum*, *Fragaria ananassa*.

1. Introduction

Plant transformation methods rely on the delivery, integration and expression of defined genes in plant cells, which can be regenerated into plants. Selection is necessary to separate transformed cells from non-transformed ones because the efficiency of stable transformation is low. Selectable marker genes are used to confer positive or negative selection and ensure growth of only genetically modified cells. Selectable marker genes that are widely used encode for resistance against the antibiotics kanamycin and hygromycin and originate from bacteria. In response to the negative image of these markers in green biotechnology, scientists have developed alternative selection strategies to avoid the use of antibiotics, although the reason for this is political rather than scientific (Ramessar *et al.,* 2007). Examples are the use of marker gene-free vectors (de Vetten *et al.,* 2003) or removable marker gene systems like pMF-1 (Schaart *et al*., 2004). Alternative selection marker genes that result in the production of plant-based products, which are non-toxic and easy to score, have also been considered (Miki and McHugh, 2004). The red and purple coloured anthocyanins seem to be promising since they are visible by the human eye, do not require addition of substrate, are not essential for development and are non-toxic. Recent reports (Butelli *et al.,* 2009) have even shown that consumption of anthocyanins can be beneficial for health, such as anti-cancer, which could make crops with increased anthocyanin content attractive from a consumer's point of view.

This paper describes the replacement of resistance to the antibiotic kanamycin with anthocyanin formation as a selectable marker for plant transformation. Previous efforts in wheat and sugarcane with the anthocyanin regulatory genes *C1* and *B* from maize failed due to the inability of the anthocyanin-producing cells to divide (Ludwig *et al.,* 1990, Chawla *et al.*, 1999) or for regenerating shoots to grow beyond a certain developmental stage (Bower *et al.*, 1996). Recently, wheat embryo-specific expression of maize transcription factor genes for anthocyanin was described by Doshi *et al.* (2007). The *PAP1/myb75* transcription factor gene from *Arabidopsis* was expressed in tobacco cell cultures and resulted in dark red cells (Zhou *et al.,* 2008), and the maize *Lc* gene expressed in apple (Li *et al*., 2007) resulted in high anthocyanin accumulation in transgenic plants, showing the feasibility of obtaining plants transformed with these transcription factor genes. However, none of these reports showed the use of anthocyanin formation as selection criterion or the use of the promoter of the transcription factor gene itself to drive expression.

Espley *et al.* (2007) isolated an apple transcription factor gene, *MdMYB10,* responsible for skin colour of apple fruits. A mutant allele was found in the apple cultivar (cv) Red Field that results in purple-stained skin and flesh of fruits. The

mutant allele contains an insertion/repeat in the promoter region that causes ectopic expression of anthocyanins (Espley *et al.,* 2009). The apple plants of cv Red Field accumulate anthocyanin in the leaves, stems, flowers and fruits (Espley *et al*., 2007).

Here we describe the use of the mutant *MdMYB10* allele from cultivar Red Field as a selectable marker gene. The *MdMYB10* gene, under the control of its own promoter and terminator, was transformed into apple, strawberry (another member of the *rosaceous* family) and potato as model crop species. Regenerated shoots were harvested from explants without any antibiotic selection or on kanamycin selection. For comparison, a *GUS* reporter gene construct was transformed in parallel.

2. Materials and methods

2.1 Plasmids used for transformation

Two binary plasmids were used for transformation experiments. The T-DNA region of plasmid pART27-21 carries the apple *MdMYB10* genomic region from cultivar Red Field, including 1.8 kb upstream of the translational start, the coding region and 2 kb after the translational stop codon in plasmid pART27 (Gleave, 1992) as well as the *NPTII* gene (Fraley *et al*., 1983) The plasmid $pK_2GW7.0$ (Karimi *et al.*, 2002) carries the *GUS* gene (Joersbo and Okkels, 1996) under the control of the 35S Cauliflower Mosaic Virus promoter (Odell *et al.,* 1985) and the *NPTII* gene for resistance against kanamycin.

2.2 Plant material, transformation procedure and selection of transformed plants

Plant material of apple (cv Gala), strawberry (cv Calypso) and potato (cv Desiree) were obtained from established tissue cultures in the lab. For transformation purposes, these were micro-propagated on medium including macro- and micronutrients, vitamins (Murashige and Skoog, 1962) and 30 g/l sucrose unless stated otherwise. All plant material was placed in a climate chamber with a day/night cycle of 16/8 hours and set at 24 ˚C at 15 μE /m^2/s unless stated otherwise. *Agrobacterium tumefaciens* strain AGL-0 (Lazo *et al.,* 1991), with either binary plasmid pART27-21 or $pK_2GW7.0$, was used for all transformation experiments.

For apple, leaf material of cultivar Gala was used for transformation, according to the method described by Yao *et al.* (1995). Leaf explants were cocultivated with the bacteria for 3 days and then placed on callus induction medium SIM (including MS salts with vitamins and 3% w/v sorbitol) and incubated in the dark at 24 ˚C, either with or without kanamycin (50 mg/l). After 4 weeks, calli were harvested, put on fresh medium and placed in the light (day/night cycle of 16 hrs light, 8 hrs dark). Emerging shoots were placed on SEM medium (including MS

salts with vitamins, 2% sucrose, 1% galactose and BAP/GA3) with or without kanamycin. Shoots with several leaves were placed on SPM including MS salts and vitamins, 3% sucrose and 96 mg/l FeEDDHA (ferric ethylenediaminedi (hydroxyphenylacetate).

Strawberry cultivar Calypso leaf explants were cocultivated for 3 days with *A. tumefaciens* carrying either the pART27-21 or $pK_2GW7.0$ plasmid, according to the method described by Schaart *et al.* (2002) with or without kanamycin (50 mg/l). Shoots regenerated from the explants without a visible callus phase and were harvested 12-16 weeks after start of the experiment.

Potato stem segments of cultivar Desiree were cocultivated for 3 days with *A. tumefaciens* carrying the pART27-21 or $pK_2GW7.0$ plasmid, according to the method described by Visser (1991). Regenerated shoots were harvested from stem segments, with minimal callus formation, after 4-10 weeks and cultured on MS medium including 3% sucrose, with or without kanamycin (50 mg/l).

2.3 Histochemical assay and microscopic analysis

Plant tissues were analysed for *ß*-glucuronidase activity according to the method described by Jefferson *et al.* (1987), using a modified buffer containing 100 mM sodium phosphate buffer, pH 7.5, 10 mM EDTA, 0.1% Triton X-100, 1 mM $K_3[Fe(CN)_6/K_4[Fe(CN)_6.3H_2O]]$ and 2 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid). Tissues were incubated overnight at 37 ˚C, and chlorophyll was extracted for 2 hours in 80% ethanol. Blue staining was detected by eye and with the help of a microscope.

2.4 PCR detection of *MdMYB10*, *GUS* and *VirG*

Genomic DNA was isolated from regenerated shoots with a CTAB-based extraction buffer according to the protocol described by Rogers and Bendich (1988). PCR primers, designed to amplify a 500 base pair region of the *MdMYB10* promoter containing the R6 repeat and a 400 base pair region of the endogenous promoter, have been described by Espley *et al.* (2009). PCR products were analysed by agarose gel electrophoresis according to standard molecular biology protocols (Sambrook *et al.,* 1989). Primers used to amplify a 819 bp region of the *VirG* gene to detect the presence of *Agrobacterium* were: forward primer (5' to 3') CCGCGGTCAGCCGCAATTCT, and reverse primer (5' to 3')CCTGCACGTCCGCGTCAAAGAAATA.

2.5 Extraction and determination of anthocyanins

Total anthocyanin content was determined spectrophotometrically according to the method described by Fuleki and Francis (1968). At least 100 mg of leaf or root material was cut from plants grown *in vitro* and incubated overnight at 4˚C in a solution of 1% (w/v) hydrochloric acid in methanol. The supernatant was collected after centrifugation and transferred to a fresh tube. Absorbance was measured at 530 nm and 657 nm (to correct for chlorophyll) and expressed in absorbance units as anthocyanin content per gram fresh weight.

For determination of the total amount and the composition of the anthocyanins, 1-10 grams of plants grown *in vitro* was freeze-dried and ground in liquid nitrogen. Anthocyanin was extracted in 2 ml of 1% (w/v) acetic acid in methanol with 0.1 % butylated hydroxyanisol (BHA). After centrifuging for 5 minutes, the supernatant was diluted 1:1 with milliQ water and a 100 µL aliquot was separated by reverse phase HPLC using a C18 Thermo column with a gradient of 1% H_3PO_4:H_3PO_4 in formic acid/TFA in acetonitrile. For hydrolysis, 2 volumes of 2 N HCl were added, and the sample was heated to 99 ˚C for 2 hours. Extraction was carried out twice. Samples were run on the column twice. The anthocyanidin standards (cyanidin, pelargonidin, peonidin and delphinidin chloride) were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands).

2.6 pH measurement of leaf homogenates

100 mg of leaf material was ground in liquid nitrogen. Six ml of distilled water was added and the pH was measured immediately with a pH electrode (pH meter CG840, Schott).

2.7 Statistical analysis

Statistical analysis was performed with Excell software version 2003.

3. Results

3.1 Accumulation of anthocyanin after transformation of apple with the *MdMYB10* gene

In a pilot experiment apple leaf explants were incubated after transformation on plates without kanamycin and kept in the dark at 24 ˚C for callus induction. Later, shoots regenerated from the calli. Fig. 7.1 shows different stages of regeneration after transformation of apple explants with the *MdMYB10* gene under standard conditions. There was localised accumulation of anthocyanins in some calli (Fig.

7.1a and 7.1b), in shoot-like structures (Fig. 7.1c), in explants with emerging shoots (Fig. 7.1d) and in regenerated plantlets (Fig. 7.1e).

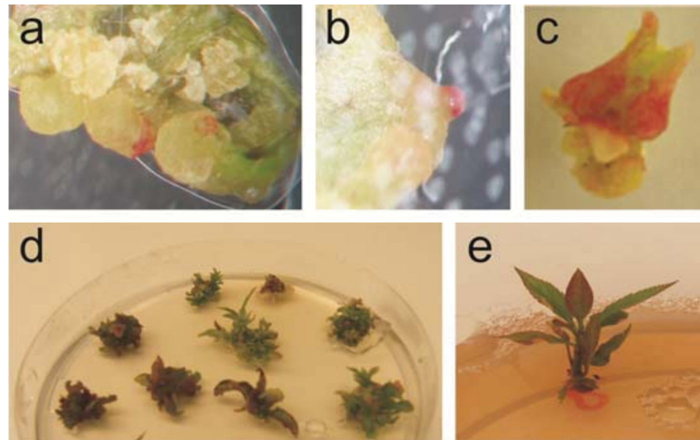

Fig. 7.1 Anthocyanin accumulation at different stages of apple regeneration. a and b: calli on explants transformed with the *MdMYB10* gene construct 4-8 weeks after transformation. c: shoot-like structure forming on a callus, approximately 8-12 weeks after transformation. d: regenerated shoots on explants on a Petri dish, 12-16 weeks after transformation and e: a regenerated plantlet on propagation medium, 20 weeks after transformation.

3.2 Light intensity, non-specifically, influences accumulation of anthocyanin

To determine in which phase of tissue culture (as early as callus or shoot) and under what conditions phenotypic selection of putative transgenic plants was possible, 600 apple explants were transformed with the *MdMYB10* gene construct in the dark. Four weeks after transformation, 563 calli were harvested, of which a low number of nine showed anthocyanin accumulation (Table 7.1a). These nine anthocyanin accumulating calli were placed at standard light condition for regeneration. Two of those nine calli developed a shoot of which one survived that was completely red. In the dark grown calli harvested from explants transformed with the *GUS* gene construct or the untransformed controls did not show any anthocyanin accumulation (Table 7.1a). So, the *MdMYB10* gene transformation showed infrequently specific accumulation of anthocyanin in, dark initiated and grown, calli.

Table 7.1a Occurrence of red calli four weeks after transformation of the apple explants, incubated in the dark

Gene	Number of explants	Number of calli analyzed	Number of red calli
MdMYB10	600	563	9
GUS	600	493	0
Untransformed control	180	164	0

In the dark initiated green calli from the transformation experiment mentioned in Table 7.1a were exposed to different light intensities: low (only reflected; 15 µE), standard (50 µE) and high (100 µE). Twenty-four hours after the calli were exposed to these different light conditions; they were scored for visible anthocyanin accumulation.

Table 7.1b The change of green to red apple calli after exposure to different light intensities

Gene	Light condition	Number of calli tested	Number of red calli after 24 hrs exposure to light
MdMYB10	low	97	0
	standard	93	12
	high	97	3
GUS	low	78	0
	standard	88	4
	high	96	8
Untransformed control	low	30	0
	standard	30	2
	high	30	1

A callus was considered red if it was completely red or showing red sectors. The results are presented in Table 7.1b, indicating that anthocyanin accumulation in a low frequency of green calli was observed after transformation with the *MdMYB10* construct but also with the *GUS* construct and even in the untransformed control. Red colouration of calli in the light was more a-specific. The callus material was further incubated in the climate chamber for regeneration of shoots.

3.3 Red shoots always contain the inserted *MdMYB10* gene and green shoots sometimes

At a later stage after regeneration, shoots were harvested from explants with red and green calli and scored for their phenotypes. PCR analysis of genomic DNA (gDNA) extracted from the red and green shoots showed that red shoots always contained the construct, as represented by a 500-bp PCR product (Online Resource 1). The 400-bp band is representative of the endogenous *MdMYB10* gene. In a pilot experiment 29 shoots were harvested from 50 leaf explants transformed with the pART27-21 construct not subjected to kanamycin selection. Of the harvested shoots, all 29 were positive when tested by PCR for the endogenous 400 bp band. As is shown in Online Resource 1, 20 out of 29 shoots were also PCR positive for the 500 bp band representing the pART27-21 construct. All 9 red coloured shoots were PCR positive as expected, whereas 11 out of the 20 green shoots also showed the same specific PCR product. So all the shoots, visibly accumulating anthocyanin, showed the inserted gene but not vice versa, indicating that shoots containing the specific PCR product of the inserted gene, were either red or green. PCR analysis on the *VirG* gene showed the absence of *Agrobacterium* contamination in all of the analyzed shoots.

3.4 *MdMYB10* induces anthocyanin accumulation in strawberry and potato

To determine the application range of the *MdMYB10* gene as a selectable marker, this gene was also transformed into the plant model species potato and another member of the *rosaceous* family, strawberry. Shoots regenerated in both cases directly from the explants, without a clear callus phase. Regenerated shoots emerging from strawberry explants transformed with the *MdMYB10* gene were either red or green. Red-leafed plantlets produced red roots (Fig. 7.2 a-d), which did not occur when the *GUS* gene was used during transformation. PCR analysis of gDNA extracted from red rooted plants showed a direct relation between anthocyanin accumulation and the presence of the inserted *MdMYB10* gene: all anthocyanin accumulating plants were PCR positive for the inserted gene. However, not all regenerated plants that contained the inserted *MdMYB10* gene showed anthocyanin accumulation. PCR analysis on the *VirG* gene showed the absence of *Agrobacterium* contamination in all but one of the regenerated shoots. Potato shoots regenerated from stem segments and further propagated *in vitro* did not show visible accumulation of anthocyanin after transformation with the *MdMYB10* gene, neither in leaves nor in roots (Fig. 7.2 e and f), although the presence of the inserted gene was confirmed by PCR. Absence of a PCR product for the *VirG* gene in 24 tested

regenerated plants indicated this was not due to *Agrobacterium* contamination. Overnight incubation of leaves or shoots of *MdMYB10*-transformed plants in acid methanol showed that they did contain extractable pigments (Fig. 7.2g).

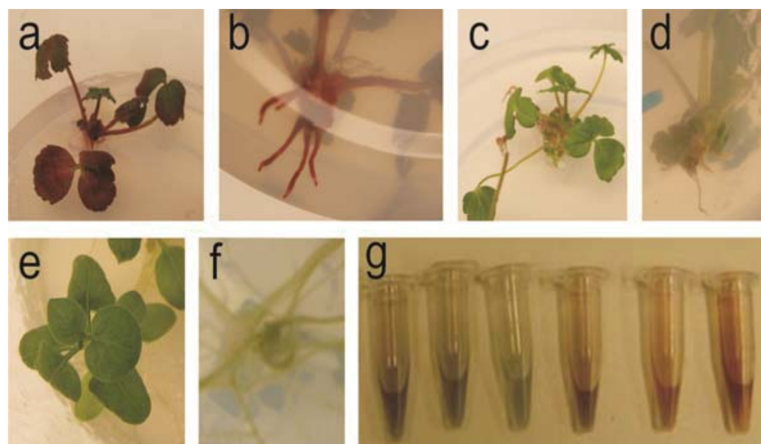

Fig. 7.2 Visible anthocyanin accumulation in strawberry and potato plants transformed with the *MdMYB10* gene. Fig. 7.3a and b show the shoots and roots of strawberries transformed with the *MdMYB10* gene. c and d show the shoots and roots of a control line. e and f show a potato line transformed with the *MdMYB10* gene without visible anthocyanin accumulation. g shows the extractable pigments after overnight incubation of roots and leaves of different potato lines transformed with the *MdMYB10* gene construct.

The absorbance of the extracts was measured and the formula A_{530}-0.25*A_{657} was used to roughly estimate the concentration of presumably anthocyanins. Roots and/or shoots of transgenic potato plants contained up to four times more anthocyanin compared to (un)transformed control (Online Resource 2). Statistical analysis (Anova single factor) of the data showed that the group of PCR+ plants differed significantly from the group of PCR- plants (and the untransformed control) for anthocyanin content both in the root and shoot.

3.5 Anthocyanin content and composition in lines accumulating visibly the highest anthocyanin

From each species we selected a line that visibly accumulated the highest amount of anthocyanin. These selected lines were PCR+ for the inserted gene construct and were multiplied *in vitro*. Harvested plant material was analyzed by HPLC to determine total anthocyanin content and anthocyanin composition after acid hydrolysis. The results are shown in Fig. 7.3. Total anthocyanin content was statistically significantly higher in the *MdMYB10* transformed plants compared to the control lines. P-values using Anova single factor from the analysis tools in Excell software were 0.0137 for apple, 0.0098 for strawberry and 0.022 for potato, respectively.

In untransformed control apple and strawberry lines, only cyanidin was detected, whereas potato mainly contained pelargonidin and peonidin. The transgenic apple and strawberry lines still accumulated mostly cyanidin-based anthocyanins in addition to detectable levels of pelargonidin and peonidin. Potato plants transformed with the *MdMYB10* gene still accumulated predominantly pelargonidin and peonidin in addition to a considerable amount of the blue delphinidin.

The HPLC analysis was performed with additional *MdMYB10* transformed, anthocyanin accumulating apple, strawberry and potato lines with similar results (data not shown).

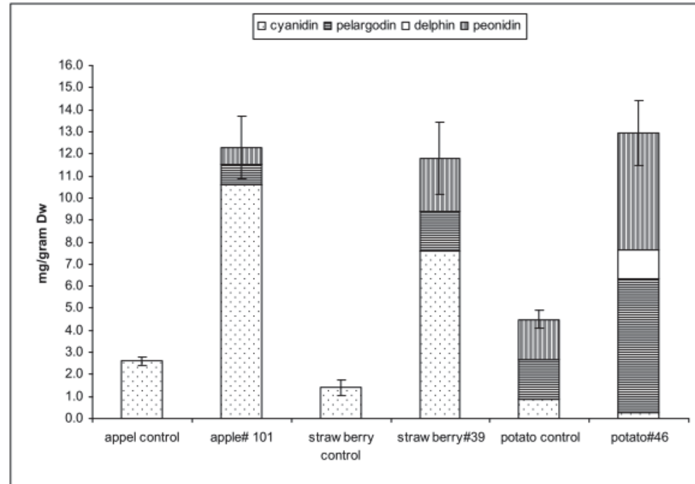

Fig. 7.3 Anthocyanin content expressed as milligram per gram of dry weight (dw) and anthocyanin composition of hydrolysed extracts determined by HPLC of apple, strawberry and potato transgenic line, carrying the *MdMYB10* gene and their (un)transformed control. Anthocyanin chloride standards were used to quantify the anthocyanin aglycones for the hydrolysed samples.

3.6 Transformation efficiency

Table 7.2 shows the number of explants, the number of regenerated shoots and PCR positive shoots, and the number of regenerated shoots with a visible phenotype: either *GUS* blue staining or visible accumulation of anthocyanin. A shoot was considered *GUS* positive when blue spots or sectors were observed.

It was generally observed in all 3 plant species that the number of PCR positive shoots after *MdMYB10* transformation was higher compared to the number of shoots with a visible phenotype indicating that not all PCR+ shoots showed a visible phenotype. This was not dependent on the presence or absence of kanamycin selection. Secondly, we observed that transformation efficiencies, expressed as percentage of explants resulting in a shoot with a visible phenotype, were higher when kanamycin was included in the regeneration medium for both the *MdMYB10* and the *GUS* gene construct in apple, strawberry and potato.

Interestingly, in apple, the transformation efficiency with the *MdMYB10* gene was 9% and with the *GUS* gene 2% in the regeneration medium without kanamycin and 20 and 5% respectively when kanamycin was included. A similar trend was observed when strawberry explants were transformed with the *MdMYB10* gene or the *GUS* gene. After transformation with the *MdMYB10* gene, 6% of the explants produced a shoot with visible anthocyanin accumulation.

When transforming the *GUS* construct into explants, no *GUS* positive shoots were found. In case kanamycin was included in the regeneration medium, a single shoot was found that stained blue for *GUS* while 10% of the explants produced shoots with visible anthocyanin accumulation. In potato, the transformation efficiencies for the different constructs were of the same order, 7% for the *MdMYB10* gene (determined after overnight acid methanol of leaf material from transformed shoots) compared to 9% for the *35S:: GUS* construct (positive staining spots) when kanamycin was omitted. When kanamycin was included the transformation efficiencies were 17% for *MdMYB10* and 24 % for *GUS*.

3.7 Detection of chimerism

During the selection for anthocyanin accumulating shoots, those with an overall uniform anthocyanin accumulation were initially chosen for multiple *in vitro* propagation cycles. Ten apple lines, 12 strawberry lines and 21 potato lines were propagated every 4-6 weeks by cutting the shoot apex and placing that on fresh medium. The plants have been multiplied *in vitro* for almost two years, and after 21 rounds of propagation the apple and strawberry plants still showed uniform red colouration and potato, after extraction in acid methanol, anthocyanin accumulation.

Table 7.2 Analysis of regenerated shoots from explants transformed with *MdMYB10* or *GUS* gene construct and selected on kanamycin (50 mg/l) or not. These shoots were checked for visible phenotype red anthocyanin accumulation or blue *GUS* staining, and analysed by PCR for the presence of the *MdMYB10* gene construct (number in brackets).

species	gene	kanamycin added	no. of explants inoculated	no. of shoots tested	no. of shoots with visible phenotype (PCR+)	no. of shoots without visible phenotype (PCR+)	% transformation efficiency for visible phenotype*
apple	*MdMYB10*	no	150	29	14[a] (14)	15 (6)	9
apple	*MdMYB10*	yes	95	25	19[a] (19)	6 (3)	20
apple	*GUS*	no	150	53	3[b]	50	2
apple	*GUS*	yes	95	6	5[b]	1	5
strawberry	*MdMYB10*	no	50	23	3 (3)	20 (4)	6
strawberry	*MdMYB10*	yes	50	31	5 (5)	26 (7)	10
strawberry	*GUS*	no	50	18	0	18	0
strawberry	*GUS*	yes	50	16	1	15	2
potato	*MdMYB10*	no	150	27	10** (10)	17 (4)	7
potato	*MdMYB10*	yes	75	28	13** (13)	15 (6)	17
potato	*GUS*	no	150	30	13	17	9
potato	*GUS*	yes	75	33	18	15	24

*Percentage of explants producing a shoot with a visible phenotype

**After incubation in acid methanol

Values with [a] differ significantly from [b] with P value< 0.05

4. Discussion

4.1 *MdMYB10* can be used as a selectable marker gene in apple, strawberry and potato

The results presented in this manuscript show that it is possible to use the *MdMYB10* gene from apple as a selectable marker gene for transformation of apple, strawberry and potato plants using standard transformation protocols. For apple and strawberry anthocyanin accumulation is directly visible *in planta*, for potato additional extraction in acid methanol is required in order to visualize increased anthocyanin synthesis by red colouration of the extract.

Red shoots, accumulating anthocyanin, always tested positive for the presence of the *MdMYB10* construct. However, not all shoots that are PCR positive for the introduced *MdMYB10* gene showed an anthocyanin accumulating phenotype. This type of variation among individual transgenic plants is often found in plant transformation experiments and can be explained by variation in expression of the introduced *MdMYB10* gene caused by copy number, position of integration or silencing of the transgene (Butaye *et al.,* 2005).

4.2 The possibility of chimerism

When the *GUS* construct was used for transformation, several plants with blue-staining sectors or spots were observed. This patchy *GUS* staining was observed in regenerating shoots both when kanamycin was omitted and when it was included in the medium (Online Resource 3). This non-uniform *GUS* staining can be attributed to the staining procedure, gene silencing, or chimerism of the regenerated shoot. Chimeras in apple have been reported in transformants of apple obtained without use of a selectable marker (Malnoy *et al.,* 2010) at a low frequency. In two out of 26 transgenic apple lines that were initially selected on kanamycin but propagated for four years *in vitro* under non selective conditions (Flachowsky *et al.,* 2008) chimerism was detected as well. Similarly, in tobacco transformation under non selective conditions, one third of the recovered shoots were shown to be chimeric (Li *et al*., 2009) as opposed to 6-7% when kanamycin was used as selection marker. So chimerism, not observed here in our experiments, is expected to occur occasionally. An additional phase after selecting the putative transgenic plants, such as re-regeneration could reduce the problem of chimerism.

In our study, 10 apple, 12 strawberry and 21 potato lines were multiplied for 21 rounds of propagation and the plants still showed uniform red colouration. On the basis of red colouration these are expected to be uniformly transformed, but the

ultimate proof will include future experiments where several rounds of regeneration of transformed plants will be analysed for their possible chimerical nature.

4.3 Conditions for selecting putative transgenic plants

Our results showed that for apple a possible early phase for selecting putative transformants is callus initiated in the dark. Red calli were observed that produce a red shoot, although this happened at very low frequencies. Upon transformation of potato or strawberry there is a very short callus phase so the shoot stage is the earliest opportunity for testing.

Environmental factors, especially light and temperature are also able to induce the production of anthocyanins (Rabino and Mancinelly, 1986). Exposing regenerating plants to high light intensity usually was accompanied by an increased anthocyanin accumulation, even in control plants.

Selection for anthocyanin accumulating shoots in the dark would prevent light induction of endogenous anthocyanins but unfortunately also causes a decrease in shoot outgrowth (Predieri and Malavasi, 1989). Using our standard transformation conditions we were able to select transgenic plants when we selected for anthocyanin accumulating shoots after regeneration in the light.

Here, in apple and strawberry regenerating shoots with very high anthocyanin accumulation died. Apparently anthocyanin accumulation can be toxic- possibly by influencing the redox state of the cell or by sequestering all available carbon and nitrogen. Similar results were described by Bower *et al.*, 1996, who described constitutive expression of the maize *R* and *C1* gene to be lethal for regenerating sugarcane shoots beyond a certain developmental state. The differences between the experiments are in the promoter that was used to drive expression of the anthocyanin inducing transcription factor genes (35S CaMV versus *MdMYB10*), the origin of the coding part of the genes (maize versus apple) and the method to deliver the genes into the genome (particle gun bombardment versus *Agrobacterium*), and the host plant themselves (sugarcane versus apple/strawberry/potato).

In potato, visible accumulation of anthocyanins in intact plants transformed with the *MdMYB10* gene was observed after overnight incubation of leaf or root material in acid methanol. The total anthocyanin content of the transformed apple, potato and strawberry plants detected by HPLC was similar, around one percent of the total dry weight (Fig. 7.3) for the plants visually showing the highest anthocyanin accumulation. The structural composition of anthocyanidins from the different sources varied, apple and strawberry mainly contained cyanidin and potato mainly contained the red pelargonidin. In contrast to control plants, *MdMYB10* transformed apple and strawberry accumulated detectable amounts of pelargonidin and peonidin. Potato

transformed with the apple *MdMYB10* gene accumulated detectable amounts of delphinidin. The differences in anthocyanin amount and composition were not likely to be the reason for the lack of red colour in the potato plants. Colour is not only determined by the amount and chemical structure of the flavonoids, but also by the presence of other coloured pigments and by the vacuolar pH (Tanaka and Ohmiya, 2008). It is possible that the anthocyanins formed in potato were not transported to the vacuole or that the vacuolar pH was too high. The pH of ground potato leaf material in water was measured with a pH electrode to be 5.75 which is consistently higher compared to the pH 5.45 of ground strawberry leaf material. Submersion of transgenic potato plants in acidic methanol, but not methanol without acid, immediately turns the material red suggesting that the lack of red colour in intact transgenic potato plants is dependent on a relative high pH.

4.4 Transformation frequencies for *MdMYB10* and *GUS* gene constructs

Analysis of the results of transformation frequencies (Table 7.2) showed that the percentage of transformed plants with the *MdMYB10* gene was higher relatively to the ones transformed with the *GUS* construct. According to Yao *et al.,* (1995), the transformation efficiency of apple cultivar Royal Gala was described to be 2.8%, which is comparable to the transformation frequency found using the *GUS* gene (2-5%). When using the *MdMYB10* construct, the transformation frequency was 9-20%, suggesting that the presence of the *MdMYB10* gene promotes regeneration. The same was found in strawberry transformation experiments; higher transformation frequency for the *MdMYB10* gene, though in potato the transformation frequency when using the *MdMYB10 g*ene stayed similar to that of the *GUS* gene. Logistic regression analysis was performed for experiments with binominal output (success/no success) where a shoot with a phenotype, either *GUS* positive staining or visible anthocyanin accumulation was defined as success. Possible explaining factors considered were: kanamycin added, species (apple, strawberry, potato) and gene (*MdMYB10, GUS*). The P values were 0.06 for kanamycin added, 0.418 for species and 0.314 for gene type, respectively. From this we conclude that the differences observed in transformation frequencies were not statistically significant. More experiments will have to be carried out to determine whether the transformation efficiency increases when the *MdMYB10* gene is used as a selectable marker or not. Speculation about a possible explanation for increased transformation frequency when using the *MdMYB10* gene is, that the additional anthocyanins help to protect the regenerating cells against oxidative stress by acting as antioxidants (Terahara *et al.,* 2001). Although the precise mechanism is not fully understood, antioxidants in tissue culture improve plant transformation (Dan,

2008) probably by protecting tissue against reactive oxygen species (ROS), which is the normal initial plant response after pathogen attack.

Another explanation for the differences between regeneration frequencies may lie in the structure of the vectors used, pART27 versus pK2GW7. To prove that anthocyanin accumulation is a result of expression of the *MdMYB10* gene and not that the binary vector structure is primarily responsible for a higher transformation frequency, experiments will have to include the same binary vector for transformation.

4.5 *MdMYB10* gene as a cisgenic or intragenic selectable marker

For apple, the *MdMYB10* gene can even be used as what is referred to as a cisgenic selectable marker. Cisgenesis is a new emerging technology based on the use of natural genes that originate from the target plant itself (Schouten *et al.,* 2006) or from crossable species that could be introgressed through sexual crossing. Similarly, intragenesis is a method based on using all native plant derived functional DNA from the same species or crossable wild species for transformation vectors (Rommens, 2004). Both, cisgenesis and intragenesis, combine the benefits of genetic modification while at the same time avoiding many of the societal issues about the expression of foreign genes and proteins in food crops. The application of cisgenic and intragenic technology requires that crop-specific selectable marker genes must be identified. Anthocyanin regulatory and biosynthetic genes seem to be ideal because the biosynthetic pathway is conserved among plant species and a large number of genes involved in the pathway have been identified. Natural mutants with colour phenotypes and altered anthocyanins have been identified in many species, such as grape, apple, pear, potato, maize and petunia (Winkel-Shirley, 2001 and references therein), and would be likely candidates to be used as selectable marker genes. Recently, the sweet potato *IbMYB1* gene was evaluated for being used as a intragenic marker for sweet potato transformation (Kim *et al.,* 2010), showing the potential application of visible marker genes in transformation of different plant species.

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Chapter 8

General Discussion

In this thesis, the first steps in developing more targeted approaches for breeding apple with improved quality traits like taste and composition of potentially health promoting compounds were established. Apple (*Malus* x *domestica* Borkh) is an important fruit of the temperate regions. It is used mainly as a fresh fruit while a small part is processed into products such as apple juice and apple sauce.

8.1 Apple fruit and human health

The famous saying 'An apple a day keeps the doctor away' has encouraged the researchers to find the magic ingredients that apple contains (Ridgway *et al.*, 1997). It is a rich source of various secondary metabolites such as flavonoids. These metabolites have been found to be the major source of antioxidants (Wolfe *et al.*, 2003). Several of these metabolites such as chlorogenic acid, quercetin, and phloridzin have been reported to have potential health benefits and are involved in the protection of many human diseases. For example, apple eating has been associated with the potential reduction of chances of becoming victim to cancer, particularly prostate, liver, colon, and lung cancers, and cardiovascular diseases (Eberhardt *et al.*, 2000; Mcghie *et al.*, 2005).

8.2 Metabolic diversity in apple

Metabolic diversity in fruits of *Malus* germplasm (wild, advanced selections and a segregating F1 population) was evaluated as a part of this study (Chapter 3). Wild germplasm was compared to advanced selections and also to a segregating F1 population, including the crossing parents, to study the diversity of metabolites and their inheritance into individual offspring. The metabolic composition may be an important factor while producing apple cultivars, as many of these metabolites have potential health benefits (Leccese *et al.*, 2009; Valavanidis *et al.*, 2009).

Liquid chromatography-mass spectrometry (LC-MS) was used for metabolic profiling in this study. LC-MS is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. LC-MS can potentially measure hundreds to thousands of secondary metabolites in parallel and thus gives a wide range of metabolic profiles of various secondary metabolites. In this way, the metabolic profile for the available apple germplasm could be evaluated in a wider and much detailed pattern. It appeared in our study that the wild germplasm of apple contained a much higher amount of different metabolites than the advanced selections or the F1 segregating population and covers wide metabolic diversity (Chapter 3, Fig. 3.1, 3. 2). This was, however, not surprising as it is generally known that wild species cover a wider genetic and metabolic diversity than advanced

selections. This information, may be used in future to detect within wild or advanced selections, individuals with desirable metabolites.

One potential application of the selection for such species could be for their use as antioxidants (Leccese *et al.*, 2009; Valavanidis *et al.*, 2009) and their potential role as protective agents in various human diseases such as cancers, particularly prostate, liver, colon, and lung cancers (Xing *et al.*, 2001), cardiovascular diseases (Hyson *et al.*, 2000; Hertog *et al.*, 1993; Hertog, 1992), coronary heart diseases, asthma, type-2 diabetes, thrombotic stroke, and ischemic heart disease (Mcghie *et al.*, 2005). A limitation, however, in dealing such data would be the annotation of metabolites and their properties. At the moment it is not possible to annotate all the metabolites from LC-MS (Keurentjes, 2009). In this study, rather a small set of metabolites was annotated. Hopefully in near future it would be possible to annotate far more metabolites, which will give a much better understanding of the metabolic profile of apple or of any other commercially important crop. The metabolic profile could be used to locate genes on the genome that cause phenotypic differences in metabolic pathways such as phenylpropanoid pathway of plants.

During this study it was found that while crossing two cultivars, certain metabolites could be lost and certain other metabolites could be gained. One quarter of the progeny of the segregating F1 population showed this loss for about one quarter of the metabolites. On the other hand some metabolites were found to have significantly higher levels in some of the progeny than in both of the parents, this we called gain of metabolites. Therefore, in future it should be considered more generally that even normal crosses in plant breeding could be accompanied with loss and gain of metabolites.

8.3 QTL mapping of secondary metabolites

A quantitative trait is defined as a characteristic whose value varies across individuals in degree rather than in category. The precise phenotype of such a trait is determined by polygenes and environmental effects, e.g., yield and yield-related traits, quality traits, resistance to diseases and insects, and abiotic stress tolerance (Zhang and Gai, 2009). In contrast to that we reported in this thesis that the metabolites are also quantitatively inherited, however, not polygenic but monogenic. This was showed in Chapter 4 and Chapter 5 where only one gene, called *MdLAR1* caused the mQTLs for several metabolites. Another example of monogenetically regulated metabolites was found at one locus on LG8, it regulates the glycosylated forms of β-glycols (Chapter 3).

A pre-requisite for QTL mapping is the availability of a dense and high quality genetic linkage map on the one hand and equally important a good phenotypic data set of the same segregating population used in genetic mapping on

the other hand. The genetic linkage map of 'Prima' and 'Fiesta' used in the present study is one which was first constructed by Maliepaard et al (1998). In this thesis, this genetic linkage map was further enriched with integration of 240 DArT markers (Schouten *et al.*, 2011). This improved map was an important help in several other chapters of this thesis. LC-MS was used for creating a phenotypic data set of secondary metabolites. Metabolomics is developing as an important functional genomics tool in crop plants, including fruit trees (Moco *et al.*, 2006; Carrari and Fernie, 2006). In our study, we were able to determine and map 418 metabolites in apple peel and 254 metabolites in apple flesh.

For mapping metabolites, currently two kinds of approaches are used in plants, namely the targeted and the untargeted one. In the targeted approach a selected number of metabolites of specific interest are mapped one by one. This is possible with mapping software such as MapQTL® (Van Ooijn, 2009). In the untargeted approach, hundreds of metabolites can be mapped simultaneously. Such an analysis may be performed with mapping software MetaNetwork (Fu *et al.*, 2007). This provides a genome-wide screening for hotspots of QTLs. However, MetaNetwork could not make use of co-factors. As MetaNetwork has been designed for self-pollinating species, and not for highly heterozygous parents that segregate in the F1, an algorithm to make MetaNetwork also suitable for a segregating F1 population from cross-pollinators was developed. The advantage of using MapQTL® is that it also gives information on the allele pairs and allele combinations to the trait. Co-factor analysis can be performed to filter out the effect of strong QTLs and account for the unbalanced allele sampling and reduce the residual variation of the population.

In this study, four linkage groups (LGs) i.e. LG1, LG8, LG13 and LG16 were found to have mQTL hotspots. The annotation revealed that these metabolites belong to mainly the phenylpropanoid pathway of secondary metabolites. The main mQTL hotspot appeared to be at the top of LG16 for many metabolites such as catechin, epicatechin and procyanidins. These mQTLs are shown in Chapter 4, Fig. 4.5. The mQTL hotspot on LG16 was further investigated and for this purpose metabolites with clear segregation in the progeny were mapped. In this way a genetic window was constructed. This genetic window was searched for candidate genes using the sequence information of cv. 'Golden Delicious' (Velasco *et al.*, 2010). It appeared that regarding the phenylpropanoid pathway, only the structural gene coding for *leucoanthocyanidin reductase* (*MdLAR1*) was located in this genetic window as were seven transcription factor genes that might regulate this pathway. We wanted to study the expression profile of these genes to see whether these genes were directly involved or not. For this purpose q RT-PCR analysis was performed with selected genes as will be explained in next section.

8.4 Expression profiling of candidate genes

Once the chromosomal region or window of a QTL is detected, the underlying potential genes could be determined and studied by locating the flanking molecular markers sequences in the available whole genome sequence. This may yield several genes in the QTL region, although only one or a few of these candidate genes could then be the real causal gene(s). In this study, the whole genome sequence of cv. 'Golden Delicious' (Velasco *et al.*, 2010) was used to locate the possible candidate genes in the mQTL. However, we have to be careful as these are putative predicted candidate genes without any functionally test performed. Therefore, before drawing any firm conclusion about these genes, the functionality of these genes should be confirmed.

One approach of checking the potential functionality of the candidate genes was to study the expression profile of these genes in the target samples picked at different time points of fruit development. For this purpose, q RT-PCR was used to investigate if there was any differential expression of one or more of the candidate gene(s) which could be correlated to the increased metabolite level observed in developing apple fruits. In this study it was found that out of 36 tested genes, only the structural gene *MdLAR1* showed a differential expression for metabolite level in different genotype classes in the mQTL hotspot on LG16 (Chapter 5, Fig. 5.2). *MdLAR1* expressed in a rather similar pattern in peel and flesh tissues (Chapter 5, Fig. 5.2).

Moreover, a positive correlation between its relative expression and the expected increase in metabolite level was observed. We could not find a good correlation for any of the transcription factor genes present in the mQTL hotspot with the metabolite levels, which showed, for the investigated developmental stages of apple fruit, that these transcription factor genes were probably not involved in the regulation of these metabolites in the mQTL hotspot window on LG16.

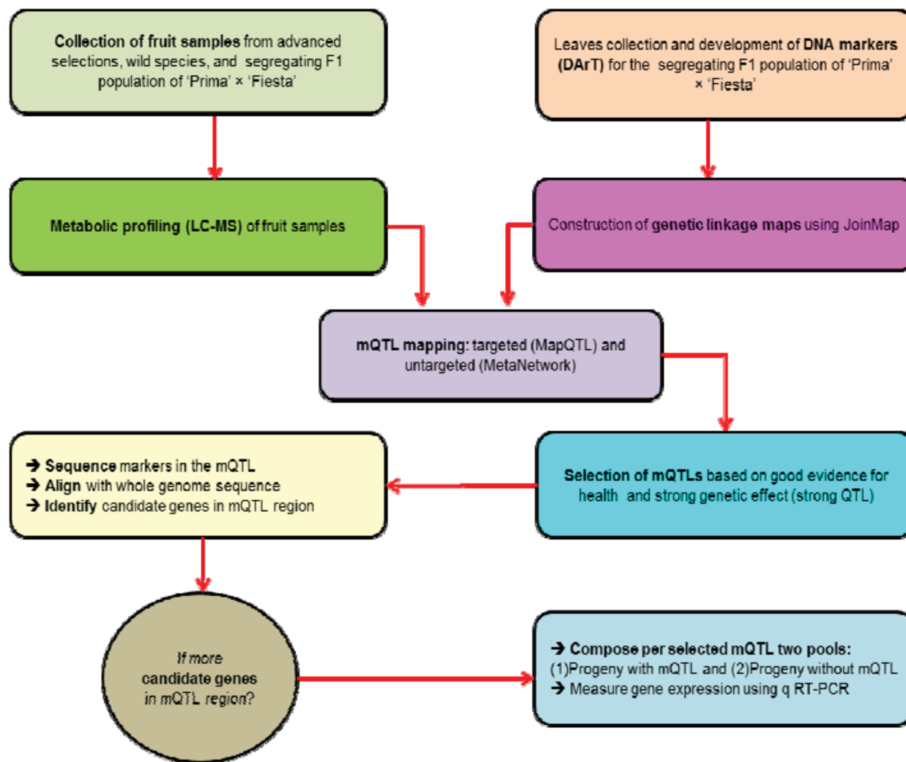

Fig. 8.1 Schematic view of the genetical metabolomics approach applied in this thesis.

8.5 Acidity is an important determinant for fruit quality

Acidity of apple fruits is directly related to its taste. Sensory traits like taste determine to a large extent the consumer preference for eating fruits (Chapter 6, Fig. 6.9). In apple malic acid is known to be the major organic acid responsible for the acidity and has a huge impact on the pH in fruits and its taste. Optimal taste is determined by a proper balance between sugar and acidity (Visser and Verhaegh, 1978).

In apple, the acidity decreases with time and sugar increases with time. However, when the fruit is fully ripe then the sugar content is rather stable and no more increase occurs (Visser *et al.*, 1968). Low acidity in apple is genetically a recessively inherited trait (*mama)* (Maliepaard *et al.*, 1998; Visser *et al.*, 1968). Most apple varieties are heterozygous (*Mama*) for acidity (Visser *et al.*, 1968) and after intercrossing, it is expected that one quarter of the progeny will have low acidity (*mama*) which for taste is undesirable. Maliepaard et al. (1998) mapped the pH trait of apple fruits on LG16 within the same segregating F1 population as used in this thesis. They observed monogenic inheritance for low versus high acidity and mapped pH on LG16. Remarkably, both parents had one dominant allele for low pH at the LG16. They denoted the locus as *Ma* for malic acid, being the major acid in apple, although they measured pH rather than malic acid itself (Maliepaard *et al.*, 1998).

In our study, two potential candidate malic acid transporter genes were detected using the whole genome sequence information. Only the *MdALMT2* gene expression showed a positive correlation with the malic acid content at different developmental stages of fruit growth (Chapter 6, Fig. 6.6). Also only the *MdALMT2* gene gave a clear differential expression in three different pH genotype classes (Chapter 6, Fig. 6.8). These classes were *MaMa*: homozygous dominant class where the progeny received a dominant allele from both parents; *Mama*: heterozygous dominant class where the progeny received a dominant allele from one parent and a recessive allele from another parent; *mama*: the homozygous recessive class, in this case the progeny received one recessive allele from each of the parents. This showed that only *MdALMT2* gene was found as a candidate gene causing the differences in acidity of apple fruits (Chapter 6, Fig. 6.8). Apparently, the *MdALMT2* gene regulates acidity of apple fruits by transporting malic acid into the cell vacuole. In next step an allele specific selection marker for this *MdALMT2* could be developed.

8.6 Genetic modification of plants through anthocyanin content

Anthocyanin can be used as a selection marker to modify apple. For instance, Chagne et al. (2007) produced apples with red flesh. Moreover, as anthocyanin is a strong antioxidant, it could improve apple as a source of health benefits for consumers. In tomato, for instance, it has been observed in a pilot test that cancer-susceptible *Trp53*$^{-/-}$ mice fed with a diet supplemented with the high-anthocyanin tomatoes caused a significant extension of life span (Butelli *et al.*, 2008). This indicates a potential role of anthocyanin in human diseases also. Anthocyanins as colourful pigments, give attractive appearance to many fruits, including apple,

which could motivate consumers for buying and eating more fruits. This will have positive implications for the human health in society in the long run.

In this study, the mutant dominant allele of the transcription factor gene *MdMYB10* has been introduced as a selectable marker for plant transformation (Chapter 7). Due to a mutation in its promoter region it is constitutively expressed, giving red colouration to many plant tissues including fruits and leaves. The use of anthocyanin as a selectable marker will enhance the transformation of different crops for desired traits by providing cheaper, efficient and quick identification of the transformation event (Chapter 7). It will also avoid the use of antibiotic based selectable marker genes such as kanamycin and hygromycin resistance genes. These antibiotics are concerns for GMO regulations and often are not accepted by the consumer. *MdMYB10* is a dominant cisgene, from apple itself, it is expected that there is less concern from consumers. It is indicated earlier in the Eurobarometer (2010) (http://ec.europa.eu/ research/ science-society/document_ library/pdf_06/ europeans- biotechnology -in- 2010_ en.pdf) that people are more in favour of cisgenic resistance genes to scab. Perhaps, in future, GMO regulations could be exempted for cisgenic apples, using apple genes in order to improve apple for different important traits such as disease resistance e.g. against apple scab or quality traits such as taste and metabolic composition.

8.7 Potential challenges in genetical metabolomics

Genetical metabolomics is comparatively a new field for plant breeding and has been recently started. Thousands of metabolites can be found in a single cell yet it is just the beginning to explore the metabolome (Keurentjes, 2009; Fu *et al.*, 2009). Earlier on Jansen and Nap (Jansen and Nap, 2001) reported on ‘genetical genomics’ approach in *Arabidopsis* by combining the genetics to the genomics information. Attempts have been made to identify the genetic factors involved in the metabolic composition for improved properties through metabolic engineering (Trethewey, 2004). As a new field it has several potential challenges: 1. it requires a sound genotypic data set, and 2. it needs sounds phenotypic data. The required software should be highly sophisticated in order to understand the genetics of complex traits such as biochemical pathways of secondary metabolites as shown in this study.

One challenge is the phenotyping. Nowadays, the genotyping is a smaller problem because of the availability of various platforms for this purpose. However, phenotyping can still be a challenge, because of several reasons such as intensive look after in case of field crops. Also it needs to be done in controlled conditions which is often a challenge such as same temperature, humidity and nutrients level to every individual plant of the population. The other problem in phenotyping is that it may need the use of specialized equipment for fast and quick data recording of the

individual plants. Therefore, in future more focus should be given to phenotyping related to many traits. Another potential challenge is the annotation of a large number of metabolites that the advanced metabolomics tools provided us with. Currently identification and classification of the numerous compounds that we get with advanced metabolic profiling technologies, is the limiting step to link metabolite to phenotype (Keurentjes, 2009). The annotation of such a large data set will remain a challenge at least for several years. However, with the rapid advancement in the metabolomics technologies, probably in near future more and more metabolites could be annotated. Once more annotation become possible, this would reveal the hidden treasure of plant metabolites and will help in understanding the important plant metabolic pathways. In this way the genes responsible for the metabolic pathways can be identified, isolated, and cloned. Later on, these can be used for genetic engineering of important compounds in various crops, including apple.

8.8 Conclusions

The main conclusions from this thesis are: 1.The structural gene *MdLAR1* was found as the causal gene of mQTL hotspot on LG16 for phenolic compounds of the phenylpropanoid pathway. 2. *MdMYB10*, a dominant transcription factor allele was successfully inserted into apple, strawberry, potato and was reported as a selectable marker for plant transformation. 3. *MdALMT2*, a malic acid transporter was detected on LG16. This gene probably regulates the acidy in apples. 4. A locus on LG8 was detected that genetically regulates glycosylated forms of β-glycols: R-octane-1,3-diol and its unsaturated form R-5-(Z)-octene-1,3-diol in apple fruits.

8.9 Future prospectives

The available genomics tools can be combined with metabolic profiling technologies to identify important genes that could be used as selection marker for the production of improved apple varieties and after cloning engineered for direct improvement of certain existing varieties.

In the present study, *MdLAR1* and *MdALMT2* genes were detected and found to be located genetically very close to each other on LG16. Apparently, the dominant allele for high acidity was in repulsion phase to the dominant allele for high levels of metabolite content in both parents. In both parents the dominant alleles for high levels of metabolites are genetically tightly linked to the recessive alleles for high pH. This has clear implications in apple breeding for improving existing cultivars by transformation. In Northern Europe, apples with a low pH are usually preferred to those with a higher pH, and therefore should contain the dominant allele for low pH. As the dominant allele for low pH is in repulsion phase

to the dominant allele for high metabolites, at least in the genotypes we investigated, the selection for the dominant low pH allele implies the selection for the recessive allele for low levels of the phenolic compounds. Therefore, progeny that are more acidic, have higher chances of having lower levels of procyanidins and other phenolic compounds. This can be solved by the selection of progeny that have one dominant allele for low pH from one parent and one dominant allele for high metabolite levels from the other parent. In the southern countries of Europe and in Asia, consumers usually prefer a higher pH. In that case, the desired absence of the dominant allele for pH is automatically combined with the presence of the dominant allele for high levels of metabolites. As indicated earlier, selection of the desired progeny for these fruit traits is feasible already at a very young stage, using DNA from leaves of seedlings and DNA markers.

Next step in this regard would be the development of a molecular marker for *MdALMT2* gene so that desired allele(s) of this gene can be selected for in classical breeding. In this way the progeny with undesirable high acidity or sweet level could be discarded at a much earlier stage of selection. This will highly reduce the selection costs for breeders. They usually spend huge amounts of money to grow, test and negatively select many genotypes after eight or more years in the field when the tree is flowering and yielding apple fruits. They could screen the experimental populations for basic quality traits like acidity, much earlier at seedling stage and thus reduce the costs and labour for developing advanced populations. In this way they could develop populations with a much higher frequency of individuals with favorable quality traits. Another option is that we can either silence the *MdALMT2* in order to reduce the level of acidity or we can insert this gene into existing sweet apple cultivars to make them sourer. The same can be said for the *MdLAR1* gene. This gene can also be selected at seedling stage using MAS. Another option is to insert *MdLAR1* to increase the level of potential health beneficial metabolites into existing cultivars which have low level of these metabolites.

The gene specific markers could be used in marker assisted selection programmes. This will enhance the process of selection for desired traits during breeding. In this way the breeders can also reduce the costs of production of new varieties by removing the undesired plants at seedling stage. The isolated genes could be transferred into promising existing apple cultivars by different plant transformation technologies including cisgenesis (Schouten *et al.*, 2006a, b). For cisgenesis marker free transformation is a prerequisite. An alternate would be the use of the dominant cisgenic marker i.e. *MdMYB10* gene in apple, as described in this thesis.

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Summary

The aim of this thesis was finding genes that control the production of potentially health beneficial metabolites in apple fruits. The approach was genetic mapping of secondary metabolites such as phenolic compounds in an F1 progeny, leading to the detection of genetic loci that controlled these metabolites. At these genetic loci candidate genes were identified, using the whole genome sequence of apple, and it was investigated whether the expression of these candidate genes in the F1 progeny correlated with the metabolite levels.

The cultivated apple (*Malus x domestica* Borkh) is among the most diverse and ubiquitously cultivated fruit species. It belongs to the family of Rosaceae which includes many commercial fruit species such as pear, strawberry, cherry, peach, apricot, almond, black cherry, and crab apple. Apple has a haploid chromosome number of 17. It is a self-incompatible and highly heterozygous crop. The breeding is further hampered by the long juvenile period which makes breeding in this crop a very slow process.

The saying "An apple a day keeps the doctor away" has encouraged many researchers to search for the "magic" ingredients found in apple. Due to the beneficial role of apple phenolics, it is also called as a "new agrochemical crop". Apple possesses many health beneficial properties for human beings as it is a rich source of phenolic compounds. It has been associated with reducing the risks of certain diseases such as cancers, particularly prostate, liver, colon, and lung cancers, cardiovascular diseases, coronary heart diseases, asthma, type-2 diabetes, thrombotic stroke, and ischemic heart disease.

The second chapter of this thesis describes the construction of genetic linkage maps of the parents of a segregating population derived from the cross between the cultivars 'Prima' and 'Fiesta'. For this purpose the already available linkage maps, as described in this chapter, were made denser by inclusion of 240 Diversity Array Technology (DArT) markers. Thus the total number of markers for 'Prima' and 'Fiesta' integrated map reached to 820. DArT-markers are hybridization based dominant DNA-markers. DArT provides a high-throughput whole genome genotyping platform for the detection and scoring of hundreds of polymorphic loci without any need for prior sequence information. This is the first report on DArT in horticultural trees. Genetic mapping of DArT markers in two mapping populations and their integration with other marker types showed that DArT is a powerful high throughput method for obtaining accurate and reproducible marker data, at low cost per data point. This method appears to be suitable for aligning the genetic maps of different segregating populations. Sequencing of the marker clones showed that they are significantly enriched for low copy, gene rich regions.

Chapter 3 describes metabolic diversity of *Malus*. Wild germplasm was compared to advanced breeding selections and to the segregating F1 population from the cross between the cultivars 'Prima' and 'Fiesta'. The metabolic profiles were analyzed by means of liquid chromatography-mass spectrometry (LC-MS). LC-MS is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. This resulted in the detection of 418 putative metabolites in the peel and 254 in the flesh. Fruits from 23 wild species, eight advanced selections and the segregating F1 population were analyzed. The data were subjected to Principle Components Analysis (PCA). Variance analysis of the first PC showed that genetic variation accounted for 96.6 % in peel and 97.4 % in flesh of the total metabolic variation. Technical variation accounted for 1.4 % and 0.8%, while environmental variation accounted for 2.0% and 1.8% in peel and flesh respectively. The genetic variation between wild genotypes was very large, compared to the advanced selections and the F1 progeny. Only 8 % of the genetic variation of the first principle component was captured by the advanced selections. This indicates strong genetic erosion during breeding. This genetic erosion was mainly caused by reduction of the levels of several flavonoids including catechin, epicatechin and procyanidins. PCA of the F1 progeny of the 'Prima' x 'Fiesta' cross showed a clear 3:1 Mendelian segregation of metabolites. These metabolites were 4.2 fold less in both peel and flesh in progeny that had inherited the recessive alleles of a gene at the top of Linkage Group 16 (LG16) from the heterozygous parents.

We found a separate group of 11 metabolites in peel and 12 in flesh. These metabolites were putatively identified as glycosylated forms of β-glycols: R-octane-1, 3-diol and its unsaturated form R-5-(Z)-octene-1, 3-diol which have a potential role in controlling infection by microorganisms and influence the aroma of some ciders. The levels of these metabolites were up to 50 fold more abundant in some progeny compared to both parents. Genetic mapping showed that this strong increase was caused by one locus at the top of LG8, in progeny that had inherited only the recessive alleles of that locus from the heterozygous parents. This research illustrates not only the strong genetic erosion in apple breeding regarding metabolic diversity, and strong reduction of flavonoids in some progeny, but also shows that inbreeding can lead to a strong increase of metabolites that were present at much lower levels in both parents and advanced selections. This loss and gain of metabolites was especially observed in case of accumulation of recessive alleles during inbreeding.

The genetic factors controlling metabolite composition were studied in more detail in Chapter 4. We investigated the genetic factors of the quantitative variation of these potentially beneficial compounds (Chapter 3, 4), by combining the genetic maps (Chapter 2) with the LC-MS data for the segregating F1 population

from the cross 'Prima' x 'Fiesta'. This resulted into metabolite quantitative trait loci (mQTLs). When using the software MetaNetwork, 669 significant mQTLs were detected: 488 in the peel and 181 mQTLs in the flesh. Four linkage groups (LGs) i.e. LG1, LG8, LG13 and LG16 were found to contain mQTL hotspots, mainly regulating metabolites that belong to the phenylpropanoid pathway. These include various metabolites i.e. sinapate hexoside, coumaroyl hexoside, phloridzin, quinic acids, phenolic esters, kaempferol glycosides, quercetin glycosides, cyanidin pnetoside, flavan-3-ols (catechin, epicatechin), and procyanidins. The genetics of annotated metabolites was studied in more detail using MapQTL®. It was found that quercetin conjugates had mQTLs on LG1 and LG13. The most important mQTL hotspot with the largest number of metabolites was, however, detected at the top of LG16: mQTLs for 32 peel-related and 17 flesh-related phenolic compounds. The metabolites that mapped in the mQTL hotspot on LG16 all belong to the phenylpropanoid pathway of secondary metabolites. These compounds showed a monogenic Mendelian inheritance in a 3:1 segregation ratio. Procyanidins dimer II was used as a representative of the numerous compounds that mapped at the LG16 mQTL hotspot. By means of graphical genotyping of this monogenic trait, a genetic window could be made in which the gene that caused the mQTL hotspot should reside. We located structural genes involved in the phenolic biosynthetic pathway, using the genetic map together with the published whole genome sequence of apple. The structural gene *leucoanthocyanidin reductase* (*MdLAR1*) was detected in the mQTL hotspot window on LG16, as were seven transcription factor genes. To our knowledge, this is the first time that a QTL analysis was performed on such a high number of metabolites in an outbreeding plant species.

The expression of the candidate genes found in the mQTL window on LG16 was studied and discussed in Chapter 5. qPCR was used for this purpose and it was found that the expression of only the structural gene *MdLAR1* was strongly positively correlated with the metabolite procyanidin dimer II content. Neither the expression profiles of other structural genes of the phenylpropanoid pathway, the transcription factor genes at the mQTL hotspot, nor of transcription factor genes outside the mQTLs hotspot, showed any significant correlation with the procyanidin dimmer II content that mapped at the mQTL hotspot. This indicates that *MdLAR1* was the gene, which caused this mQTL hotspot (Chapter 5). The progeny that had inherited one or two copies of the dominant alleles (*Mm, MM*) showed on the average a 4.4 and 11.8 fold higher expression level of *MdLAR1* respectively, compared to the progeny that had inherited the recessive alleles only (*mm*). This led to a 4.0 fold increase of procyanidin dimer II level at the ripe stage.

Strikingly, at the mQTL hotspot at the top of LG16, there is also a locus that controls acidity of the ripe fruits. However, the dominant alleles for acidity

appeared to be in repulsion to the dominant alleles for high metabolite levels (Chapter 6). This shows that acidity is controlled by another gene than the metabolite levels. The combination of the genetic position based on the whole apple genome sequence, annotation of potential genes, and expression profiling indicated that the malic acid transporter gene *MdALMT2* was responsible for the clear differences in malic acid content and pH in mature apple fruits of the segregating F1 population. The genetic inheritance of at least one dominant allele (*MaMa*/*Mama*) of this gene sufficed for a three-fold increase of the malic acid concentration and a reduction of the pH from 4 to 3 in ripe apples, compared to the presence of only the lower expressed recessive allele *(mama*). This malic acid transporter gene is located at the top of LG16. Malic acid is the predominant organic acid associated with the pH in apple fruits. It is synthesized in the cytoplasm and transported into the cell vacuole. The concentration of malic acid in the cell vacuole determines the pH of the cell. pH is very important for the overall taste of many fruits, including apple, and has profound effects on the organoleptic quality of apples. The pH of mature apples was genetically mapped on LG16 in the segregating population from the cross 'Prima' x 'Fiesta'. To our knowledge, this is the first time that the genetic segregation of the pH in apple is assigned to a specific gene. Further, this gene has not been reported yet in conjunction to pH of apples or other fruits. After cloning of the *MdALMT2* gene, it can be used for, proof of principle, influencing the acidity of existing varieties either by silencing this gene in more acidic cultivars or by inserting this gene into the low acidic cultivars. Another step would be to develop an allele specific molecular marker for selection (Marker Assisted Selection) of the acidity of fruits already at seedling stage, five years before the trees carry fruits.

In another study, a dominantly mutated allele of the transcription factor gene *MdMYB10*, including its upstream promoter, coding region and terminator sequence, was introduced by transformation into apple, strawberry and potato plants. The dominantly inherited mutant allele of *MdMYB10* from apple induces anthocyanin production throughout the plant, also at the early stage after transformation. The aim was to determine whether *MdMYB10* could be used as a visible selectable marker for plant transformation as an alternative to chemically selectable markers, such as kanamycin resistance. After transformation, the colour of calli, shoots and well-growing plants were evaluated. Red and green shoots were harvested from apple explants and examined for the presence of the *MdMYB10* gene by PCR analysis. Red shoots of apple explants always contained the *MdMYB10* gene but not all *MdMYB10* containing shoots were red. Strawberry plants transformed with the *MdMYB10* gene showed anthocyanin accumulation in leaves and roots. No visible accumulation of anthocyanin could be observed in potato plants grown *in vitro*, even the ones carrying the *MdMYB10* gene. However, acid methanol extracts

of potato shoots or roots carrying the *MdMYB10* gene contained up to four times higher anthocyanin content than control plants. Therefore, anthocyanin production as a result of the dominant *MdMYB10* gene can be used as a selectable marker for apple, strawberry and potato transformation, replacing kanamycin resistance gene such as *nptII*. We reported this *MdMYB10* as a cisgenic selectable marker gene for apple transformation (Chapter 7). The results from all experimental chapters have been discussed in a broader sense in the general discussion (Chapter 8). The future prospectives and potential challenges in the genetical metabolomics are also highlighted. The approaches we developed in the current thesis could be used not only for developing potentially a more healthy and improved apple but can also be applied for the genetical metabolomics studies in other important crops.

Samenvatting

Doel van het promotieonderzoek was genen in appel te vinden die verantwoordelijk zijn voor de productie van metabolieten met potentieel gezondheidsbevorderende eigenschappen voor de consument. De gekozen benadering was secundaire metabolieten, zoals fenolische verbindingen, in een F1 kruisingspopulatie van appel genetisch te karteren. Daardoor werden bij de genetische kaart op diverse chromosomen gebieden gevonden waarin de genen liggen die voor de productie van mogelijk gezondheidsbevorderende metabolieten in het fruit verantwoordelijk zijn. De expressie van kandidaatgenen, die in de specifieke chromosoomgebieden gevonden werden, werd in de F1 populatie, onderzocht en vergeleken met de niveaus van de betrokken metabolieten.

De gedomesticeerde appel (*Malus x domestica* Borkh) wordt in vele delen van de wereld geteeld. Appel behoort tot de familie der Roosachtigen, waarin allerlei fruitsoorten vallen, zoals peer, aardbei, perzik, abrikoos, amandel en kers. Appel heeft een basis aantal van 17 paar chromosomen. Omdat appelbomen zichzelf niet kunnen bevruchten, is appel genetisch sterk heterozygoot. De veredeling van appel wordt verder vertraagd door de lange jeugdfase.

Het Engelse spreekwoord '"An apple a day keeps the doctor away" heeft vele onderzoekers aangemoedigd het 'magische' ingrediënt voor dit gezondheids-bevorderende effect in appel te zoeken. De consumptie van appel is vaak in verband gebracht met verminderd risico op ziekten zoals sommige vormen van kanker, met name prostaat-, lever- en longkanker, hart- en vaatziekten, astma, type-2 diabetes en trombose.

Het tweede hoofdstuk van dit proefschrift beschrijft de ontwikkeling van een genetische koppelingskaart voor appel, gebruik makend van DNA-merkers in de F1 populatie uit de kruising tussen de rassen 'Prima' en 'Fiësta'. Hierbij bouwden we voort op de al bestaande kaarten van Maliepaard en co-auteurs, door daaraan 240 DArT merkers toe te voegen. Het totaal aantal DNA-merkers voor de geïntegreerde kaart kwam daarmee op 820. DArT merkers zijn gebaseerd op hybridisatie van DNA-fragmenten op micro-arrays, en erven dominant over. De resultaten lieten zien dat DArT een krachtige, betrouwbare merker-methode is, met lage kosten per data-punt, en geschikt voor het maken van kaarten in diverse populaties van appel. Uit de DNA-sequenties van de DArT-merkers bleek dat DArT merkers met name voorkomen in gen-rijke gebieden.

Hoofdstuk 3 beschrijft de diversiteit van metabolieten in *Malus.* Wild materiaal werd vergeleken met elite veredelingsmateriaal en de F1 populatie van 'Prima' X 'Fiësta'. De metabolietensamenstelling werd geanalyseerd met behulp van een combinatie van scheiding door chromatografie en massa spectometrie (LC-MS). Dit resulteerde in de detectie van 418 metabolieten in de schil en 254 in het vruchtvlees.

Appels van 23 wilde soorten, acht geavanceerde veredelingsselecties, en de F1 populatie werden geanalyseerd. De data werden geanalyseerd met Principale Componenten Analyse (PCA). Variantieanalyse van de eerste principale component liet zien dat de technische variatie zich beperkte tot 1,4 % en 0,8 % in respectievelijk schil en vruchtvlees. De milieuvariatie tussen bomen van hetzelfde genotype was 2 %. De resterende 97 % van de variatie in de eerste principale component kon verklaard worden door genetische verschillen tussen genotypen. Van deze genetische variatie was slechts 8 % aanwezig in de elite-selecties. Dit wijst erop dat er een sterke genetische verarming heeft plaats gevonden tijdens de veredeling. Deze genetische erosie bleek met name uit reductie van gehaltes van diverse flavonoïden, zoals catechine, epicatechine en procyanidine.

De PCA van de F1 populatie liet een duidelijke Mendeliaaanse 3:1 uitsplitsing zien voor een groep metabolieten. Deze metabolieten waren een factor 4.2 verlaagd in nakomelingen die alleen de recessieve allelen hadden geërfd van een gen dat bij de top van chromosoom 16 gelokaliseerd is.

Verder vonden we een andere groep van 11 metabolieten in de schil en 12 in het vruchtvlees, behorend tot geglycosyleerde vormen van vluchtige alcoholen: R-octaan-1, 3-diol en de onverzadigde vorm R-5-(Z)-octene-1, 3-diol. Deze stoffen spelen een rol bij afweer tegen micro-organismen, en bij het aroma van cider. De gehaltes van deze stoffen liep in sommige nakomelingen tot 50 keer zo hoog op ten opzichte van beide ouders. Genetische kartering liet zien dat een gen bovenin chromosoom 8 verantwoordelijk was voor deze stijging. De stijging trad op in nakomelingen die de recessieve allelen van beide ouders geërfd hadden.

Dit hoofdstuk laat zien dat er niet alleen een sterke genetische erosie heeft plaats gevonden in de appelveredeling, maar ook dat inteelt kan leiden tot een sterke afname of toename in metabolieten ten opzichte van beide ouders. Deze toename en afname trad vooral op als recessieve allelen accumuleerden.

De genetische factoren die verantwoordelijk waren voor de samenstelling van metabolieten, werden nader onderzocht, door de kwantitatieve variatie in metabolieten in de F1-populatie (Hoofdstuk 3) te combineren met de genetische merkerkaart van deze populatie (Hoofdstuk 2). Dit resulteerde in detectie van chromosoomgebieden die verantwoordelijk waren voor verschillen in metabolietgehaltes in de nakomelingen (mQTLs), zoals beschreven in Hoofdstuk 4. Met de software MetaNetwork werden 669 significante mQTLs gevonden: 488 in de

schil en 181 in het vruchtvlees. Voor zover wij weten is dit de eerste genetische studie aan zo'n hoog aantal metabolieten in kruisbestuivende plantensoorten. Vier chromosomen (chromosoom 1, 8, 13 en 16) bevatten gebieden met mQTL concentraties, met name voor fenolische verbindingen, zoals sinapoyl hexoside, coumaroyl hexoside, floridzine, kinine zuren, fenolische esters, kaempferol glycosiden, quercetine glycosides, cyanidine pentoside, flavan-3-olen (catechine, epicatechine) en procyanidines. De genetica werd in meer detail onderzocht met JoinMap. Quercetineconjugaten bleken op chromosoom 1 en 13 mQTLs te hebben. Het belangrijkste concentratiegebied van mQTLs lag echter bovenin chromosoom 16: mQTLs voor 32 metabolieten in de schil en 16 metabolieten in het vruchtvlees vielen samen in dit gebied. Deze metabolieten lieten een monogene Mendeliaanse uitsplitsing van 3:1 zien. Alle deze metabolieten bleken te behoren tot de fenylpropanoïde route. Een procyanidine dimeer werd gebruikt als representant van deze metabolieten, voor afbakening van het genetische gebied waarbinnen het oorzakelijke gen voor deze concentratie van mQTLs moet liggen. In dit gebied bleek het structurele gen coderend voor leucoanthocyanidine reductase (*MdLAR1*) te liggen, evenals zeven transcriptiefactoren.

In Hoofdstuk 5 wordt de expressie beschreven van kandidaatgenen voor de concentratie van mQTLs bovenin chromosoom 16. Procyanidine werd gebruikt als representant van de metabolieten die hier gekarteerd waren. Alleen de expressie van het structurele gen *MdLAR1* bleek sterk positief gecorreleerd te zijn met het gehalte aan procyanidine. De expressie van geen enkel ander structureel gen van de fenylpropanoïde route bleek gecorreleerd te zijn met het gehalte aan procyanidine. Evenmin bleek de expressie van transcriptiefactorgenen, noch die binnen het mQTL-gebied, noch die buiten dit gebied, significant geassocieerd te zijn met procyanidine. Dit geeft aan dat *MdLAR1* de concentratie van de vele mQTLs op chromosoom 16 heeft veroorzaakt. De nakomelingen met één of twee kopieën van het dominante allel van *MdLAR1* (*Mm, MM*) lieten respectievelijk een 4,4 en 11,8 maal hogere expressie zien van *MdLAR1,* ten opzichte van de nakomelingen met alleen het recessieve allel (*mm*). Dit leidde tot een viervoudig hoger gehalte aan procyanidine in rijpe appels.

Opvallend was dat in het gebied van de mQTL concentratie op chromosoom 16 ook een locus werd gevonden voor zuur in rijpe appels. Echter, het dominante allel voor zuur bleek in afstotingsfase te verkeren ten opzichte van het dominante allel voor verhoogde gehaltes voor procyanidine (Hoofdstuk 6). Dit laat zien dat de zuurgraad door een ander gen wordt veroorzaakt dan die voor de hoge procyanidine-niveaus. De combinatie van de genetische positie, de gepubliceerde genoomsequentie van het appelras 'Golden Delicious', annotatie van potentiële genen en expressieprofielen van kandidaatgenen, wees erop dat het appelzuurtransportgen *MdALMT2* verantwoordelijk was voor de duidelijke

verschillen in appelzuurgehaltes en pH in appels in de F1 populatie. De erfenis van minimaal één dominant allel (*MaMa, Mama)* van dit gen, was voldoende voor een drievoudige toename van de appelzuurconcentratie en een verlaging van de pH van 4 naar 3 in rijpe appels, ten opzichte van de erfenis van alleen het recessieve allel (*mama*).

Appelzuur is het belangrijkste zuur in appels. Het wordt geproduceerd in het cytoplasma en wordt getransporteerd naar en opgeslagen in de vacuole. De concentratie van appelzuur beïnvloedt sterk de pH van appels. Verder heeft de pH een sterke invloed op de smaakbeleving van appels. Voor zover wij weten is dit de eerste keer dat de genetische uitsplitsing van de pH gekoppeld is aan een specifiek gen in appel. Verder is dit gen nog niet eerder gekoppeld aan pH van appels of ander fruit. Na kloneren van dit gen kan het worden gebruikt voor een functionele toets door het in rassen met een hoge pH te brengen, of door het gen stil te leggen in zure rassen. De kennis kan nu al toegepast worden door een allel-specifieke merker te ontwikkelen voor merkergestuurde selectie. Op basis van deze merkerinformatie zouden nakomelingen al in het zaailingstadium kunnen worden geselecteerd op zuurgraad van de appels, die pas vijf of meer jaren later aan de bomen zouden gaan groeien. Dit kan een forse tijdwinst opleveren in de selectie.

In een andere studie (Hoofdstuk 7) werd een dominant gemuteerd allel van het *MdMYB10* gen uit appel met de eigen natuurlijke promoter, coderende sequentie en terminator in appel, aardbei en aardappel gebracht door middel van transformatie. Dit mutante allel induceert productie van de rode stof anthocyaan in de gehele plant bij appel, al vanaf de transformatie. Met dit experiment wilden we nagaan of *MdMYB10* gebruikt kan worden als visuele selectiemerker voor getransformeerde regeneranten, als alternatief voor selectiemerkers zoals het kanamycine-resistentiegen. Na transformatie werd de kleur van het callus, de scheuten en groeiende planten beoordeeld. Rode en groene scheuten werden getoetst op aanwezigheid van het *MdMYB10* gen met behulp van PCR analyse. Alle rode scheuten van appel hadden het *MdMYB10* gen, maar niet in alle groene appelscheuten ontbrak dit gen. In aardbei bleek het *MdMYB10* gen ook te leiden tot roodverkleuring, ondanks dat dit gen uit appel afkomstig was. In aardappel was geen roodverkleuring zichtbaar. Echter, extractie met zure methanol van aardappelscheuten en –wortels liet zien dat planten met het *MdMYB10* gen een viermaal hoger anthocyaangehalte hadden dan de controleplanten van aardappel. Daarom kan worden geconcludeerd dat het *MdMYB10*-gen gebruikt kan worden als visuele selectiemerker in appel, aardbei en aardappel. In appel kan het dienen als cisgene selectiemerker.

De resultaten van de experimentele hoofdstukken worden in een breder kader besproken in de Algemene Discussie (Hoofdstuk 8). De perspectieven en uitdagingen van 'genetical metabolomics' worden er bediscussieerd. De benaderingen die in dit promotieonderzoek zijn ontwikkeld zijn niet alleen toepasbaar voor mogelijke ontwikkeling van nieuwe appelrassen of verhoging van gehaltes aan gezondsheidsbevorderende metabolieten in bestaande appelrassen, maar kunnen ook worden toegepast in 'genetical metabolomics' bij andere belangrijke gewassen.

Supplementary Materials

The supplementray materials for different chapters can be access through the following weblinks.

Chapter 3

Figure S3.1 Peel: Metabolites having much higher level in some progeny that both parents.
Link:
http://dl.dropbox.com/u/51692666/Supplementary%20Figure%20S3.1.Peel.docx

Figure S3.2 Flesh: Metabolites having much higher level in some progeny that both parents.
Link:
http://dl.dropbox.com/u/51692666/Supplementary%20Figure%20S3.2.%20Flesh.docx

Chapter 4

Table S4.1 Annotation of metabolites found in apple peel
Link:
http://dl.dropbox.com/u/51692666/Spplementary%20Table%20S4.1%20%20%20Annotation%20of%20metabolites%20found%20in%20apple%20peel.xls

Table S4.2 Annotation of metabolites found in apple flesh
Link:
http://dl.dropbox.com/u/51692666/Spplementary%20Table%20S4.2%20%20Annotation%20of%20metabolites%20found%20in%20apple%20flesh.xls

Tables S4.3,4.4 mQTL mapping of metabolites in apple peel and flesh
Link:
http://dl.dropbox.com/u/51692666/SupplenmentaryTables%20S4.3%20S4.4%20mQTL%20mapping%20of%20metabolites%20in%20apple%20peel%20and%20flesh.xls

Table S4.5 Correlation of metabolites found in peel of apple fruits
Link:
http://dl.dropbox.com/u/51692666/Spplementary%20Table%20S4.5.%20Correlation%20of%20metabolites%20found%20in%20peel%20%20of%20apple%20fruits.xls

Table S4.6 Correlation of metabolites found in flesh of apple fruits
Link:
http://dl.dropbox.com/u/51692666/Supplementary%20Table%20%20S4.6.%20Correlation%20of%20metabolites%20found%20in%20flesh%20of%20apple%20fruits.xls

Table S4.7 Additional simple sequence repeats (SSR) loci tested for LG16
Link:
http://dl.dropbox.com/u/51692666/Supplementary%20Table%20S4.7.%20Additional%20simple%20sequence%20repeats%20%28SSR%29%20loci%20tested%20for%20LG16.doc

Chapter 5

Table S5.1 Genotypes used for measuring relative expression of phenylpropanoid pathway genes and the candidate genes in the mQTL hotspot. Average sizes of eight fruits per tree of genotype are also given at each stage.
Link:
http://dl.dropbox.com/u/51692666/Supplementary%20Table%20S5.1%20Genotypes%20used%20for%20measuring%20relative%20expression%20of%20phenylpropanoid%20pathway%20genes.docx

Table S5.2 Correlations between the expression levels of the transcription factor genes that were detected in the mQTL hotspot
Link:
http://dl.dropbox.com/u/51692666/Table%20S5.2%20Correlation%20between%20relative%20expression%20of%20diffreent%20genes%20of%20the%20phenypropanoid%20pathway.xlsx

Chapter 6

Table S6.1 Genotypes used for measuring malic acid content and expression of three candidate genes. Average sizes of eight fruits per tree of a genotype are given at each stage. The malic acid content has been normalized to ribitol.
Link:
http://dl.dropbox.com/u/51692666/Supplementary%20Table%20%20S6.1%20Genotypes%20used%20for%20measuring%20malic%20acid%20content%20and%20gene%20expression.docx

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It was January, 2007, when I came to know that my utmost ambition and desire of doing PhD from an abroad well-known and advanced University in the form of Wageningen University can turn into reality. It is still very fresh in my memory when I received the PhD offer letter from Wageningen University and started my PhD on 19th October, 2007. Time flies like an arrow and it has been already now more than four years.

Many people however, have great contributions in fulfilling this dream of mine. I am very thankful to my supervisor **Prof. Dr Evert Jacobsen** for his all-time guidance. Dear **Evert**! you were the very first person who responded to my email mentioning I am interested in doing PhD in the Department of Plant Breeding. You forwarded that to Dr. Henk Schouten who was probably on holidays at that moment. Finally I received an offer letter from Henk that he is interested in my stay doing PhD in apple group. Thus the first official tie was made.

Dear **Dr. Henk**, my co-supervisor, I am very thankful for your support. I was very pleased when I received the first phone call from you but we could not properly communicate and then you sent me an email about my possible PhD in Wageningen. After all the official things done, I arrived in the Netherlands at the time when you were still sleeping. You were certainly surprised by my call. Thanks that you came and picked me up from the airport. You always encouraged me during my research and replied all my emails immediately. I am extremely thankful for all your technical guidance and support throughout my PhD. You also helped me in bringing my wife to Wageningen. Indeed I am thankful to you for that as well.

I am also very thankful to my external supervisor **Prof. Dr. Linus van der Plas** for his useful discussions during my PhD research.

I want to extend my acknowledgements and thanks to **Prof. Dr. Richard Visser**, head of the Department of Plant Breeding for giving me the opportunity to do PhD in his department. I always found it very convenient to talk to you and to settle any departmental issue. I am also very thankful to **Dr**. **Rene Smulders**, the group leader for research group of biodiversity and genetic variation, for his all times encouragement and the useful discussions during my PhD period. I enjoyed my time being part of this research group. Thanks to all the members of this group.

Many thanks to the secretariat of Plant Breeding consists of Annie, Letty, Janneke, Nicole and the former secretary Mariame Gada for their help in the administrative work. Special thanks to Annie for her help during my arrangements for conferences and courses.

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Thanks to all the collaborators namely **INOVA Fruit B.V**., people at IASMA in Italy, and Andy and Roger Hellens at Horticultural Research in New Zealand. Special thanks to **Higher Education Commission (HEC)** of Pakistan for my fellowship funding and **Netherlands Organisation for International Cooperation in Higher Education (NUFFIC)** for handling all the administrative and financial matters during my PhD period.

I enjoyed my time in a very internationalised community both in and outside the department. I am thankful to all international friends and colleagues such as Anitha, Madhuri, Sharmistha, Animesh, Paula, Cesar, Antoine, Ali Reza, Suxian, Vani, Nasim, Arwa, Reza Aghnoum, Yuni, Ram Kumar Basnat, Yusuf, Loius, Heba, Bjorn Klosterman, Bjorn Dhoop, Weicong, Xi, Jan Uitdewilligen, Maria, Xing Feng, Christos, Peter, Freddy, Manoj, Susana, and Junfei Gu.

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I am also thankful to my friends outside the Netherlands who continuously helped and encouraged me throughout my PhD. They include Niamat Khan, Meraj Ali, Saeedullah, Zahid Usman, Hazrat Umar, Abdul Qayyum, Minhaj, Tariq, Fakhar ul Islam, Zahid Hanif, Younas, Sir Ijaz and Shakir Afridi.

I am extremely thankful to my family and relatives including my **beloved parents**, **brothers**, **sisters**, **nephews**, **nieces**, **uncles**, **aunts** and **cousins** for their continuous support and good wishes throughout my studies. Thanks to my eldest brother **Farid Gul** who took up the responsibility in the very earlier age and looked after me and my other brothers and sisters to the best of his efforts despite the limited resources. Special thanks to my parents who always take care of me and supported me in every sphere of life to the best that they can. My sweet **MOTHER**!!! I will always remember your words of encouragement. You always supported me and made everything apparently much easier for me. I enjoyed all the phone calls with you especially during my weekends. I am especially very thankful to my wife for her full support, patience, undersatnding and encouragement during my PhD study.

Last but not the least, I would like to extend my thankfulness to all those who have helped me in one way or the other and I forgot to mention them by name.

Finally thanks GOD for giving me the strength to complete my PhD studies in due time course and with good health.

Sabaz Ali Khan
Wageningen University
12th January, 2012

About the author

Sabaz Ali Khan was born on 10th April, 1981 in Nowshera, Khyber-Pakhtunkhwa, Pakistan. He received his Bachelor degree in Agriculture from Khyber-Pakhtunkhwa Agricultural University Peshawar, Pakistan in 2004. He obtained his M.Phil. in Plant Physiology from Quaid-i-Azam University Islamabad, Pakistan in 2006. He worked as research officer from 2006-2007 in the Agricultural Biotechnology Institute (ABI), National Agricultural Research Centre (NARC), Islamabad, Pakistan. He started his PhD research in 2007 at the Department of Plant Breeding, Plant Research International (PRI), Wageningen University and Research Centre under the supervision of Prof. Dr. Ir. Evert Jacobsen and Dr. Ir. Henk J. Schouten. In his PhD he worked on "Genetical metabolomics in apples (*Malus* x *domestica* Borkh)" which resulted in the outcome of this thesis.

Publications

A. J. Kortstee, **S. A. Khan**, C. Helderman, L. M. Trindade, Y. Wu, R. G. F. Visser, C. Brendolise , and A. Allan, H. J. Schouten and E. Jacobsen. Anthocyanin production as a potential visual selection marker during plant transformation. Transgenic Res (2011) 20:1253–1264.

Henk J. Schouten, W. Eric van de Weg, Jason Carling, **Sabaz Ali Khan**, Steven J. McKay, Martijn P. W. van Kaauwen, Alexander H. J. Wittenberg, Herma J. J. Koehorst-van Putten, Yolanda Noordijk, Zhongshan Gao, D. Jasper G. Rees, Maria M. Van Dyk, Damian Jaccoud, Michael J. Considine and Andrzej Kilian. Diversity arrays technology (DArT) markers in apple for genetic linkage maps.
Mol Breeding: DOI 10.1007/s11032-011-9579-5

Submitted

Sabaz Ali Khan, Pierre-Yves Chibon, Ric C.H. de Vos, Bert A. Schipper, Evert Walraven, Jules Beekwilder, Thijs van Dijk, Richard Finkers, R.G.F Visser, Eric W. van de Weg, Arnaud Bovy, Alessandro Cestaro, Riccardo Velasco, Evert Jacobsen and Henk J. Schouten. Genetical metabolomics in apple indicates an mQTL hotspot for phenolic compounds on Linkage Group 16.

Sabaz Ali Khan, Jules Beekwilder, Jan G. Schaart, Roland Mumm, Evert Jacobsen and Henk J. Schouten. Differences in acidity of mature apples are mainly caused by a malic acid transporter gene on LG16.

In preparation

Sabaz Ali Khan, Yury Tikunov, Pierre-Yves Chibon, Chris Maliepaard, Jules Beekwilder, Evert Jacobsen and Henk J. Schouten. Loss and gain of metabolites during apple breeding.

Sabaz Ali Khan, Jan G. Schaart, Jules Beekwilder, Andrew C. Allan, Evert Jacobsen and Henk J. Schouten. The *leucoanthocyanidin reductase* gene causes an mQTL hotspot on LG16 for phenolic compounds in apple fruits.

Education statement of the Graduate School Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Sabaz Ali Khan
Date: 12 January 2012
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Identification of genes responsible for health beneficial polyphenolic compounds in apple fruits	Apr 18, 2008
► **Writing or rewriting a project proposal**	
Identification of genes responsible for health beneficial polyphenolic compounds in apple fruits	2008
► **Writing a review or book chapter**	
► **MSc courses**	
Cell Physiology and Genetics	Jan-Feb 2008
Gene Technology	Apr-Jul 2008
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Feb 26, 2009
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, Wageningen University	May 20, 2011
► **EPS theme symposia**	
EPS theme 4 'Genome Plasticity', Wageningen University	Dec 12, 2008
EPS theme 4 'Genome Plasticity', Radboud University Nijmegen	Dec 11, 2009
EPS theme 4 'Genome Plasticity', Wageningen University	Dec 10, 2010
EPS theme 4 'Genome Biology', Wageningen University	Dec 09, 2011
► **NWO Lunteren days and other National Platforms**	
Meeting 'Expiremental Plant Sciences', Lunteren	Apr 07-08, 2008
Meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
Meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
► **Seminars (series), workshops and symposia**	
European Flying Seminar - Prof.dr. Hiroo Fukuda,Prof.dr. Hiroo Fukuda, Dept. of Biological Sciences, The University of Tokyo, Japan.Title: 'Short-distance intercellular signaling machineries to form organized vascular tissues	Nov 26, 2007
Seminar 'Proteomics of ripening initiation in wine grapes: a closer look at anthocyanin biosynthesis' by Dr. Joost Lucker	Dec 18, 2007
European Flying Seminar Prof.dr. Simon Gilroy, Title: 'How do plants feel? Mechanical Signaling in Arabidopsis'	May 19, 2008
Seminar on "partitioning the genome: mechanisms that ensure accurate chromosome segragation in cell division" by Dr Michael Lampson, departmant of biology university of pannsylvania, USA.	Feb 01, 2008
Research Day plant breeding, Hof van Wageningen	Jun 17, 2008
Prof. Harro Bouwmeester, Title "The physiology of the interaction of plants with their biotic and abiotic environment"	Nov 30, 2007
Mechanism and function of active DNA demethylation in Arabidopsis, Prof. Jian-Kang Zhu	Nov 03, 2008
'Science from an editor's view; science organization, tips about being an author and a referee, programs at AAAS, Alternative careers etc" by Dr. Pamela J. Hines, USA.	Nov 06, 2008
Towards an individual based model for simulating introgression of abiotic stress tolerance transgenes in wild relatives"by Dr D.A.P. Hooftman, university of Amsterdam.	Dec 01, 2008
Colloqia Joinmap 4.0	Dec 15-20, 2008
Seminar on the activities of AVRDC by dr. Dyno Keatinge	Jun 18, 2009
Linkage disequilibrium and association mapping – helping to overcome the paradox of modern plant breeding, by Dr Wallace Cowling	Jun 26, 2009
Seminar, "Seeds, microRNA and Darwin?" by Dr. Hiro Nonogaki , Seed Biology at Oregon State University, USA.	Sep 17, 2009
EPS symposium "Ecology and Experimental Plant Sciences 2"	Sep 22, 2009
Connecting genetics and genomics of pathogenicity and behavior in root-knot nematodes, Valerie Williamson – Dept of Nematology, University of California, Davis, US	Oct 23, 2009
Research Day plant breeding, Hof van Wageningen	Feb 08, 2010
First Agrigenomics workshop, by Illumina	Mar 03, 2010

Genetic, functional analysis of disease resistance in Brassica by Régine Delourme from INRA	Oct 05, 2011
CBSG technology symposium "advances in life-science technologies" Hof van Wageningen	Nov 25, 2010
Fluidigm demo lecture, Room W 0.1 Radix building	Feb 08 2011
Research Day plant breeding, Hof van Wageningen	Mar 08, 2011
The genome of Dothistroma septosporum, a close relative of Cladosporium fulvum; what have we learnt so far? Prof. dr. Rosie Bradshaw, Associate Professor of Genetics, Institute of Molecular BioSciences, Massey University, Manawatu Campus, Palmerston North, New Zealand	Aug 04, 2011
'How to efficiently measure circadian hyponastic movements of single Arabidopsis leaves?' by Dr. Thomas Greb	Oct 12, 2011
EPS Mini symposium 'Plant Breeding in the genomics era'	Nov 25, 2011
Minisymposium on sequencing technologies organised by ServicesXS	Dec 08, 2011
Short course on "Reviewing a Scientific paper" organised Graduate School Wageningen UR	Dec 20, 2011
► **Seminar plus**	
I attended a separate meeting with Prof. Hiroo Fukuda	Nov 26, 2007
► **International symposia and congresses**	
First international symposium on biotech fruits, Dresden (Germany)	Sep 01-05, 2008
Technology Workshop on Marker Assisted Selection: from discovery to application	Feb 09-10, 2010
Cost 864 pome fruit health working group 4 meeting	Feb 11-12, 2010
5th Rosaceous Genomics Conference, South Africa	Nov 14-17, 2010
Cost 864 pome fruit health working group 4 meeting	Feb 01-03, 2011
► **Presentations**	
EPS PhD student day, Leiden University, poster presentation	Feb 26, 2009
Plant breeding research day, poster presentation	Mar 03, 2009
Technology Workshop on Marker Assisted Selection: poster presentation from discovery to application	Feb 09-10, 2010
Cost 864 pome fruit health working group 4 meeting, oral presentation	Feb 11-12, 2010
EPS PhD student day, Utrecht; poster presentation	Jun 01, 2010
5th Rosaceous Genomics Conference, South Africa' oral presentation'	Nov 14-17, 2010
Cost 864 pome fruit health working group 4 meeting, oral presentation	Feb 01-03, 2011
► **IAB interview**	Dec 04, 2009
► **Excursions**	
Subtotal Scientific Exposure	*20.4 credits**
3) In-Depth Studies	date
► EPS courses or other PhD courses	
Basic Statistics	Dec 15-22, 2009
Bioinformatics: A User Approach	Mar 15-19, 2010
Transcription Factors and Transcriptional Regulation	May 09-11, 2011
► Journal club	
Member of literature discussion group of Plant Breeding	Oct 2007- Nov 2011
► Individual research training	
Subtotal In-Depth Studies	*7.2 credits**
4) Personal development	date
► **Skill training courses**	
PhD Competence Assessment	Feb 19, 2008
Information literacy fo PhD, including introduction Endnote	Jun 09-10, 2009
Workshop on "Scientific Publishing"	Nov 19, 2009
English Academic Writing II	Sep 23-Feb 02, 2010
Mini symposium "how to write a world class paper" Wageninen UR Library	Oct 26, 2010
Techniques for writing and presenting a scientific paper	Feb 15-18, 2011
Training - Speed reading By KLV	Oct 04, 2011
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.5 credits**
TOTAL NUMBER OF CREDIT POINTS*	**45.6**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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