# Genetic dissection of the dwarfing effect of the apple rootstock 'M.9'

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## ABSTRACT

The dwarfing phenomenon in apple is mainly characterized by the ability of a rootstock to reduce the vegetative growth and ultimate size of the scion grafted onto it. Many hypotheses have been proposed to explain the dwarfing effect of rootstocks, from the production and translocation of hormones to the altered anatomy of the graft union. However, despite the numerous studies undertaken, none of the studies support a single hypothesis. This study focussed on identifying genetic markers for genomic regions influencing the dwarfing effect of 'Malling 9' ('M.9').

Two rootstock populations derived from crosses between 'M.9' and the vigorous rootstock 'Robusta 5' ('R5') were propagated and phenotyped at the HortResearch Havelock North Research Centre (New Zealand). Bulked segregant analysis (BSA) and QTL analysis were performed using phenotypic data collected from scions grafted onto the rootstock populations. Several genomic regions in 'M.9' and 'R5' were found to have a significant influence on the dwarfing phenotype and other related traits. The percentage of variation explained by these QTLs ranged from 4.2% to 57.2%. A large number of traits had significant variation associated with the major dwarfing QTL *DW1* (identified previously by BSA), confirming the influence of this gene on tree architecture.

To identify the genes responsible for the dwarfing effect, a microarray analysis on RNA extracted from bark tissues was performed to detect genes differentially expressed among dwarfing and vigorous rootstocks derived from the 'M.9' × 'R5' family. Following the mapping of 16 markers developed from 12 candidate genes, their position was compared with those of the QTLs identified previously and co-localisations among genes and QTLs were identified. Results to date indicate that none of these particular CGs co-segregate with *DW1*.

In order to estimate the number of different genetic sources of dwarfing present in commercial rootstocks, two SSR markers mapping about 0.5 cM away from the dwarfing QTL *DW1* were screened over 58 rootstock accessions. The majority of the dwarf and semi-dwarf accessions screened carried the locus *DW1*, indicating that there may be only a single genetic source of dwarfing in apple rootstocks.

The identification of markers for dwarfing will have a major impact on apple rootstock breeding, which currently relies on laborious phenotyping of individuals in breeding populations that presently takes at least 5-7 years to adequately perform. The identification of the genetic function of *DW1* would provide an opportunity to develop

dwarfing rootstocks for other members of the Rosaceae family for which such rootstocks have not yet been developed.

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# List of abbreviations

| | |
|---|---|
| ABA | abscisic acid |
| ANOVA | analysis of variance |
| AFLP | amplified fragment length polymorphism |
| BAC | bacterial artificial chromosome |
| bp | base pair |
| BSA | bulked segregant analysis |
| °C | degrees Celsius |
| cDNA | complementary DNA |
| CG | candidate genes |
| cm | centimetre |
| cM | centiMorgan |
| cv. | cultivar |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| *DW1* | dwarfing locus 1 |
| *DW2* | dwarfing locus 2 |
| DW% | overall dwarfing phenotype |
| EDTA | ethylenediaminetetraacetic acid |
| EST | expressed sequence tag |
| FDR | false discovery rate |
| GAs | gibberellins |
| gDNA | genomic deoxyribonucleic acid |
| GDR | Genome Database for Rosaceae |
| IAA | indole-3-acetic acid |
| indel | insertions and deletions |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| Kb | kilo base |
| KS | Kolmogorov-Smirnov |
| L | litre |
| LG | linkage group |
| LOD | logarithm of odds |
| m | metre |
| 'M.9' | 'Malling 9' |
| MAS | Marker assisted selection |
| mg | milligram |

| | |
|---|---|
| min | minute |
| ml | millilitre |
| mM | millimolar |
| ng | nanogram |
| MQM | multiple QTL model |
| mRNA | messenger RNA |
| ORFs | open reading frames |
| PCR | polymerase chain reaction |
| qPCR | quantitative PCR |
| QTL | quantitative trait loci |
| 'R5' | 'Robusta 5' |
| RAPD | randomly amplified polymorphic DNA |
| Rep | repetition |
| RFLPs | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| s | second |
| SCAR | sequence characterized amplified region |
| SIM | simple interval mapping |
| SNP | single nucleotide polymorphism |
| SSR | simple sequence repeat |
| STSs | sequence tagged site |
| TAE | tris acetic acid EDTA |
| TAIR | The Arabidopsis Information Resource |
| TCA | trunk cross-sectional area |
| TIGR | The Institute for Genomic Research |
| Tris | tris(hydroxymethyl)aminomethane |
| Ty medium | tryptone yeast extract media |
| µg | microgram |
| µl | microlitre |
| µM | micromole |
| UTR | untranslated region |
| UV | ultra violet |
| Xgal | 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside |
| YAC | yeast artificial chromosome |

# 1- INTRODUCTION

## 1.1 Apple and apple rootstocks

### 1.1.1 Overview of apple and apple rootstocks

#### 1.1.1.1 Apple production

Apple is the most ubiquitous species among the temperate fruit crops and grows in high latitude regions of the world, where minimum temperatures may reach -40°C, up to a high elevation, as well as in the tropics where two crops may be grown in a single year (Janick, 1974). In terms of production, apples are the third biggest fruit crop in the world, with an estimated 62 million metric tonnes (MT)), behind banana (68 million MT) and grapes (66 million MT) (FAO 2005). Apple production relies on very few cultivars, mainly 'McIntosh', 'Jonathan', 'Cox's Orange Pippin', 'Granny Smith', 'Delicious', 'Golden Delicious', 'Braeburn', 'Royal Gala', 'Jonagold', 'Fuji' and 'Elstar'.

#### 1.1.1.2 Taxonomy

Apple (*Malus*) belongs taxonomically to the Rosaceae family, along with other economically important temperate fruit crops. Together with other closely related fruit (*Pyrus* and *Cydonia*), and ornamental (*Amelanchier*, *Aronia*, *Chaenomeles*, *Crateagus*, *Pyracantha*, *Sorbus*, *Cotoneaster*) genera, they form the subfamily *Maloideae*. The genus *Malus* comprises some 55 species, although between eight and 79 species have been recognized (Harris *et al.* 2002). The scientific names that have been applied to the domesticated apple include *Malus pumila* Miller, *M. communis* Desf., *M. sylvestris* (L.) Miller, and *Malus domestica* (Borkh) (Mabberley *et al.* 2001, Harris *et al.* 2002). In this thesis, following Korban and Skirvin (1984), I will use *Malus domestica* (Borkh) when referring to apple, although most recent published opinion (Kartesz and Gandhi, 1992) argues for *M. sylvestris* (L.) Miller.
The origin of the *Maloideae* subfamily has also been the subject of some debate. The basic chromosome number x = 17 suggests a polyploid origin since other Rosaceae have x = 7, 8 or 9. Autopolyploidy was suggested by Darlington and Moffet (1930) but more recently the possibility of an allopolyploid origin with *Amygdaloideae* (x = 8) and *Spiraeoideae* (x = 9) has been advanced (Phipps *et al.* 1991; Lespinasse *et al.* 1999).

The common domesticated apple is usually designated *Malus* × *domestica* Borkh. (Korban and Skirvin, 1984) or *M. domestica* Borkh (Phipps *et al.* 1990). Most *Malus* species are diploids (2n=34), but triploid and tetraploid species have been observed (Way *et al.* 1989).

### 1.1.1.3 The apple tree

A whole apple tree used for commerce usually constitutes a rootstock onto which a scion is grafted. Occasionally, a third distinct component, the interstock, is grafted between the rootstock and the scion (Webster and Wertheim 2003) (Figure 1.1). Rootstocks have been developed by horticulturists originally as an aid in the propagation of selected scions but with apple today, also largely for control of scions growth.

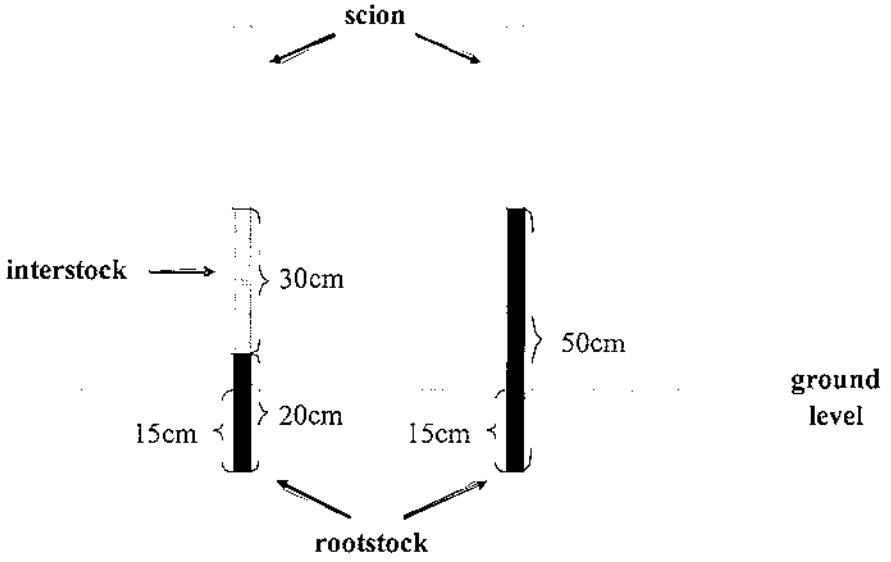



**Figure 1.1. Standard size and position of rootstock/interstock segments of apple tree.** Trees with both rootstock and interstock (left) and rootstock only (right) in the apple rootstock/interstock system are illustrated (Figure provided by Alla Seleznyova).

Most apple rootstock clones are derived from *Malus* species including *M. prunifolia* Willd., *M. baccata* (L), Borkh., *M. pumila* (Malling series), *M. sylvestris* Miller, *M. micromalus*, *M. niedzwetskyana* ('Red Standard'), *M. floribunda*, *M.* × *robusta* ('Robusta 5') and *M.* × *domestica* ('Northern Spy') (Ferree and Carlson 1987; Webster 2002).

#### 1.1.1.4 Origins and History

Although the origin of apple is not entirely clear, it probably evolved from extensive forests of apples in central Asia, particularly in Kazakhstan, Kyrgystan, Uzbekistan, Turkmenistan and Tajikistan (Ferree and Warrington 2003, Harris *et al.* 2002).

It is established that rootstocks have been used in the propagation of apple trees for more than 2000 years (Webster and Wertheim 2003). Historians believe that dwarfing apple rootstocks were used in Greece during the Hellenistic period (after 323 BC). Alexander the Great was reported by the Greek botanist and philosopher Theophrastus to have brought dwarfing apple trees (or seeds) from Asia Minor (Turkey, Caucasus mountains, Iran).

The extension of apple cultivation throughout Europe was realised via the expansion of the Roman Empire. By the 1500s to 1800s, apples were grown on two general types of dwarfing rootstocks in France: Doucin and Paradise. In the 19$^{th}$ century, the apple rootstock 'Jaune de Metz' later renamed by Hatton (1916) as 'Malling 9' ('M.9') was discovered and has since become the industry standard for dwarfing rootstocks in apple. The parentage of this rootstock is unknown and the sequence of events associated with its selection is obscure (Carrière 1879).

#### 1.1.1.5 Dwarfing rootstocks and interstocks

Dwarfing rootstocks are considered to be rootstocks which when grafted with scions, produce mature trees that are significantly smaller than trees grown on their own roots. Trees that grow slowly (by a reduced annual shoot growth) but at maturity are of large size are not considered as being part of the dwarfing category (Rusholme Pilcher *et al.* in press).

Interstocks were traditionally used when graft compatibility between the rootstock and the scion was a problem. Interstock use in apple is applicable when the desired dwarfing rootstock is difficult to propagate on its own roots or when the soil is not appropriate for the use of dwarfing rootstocks. The use of interstock can provide an alternative way of regulating the vigour of shoot growth on the scions (Wertheim and Callesen 2000).

### 1.1.1.6 Uses and benefits of dwarfing rootstocks and interstocks

Many of the important tree crops such as apple, peach, apricot, cherry or plum, use rootstocks as a way to improve production efficiency (Webster *et al.* 2000). Most fruit growers are seeking trees with limited height to facilitate more efficient production, harvesting, pruning and thinning operations. Smaller trees are also easier to spray, which reduces spray drift and increases efficiency of spray usage. Because they have reduced vegetative growth they can be planted close together to increase yield efficiency of orchards, especially precocious production; they require less pruning so labour cost is reduced and they produce more uniform sized and coloured fruits (Sarwar *et al.* 1998).

Clonal apple rootstocks offer a wide range of scion vigour control, ranging from super dwarfing to very vigorous. Currently, apple producers favour dwarfing selections, essentially 'M.9', resulting in a tree size of no more than three to four metres in height (Webster 2004).

In apple, a dwarf growth habit is the aim of many rootstock breeding programmes (Welander 1988). With apple, the degree of dwarfing of the scion can be influenced by the height of the graft. Increasing the length of rootstock stem between the roots and the graft union can increase the degree of dwarfing in the scion, especially with dwarfing and semi-dwarfing rootstocks (Parry 1986). Insertion of a dwarfing interstock between a combination of vigorous rootstock and scion reduces the scion vigour (Felius and Toorenaar 1959; Carlson and Robitaille 1970). The dwarfing effect increases with the length of the interstock, to an upper limit of 20 cm, beyond which no further dwarfing is attained (Kamboj *et al.* 1999a). In some cases, the vigour of trees using an interstock can be the same as if the same trees were grafted onto the corresponding rootstocks (Parry and Rogers 1972). All these observations imply that in apple, the dwarfing effect is partly due to the rootstock stem. It also suggests that these stems modify the exchange of hormones, water and nutrients between the root system and the scion crown and *vice versa.*

Dwarfing rootstocks and interstocks influence floral precocity (Tydeman 1937), either directly or indirectly by their effects on scion branching. Precocity is defined as the capacity to bear prolific quantities of blossom and comparatively heavy crops of fruit relatively early in the life of the tree (Tydeman 1937). The juvenile period, determined by the appearance of the first inflorescences, can be very long in apple and last up to 12 years (Fischer 1994). A short non-flowering phase is required to reach productivity in the three to four years after grafting. Using dwarfing rootstocks

has proven to be an efficient way to shorten the time until first flowering (Visser 1970; Aldwinckle 1975; Fischer 1994). All dwarfing rootstocks appear to have the effect of reducing the duration of the non-flowering phase (Tustin, personal communication). The precocity of flowering, and the ability of those flowers to set, retain, and size fruits, is crucial to the profitability of commercial tree fruit orchards. Substantial yields of high quality fruits early in the life of an orchard is essential if investments in fruits growing are to be worthwhile, particularly with high density plantings on dwarfing rootstocks (Webster 1995).

Rootstocks and interstocks are not the only means available to horticulturalists for the control of tree size. Each scion has an intrinsic vigour that influences its growth. Climatic factors, such as temperature, light and rainfall, as well as soil factors, like depth, mineral content and water availability must be taken into account. Management factors such as irrigation, nutrition, pruning (including root pruning) and weed control play an important part in the control of scion development. Tree factors like tree health and crop loading, which influences competition between fruit and shoot development for water, minerals, and assimilates also play a part in the architectural vigour of the developed scion (Webster 1995).

#### 1.1.1.7 Overview of the major apple rootstocks and breeding programs

At the end of the 19$^{th}$ century, the number of different rootstocks used throughout Europe became relatively important, and because of the confusion arising due to their various effects on grafted scions, it became essential to classify them (Manhart 1995).

##### *1.1.1.7.1 The Malling Apple rootstocks*

In 1912, at the East Malling Research Station in Kent, England, all the different rootstocks known were gathered together and planted out for propagation. These rootstocks were standardized lines of well known vegetatively propagated rootstocks, some of them several centuries old.

From 1913 to 1935, R. Wellington followed by Lord Hatton and Dr. Tydeman identified 26 types of rootstocks, from 'M.1' to 'M.26' (Manhart 1995). Because of the various locations where the rootstocks were collected, the relationship among them is unknown. The dwarfing rootstock 'Malling 9' originates from this collection. 'Malling 9' ('M.9'), also called 'Jaune de Metz', 'Yellow Metz', 'Yellow Paradise of Metz', and 'Dieudonne', was selected as a chance seedling in France in 1879. The

leaves are large, shiny and flat (Figure 1.2). The shoots are straight, reddish yellow with silvery sheen and brittle. Scions grafted to this rootstock are dwarfed, about two metres high, and have a short juvenile phase (Webster 1995). 'M.9' is susceptible to woolly aphid (*Eriosoma lanigerum* Hausm.) and fire blight (*Erwinia amylovora* (Burrill) Winslow *et al.*), but resistant to collar rot.

**Figure 1.2. Illustration of a shoot (left) and leaf (right) of a 'M.9' tree**
(http://www.agric.nsw.gov.au/reader/7285)

*1.1.1.7.2 The Malling-Merton apple rootstocks*

In 1922, the John Innes Horticultural Institute then at Merton (England) and the East Malling Research Station at East Malling, began to raise a series of rootstocks by systematic plant breeding. This project was aimed at improving the range of stocks available, especially by introducing a resistance to woolly aphid which was then an important problem for fruit growers in the Southern Hemisphere former British colonies of South Africa, Australia and New Zealand. 'Northern Spy' was used as a parent for its resistance to woolly aphid, and various 'Malling' and other rootstocks were used as the other parent. The two stations released 'M25', 'M26', 'M27' and the 'Malling-Merton' ('MM') series, 'MM101' – 'MM115' (Cummins and Aldwinckle 1983). 'Merton 793', which is widely used, was also developed by the two institutes.

*1.1.1.7.3 The Cornell Geneva ('CG') series*

In 1968 the New York State Experimental Station in Geneva started a breeding programme principally aimed at resistance to fire blight and *Phytophthora*. Some rootstocks derived from this programme have recently been released. They cover a wide range of tree size control with improved winter hardiness, such as 'CG 202',

'CG 210' and 'CG 179'. Moreover, 'CG 179', 'CG 202' and 'CG 210' are resistant to fire blight, woolly apple aphid and *Phytophthora* (Wertheim 1998).

#### *1.1.1.7.4 Important accessions from other breeding programmes*

The other important rootstocks utilized in breeding programmes include 'Northern Spy' (originated at East Bloomfield in western New York in 1828), *Malus* 'Robusta 5', 'Ottawa 3' and 'Vineland 1' (from V. series) rootstocks developed in Canada, 'Bemali' selected in Balsgard in Sweden, 'Marubakaido' selected in Japan, and finally 'Aotea' (or 'Aotea 106') bred in New Zealand (Wertheim 1998).

### 1.1.1.8 Classification of rootstock according to their dwarfing effect

Rootstocks are traditionally classified into six categories: very dwarfing; dwarfing; semi-dwarfing; intermediate (also known as semi-vigorous); vigorous and very vigorous. Recently a new method of classification has been proposed. It consists in classifying rootstocks based on the percentage of growth of the scion compared to a tree grown on its own roots. However this method is suitable for a specific location only as the effects of the rootstocks vary depending on the environment in which the tree is grown (Fazio, personal communication).

The figure 1.3. represents a classification of the Malling series, the Malling-Merton series as well as some accessions originating from Cornell Geneva and other breeding programmes. These accessions have been classified into the six categories mentioned above, with the help of tree physiologist Stuart Tustin, according to growing conditions in the apple growing region of Hawkes Bay (North Island, New Zealand).

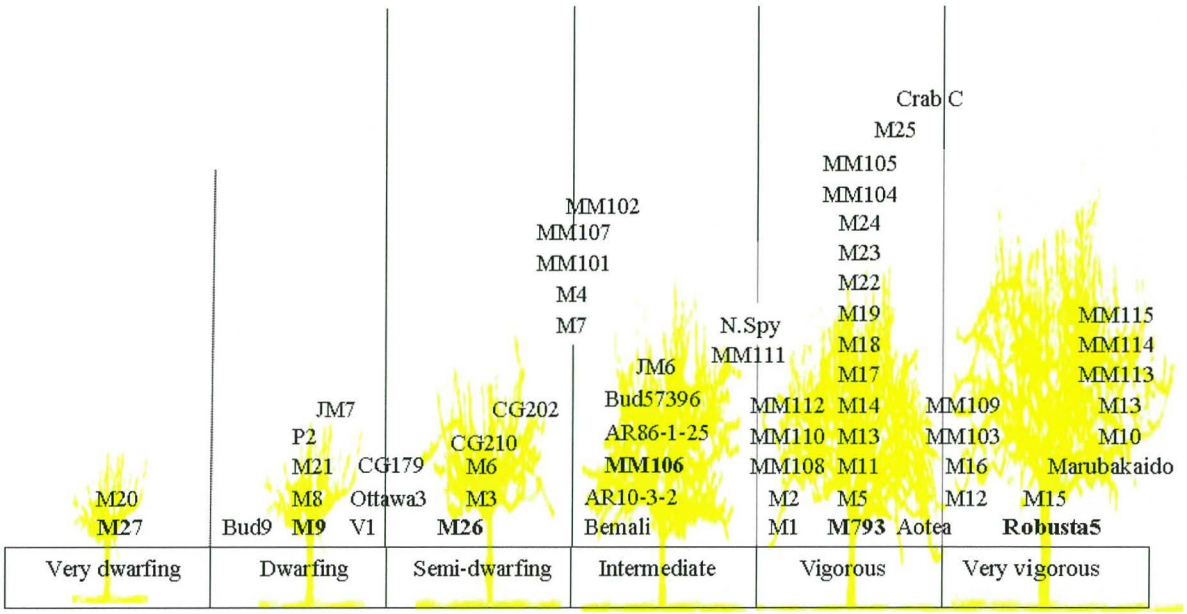



**Figure 1.3. Classification of major apple rootstocks according to their dwarfing effect.** Classification based on the growing conditions of Hawkes Bay (North Island, New Zealand). Rootstocks are organized in the order of their dwarfing effect on apple scions. The standard rootstock of each category is indicated in bold (ex. 'M.9' for the dwarfing category).

#### 1.1.1.9 Dwarfing rootstocks in other members of the Rosaceae family

Dwarfing rootstocks for other deciduous (temperate) fruit species are of more recent origin than apple (Webster 2004). Rootstocks used for propagating stone fruits such as peaches, apricots, cherries and plums are very often of different species from the fruiting scion species, and some of these rootstocks may be hybrids (Webster and Wertheim 2003). It is only in recent years that dwarfing rootstocks have been reported for these crops and are commercially available.

Pear cultivars, particularly European pears, are mainly propagated by means of common pear (*Pyrus communis*) or quince (*Cydonia oblonga*) rootstocks. Some pear rootstocks such as 'Kosui' and 'La France' have a dwarfing growth habit when grafted to selected scions (Robbani *et al.* 2006).

### 1.1.2 Rootstock effect on scion development

#### 1.1.2.1 Factors influencing scion development

The mechanisms by which rootstocks influence tree size in apple have not been fully understood. Over the years, several theories have been proposed in an attempt to explain, in anatomical, nutritional, hormonal, or other physiological terms, the dwarfing effect of rootstocks on scion performance (Webster 2004). The majority of

these theories suggest that the rootstock and interstock bring about their effect upon the scion by influencing the amount and/or ratios of growth promoting and inhibiting endogenous hormones and their distribution within the tree. The movement of assimilates (sugars and amino acids) or mineral elements between the scion and rootstock, and the amount of water taken up and moved through the rootstock/interstock to the scion have also been considered (Lockard and Schneider 1981).

#### 1.1.2.2 Effects of dwarfing rootstocks on scion development

The dwarfed growth habit is characterized by a reduced number of vegetative shoots and a corresponding increase in the number of fruit spurs (Eaton and Lapin 1970). Similarly, Costes and Garcia-Villanueva (2007) found that the two main effects of dwarfing rootstock on the aerial development of trees were a reduction in the number of axes developed per tree and an enhancement of flowering. Seleznyova *et al.* (2003) determined that the dwarfing effect of 'M.9' is mainly due to a reduced number of nodes per extension growth unit of the scion. As the number of extension growth units produced in the next cycle is proportional to this, it results in the expression of the dwarfing phenotype over time.

This reduction in the number of nodes per extension growth unit is not the only effect brought about by the rootstock and many other outcomes have been documented (Webster 2004). Rootstocks can delay scion vegetative budburst and the onset of shoot extension in spring, resulting in a shorter shoot growth period. They can significantly influence both rates of shoot extension and the timing of termination of active extension growth (Webster 1995). Rootstocks influence tree development by having a significant effect on the degree of scion branching, the individual length of shoots (Warner 1991) and the angle of branching for scions with upright growth habit (as opposed to spreading growth habit) (Tworkoski and Miller, 2007). Seleznyova *et al.* (in press) also demonstrated that dwarfing rootstocks and interstocks accelerate the natural process of tree aging by reducing both the proportions of extension shoots and their cyclicity commencing in the second year of tree growth. Many studies have shown that branches grow less vigorously when they are inclined, either by tree training or naturally, towards the horizontal (Webster 2004). Rootstocks may also have an indirect consequence on tree habit by affecting the quantity of blossoms produced and the precocity of cropping: heavy or light fruit

#### 1.1.2.4 Rootstock effect on resistance to diseases and gene expression in apple tree scion

The susceptibility of different rootstocks and scion cultivars to various diseases can vary substantially (Wilcox 1994). A given cultivar can have different levels of disease resistance depending on the rootstock to which it is grafted. For example, 'Delicious' cultivar scions grafted onto 'M.9' rootstocks have a higher susceptibility to fire blight compared to the same cultivar grafted onto 'M.7' rootstocks (Carlson *et al.* 1970; Cline *et al.* 2001). In general, it is recommended that scion cultivars susceptible to fire blight be grafted to resistant rootstocks (Jensen *et al.* 2003). This indicates that the rootstock may influence the expression of resistance genes in the grafted scion. Jensen *et al.* (2003) studied the level of expression of genes in 'Gala' scions grafted onto different rootstocks. Scions grafted to the dwarfing rootstock 'M.9 T337' (clone of 'M.9') showed higher expression of genes related to photosynthesis, transcription/translation and cell division, while scions grafted to the intermediate rootstock 'M.7 EMLA' showed increased stress-related gene expression. Some of these differentially expressed genes may be linked to the dwarfing effect of the 'M.9' rootstock.

#### 1.1.2.5 Effect of rootstock organs on scions

When considering possible mechanisms of rootstock influence on scion development, it may be useful to evaluate which parts of the rootstock may be involved in the dwarfing effect.

The roots are the most important part of a rootstock and may contribute significantly to the dwarfing effect. Roots of dwarfing rootstocks have increased proportions of phloem and bark and reduced proportions of xylem (Beakbane 1949; 1953). Also many dwarfing rootstocks have smaller and shallower root systems than invigorating rootstocks.

Reports in the literature have often suggested the implication of the graft union in the dwarfing effect of some rootstocks. Its potential role in the dwarfing effect is presented in section *1.1.3.3.1.*

The stem or shank of the rootstock contributes significantly to the dwarfing effect, and its role is described below (section *1.1.3.3.2.*).

### 1.1.3 Hypotheses explaining the dwarfing effect of apple rootstocks

#### 1.1.3.1 Changes in hormones production and translocation

The dwarfing effect of apple rootstocks is a complex subject that has been the object of a multitude of studies. The most investigated hypotheses concern the changes in hormone concentrations between the rootstock and the scion.

Plant hormones, also known as phytohormones, plant growth substances or plant growth regulators, are naturally occurring substances which, in low concentrations, regulate plant functions. Plant hormones encompass all compounds, natural and synthetic, which when applied to plants evoke a specific physiological response (Tomić, 1998). Five 'classical' endogenous plant hormones (groups) exist: auxins, gibberellins, cytokinins, abscisic acid and ethylene. All these hormones influence vegetative growth mainly by affecting cell division and elongation (Kende and Zeevaart, 1997).

Hartmann and Kester (1990) have hypothesised that dwarfing rootstocks would limit scion growth because of their reduced production of growth promoting hormones (mainly auxins, cytokinins and gibberellins), or by lowering the basipetal auxin transport (from the stem to the roots) in their tissues.

##### *1.1.3.1.1 Auxins*

###### 1.1.3.1.1.1 Site of synthesis and role in plant development

Auxins, of which indole-3-acetic acid (IAA) (Thimann 1977) is the most abundant, are synthesized predominantly from tryptophan in growing regions of plants such as apical meristems and young roots (Kerr and Bennett 2007).

Auxins have a wide range of effects on plant development. They influence vascular tissue differentiation, flowering and fruit development, root and shoot development, phototropism, gravitropism and senescence, by up-regulating the transcription of genes whose promoter regions contain auxin-responsive elements (Kerr and Bennett 2007). This hormone may thus play a significant role in growth regulation of dwarfing apple rootstocks and scions.

###### 1.1.3.1.1.2 Auxin translocation and effect on stem vascular differentiation

One of the most studied roles of auxins in dwarfing apple rootstocks is their effect on the rootstocks stem vascular tissues. Auxins have been shown to be important in stimulating cambial activity and xylem development in many species (Digby and

Wareing 1966; Zarkrewski 1983). In 'M.9' rootstocks, the xylem linking the bud to the rootstock contains fewer and smaller vessels (Figure 1.4) than in a rootstock with intermediate vigour such as 'MM106' (Soumelidou *et al.* 1994 a). This difference may indicate that the level of growth regulators, such as auxins, reaching these tissues in 'M.9' is lower than that in 'MM106'.

To summarize this hypothesis, it may be a failure of auxins from the bud to enter rootstock tissues, via the graft union, in sufficient quantities that is responsible for the low levels of xylem formation in the rootstock. Dwarfing rootstocks may also have an inactivation mechanism for metabolising auxin into an inactivate form (Soumelidou *et al.* 1994b), resulting in low levels of xylem formation. This in turn leads to a reduced supply of water and minerals to the scion with consequent reduction of scion growth (Soumelidou *et al.* 1994 a).

1.1.3.1.1.3 Auxins effect on root growth

The level of auxins in root tissues influences root growth by stopping or promoting organ growth at various stages of development (Celenza *et al.* 1995), which in turn influences the synthesis of other hormones such as cytokinins and gibberellins, which are then exported to the shoot via the xylem (Muday and Haworth 1994).

As plants maintain a constant shoot-root ratio, auxins produced in the shoot and translocated to the roots may be a mechanism for control of root growth by the shoot (Torrey 1976; Goodwin *et al.* 1978). A reduction in the level of shoot synthesized auxin reaching the root tissue, via a disorganised vascular system or through metabolic inactivation by the rootstock stem tissues (see section 1.1.3.1.1.2), may reduce root growth which would in turn reduce scion development.

1.1.3.1.1.4 Auxin effect on cell extensibility and metabolite availability

One important role of auxins is to regulate cell extensibility. Auxins may activate the enzyme dextranase that would break down a highly branched compound (dextran) located in the cell walls. The break down of this compound would result in an increase of the wall elasticity that is necessary for the cell elongation (Heyn 1970). A decrease in auxin concentration would affect cell extensibility and thus shorten internodes, which may be a factor contributing to dwarfed scions.

Auxins are also involved in carbohydrate metabolism (Colby 1935) and nutrient translocation (Davies and Wareing 1964). A reduction in auxin concentration would upset these mechanisms and potentially lead to a reduced plant growth (Figure 1.4).

*1.1.3.1.2 Cytokinins*

1.1.3.1.2.1 Site of synthesis and role in plant development

Cytokinins are synthesized primarily in roots and are translocated through the xylem sap to the shoot buds where they promote shoot growth and development (Kende 1965; Carr 1966; Itai and Vaadia 1965; Torrey 1976). Zeatin and zeatin riboside (ZR) constitute a significant proportion of xylem-mobile cytokinins in many plant species, including apple (Kamboj *et al.* 1999a).

Cytokinins mainly promote cell division and differentiation (organogenesis). These hormones enhance the "sink" effect of the tissues (Morris and Winfield 1972), which makes the shoot tip a more efficient competitor for carbohydrates and amino acids. Cytokinins may also affect cell membrane integrity (Shaw and Manocha 1965) and facilitate the movement of compounds in the tip region (Turvey and Patrick 1979). Despite numerous studies, little is known about the response on the whole plant to cytokinins as its actions are often dependant on interactions with other hormones (Hooijdonk *et al.* 2006).

1.1.3.1.2.2 Interaction of cytokinins with auxins

Cytokinins interact with other phytohormones, primarily with auxins. Production and translocation of cytokinins acropetally through the xylem is dependant on the amount of shoot-synthesized auxins reaching the roots (Lockard and Schneider 1981). Cytokinins and auxins levels are inversely correlated *in vivo* (Eklof *et al.* 2000) and auxin treatment can rapidly inhibit cytokinins by suppressing both the pool size and the synthesis of these hormones (Nordstrom *et al.* 2004).

1.1.3.1.2.3 Effects on scion growth

Several authors have observed a positive correlation between the growth potential of a rootstock and cytokinin levels in its xylem sap (Jones 1986; Kamboj *et al.* 1999a). The growth potential of apple rootstocks shows a positive correlation with the rate of cytokinin export from their roots (Kamboj *et al.* 1999a) (Figure 1.4). The differences in concentrations of cytokinins observed in the xylem sap among rootstocks could result from either selective transport or selective synthesis of cytokinins in the rootstocks (Kamboj *et al.* 1999a). In *Prunus*, the most vigorous rootstocks have a higher level of cytokinins compared to dwarfing rootstocks (Sorce *et al.* 2001).

In ungrafted rootstocks, shoot and root growth are well balanced, and the level of cytokinins is only slightly lower than auxins. In grafted plants, a dwarfing rootstock could upset such a balance, resulting in the long term in a reduced stature of the scion grafted onto it (Sorce *et al.* 2001).

#### *1.1.3.1.3 Gibberellins*

1.1.2.1.3.1 Site of synthesis and role in plant development

Gibberellins (GAs) are a complex family of tetracyclic diterpenoid plant hormones that mediate environmental and developmental signals (Bulley *et al.* 2005).
GAs are synthesized in shoot and root tips and have been detected in the phloem and xylem of a wide range of plants. GAs act by regulating cellular processes such as cell elongation and division and control seed germination, stem elongation, leaf expansion, trichome development as well as fruit and flower development. The level of bioactive GAs is controlled by several mechanisms such as transcriptional regulation of genes encoding enzymes from both catabolic and biosynthetic pathways (Olszewski *et al.* 2002).

1.1.2.1.3.2 Effect on scion growth

Many of the growth, flowering and fruiting characteristics of dwarfed apple trees, and their response to applied GAs, suggest that reduced levels of endogenous bioactive GAs are involved in the dwarfing mechanism (Robitaille and Carlson 1971; Richards *et al.* 1986) (Figure 1.4).
Richards *et al.* (1986) established that the whole of the 'M.9' dwarfing interstock is involved in the reduced transport of GA and its metabolites to the shoot. 'M.9' dwarfing interstocks have a tendency to overgrow rootstock and scion, indicating that 'M.9' tissues are either especially responsive to elevated GA levels or retain more GAs (Richards *et al.* 1986).
The down regulation of GA 20-oxidase, which catalyses the penultimate step in the formation of bioactive GAs, results in dwarfing of an apple scion independent of the rootstock on which it is grafted (Bulley *et al.* 2005). The dwarfing effect, in this transgenic plant, is a result of reduced level of bioactive GA in shoot tips. When transgenic scions were grafted onto vigorous rootstocks, the dwarfing effect was maintained, demonstrating that scion vigour can be controlled independently from the rootstock.

*1.1.3.1.4 Abscisic acid*

1.1.2.1.4.1 Site of synthesis and role in plant development

Abscisic acid (ABA) is a 15-carbon compound (a sesquiterpene), which is synthesized in a pathway that involves carotenoid intermediates. This hormone can be synthesized throughout the plant, where there are plastids (any of several pigmented cytoplasmic organelles found in plant cells) (Milborrow 2001), but is primarily made in roots. Following its synthesis, ABA can be transported through the xylem or the phloem (Hartung *et al.* 2002).

ABA has been reported to have multiple roles during the life cycle of plants, such as growth inhibition, gravitropism, stomatal closure and water relations, seed development and bud dormancy (Zeevaart and Creelman 1988).

1.1.2.1.4.2 ABA and relation to dwarfing

In apple, concentrations of ABA-like substances in some dwarfing rootstocks are reportedly higher than those in vigorous rootstocks (Yadava and Dayton 1972). Higher ABA levels have also been measured in stems of dwarfed apple scions compared to vigorous ones (Robitaille and Carlson 1976). These observations point to a possible implication of ABA in the dwarfing effect of apple rootstocks (Figure 1.4). Furthermore applying ABA by injection into the stem reduces shoot size in apple (Kim *et al.* 1984). High concentrations of ABA in dwarfing rootstocks could inhibit the transport or reduce the effects of other hormones such as auxins, cytokinins and gibberellins (Basler and McBride 1977; Jacqmar *et al.* 1995).

High ABA concentrations, which in turn increases the ABA:IAA ratio, also increase the synthesis of the rootstock bark (Kamboj *et al.* 1999b) caused by a greater differentiation of phloem and related tissues. The high bark to root ratio resulting from the increase in ABA concentrations has been used as a marker for the early selection of dwarfing rootstocks in rootstock breeding programmes (Rogers and Beakbane 1957).

1.1.3.2 Influence of phenolic compounds

Phenols are low molecular compounds ubiquitous in all tissues of higher plants which have a significant impact on plant development (Makoi and Ndakidemi 2007). Some phenolic compounds such as tyrosine and 4-hydroxybenzoic acid have been

found to promote bud formation in presence of kinetin and IAA, while ferulic and protocatechuic acids strongly inhibited it (Lee and Skoog 1965).

In apple, phenols are concentrated mainly in the bark of trees and have been regarded as possible growth controlling compounds in the control of tree size by dwarfing rootstocks. Some phenolic acids can enhance oxidative decarboxylation of auxins, resulting in the inhibition of tissue growth (Tomaszewski and Thimann 1966). The same hypothesis has been developed to explain the dwarfing effect of some citrus rootstocks (Mendel and Cohen 1962).

Yu and Carlson (1975) proposed that phenols may be involved in graft incompatibility. Noggle (1979) suggested that when freshly cut surfaces of stock and scion are brought together, the cells of the adjacent graft may be subjected to foreign phenolic compounds and may not have the appropriate enzymes to break down these compounds or convert them into non-toxic forms. These free phenols may then inhibit cell division, which would have the effect of a non-union or bad union of the graft (Figure 1.4). This would lead in the first case to the death of the plant, and in the second case to a disorganisation in the graft union that would disrupt the formation of xylem and phloem, and as a consequence disturb the translocation of hormones, nutrients and water.

### 1.1.3.3 Anatomical and physiological hypotheses

#### *1.1.3.3.1 Anatomy of the graft union: role in mineral and water translocation*

Abnormal features in the graft unions of apple trees have been reported to be associated with different degrees of dwarfing imposed by the rootstocks. Studies on the graft union showed that the vascular tissues that develop between the stock and the scion are arranged in a twisting pattern, and become necrotic during subsequent growth of the plant. It has been suggested that these atypical anatomical features are the result of incompatibility of the bud and rootstock tissues (Simon and Chu 1984).

Studies on the total solute and nutrient content of the xylem sap below and above the graft union have revealed that there is a depletion of sap nutrients, together with cytokinins, at the graft union with dwarfing rootstocks and interstocks (Jones 1984). The graft union clearly has a role to play in the dwarfing phenomenon.

#### *1.1.3.3.2 Root anatomy of dwarfing rootstock*

Many dwarfing rootstocks have naturally small root systems, which reduces the uptake of water and minerals and the production of hormones (Figure 1.4). Young apple trees grown within root restriction membranes are very similar in size irrespective of whether grown on 'M.9' or 'MM106' (Webster 1995). These results demonstrate the importance of the root system in the mechanism by which rootstocks dwarf scions.

The hydraulic conductivity of roots from apple dwarfing rootstocks is lower than those measured from vigorous rootstocks (Atkinson *et al.* 2003). By consequence, the amount of water and minerals reaching the shoot would be lower for trees grafted on dwarfing rootstock, and would result in a slower growth of the scion. The same result was found by Syvertsen and Graham (1985) on citrus dwarfing rootstocks.

#### 1.1.3.4 Pathological hypotheses

Virus and mycoplasma-like organisms have been shown to affect the growth, yield and quality of apple cultivars (Posnette *et al.* 1963). Scions of the same cultivar are more vigorous when grafted onto virus-free rootstocks compared to their equivalent virus infected clones (Campbell 1980). However, smaller infected trees crop as well as the larger healthy ones in proportion to their size. Nevertheless, the effect of virus and micoplasma-like organisms is not consistent with the inheritance of the dwarfing character, and may only be a contributing factor to the dwarfing effect of some apple rootstocks.

#### 1.1.3.5 Dwarfing hypotheses: summary

Despite all the studies and hypotheses considered over the past 100 years, the dwarfing phenomenon is still not fully understood and the cause and genetic basis of this effect have not yet been revealed. Dwarfing effects are probably associated with disturbances in the metabolism and translocation of auxins, cytokinins and gibberellins. Although their action is still unclear in the dwarfing response, growth inhibitors such as ABA and phenolic compounds possibly have a supplementary role to play. Rootstock anatomy, which includes the size of the root system, the stem and the graft union, may also partially explain the dwarfing effect of some rootstocks. Finally a form of incompatibility might explain some growth control associated with certain rootstocks but this cannot explain the effects induced by the majority of dwarfing rootstocks (Yu and Carlson 1975).

It is possible that the cause of dwarfing is not controlled by a single factor, and a combination of hypotheses may prove to be true. Any alteration in the rootstock/scion system, combined with the intrinsic effect of the rootstock can lead to a cascade of reactions that can dramatically transform the physiology of the plant and lead to the development of a dwarfed tree.

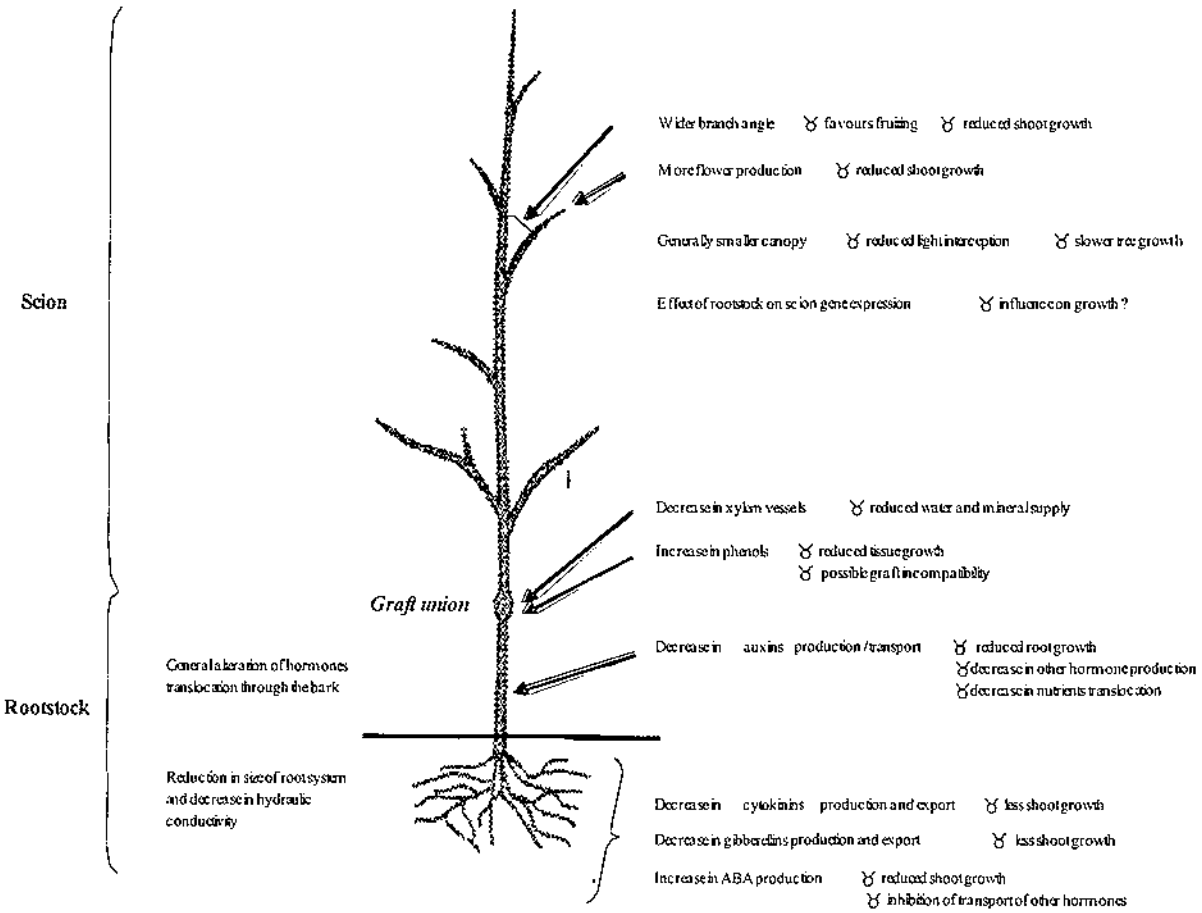



**Figure 1.4. Summary of the major dwarfing hypotheses.**

Until now, only physiological aspects of the dwarfing effect of some apple rootstocks have been considered, each author demonstrating the viability of their own hypotheses. Such approaches may have led to a restricted overview of the dwarfing phenomenon, and the variations observed by these authors between dwarfing and vigorous rootstocks may only be direct consequences of a more subtle alteration. A more general approach is certainly required to understand this complex phenomenon, which would require no underlying assumption concerning the cause of this effect. Such study could be conducted with the aim of describing the process

by which the scion is dwarfed. This type of study would have the advantage of not having a specific hypothesis of what the control mechanism may be, but would examine all the contributing factors of a plant structure and anatomy that may be implicated. Studies could also be conducted with the aim of understanding the genetic basis of dwarfing, which would eventually lead to an understanding of the physiology of the trait. Methods such as genetic mapping and other genomic approaches (closely linked with parallel physiological studies), which were unknown to researchers only a few decades ago, are now available. These methods, which include bulked segregant analysis, QTL analysis and candidate gene approach via microarray analysis will be described in the next paragraphs of this thesis.

1.1.4. Knowledge on the genetic control of the dwarfing effect by the apple rootstock 'M.9'

Bulked segregant analysis (BSA, see section 1.2.3.1) using randomly amplified polymorphic DNA (RAPD, see section 1.2.1.1) primers has already been performed in an F1 population derived from a cross between the dwarfing rootstock 'M.9' and the vigorous rootstock 'R5', and several markers linked to a major dwarfing locus (*DW1*) have been identified (Rusholme Pilcher *et al.* in press). Using simple sequence repeat (SSR, see section 1.2.1.4) markers developed by Liebhard *et al.* (2002), *DW1* was mapped to the top of the linkage group 5 (LG5) on the apple genome. However, the mapping of *DW1* was determined using only individuals with extreme phenotypes, and vigorous individuals amplifying a marker linked to *DW1* were not included in the analysis, which may have led to an incorrect estimation of the genetic distance between *DW1* and its closest markers (CH03a09).

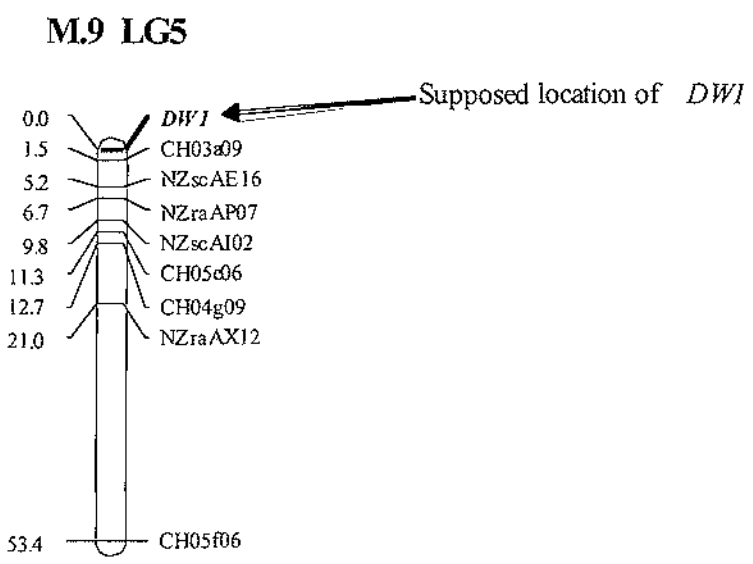



**Figure 1.5. Location of *DW1* on the linkage group 5 of 'Malling 9'** (from Rusholme *et al.* 2004). The names of the SSR markers (CH) from the literature were not changed. The sequence characterized amplified regions (SCAR) markers developed in HortResearch are prefixed with 'NZsc'. The RAPD markers are prefixed with 'NZra' followed by the Operon primer code. Numbers on the left side of the linkage group represent the distance in cM.

The absence of genetic markers on both sides of DW1, together with the fact that DW1 alone does not explain all the variation in the scion architecture, does not permit the implementation of a robust marker assisted breeding programme.

## 1.2 Genetic mapping and apple genetics

### 1.2.1 Molecular markers and mapping

Molecular markers are genetic markers that are based on DNA sequences, as opposed to morphological (expression of a trait), biochemical (chemical composition) and protein (isoenzymes) markers (Jones *et al.* 1997a). Markers occupy specific positions in the genome which are called loci (singular locus). Molecular markers can be coding or non-coding, they can be anonymous or of known sequence, they can be based on their length or on variations in their sequence and they can be dominant or codominant. In the following paragraphs, I will limit my description to the markers used in the context of this thesis, with a special emphasis on microsatellite markers.

#### 1.2.1.1 Randomly amplified polymorphic DNA (RAPD)

RAPD markers are generated from PCR primers of 10 arbitrary nucleotides in length. Each product is derived from a region of the genome that contains two short segments in inverted orientation, on opposite strands, that are complementary to the primer and sufficiently close together for the amplification to work. RAPD primers generally amplify 5 to 12 DNA strands of between 200 and 2000 bp long, which lie between two inverted copies of the primer, one copy binding to each strand of the DNA. Priming occurs statistically once every million base pairs (Jones *et al.* 1997b). Polymorphisms arise because of sequence variations in the genome that alter the primer binding sites. RAPD markers are termed dominant markers because of their presence or absence at particular loci. Amplification products are electrophoretically resolved on agarose gels with staining using ethidium bromide.

RAPD markers are simple and less expensive than other markers such as Restriction Fragment Length Polymorphism (RFLPs) (based on the use of labelled DNA probes annealing genomic DNA cut by restriction enzymes) for the reason that no prior knowledge of sequence is required. There is no limit to the number of RAPD markers in a genome, making them very useful for mapping (Jones *et al.* 1997b). However, these markers have disadvantages: they cannot easily be transferred among species, because of the random nature of their generation and their short primer length, they have poor reliability and reproducibility, and they have a high sensitivity to experimental conditions (e.g.: concentration of salts, dNTPs, DNA) (Karp *et al.* 1996). Nevertheless they have proved useful in many studies in apple for the identification of molecular markers linked to resistance genes such as apple scab (*Venturia inaequalis*) (Yang *et al.* 1997; Gygax *et al.* 2004) and powdery mildew (*Podosphaera leucotricha* (Ell. *et* Ev.) Salm.) (Dunemann *et al.* 2004; James and Evans, 2004). RAPD markers have also been used for genetic map construction in apple (Hemmat *et al.* 1994).

#### 1.2.1.2 Sequence characterized amplified regions (SCAR)

SCAR markers are derived from the sequencing of PCR products obtained from RAPD markers. Knowledge of the DNA sequence allows longer primers (from 20 to 25 bp) to be designed with the aim to make the amplification more specific. DNA sequence differences are manifested by the presence or absence of a single unique band. SCAR markers are more reproducible than RAPD markers and are usually

dominant markers (Staub *et al.* 1996). Like RAPD, SCAR markers are resolved using electrophoresis on agarose gels with staining using ethidium bromide.

#### 1.2.1.3 Single nucleotide polymorphism (SNP)

Single DNA base differences between homologous DNA fragments, including small insertions and deletions (indels), are referred to as single nucleotide polymorphisms (SNP) (Zhu *et al.* 2002). In maize, the average is one SNP per 48 bp in non-coding regions, and one SNP per 131 bp in the coding regions (Rafalski 2002). Indel polymorphisms are present on average one in 126 bp, but almost exclusively in non-coding regions (Rafalski 2002). In apple, the average occurrence of SNP markers in coding regions is about one per 107 bp (HortResearch, unpublished data). SNP markers are usually bi-allelic and are very abundant throughout the genome. Genotyping of SNP generally involves the generation of allele-specific products of SNP of interest followed by their detection for genotype determination (Kim and Misra 2007). The majority of current genotyping technologies require a PCR amplification step. Several SNP genotyping technologies based on allele discrimination strategies have been described to characterise SNP polymorphism. These technologies are based on allele-specific PCR amplification or allele-specific biochemical reactions. Four popular methods have been developed: primer extension, hybridization, ligation and enzymatic cleavage (Kim and Misra 2007).
SNP markers have a wide range of applications such as construction of genetic maps, candidate gene mapping, genetic diagnostics and analysis of the genetic structure of populations (Batley and Edwards 2007).

#### 1.2.1.4 Simple sequence repeats (SSR)

Simple Sequence Repeats (SSR) or microsatellites have been detected in the genome of every organism analysed so far, and are found at frequencies much higher than would be predicted purely on the grounds of base composition (Epplen *et al.* 1993). SSR markers are relatively rare in protein-coding regions but constitute a large fraction of non-coding DNA (Li *et al.* 2002a). They are an important class of DNA markers because of their abundance and length hypervariability. SSR markers have been used in cultivar identification, genetic analysis, genetic diversity analysis and genetic mapping (Guilford *et al.* 1997).

*1.2.1.4.1 SSR definition and putative function*

SSR markers are short tandem repeats of one to six base pairs with a maximum number of repetitions rarely greater than 60 (Taylor *et al.* 1999). SSR markers are generally classified in three categories following the nomenclature of (Weber 1990): 'perfect', when the motif is repeated without any point mutation (Figure 1.5 a); 'imperfect', where a point mutation is present in the repeat (Figure1.5 b); and composite (or compound), where the SSR is made up of two or more different types of motif (Figure 1.5 c).

(a)- Perfect dinucleotide repeat.

GTGTGTGTGTGTGTGTGTGT
CACACACACACACACACACA

(b)- Imperfect nucleotide repeat: contains a point mutation.

GTGTGTGTGTGGGTGTGTGT
CACACACACACCCACACACA

(c)- Composite SSR: transition from GT/CA to GA/CT.

GTGTGTGTGTGTGAGAGAGAGAGA
CACACACACACACTCTCTCTCTCTC

**Figure 1.6. Diagram representing the different types of SSR**. Panel (a) illustrates perfect repeats, where the SSR consists of a repeated motif; panel (b) illustrates imperfect repeats, where a point mutation is present (highlighted); and panel (c) illustrate a composite SSR repeat where there is a transition to another repeat motif (highlighted).

Even though SSR markers are generally considered as evolutionary neutral DNA markers, critical tests in various biological phenomena have proved the functional significance of a substantial part of them. Some SSR markers are probably involved in chromatin organisation, in the regulation of DNA metabolic processes (DNA

replication and recombination) and in the regulation of gene activity through transcription, protein binding and translation (Li *et al.* 2002b).

*1.2.1.4.2 SSR Polymorphisms and mutation*

The high polymorphism found in SSR markers is due to their high mutation rate. In plants this mutation rate is often quoted in the range of $10^{-2}$ to $10^{-6}$ per locus per generation. To make a comparison, eukaryotic DNA sequences mutate at a rate of approximately $10^{-9}$ per nucleotide per generation (Crow 1993). Mutation rate can vary among species as well as among loci. One major determinant of this variation is the length of the individual SSR. Long SSR repeats mutate more often than short ones. However other factors affect the rate of mutations at SSR loci including C/G content in flanking DNA, chromosome position, cell division (mitotic vs. meiotic), sex and genotype (Li *et al.* 2002b).

The majority of SSR mutations represent gains and losses of entire repeat units. Two mutational mechanisms can be invoked to explain these high rates of mutation. The first and the most important one involve DNA slippage during DNA replication. The second involves recombination between DNA strands.

Polymorphism of SSR markers depends on the size of the amplified fragments. Bands of different size for the same SSR primers can be considered alleles of that specific locus (Serrano *et al.* 2002).

*1.2.1.4.3 Repeat type and repeat length*

The majority of SSR markers found in many species are dinucleotides (48-67%) (Schug *et al.* 1998). The dinucleotide repeat SSR markers occurring with the highest frequency in plants are (AT)/(TA), with (AG)/(CT) and (AC)/(GT) as the second and third most frequent (Wang *et al.* 1994). Trinucleotide and tetranucleotide repeats are not as common but tend to generate fewer stutter bands, which makes them easier to score.

Di- and tri-nucleotide SSR markers occur in the apple genome at a frequency comparable with other plant species. The (GA) motif for example occurs about every 120 kb, compared to about every 225 kb in rice and 100 kb in tropical tree genomes (Condit and Hubbell 1991; Wu and Tanksley 1993). The GT repeats occur in the apple genome about every 190 kb (Wu and Tanksley 1993).

*1.2.1.4.4 SSR amplification*

SSR markers are flanked by specific regions that can be used to develop primers in PCR reactions (Serrano *et al.* 2002). In apple, the flanking regions of SSR markers tend to be conserved and the markers already developed work reliably on a wide range of cultivars (Liebhard *et al.* 2002).

SSR alleles can be distinguished from PCR artefacts by the presence of stutter bands. These stutter bands appear more often in markers that contain simple di-nucleotide repeats. These bands are amplified because of a slippage of the polymerase during the amplification, which result in the production of fragments that are reduced in length by a multiple of repeat units (Smeets *et al.* 1989).

*1.2.1.4.5 Null alleles and heteroduplex formation*

As mentioned above, SSR detection is PCR based. It is a requirement that both PCR primers match the flanking regions of all alleles. If a point mutation or a deletion is present in the primer binding site of a specific allele, that allele will not be amplified. Such alleles are called 'null alleles' (Schlotterer 1998).

Heteroduplex formations are generated by base pairing between complementary single strands derived from the different parental duplex molecules generated during genetic recombination (Ayliffe *et al.* 1994). The use of denaturating gels such as polyacrylamide gels eliminates heteroduplex formations, resulting in an easier analysis of the results. With non-denaturating gels, such as agarose gels and other types of gels such as the one used in the capillary electrophoresis system CePro 9600™ (Combisep, Ames, Iowa), heteroduplexes can be visualised as extra bands, usually of higher molecular mass. This apparent shift in the molecular mass of the heteroduplexes is not due to an increase in the length of the amplification products, but rather to the formation of nucleotide loops (because of the annealing of two alleles of different length), which slow down the migration of DNA strands through the gel during electrophoresis.

*1.2.1.4.6 Methods for obtaining SSR flanking primers*

1.2.1.4.6.1 Markers developed from SSR libraries

The traditional method used to obtain SSR flanking regions is to screen by probing genomic libraries with SSR sequences (Rassmann *et al.* 1991). Positive clones are then sequenced to identify the SSR and flanking regions for primer design. This

method has a low rate of SSR recovery and is very expensive and time consuming (Zane *et al.* 2002). Other methods involving repeated enrichment of a given library followed by cloning and sequencing have been widely used for SSR primer design (Zane *et al.* 2002). The majority of SSR developed in apple were developed using these methods (Liebhard *et al.* 2002; Silfverberg-Dilworth *et al.* 2006). SSR markers developed from anonymous genomic sequences often correspond to non-coding sequences.

1.2.1.4.6.2 Markers developed from Expressed Sequence Tags (EST) sequences

ESTs are sequenced portions of complementary DNA copies of mRNA. They represent part of the transcribed portion of the genome of an organism grown under various experimental conditions (Poncet *et al.* 2006). Polymorphisms associated with these sequences have been found, including SNP markers, introns and SSR markers. Sequence analyses revealed a range from 2.65 to 16.82% of ESTs containing SSR motifs in dicotyledonous species (Kumpatla and Mukhopadhyay 2005). In apple, over 250,000 EST sequences are now publicly available (Genome Database for Rosaceae (GDR)). SSR markers are particularly common in the 5'-untranslated region (UTR) and, to a lesser extent, in the 3'-UTR of transcribed plant sequences (Morgante *et al.* 2002). Out of the 160,719 apple EST sequences analysed by Han *et al.* (2006), more than 2,000 (12.5%) were found to contain an SSR. Of the 150,000 ESTs recently added to the database, Newcomb *et al.* (2006) found that 17% of the apple sequences contained one or more di-, tri- or tetranucleotide SSR markers. These ESTs represent an extensive source of ready to use SSR markers that is still mostly unexploited.

Together with the qualities common to all SSR markers, EST-SSR markers can be used to cross-reference genes among species for enhancing the resolution in comparative genomic studies and identifying conserved genomic regions among species and genera (Brown *et al.* 2001; Decroocq *et al.* 2003).

*1.2.1.4.7 Transfer of SSR markers among species*

For genomes with no or little DNA sequence available, the development of SSR markers can be expensive and time consuming. An alternative approach is to use SSR markers developed in other related species. This approach has been successful in many different plant species including *Eucalyptus* (Yasodha *et al.* 2005), *Pinus* (Chagné *et al.* 2004) as well as different species of bird (Eggert and Fleischer 2004)

and fish (Yue and Orban 2004). The major problems in transferability are the possible point mutations in the primer binding sequence and more drastically the complete absence of the locus.

Within the Rosaceae family, this approach has been successfully used. Several *Malus* SSR markers have been mapped in *Pyrus* (Yamamoto *et al.* 2004a) and *Cydonia* (Yamamoto *et al.* 2004b). *Prunus* SSR markers have been used in *Pyrus* (Yamamoto *et al.* 2004a) and *Fragaria* (Santiago *et al.* 2007) for the purpose of map alignment.

*1.2.1.4.8 SSR application to apple*

SSR markers have proved valuable in apple for framework map construction, marker assisted selection and cultivar fingerprinting. To date, over 300 SSR markers have been developed in apple, mainly from anonymous sequences (Hokanson *et al.* 1998; Liebhard *et al.* 2002; Silfverberg-Dilworth *et al.* 2006). As the majority of commercial cultivars of *Malus* × *domestica* are diploid, no more than two SSR alleles should be expected (Guilford *et al.* 1997). However, some SSR primer pairs can amplify more than two alleles for individual cultivars. These banding patterns may reveal ancestral chromosome duplication events (see section 1.2.2.4).

## 1.2.2 Gene mapping

### 1.2.2.1 Principle

Mapping implies measuring the relative genetic distances between markers and assigning them to their linkage groups on the basis of the recombination values from all their pairwise combinations. Mapping is based on the principle that genes and markers segregate via chromosome recombination (designated as crossing-over) during meiosis (i.e. sexual reproduction) (Collard *et al.* 2005). Recombination is the process by which new combinations of parental genes or characters arise. It occurs by independent segregation of unlinked loci or by crossover between loci that are linked (Jones *et al.* 1997a). The distance between two markers is proportional, although not linearly related (Hartl and Jones 2001), to the recombination frequency between them, and is measured in centiMorgan (cM). The number of different linkage groups found corresponds, given enough markers, to the basic chromosome number of the species.

The construction of a linkage map requires a segregating population. Ideally the parents selected for this mapping population differ for one or more traits of interest. The number of individuals composing the segregating population generally ranges from 50 to 250, but larger populations are required for high-resolution mapping (Mohan *et al.* 1997).

The next step in the construction of a linkage map is to identify molecular markers that reveal differences between parents and that segregate among the individuals composing the progeny. Depending on the size of the genome to be mapped (in cM), the number of markers required to saturate the genome will vary. A genetic map is considered saturated when each marker in the genome is linked to at least one other marker on the map and when the number of linkage groups identified is equivalent to the number of chromosome in the genome studied (Mohan *et al.* 1997).

Once polymorphic markers have been identified and screened over the segregating population, mapping software programmes such as Mapmaker/EXP (Lander *et al.* 1987; Lincoln *et al.* 1993), MapManager QTX (Manly *et al.* 2001) and JoinMap (Stam and Van Ooijen 1995) are used to construct the genetic map. Linkage between markers is usually calculated using odds ratios (i.e. the ratio of linkage versus non linkage). This ratio is more commonly expressed as the logarithm of the ratio and is called logarithm of odds (LOD) value or LOD score (Risch 1992). The LOD score is a ratio obtained by dividing the probability that two loci are linked by the probability that they are not. LOD values >3 are usually used to construct linkage maps. As an example, a LOD value of three between two markers indicates that linkage is 1000 times more likely than no linkage. LOD values can be lowered in order to detect a greater level of linkage or to place additional markers within maps constructed at higher LOD values.

High-density linkage maps are an essential tool for the determination of marker-trait associations using the genome scanning approach (Patocchi *et al.* 2005). Genetic maps permit localisation of genes of interest, and the identification of quantitative trait loci (QTL), by providing the framework to understand the biological basis of complex traits (Tanksley *et al.* 1989). When multilocus markers such as SSR, RFLP and SNP markers are employed, genetic maps are invaluable for identifying homoeologous chromosomal regions (Liebhard *et al.* 2002; Maliepaard *et al.* 1998a). Furthermore, the use of orthologous markers (e.g. transferable microsatellites or EST-based markers) can make it possible to align framework maps to other species

maps using comparative genome mapping. Finally, linkage maps enable marker assisted selection (MAS) of favourable alleles in parents and progenies. A major advantage of the use of markers is that they increase breeding efficiency by enabling early selection for adult traits. MAS also enables simultaneous selection for multiple traits, including resistance gene pyramiding, and selection for traits that are expensive to phenotype (Gardiner *et al.* 2007).

#### 1.2.2.2 Mapping strategy used in apple

Unlike many other crop species, where segregating populations are commonly derived from backcross ($BC_1$) or $F_2$ crosses, in apple and other outbreeding species, full-sib families are used for genetic analysis. This substitute mapping method is called double pseudo-testcross mapping method (Weeden 1994). In this procedure, highly heterozygous cultivars are crossed and independent maps are constructed for loci segregating from each parent. Hence, different types of segregation for markers and QTL can occur simultaneously. If the parents are heterozygous for different alleles at marker loci, then new segregation types will occur in the progeny (Figure 1.6). For dominant RAPD, markers heterozygous in only a single parent segregate in a 1:1 present: absent ratio in the progeny (a), and markers heterozygous in both parents segregate in a 3:1 present: absent ratio (b). Doubly heterozygous markers are less informative than single heterozygous markers because the dominant allele progeny are composed of three indistinguishable genotypes: + +, + –, and – +. For co-dominant markers such as SSR markers, the two alleles of each parent can be detected and segregation types such as 1:2:1, 1:1:1:1 or 3:1 can be obtained in the progeny (c). When both parents are informative for several loci, the male and female maps can be aligned and a consensus map of the cross can be drawn (Figure 1.7).

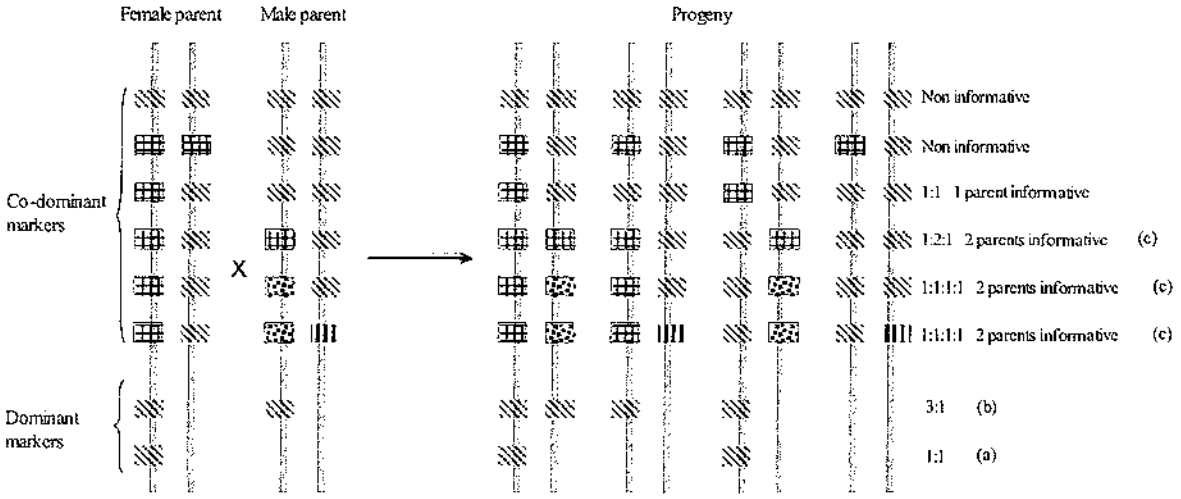



**Figure 1.7. Double pseudo-testcross mapping strategy and different marker segregation types.** ⊞ ⊠ ▯▯ ▨ represent different alleles of the loci.

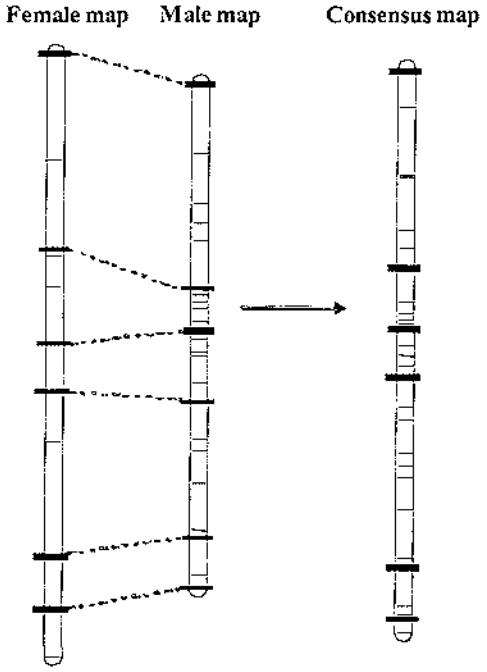



**Figure 1.8. Construction of a consensus map.** Male and female maps have been aligned using markers in common. Common markers among the maps are indicated by a bold line (━) and markers specific to each map are indicated by a simple line (—).

#### 1.2.2.3 Genetic map construction in apple

The first genetic maps of apple, developed in the USA in the 1990s, included mainly RAPD markers and a small number of isoenzyme markers (Conner *et al.* 1997; Hemmat *et al.* 1994) (Table 1.1). Because of the poor transferability of RAPD markers, these maps were specific to the genetic background of the mapping parents. Later, genetic maps were constructed with co-dominant transportable markers, mostly RFLP markers and a few SSR markers (Maliepaard *et al.* 1998a). The first SSR markers mapped in apple included some of those identified by Guilford *et al.* (1997), Hemmat *et al.* (1997), and by Horticulture Research International (HRI). The alignment of 17 linkage groups, corresponding to the 17 chromosomes of the apple genome, and the calculation of the first integrated apple map was realised in a 'Prima' × 'Fiesta' population (Maliepaard *et al.* 1998a) (Table 1.1).

Currently, a genetic map constructed in a 'Fiesta' × 'Discovery' population of about 250 individuals contains the largest core of SSR markers. This map includes over 300 SSR markers identified by Liebhard *et al.* (2002), Gianfranceschi *et al.* (1998) and Silfverberg-Dilworth *et al.* (2006). The majority of these SSR markers are derived from enriched libraries, the rest is composed of apple EST-SSR and genomic SSR markers developed in *Pyrus* (Yamamoto *et al.* 2004a; Yamamoto *et al.* 2002a; Yamamoto *et al.* 2002b; Yamamoto *et al.* 2002c). The maps span a total of 1,145.3 cM ('Fiesta') and 1,417.1 cM ('Discovery') and the coverage is close to 100% (Silfverberg-Dilworth *et al.* 2006).

**Table 1.1. Summary of published apple genetic maps**. The population size (pop. size), number of markers for each parent, number of SSR markers mapped and length of the map (cM) for each parent is given.

| Cross | Pop size | Number of markers | | SSR markers mapped | Length of map (cM) | Reference |
|---|---|---|---|---|---|---|
| | | Female | Male | | | |
| 'Rome Beauty' × 'White Angel' | 56 | 156 | 253 | - | -, 950 | Hemmat *et al.* 1994 |
| 'Wijcik McIntoch' × 'NY 75441-67' | 114 | 238 | 110 | - | 1206 (integrated), 692 | Conner *et al.* 1997 |
| 'Wijcik McIntosh' × 'NY 77441-58' | 172 | 181 | 183 | - | 1206, 898 | Conner *et al.* 1997 |
| 'Prima' × 'Fiesta' | 152 | 194 | 163 | 10 | 842, 984 | Maliepaard *et al.* 1998 |
| 'Fiesta' × 'Discovery' | 112 | 202 | 227 | 118 | 914, 1015 | Liebhard *et al.* 2002 |
| 'Fiesta' × 'Discovery' | 267 | 439 | 499 | 129 | 1140, 1450 | Liebhard *et al.* 2003b |
| 'Fiesta' × 'Discovery' | 44 | - | - | No new SSR markers | 1144, 1455 based on Liebhard *et al.* 2003b | Baldi *et al.* 2004 |
| 'Discovery' × 'TN10-8' | 149 | - | - | 62 | 1,219 (integrated map) | Calenge *et al.* 2005 |
| 'Telamon' × 'Braeburn' | 257 | 259 | 264 | 20 | 1039, 1245 | Kenis and Keulemans 2005 |
| 'Fiesta' × 'Discovery' | | | | 168 new loci; total >300 | 1145, 1417 | Silfverberg-Dilworth *et al.* 2006 |

#### 1.2.2.4 Genome organisation and homoeology

There are several hypotheses about the origin of the domestic apple (see section 1.1.1.2), each involving a certain level of duplication within its genome. During the development of genetic maps, several authors have reported the mapping of loci, from the same marker, on at least two different linkage groups. Maliepaard *et al.* (1998) reported that linked sequences detected by RFLPs on one linkage group could also be found linked at another linkage group. The same observations were made by Liebhard *et al.* (2002) and Silfverberg-Dilworth *et al.* (2006) when some SSR markers amplified two loci mapping at different locations in the genome. These results suggest almost complete homoeology between some linkage groups (i.e. LG05 and LG10; LG13 and LG16) and partial homoeology among others (i.e. top of LG04 and top of LG06, bottom of LG02 and top of LG07). A partial map indicating homoeologous portions of the genome among 14 of the 17 linkage groups of apple was recently published by Van de Weg in Gardiner *et al.* (2007). These results also indicate that the ancestors of the original hybrid were closely related (Maliepaard *et al.* 1998a).

### 1.2.3 Methods used to map genes in apple

#### 1.2.3.1 Bulked segregant analysis (BSA)

Developed by Michelmore *et al.* (1991), BSA is based on the comparison of marker amplification patterns between DNA bulks. The bulks are composed of approximately 10 individuals each, selected to have identical phenotype, and thus identical genotypes for a particular genomic region. The contrasting bulks are then screened to identify polymorphic markers differentiating them. The presence of polymorphisms between the amplification patterns of the two bulks (Figure 1.8) is expected only for those bands that are genetically linked to the gene of interest (Giovannoni *et al.* 1991) because markers differentiating the bulks are likely to be linked to the gene conferring the particular trait.

Polymorphisms between the DNA bulks result from either chromosomal changes in the amplified regions or base changes at the primer binding site (Michelmore *et al.* 1991).

Different types of markers can be employed to perform BSA, though RAPD primers provide the most efficient way of identifying new loci (Michelmore *et al.* 1991). Other more informative markers such as RFLPs, STSs (sequence tagged site) and SSR markers have successfully been used in BSA (Michelmore *et al.* 1991; James and Evans 2004; Rusholme Pilcher *et al.* in press). These markers offer the advantage of being transferable among crosses of different background and their location on the genome is known. When informative markers such as SSR markers are used in the context of BSA, the technique is often called whole genome scanning. James and Evans (2004) and Rusholme (unpublished) successfully used this technique to identify the location of some major resistance genes in apple.

The selection of individuals composing the bulks, based on their phenotype, must be meticulous because recombination between the target marker and the assayed polymorphic locus will result in diminishing distinction between the bulks (i.e. bulk of resistant individuals versus bulk of susceptible individuals). Thus, an accurate assessment of the desired trait is crucial to the success of BSA. In addition, the diminishing distinction between the two bulks may be the result of a decrease in the linkage of the marker to the gene.

Because of the relative DNA concentration of each of 10 individuals composing a bulk, segregating markers within a window of 10% recombination either side of the target locus will always be detectable, and many markers within 30% recombination window will also be detectable, at least as bands of unequal intensity (Michelmore *et*

*al.* 1991). In other terms, all markers closer than 15 cM are likely to be detected, whereas the limit of detection is located around 25 cM.

The probability of an unlinked locus being polymorphic between two bulks of 10 individuals was calculated to be $2\times10^{-6}$ (Michelmore *et al.* 1991). Therefore, the frequency of unlinked loci will increase with the use of smaller bulks.

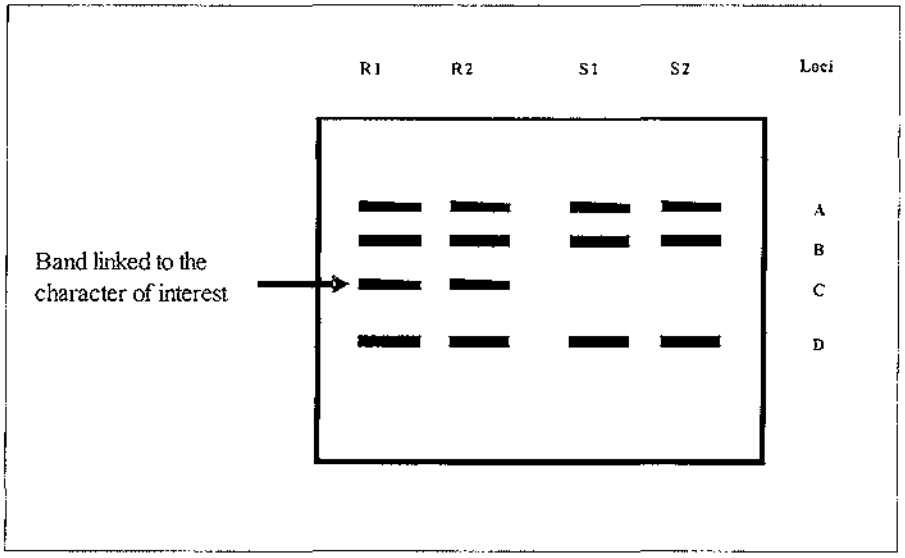



**Figure 1.9. Illustration of bulked segregant analysis.** Genotype of a RAPD marker screened on bulks derived from individuals homozygous for resistance (Bulks R1 and R2) or susceptibility (Bulks S1 and S2). The dominant allele at locus C is linked to the resistant allele (R) and therefore is polymorphic between the bulks. The other three loci (A, B and D) are not polymorphic between the 2 classes of bulks and therefore are unlinked to the R locus.

Markers showing polymorphism between the bulks are then screened over individual genotypes in the population and genetic distance between the trait and the marker is subsequently calculated (Michelmore *et al.* 1991).

BSA has been successfully used to find markers related to resistance genes in many species including lettuce (Michelmore *et al.* 1991.), tomato (Martin *et al.* 1991), bean (Haley *et al.* 1993) and apple (Markussen *et al.* 1995; Yang *et al.* 1997; Cheng *et al.* 1998; Gygax *et al.* 2004; James and Evans, 2004; Patocchi *et al.* 2004).

BSA has also been used to identify markers for major QTL (Quarrie *et al.* 1999). By grouping plants according to either high of low expression of a particular trait and extracting DNA from these two bulks, the process of genotyping the plants is reduced to only two DNA samples to be analysed instead of having to analyse DNA

independently from each of the plants composing the population (Quarrie *et al.* 1999).

#### 1.2.3.2 Quantitative trait loci (QTL): principle

Kearsey and Pooni (1996) describe 'quantitative trait' as being a character for which the observed phenotypic variation is due to the segregation of several genes, and to the interaction between these genes and their environment. QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers (Collard *et al.* 2005).

Locating QTL provides a mean to answer fundamental questions about the genetic control of quantitative traits such as the number of genes involved in the control of the trait and the intensity of the effect attributable to each of these genes.

Knowledge of the location of QTL opens many opportunities to improve selection efficiency.

QTL analysis is a multi-step procedure. Each of the major components will be described in the following paragraphs.

1- Creation of a segregating population

Most commonly, a QTL mapping population is derived from the cross of two parental lines that show marked differences for the trait of interest. A typical QTL population consists of 100 to 300 individuals. The size of a population can greatly influence the outcome of a QTL analysis study. The precision of QTL location depends more on sample size than on the density of markers (Kearsey and Pooni 1996). The larger the population, the more accurate the mapping study and the more likely it is to allow detection of QTL with small effects (Haley and Andersson 1997; Tanksley 1993). An increase in population size provides gains in statistical power, estimates of gene effects and confidence of the locations of QTL (Beavis 1998; Darvasi *et al.* 1993).

2- Development of a genetic map (or linkage map)

The identification and localisation of QTL relies on the use of a linkage map covering the entire genome, with regularly spaced markers (15 to 20 cM between markers). Large gaps between markers on a linkage group can lead to inaccurate analyses. QTL located in regions that contain gaps between markers cannot be mapped precisely as their phenotypic effect will be underestimated because distant linkage cannot be differentiated from small phenotypic effect (Lander and Botstein

1989), and QTL on unmapped regions of the genome will be unobserved. According to Kearsey and Pooni (1996), no great increase in precision is obtained with more than five well-spaced markers per chromosome.

3- Phenotypic evaluation of the trait(s)

An essential part of QTL analysis is obtaining accurate estimates of the traits of interest for each individual in the mapping population. For most traits, this involves the use of randomized, replicated designs and measurement of multiple plants per line. Growing conditions and evaluation methods must be as uniform as possible across the whole population. Often a QTL population is evaluated in multiple locations or years, to determine whether the same or distinct QTL influence a trait under different environmental conditions (George *et al.* 2003; Hittalmani *et al.* 2002).

Following the collection of a set of phenotypic data, various analyses of this set can be performed prior to the actual QTL analysis. These include:

(i) Analysis of frequency distribution: graphing the number of individuals of a population that fall into different phenotypic classes is a useful place to start in examining phenotypic data. It is important to know if the frequency distribution of the population is normal or approximately so. If the trait is not normally distributed, transformation to obtain a more normal distribution might be considered; however, transformation may modify the data in such a way that genetic relationships may be obscured. Three main characteristics describe the frequency distribution of a trait: the degree of skewness which indicates whether phenotypic data tend to cluster on one end of the distribution curve or the other, the kurtosis which refers to whether the shape of a distribution is relatively short and flat, or tall and slender, or somewhere in-between those two extremes, and the modality which refers to the number of distinct peaks that appear within a distribution.

(ii) Estimation of heritability: The most basic question to be asked about a quantitative trait is whether or not the observed variation in the character is influenced by genes at all. Variations among phenotypes in a population arise from two sources. First, there are differences among the genotypes, and second, each genotype exhibits phenotypic variance because of environmental variation. The total phenotypic variation in a population ($s^2_p$) can be broken into two portions: the variance among genotypic means, also known as genetic variance ($s^2_g$), and the

environmental variance ($s^2_e$). The degree of heritability can be defined as the proportion of the total variance that is due to genetic variance:

$$H^2 = \frac{s^2_g}{s^2_p} = \frac{s^2_g}{s^2_g + s^2_e}$$

$H^2$ is called the broad sense heritability of the character, and ranges from 0 (variation entirely due to environment) to 1 (variation entirely due to genes). This measure of genetic influence indicates what proportion of the population's variation in phenotype can be assigned to variation in genotype. The higher the heritability estimate for a trait, the greater the proportion of total variability that is due to genetic variation, rather than environment. High heritability in a QTL study means that a great percentage of phenotypic variance can be accounted for by the QTL (Griffiths *et al.* 1993).

(iii) Correlation analysis among traits measured in the segregating population: Many related phenotypic traits can vary together in an imperfect or approximate way. The usual measure of the precision of a relationship between two variables is the correlation coefficient (R). A high correlation between two traits may indicate that the same QTL influence both traits (a condition known as pleiotropy). It may also indicate that linked QTL, rather than the same genes, are associated with the traits.

4- Identification of QTL

Many programmes have been developed for detecting QTL, including MAPMAKER/QTL (Lincoln *et al.* 1992), QTL Cartographer (Basten *et al.* 1994, 1997), Map Manager QT (Manly 1997; Manly and Elliott 1991), Multimapper (Sillanpää 1998) and MapQTL (Van Ooijen 2004).

In the next section, I will focus on the three methods that I used during my QTL analysis: single marker analysis, simple interval mapping and multiple-QTL model mapping (Liu 1998; Tanksley 1993).

Single-locus association (single-marker analysis):

This test considers each marker locus separately and does not require that the marker loci be mapped relative to one another. The statistical methods used for this analysis include t-test, analysis of variance (ANOVA) and linear regression. Linear regression is most commonly used because the coefficient of regresssion ($R^2$) from

the marker explains the phenotypic variation arising from the QTL linked to the marker. The disadvantage with this method is that the further a QTL is from a marker, the less likely it will be detected. This is because recombination may occur between the marker and the QTL. It usually causes the magnitude of the effect to be underestimated (Tanksley 1993). The use of a large number of markers covering the entire genome may minimize this problem. The QTL analysis software MapQTL® 5 uses the rank sum test of Kruskal-Wallis to detect association between marker and QTL. This test is nonparametric, meaning that no assumptions are made for the probability distribution of the quantitative trait (after fitting the QTL genotype). For the same reason, Kruskal-Wallis test can also be used when dealing with ordinal traits (traits classified in categories) (Van Ooijen, personal communication). The test is performed on each locus separately and no use is made of the linkage map other than for sorting the order of loci on the linkage groups. The test ranks all the individuals according to the quantitative trait, while it classifies them according to their marker genotype. A segregating QTL linked closely to the tested marker will result in large differences in average rank of the marker genotype classes (Van Ooijen 2004).

Interval mapping (Simple interval mapping and Multiple-QTL model mapping):
Simple interval mapping (SIM):
This method requires prior construction of a genetic map and is based on the analysis of intervals between adjacent pairs of linked markers along chromosomes (Lander and Botstein 1989). With this method, for each position on the genome (every centiMorgan) the likelihood for the presence of a segregating QTL is determined (the likelihood under the alternative hypothesis, H1). At the same time the genetic effects of the QTL and the residual variance are calculated. The likelihood under H1 is compared to the likelihood for the situation when a locus with zero genetic effect would segregate, i.e. there is no segregating QTL (the likelihood under the null-hypothesis, H0). This comparison is performed with the likelihood statistic ratio LOD (or the LOD score), which is the 10-base logarithm of the quotient of the two respective likelihoods. When the LOD score exceeds the predefined significance threshold (see permutation test) somewhere on a linkage group, a segregating QTL is detected.
The position with the largest LOD on the linkage group is the estimated position of the QTL on the map. The use of linked markers for this analysis compensates for the

recombination between the markers and the QTL, and is considered statistically more powerful compared to single marker analysis.

Multiple-QTL model mapping (MQM mapping):

MQM mapping was developed by Jansen (Jansen 1993; Jansen 1994) and Jansen and Stam (1994). Following QTL identification by interval mapping, markers close to detected QTL are selected as cofactors to take over the role of the nearby QTL in the approximate multiple-QTL models used in the subsequent MQM mapping. With this MQM mapping, a search over the genome is performed by testing for a single segregating QTL, while simultaneously fitting the selected cofactors, both under H0 and under H1. By doing this, the cofactors will reduce the residual variance of the QTL already detected. When a QTL explains a large proportion of the variation, the use of a linked marker as cofactor will enhance the power in the search for other segregating QTL. Several rounds of MQM mapping may be necessary to obtain the best possible final solution, and cofactor markers can be added or dropped according to the latest results (Van Ooijen 2004). As for interval mapping, the results of this test are presented using LOD score.

For both SIM and MQM mapping, the percentage of variance explained by a QTL (or percentage of explanation) was calculated as follow:

$$\% \text{ explanation} = \frac{100 \times \text{H0 var} - \text{var}}{\text{Population var}}$$

In which H0 var is the residual variance under the current null hypothesis.

QTL significance:

In order to establish whether a QTL has a significant effect on a trait, it is possible to calculate the significance threshold of the LOD score. The permutation test (Churchill and Doerge 1994) is a method widely used to determine this threshold. In this test, the trait values are randomly permuted among the progeny (1,000 to 10,000 times), destroying the relationship between the trait values and the genotypes of the marker loci. QTL parameters and a LOD value is then estimated for each permuted data set at regular intervals throughout the genome and the maximum LOD is recorded. To determine the significance threshold, a P-value is determined for each

linkage group (generally 0.05) and the LOD corresponding to this value is taken as the threshold (Van Ooijen 2004).

5- Reporting and describing QTL detected

The most common way of reporting QTL is by indicating the most closely linked markers in a table and/or as bars on linkage maps. The chromosomal regions represented by rectangles are usually the regions that exceed the significance threshold. QTL that are detected in common regions (based on different criteria or for related traits) are likely to be important QTL for controlling the trait (Collard *et al.* 2005). If the strength of the QTL is considered, three categories can be defined. Those which explain over 20% of the variance are strong QTL and can be considered almost Mendelian. Moderate QTL are those which explain between 1% and 20% of the variance. Weak QTL explain 1% or less of the trait variance and require at least a thousand progeny to detect them (Manly and Olson 1999).

As mentioned previously, the most likely position for a QTL is the map position at which the highest LOD score is detected. However, QTL are usually reported with confidence intervals. The simplest way to calculate a confidence interval is to find the region on both sides of a QTL peak that corresponds to a decrease of 2 LOD score (Van Ooijen 1992).

6- The last step in a QTL analysis study involves the confirmation of QTL locations and influence on the trait. Such confirmation studies may involve independent populations constructed from the same parental genotype or closely related genotypes used in the primary QTL mapping study. Making comparisons among maps and QTL locations can also be a way to verify the validity of the QTL detected. To perform such comparison, anchor markers, such as SSR markers, are needed in order to correlate information from one map to another. If common markers have been incorporated into different maps, they can be aligned together and QTL locations can be evaluated. Such comparisons can potentially reveal clusters of QTL on specific chromosomic regions.

### 1.2.4 QTL mapping in apple

Since the development of saturated genetic maps in apple, QTL influencing a wide range of traits have been studied. QTL have been identified for resistance to apple scab using reference genetic maps constructed in the populations 'Prima' × 'Fiesta'

(Durel *et al.* 2003) and 'Fiesta' × 'Discovery' (Liebhard *et al.* 2003a). QTL for resistance to powdery mildew (Calenge and Durel 2006; Stankiewicz-Kosyl *et al.* 2005) and fire blight (Calenge *et al.* 2005; Peil *et al.* 2006) have also been recently mapped. Various QTL controlling fruit quality such as number of fruit, fruit weight, fruit firmness, sugar content and acidity (King *et al.* 2000; Liebhard *et al.* 2003a) have also been identified.

QTL influencing tree growth and development have also been investigated and are described in the next section of this chapter as they are of particular relevance to this study.

### 1.2.5 Tree architecture and development QTL in apple

Many morphological and developmental traits in apple are believed to be under multigenic control. Using a map developed from a segregating population derived from a cross between 'Rome Beauty' and 'White Angel', Lawson *et al.* (1995) investigated the genetic control behind branching type, reproductive budbreak and root suckering. Several QTL were identified for these traits. Further work by Conner *et al.* (1998) used a population derived from a cross between Wijcik McIntosh and NY 75447-58 to position additional QTL influencing height increment, internode length, internode number, base diameter increment, base diameter, branch number and leaf break. The QTL influencing these developmental traits identified in both studies are important but the linkage maps used by the authors cannot be aligned with the consensus map (Liebhard *et al.* 2003b) because of a lack of transferable markers.

Using the 'Fiesta' × 'Discovery' linkage map, Liebhard *et al.* (2003a) undertook the analysis of several seedling and tree traits: stem diameter and leaf size (seedling); height increment, stem diameter, blooming time, number of branches, juvenile phase length and fruit harvest date (Figure 1.9). Using a population derived from a cross 'Telamon' × 'Braeburn', Kenis and Keulemans (2007) undertook the analysis of the following growth characteristics: main axis growth rate (GR), main axis height increment (HI), main axis internode number (IN), main axis mean internode length (IL), sylleptic branch number (SBN), sylleptic branch length (SBL), proleptic branch number (PBN), total branch number (TBN), total branch length (TBL) and growth increment (GI). These measurements were taken for plants on their own roots for the first and second year of growth (respectively OR1 and OR2), and for plants grafted on 'M.9' rootstock the first year after grafting (RS1). Thanks to the use of SSR

markers this genetic map has been aligned with the consensus map. Finally, Segura *et al.* (2007), using a population derived from a cross 'Starkrimson' × 'Granny Smith', studied a series of geometric, topological and phenological traits on one year old progeny. The approximate location of all the QTL for tree growth and development is represented in figure 1.9.

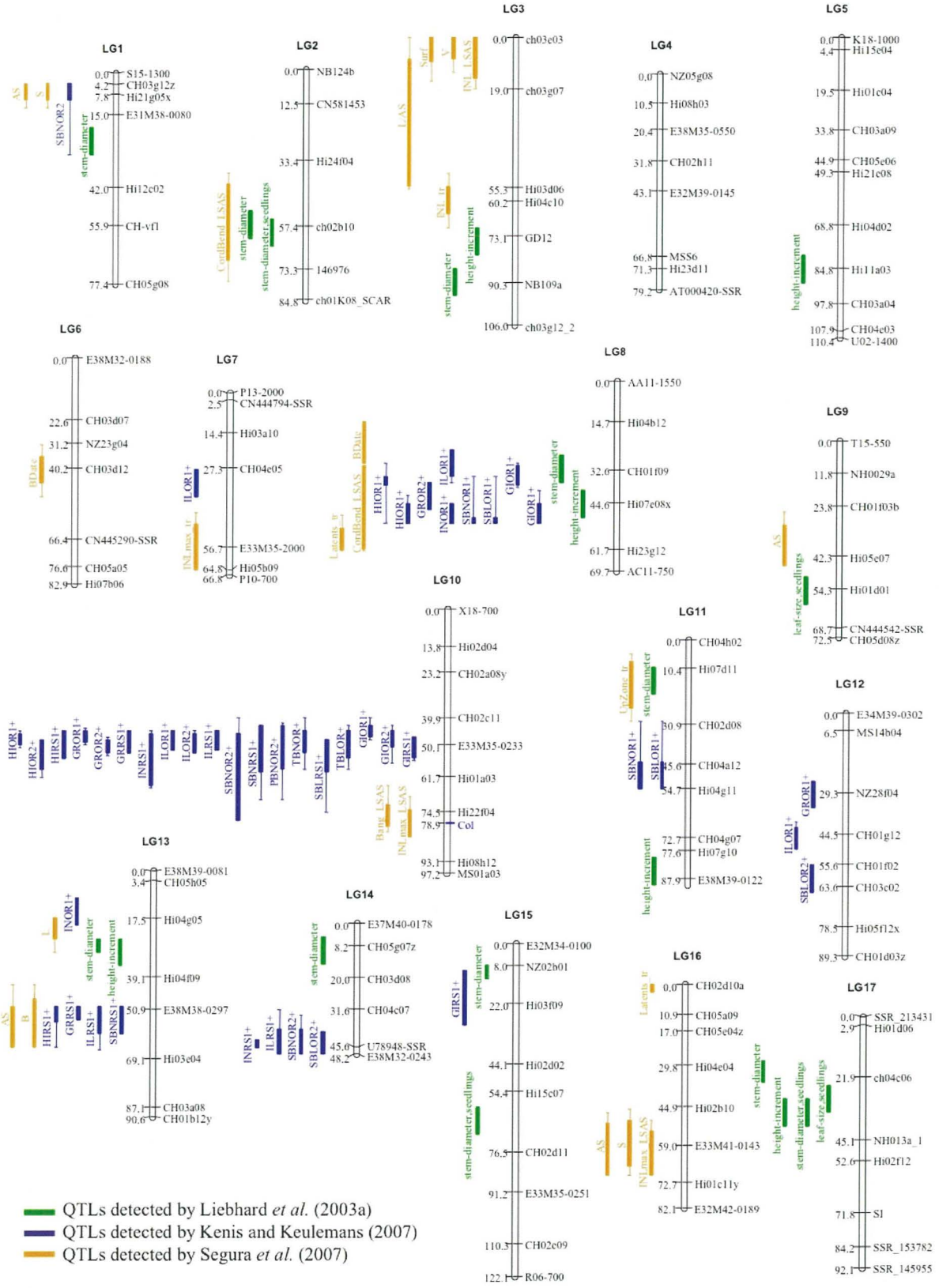



**Figure 1.10. Representation the approximate location of all the QTL for growth traits on the linkage groups of a consensus map.** LG represents the linkage groups following Liebhard *et al.* (2003b) numbering. The solid part of the bars of the QTL symbols indicate the most likely position of the QTL, the lines (when present) represent the confidence interval. QTL are located on the left side of LGs. Name of genetic markers is located on the right side of the LG.

QTL for tree architecture are located on all the LGs of the apple genome with the notable exception of LG4, LG6 and LG16. The results of these studies clearly show a clustering of the different QTL on LG8 and LG10. This is particularly relevant for the main axis trait QTL and the branching trait QTL. The main cluster is found on the LG10, in the region where a gene controlling the columnar habit had previously been mapped (Tian *et al.* 2005).

It is important to note that all the measurements in each study were made on genetically different scions grafted onto the same rootstock: 'M.27' in the case of the 'Fiesta' × 'Discovery' cross, and 'M.9' in the case of the 'Telamon' × 'Braeburn' cross. The influence of the rootstock on scion development has not been taken into account in any of these studies.

### 1.2.6 Bin Mapping: introduction and principles

A new method for mapping molecular markers and genes has recently emerged as a result of the development of saturated genetic maps. This method known as bin mapping or selective mapping was proposed by Vision *et al.* (2000) to improve the efficiency of mapping by significantly reducing the cost of genotyping new markers with a minimal loss of mapping precision. The method consists of a two-step process in which first a mapping population is used to construct a saturated framework map, and second, using a selected subset of highly informative plants, new markers are added to the map with lower precision. The number of plants in the subset is dependent on the size of the genome of the species studied, and the selection of the plants is based on the number and location of crossover sites. Ideally, for a given marker, the joint genotype of the selected subset of plants identifies a unique small bin in the genome (Figure 1.10).

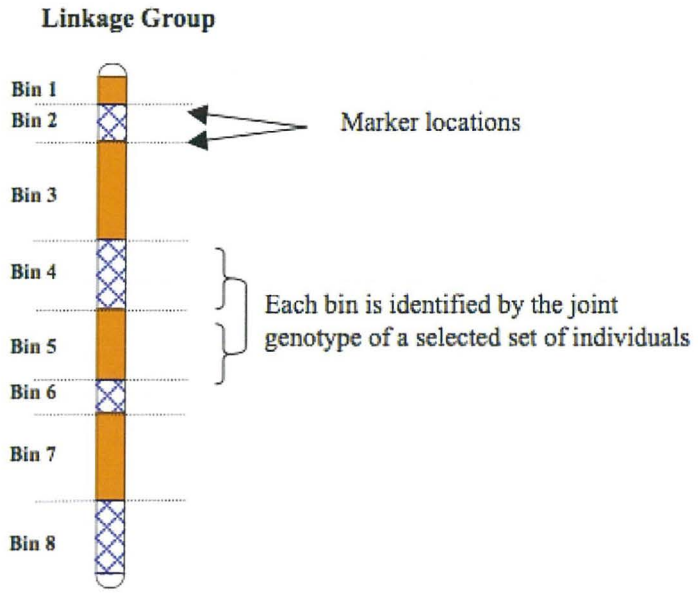



**Figure 1.11. Example of a linkage group divided into bins.** Each of the eight bin is unique and identified by the joint genotype of the individuals composing the bin set. The length of individual bins in a linkage group is dependent on the location of crossovers in individuals composing the bin set.

This method of mapping has already been successfully applied in *Prunus* (Howad *et al.* 2005), wheat (Johnson *et al.* 2007), and melon (Monforte *et al.* 2007).

### 1.2.7 The Candidate gene approach

The aim of the candidate gene approach is to identify markers within the gene directly controlling the trait of interest. This approach was made possible in apple by the creation and characterisation of 97 libraries of EST sequences (GDR, 2006) from 'Royal Gala', 'Pinkie', 'Pacific Rose', 'M.9', 'Aotea 1', 'Braeburn', 'Northern Spy', 'Goldrush', 'Jonagold', 'Granny Smith', 'Fuji', 'Suncrisp' and 'M.111'. The libraries represent 18 different tissue types, at 33 different stages of development, with a majority of them originating from different stages in fruit development (Newcomb *et al.* 2006; Arús and Gardiner 2007).

Candidate genes can be identified following two procedures. The first one consists of identifying potential candidates in the EST databases based on their homology to genes controlling traits in model plants. This procedure has already been successfully employed for various traits in many species, including members of the Rosaceae family, such as peach (Etienne *et al.* 2002), almond (Silva *et al.* 2005) and apple (Gardiner *et al.* 2003). The second is the microarray analysis approach (Pflieger *et*

*al.* 2001). Microarrays measure mRNA concentrations by labelling the sample with a dye and allowing it to hybridize to the spots on the array. Each spot contains either PCR-amplified cDNAs, or long oligonucleotides complementary to the transcribed parts of genes. The choice of spotting oligomers or cDNA sequences yields two different microarray technologies. Oligo arrays are generated by photolithography techniques to synthesize oligomers directly on the glass slide (Lipshutz *et al.* 1999). cDNA arrays are created by mechanical gridding, where prepared material is applied to each spot by ink-jet or physical deposition (Schena *et al.* 1995).

Generally, a one-to-one correspondence exists between spots and genes. However, various exceptions exist. Multiple genes may hybridize to the same spot if the DNA at that spot is not unique to a single gene; this problem is called cross-hybridization. A gene may also hybridize to more than one spot on the microarray if different spots cover different regions of the gene. Oligo microarrays can have non-specific binding, generally due to the presence of a single nucleotide polymorphism, which can alter their hybridization efficiency (Wu 2001).

The amount of hybridization to each spot is measured by the intensity of phosphorescent dye at each spot. mRNA samples are labelled with a dye before hybridization and the non-hybridized samples are washed off. The remaining hybridized and dye-labelled mRNA is then measured by a camera which records an intensity level. Problems due to artefacts arising from dust and other imperfections are handled by image processing software (Pflieger *et al.* 2001).

Data normalization:

During analysis of the microarrays, several factors have to be taken into account, such as the initial difference in mRNA concentration among samples; the concentration, brightness and relative binding affinity of the dye; the exposure time and the camera sensitivity. To correct these differences in intensity levels, a normalization, or bias correction needs to be performed (Wu 2001). In addition to these multiplicative effects, additive effects such as the background intensity level and the saturation effect in the hybridization process (amount of dye bound by an mRNA molecule) have to be estimated.

Evaluation of relative expression levels:

After data normalization, the relative expression of each gene can be evaluated according to its degree of differential expression. Because each gene is represented by a pair of expression values, the difference of expression can be evaluated by their difference or ratio. A threshold for selecting differentially expressed genes and *P* values can be calculated (Wu 2001).

Genes for which the *P* value is above the threshold can be considered as up or down regulated and consequently be evaluated as candidate genes.

Microarray analysis allows a significant initial reduction in the number of potential candidate genes that could be associated with a specific trait. Many studies in apple (Janssen *et al.* 2006; Schaffer *et al.* 2006; Schaffer *et al.* 2007) have demonstrated the power of this technology. The microarray analysis platform developed by HortResearch Ltd. (New Zealand) utilizes a 15,723 45-55 mer oligonucleotide array, representing 15,102 non redundant *Malus* sequences.

## 1.3 Aims and objectives

The overall aims of the project were to understand the genetic control of scion architectural modification induced the dwarfing apple rootstock 'M.9', and to develop genetic markers enabling an early detection of this desired phenotypic trait in apple rootstock. To achieve our goals, two populations segregating for the dwarfing characteristic were derived from crosses between 'M.9' and 'Robusta 5' (vigorous rootstock). Using these two populations, several strategies were used to achieve specific objectives.

I- In order to identify genetic markers closely linked to the major locus (*DW1*) influencing the dwarfing effect of 'M.9' (identified by Rusholme *et al.* 2004), and to uncover additional loci involved in the control of this trait, the bulked segregant analysis (BSA) strategy using RAPD primers was employed. To achieve these goals, various bulks were constructed from DNA of individuals based on their phenotype and genotype at the *DW1* locus.

II- To localize the dwarfing loci identified in objective I on the apple genome, and to gain a better understanding of the genetic control of this trait, the saturated genetic maps of 'M.9' and 'R5' were constructed using a variety of published molecular markers, as well as newly developed SSR markers from the apple EST database.

III- In order to ascertain the validity of the loci identified by BSA, and to identify additional loci with small genetic effects, a QTL analysis of the dwarfing phenotype and other related traits was performed using the genetic maps developed in objective II.

IV- With the aim of identifying the genes responsible for the dwarfing effect of 'M.9', candidate genes identified by microarray analysis were mapped and their location in the genome was compared to the position of the QTL previously identified. The identification of genes responsible for dwarfing would provide breeders with reliable markers located directly on the gene, enabling a very accurate marker assisted selection system to be implemented.

V- As it is often the case in many important food crops, apple scion cultivars are based on a very limited range of progenitor cultivars. This observation can also be applied to apple rootstocks since the majority of the commercially used rootstocks throughout the world are derived from 'M.9' or one of its seedlings. In order to determine the number of different dwarfing genetic sources, markers closely linked to the major locus *DW1* were screened over a set of rootstocks accessions, related or not to 'M.9'.

# 2- GENERAL MATERIALS AND METHODS

In the following chapter, I will present the dwarfing population that I used during this project as well as the general methods common to the chapters 3 to 7. Materials and methods specific to particular experiments are described in the relevant chapters.

## 2.1 Dwarfing populations and phenotypic assessment

Two F1 populations segregating for the dwarfing characteristic were derived from a cross between the dwarfing rootstock 'M.9' and the vigorous rootstock 'R5', using 'R5' as the pollen parent.

The first cross, performed by Stuart Tustin in 1997 generated a population of 146 seedling rootstocks. These were grown as stool-beds (aggregation of closely spaced stumps, managed for the production of one-year-old vegetative spurs) to produce multiple cuttings of each rootstock genotype, annually. 'Braeburn' scions were budded onto these rootstocks stool cuttings and each scion/rootstock combination was replicated between one and three times in the orchard in a randomised design. The first replicate was grafted in spring 1999 in nursery and planted in the orchard in 2000. In the nursery, the young apple trees were planted 30 cm apart along rows. Rootstocks were planted 15 cm deep, and the above-ground portion of the rootstock was standardised at 15 cm. When active bud growth resumed, scions were trimmed so that only one bud developed as the primary stem of the new tree. Trees were dug up after one year in nursery and planted in the orchard. These trees were spaced 1.5 m apart along rows that were spaced 4 m apart. No pruning of the trees was carried out. The general orchard management was carried out by orchard technicians and included pest, disease, weed control and nutrition of the plantings, using the standard New Zealand industry Integrated Fruit Production practices. Once trees commenced fruiting, commercial chemical thinning sprays were applied to reduce total crop levels to avoid over-cropping and limb breakage.

The second year after grafting (2001), trees from replicate one were annually phenotyped for vigour of scion growth by Stuart Tustin (Figure 2.1), until their sixth orchard year (seventh year after grafting: 2006). Replicates two (121 trees) and three (62 trees) of the 146 rootstocks were propagated and planted as one-year old grafted trees and were first assessed for vigour at the time of planting in 2001 (one year after grafting). These rootstocks were not phenotyped again until their third year in the orchard (2004), and again in their fourth and fifth years (fifth and sixth years after

grafting respectively). For all trees, annual expert visual assessments were made, classifying each tree into one of four categories: dwarfing, semi-dwarfing, intermediate and vigorous.


**Figure 2.1. Trees derived from the first 'M.9' × 'R5' cross, Rep 1, in their third year after grafting.** A blue plastic sheet has been spread out behind the trees to better observe the scion architecture. The difference in size and architecture is already evident between a scion grafted on a dwarfing rootstock (left) and a scion grafted on a vigorous rootstock (right), after only three years of growth.

A fifth phenotypic category was added for trees of replicate one in their fifth orchard year: very vigorous. For each category, a percentage of growth vigour compared to a tree grown on its own roots was assigned: dwarfing = 20%, semi-dwarfing = 30%, intermediate = 70%, vigorous = 90%, very vigorous = 100%. In the QTL analysis chapter (Chapter 5), this classification of the trees will be referred as DW%. Measurements were also made of trunk circumference 20 cm above the bud union (measures made by Shayna Ward and Daya Dayatilake (HortResearch Havelock North)) and trunk cross-sectional area (TCA) was calculated. For replicate one, estimates of the number of flower clusters per scion two year after grafting (2001) were taken and classified in five categories (1 – 5). One meaning no flower cluster and five many flower clusters.

The second cross performed in 2002 generated a population of 385 seedling rootstocks. No replicates were done for this second population. Braeburn scions were grafted to one year old rootstocks in 2004 and trees were planted in the orchard (Figure 2.2).

**Figure 2.2. Trees derived from the second 'M.9' × 'R5' cross, first year after grafting.** As illustrated on this picture, the trees are planted in the orchard at Havelock North (New Zealand). The phenotype of the scion cannot be assessed one year after grafting because they have the same vigour.

From the original 385 trees, 18 died in the orchard in the first year after grafting, and 39 grafts were unsuccessful. As for the first population, measurements of the trunk circumference 20 cm above the graft union were made for the 328 remaining trees and TCA was calculated in 2006 (second year after grafting). Twenty seven trees were considered as having an abnormally reduced growth, which was the result of a defective graft. These trees were therefore removed from further analysis. At the end, 301 trees were available for investigation. For these trees, measures of the height of the main axis for the first year of growth, internode number and average internode length on the main axis were taken.

Table 2.1 summarises the different phenotypic measurements taken for the two dwarfing populations. It also shows which population and the number of individuals used in each experiment.Visual assessment of scion vigour was performed on the three replicates of the first dwarfing population, after the third year of growth. TCA was measured for each tree of the first dwarfing population Rep 1 from the second to the seventh year of growth after grafting. For Rep 2 and Rep 3, TCA was measured from the fourth to the sixth year of growth after grafting. The number of flower clusters was only estimated the first year of growth after grafting for Rep 1 of the

first dwarfing population (phenotypic data taken by Rachel Rusholme and Stuart Tustin). For the second dwarfing population, the TCA, number of nodes of the main axis, height of the main axis and average internode length was measured the second year of growth after grafting.

The bulks used for the BSA experiment were set up using the phenotype measured from the first dwarfing population Rep1. The genetic maps of 'M.9' and 'R5' used for the QTL analysis were constructed using 135 individuals from the first dwarfing population as well as 81 individuals from the second dwarfing population.

**Table 2.1. Summary of the phenotypic measurements taken for each population.** The number of individuals used in each experiment is indicated

| | | | 1rst dwarfing population | | | 2nd dwarfing |
|---|---|---|---|---|---|---|
| | | | Rep 1 | Rep 2 | Rep 3 | population |
| Visual assessment | Dwarfing (20%) | | 18 | 13 | 12 | - |
| | Semi-dwarfing (30%) | | 11 | 15 | 10 | - |
| | Intermediate (70%) | | 22 | 23 | 14 | - |
| | Vigorous (90%) | | 47 | 69 | 26 | - |
| | Very Vigorous (100%) | | 12 | - | - | - |
| Total phenotyped | | | | 146 | | - |
| Total genotyped | | | | 137 | | 81 |
| Experiment | BSA | | 110 | 27 | - | - |
| | Map construction | | 135[a] | - | - | 81 |
| | QTL analysis | DW(%) | 135 | 121 | 62 | - |
| | | Nb flower cluster | 103 | - | - | - |
| | | TCA[c] 2nd year | 135 | - | - | 81 |
| | | 3rd year | 135 | - | - | - |
| | | 4th year | 135 | 121 | 62 | - |
| | | 5th year | 135 | 121 | 62 | - |
| | | 6th year | 135 | 121 | 62 | |
| | | 7th year | 135 | | | |
| | | Height[b] | - | - | - | 73 |
| | | Node number[b] | - | - | - | 73 |
| | | Average internodes length[b] | - | - | - | 73 |

[a]SSR analysis showed that one individual was a rogue and another one was triploid.

[b] Measurement taken on the main axis for the first year of growth after grafting

[c] First dwarfing population:

TCA 2nd year = 2001

TCA 3rd year = 2002

TCA 4th year = 2003

TCA 5th year = 2004

TCA 6th year = 2005

TCA 7th year = 2006

Second dwarfing population:

TCA 1rst year = 2006

For both populations, young leaves (less than 1.5 cm long) were harvested from the rootstocks, from the stool-beds for the first population and from the rootstock

seedling prior to grafting for the second population. Leaves were placed individually in plastic zip bags and immediately frozen in dry ice prior to transfer to a -80°C freezer.

## 2.2 DNA isolation

DNA from leaves was isolated following a protocol developed in the laboratory and published in Gardiner *et al.* (1996). Leaves were placed in a plastic zip bag and 2 ml of DNA extraction buffer (140 mM Sorbitol, 220 mM Tris-HCl pH 7.5, 22 mM EDTA, 0.8 M NaCl, 0.8% (w/v) hexacetyl trimethylammonium bromide, 1% (w/v) N-Lauroyl sarcosine, 1% (w/v) Polyvinyl-pyrrolidone 4000) was added and the zip bag was sealed. The leaves were ground to a homogeneous pulp by applying a Teflon roller to the sealed bag placed on a flat surface. A 1.6 ml sample of the pulp was extracted with 400 µl of chloroform:octanol (24:1 v/v) for 30 min at 65°C and centrifuged in a 2 ml Eppendorf tube at 15,000 rpm for 10 min. The aqueous layer was collected and DNA was precipitated by adding 1.0 ml of icecold isopropanol. DNA was recovered by centrifugation at 12 000 rpm for 5 min, the pellet washed twice with 70% ethanol, dried under vacuum and re-suspended in 50 to 100 µl of sterile distilled water.

## 2.3 DNA quantification

Isolated DNA was quantified following a protocol developed in the laboratory. The gDNA concentration was estimated using a 0.9% agarose 1xTAE (40 mM Tris-HCl pH 8.0, 20 mM acetic acid, 1 mM EDTA pH 8.0) electrophoresis gel. Two aliquots of extracted DNA (1 µl and 2.5 µl) were mixed with 1 × loading dye (416 µg $ml^{-1}$ bromophenol blue, 416 µg $ml^{-1}$ xylene cyanol, 66.6 mg $ml^{-1}$ sucrose) and loaded onto the agarose gel. Upon each line of wells several λ DNA (Invitrogen New Zealand Limited, Auckland) standards (equating to 50 ng) were loaded onto the gel. The gel was electrophoresed for approximately one hour at 70 volts. Gels were stained in 0.5 µg $ml^{-1}$ ethidium bromide solution for 30 min and examined under ultra-violet light (UV). gDNA concentrations were estimated by comparison of sample band intensity to the λ standard.

In the following chapters of this thesis, I performed all the work presented, except when mentioned otherwise.

# 3- BULKED SEGREGANT ANALYSIS USING RAPD MARKERS

## 3.1 Abstract

The use of dwarfing rootstocks in apple (*Malus* × *domestica* Borkh.) results in a reduction in the vegetative growth of the grafted scion (Seleznyova *et al.* 2003). Although this phenomenon has been exploited by horticulturalists for hundreds of years, the molecular and genetic basis of this effect is not yet well understood. The breeding of dwarfing apple rootstocks is difficult and time consuming because of the long time required to assess their phenotypes. Techniques such as marker assisted selection (MAS) would help accelerate future rootstock breeding programmes.

The aim of this study was (i) to identify additional genetic markers for the major dwarfing locus *DW1* identified by Rusholme *et al.* (2004) and located on 'M.9' LG5, using the same segregating population derived from a cross between 'Malling 9' ('M.9') (dwarfing rootstock) and 'Robusta 5' ('R5') (non-dwarfing); (ii) to locate more accurately the position of the dwarfing locus in the apple genome; and (iii) to identify other loci influencing the dwarfing effect (preliminary results from Rusholme *et al.* (2004) suggest that *DW1* is not sufficient to fully explain the dwarfing phenomenon). To achieve our goals, bulked segregant analysis (BSA) was employed with DNA bulks constructed with the help of the latest phenotypic and genotypic data available from the first dwarfing population Rep 1 (see Chapter 2). As a result, 10 new RAPD markers were identified on both sides of *DW1*, allowing a more accurate location of this locus. Furthermore, six markers were identified for a second locus (*DW2*) influencing the dwarfing effect.

Our results suggest that *DW1* and the *DW2* together are not sufficient to fully explain the dwarfing phenotype, suggesting more complex, multigenic control of this character.

## 3.2 Introduction

The phenomenon by which an apple *(Malus* × *domestica* Borkh) dwarfing rootstock induces reduced and compact growth of scions grafted onto it has been known and exploited by horticulturalists for centuries (Hatton 1916). However, despite the numerous investigations devoted to its study, the molecular and genetic basis of this effect is not yet well understood (see Chapter 1). The identification of molecular markers linked to the dwarfing effect of rootstocks could contribute to understanding the mechanism underlying this phenotype. Rootstock effects are cumulative (Costes *et al.* 2001) and affected by scion cultivar and environmental variations. Seleznyova *et al.* (2003) found that scions grafted onto 'Malling 9' ('M.9') dwarfing rootstocks possessed a reduced number of nodes per extension growth unit, and that the formation of new metamers (neoformation) in scions grafted onto 'M.9' was altered soon after bud break. This reduction in the number of nodes resulted in a smaller number of axillary annual shoots over the following year, so that after several successive annual cycles of this growth pattern, the dwarfed phenotype was expressed by the scion (Seleznyova *et al.* 2003).

Marker assisted selection (MAS) for apple offers quick and reliable prediction of the phenotype of a young seedling (Gardiner *et al.* 2007). The development of molecular markers for dwarfing in a apple rootstock breeding programme would be invaluable for breeders, as their use would avoid the lengthy and costly phenotyping of all individuals in a breeding population. Genetic markers for the dwarfing ability of the *Citrus* rootstock *Poncirus trifolia* 'Flying Dragon' have already been identified (Cheng and Roose, 1995), and it is believed that the inheritance of this *Citrus* rootstock dwarfing phenotype is controled by a single dominant gene. However, the dwarfing ability of apple rootstocks is thought to be polygenically determined (Tydemann, 1933) and complicated by its environmental context.

To identify molecular markers near a gene of interest or in a specific region of the genome, bulked segregant analysis (BSA), developed by Michelmore *et al.* (1991), has been described as very efficient in multiple studies (James and Evans 2004; Monna *et al.* 1995). This strategy has been successfully used for the identification of markers linked to major genes or quantitative trait loci (QTL) for various characters such as drought tolerance in maize (Quarrie *et al.* 1999) and grain quality in rice (Govindaraj *et al.* 2005). This technique is based on the comparison between DNA bulks (see section 1.2.3.1). Different types of markers can be employed to perform BSA, such as randomly amplified polymorphic DNA (RAPD) and amplified

fragment length polymorphism (AFLP). Despite providing more polymorphic DNA bands, the use of AFLP requires more steps, which increase the potential for errors (Chenuil 2006). For this reason, and combined with the use of an automated platform to set up polymerase chain reactions (PCR), RAPD markers provide the most efficient way for identifying new loci.

Although molecular markers linked to *DW1* have already been identified by Rusholme *et al.* (2004) (Chapter 1, Figure 1.5), many more markers in this specific region are necessary to enable the initiation of high resolution mapping around the genetic locus and subsequent cloning of this dwarfing gene, leading to a partial elucidation of the mechanism controlling the dwarfing ability of the apple rootstock 'M.9'.

In this study, we performed two rounds of BSA to identify RAPD markers around the *DW1* locus and to more precisely determine its location on LG5.

## 3.3 Materials and methods

### 3.3.1 Plant material and phenotypic assessment

The same F1 population of rootstocks used by Rusholme *et al.* (2004) was employed in this study. This population is the same as the first 'M.9' × 'R5' population described in Chapter 2 (Table 2.1). Only the phenotypes obtained from replicate one were taken into account in this study as four years of growth after grafting were assessed in this replicate at the time of this study.

### 3.3.2 Plant DNA isolation

Leaf material was harvested from the stool-beds and DNA was isolated and quantified as described in sections 2.2 and 2.3.

### 3.3.3 PCR product gel electrophoresis

PCR products were separated by electrophoresis on a 0.9% agarose (half agarose Multi-Purpose (Bioline), half agarose SFR (AMRESCO®)) 1xTAE gel, alongside a 100 bp DNA ladder or a 1 $kb^+$ DNA ladder (Invitrogen). Gels were stained in a 0.5 $\mu g\ ml^{-1}$ ethidium bromide solution for 30 min, washed with running water for 30 min, and examined under UV light.

### 3.3.4 Bulks design

To construct the set of DNA bulks for the round one of BSA, account was taken of the detailed genotyping results of Rusholme *et al.* (2004), and of the latest phenotypic data obtained from the research orchard for trees belonging to replicate one (year four after grafting). The bulks were designed as described in table 3.1. The first bulk was made of DNA extracted from 11 rootstocks showing a dwarfing effect on the grafted scion. The second bulk was composed of eight individuals with a semi-dwarfing habit. Bulks three and four were made of DNA extracted from 12 rootstocks each displaying a vigorous effect on the grafted scion.

**Table 3.1. Design of the bulks used in bulked segregant analysis round 1.** The number of individuals, phenotype and genotype at the *DW1* locus is indicated for each of the four bulks. The genotype of each individual was determined using data from Rusholme (personal communication).

| Bulks | Number of individuals in bulk | Phenotype | Genotype (*DW1*) |
|---|---|---|---|
| 1 | 11 | Dwarfing | + |
| 2 | 8 | Semi-dwarfing | + |
| 3 | 12 | Vigorous | - |
| 4 | 12 | Vigorous | - |

+ indicates the presence of markers linked to *DW1* for all the individuals composing the bulk

- indicates the absence of markers linked to *DW1*

In the second BSA round, more bulks were designed in an attempt to locate additional loci influencing the degree of dwarfing. These bulks were based on the results obtained from markers for *DW1* as well as a second locus identified (*DW2*) during the first BSA round, and phenotypic data collected from the orchard at year five. The bulks for the second BSA were designed as shown in table 3.2. Because of an insufficient number of dwarfing and semi-dwarfing rootstocks in the population, DNA from some individuals was duplicated in different bulks. Bulks one and six are composed of the same DNA extracted from rootstocks showing a dwarfing effect on the grafted scion. Bulks two and three are made of DNA from semi-dwarfing rootstocks. The DNA extracted from rootstocks inducing an intermediate vigour to the grafted scions composes bulks four and five. Bulks seven and eight are composed of DNA from nine vigorous and nine very vigorous rootstocks respectively.

**Table 3.2. Design of the bulks used in bulked segregant analysis round 2.** The number of individuals, phenotype and genotype at the *DW1* locus is indicated for each of the four bulks. The genotype of each individual was determined using data from Rusholme (personal communication) and from markers identified in BSA round 1.

| Bulks | Number of individuals in bulk | Phenotype | Genotype [a] | |
|---|---|---|---|---|
| | | | *DW1* | Other locus |
| 1 | 10 | Dwarfing | + | + |
| 2 | 10 | Semi-dwarfing | + | + |
| 3 | 8 | Semi-dwarfing [b] | + | mixed |
| 4 | 6 | Intermediate | + | mixed |
| 5 | 10 | Intermediate | - | mixed |
| 6 | 9 | Dwarfing [c] | + | + |
| 7 | 9 | Vigorous | - | - |
| 8 | 9 | Very vigorous | - | - |

[a] Presence of markers linked to corresponding locus

[b] DNA from some semi-dwarfing individuals used in bulk 3 have been used in bulk 2

[c] DNA from dwarfing individuals used in bulk 6 have been used in bulk 1

### 3.3.5 RAPD screening and mapping

BSAs were performed using 10-base RAPD primers (Operon Technologies Inc, Alameda, CA). The amplification programme and reaction mixtures were as described in Gardiner *et al.* (1996) except that the annealing temperature was 37°C and the reaction volume was 15µl. PCR reactions for BSA were set up in 384 well plates using a liquid handling robot Biomek® 2000 (Beckman, USA). The programme used was named "384BSA+MM multi". PCR reactions were performed in a Hybaid MBS 384S Thermal Cycler. Amplification products were robot loaded in 0.9% agarose gels (USB Corporation, Cleveland, OH USA) using the gel loading programme "384full-Gel-Load" developed in our laboratory by Michael Cook.

For the screening of RAPD markers over selected individuals and the whole population, PCR reactions were set up in 96 well plates and performed in Hybaid MBS 0.5G PCR machines (Thermo Electron, Walthman, MA).

All the gels were electrophoresed with TAE buffer at 110 volts and stained with a solution of ethidium bromide (0.75 mg $L^{-1}$).

For the first round of BSA, each bulk was screened with 336 RAPD primers. Primers that amplified bands in both dwarfing and semi-dwarfing DNA bulks, but not in both vigorous bulks, were screened across a selection of 46 individuals from the

'M.9' × 'R5' population. This subset of the population was composed of 10 dwarfing, 10 semi-dwarfing, 16 vigorous without markers associated with *DW1*, and 10 vigorous individuals amplifying markers associated with *DW1*.
Primers exhibiting potential linkages were then screened across the remaining individuals of the population.

For the second BSA round, each bulk was screened with 423 RAPD primers. Primers producing polymorphisms that allowed making distinctions among the different DNA bulks were screened across the same selection of 46 individuals. Markers showing potential segregation with *DW1* or another dwarfing locus were screened over the whole population.

JoinMap v3.0 software (Van Ooijen and Voorrips 2001) was used to perform a genetic linkage analysis. A LOD score of 3.0 was used to map the genetic markers. The final order was determined by extensive proof reading and by minimising double crossovers flanking loci. Recombination frequencies were converted to map distance using Kosambi's mapping function (Kosambi 1944) and $\chi^2$ tests were used to identify any segregation distortion of the parental alleles. Drawings of the linkage maps were generated with MapChart (Voorrips 2001).

## 3.4 Results

### 3.4.1 BSA first round

Out of the 336 RAPD markers screened over DNA bulks, 262 amplified DNA bands and 41 segregated between the bulks. These 41 RAPD primers were tested over the subset of 46 individuals, and seven showed patterns of interest. These markers were amplified in the majority of the dwarfing individuals and absent in most of the vigorous individuals. Following screening of the whole population, five new markers were found to be linked to dwarfing phenotype (Table 3.3.).

**Table 3.3. New markers linked to *DW1* identified after the first round of BSA.** The segregating band size and the percentage of recombination are indicated for each marker. Percentage of recombination was calculated according to the supposed location of *DW1* from Rusholme (2004). The RAPD markers are prefixed with 'NZra' followed by the Operon primer code.

| RAPD Primer | Segregating band size (bp) [+] | Percentage of recombination (%) |
|---|---|---|
| NZraAB07 | 1250 | 38.6 |
| NZraI12 | 950 | 17.0 |
| NZraAM18 | 700 | 6.3 |
| NZraAE02 | 1200 | 17.1 |
| NZraAB03 | 1800 | 14.0 |

[+] All the markers originated from the 'M.9' parent.

Two other markers, NZraI14(250) and NZraAG02(500) tended to be amplified more often on dwarfing and semi-dwarfing individuals than on vigorous. The segregating bands amplified by these two markers were present in 73% of the dwarfing and semi-dwarfing individuals, and in 35% of the vigorous individuals (Appendix I). However, these segregating bands were amplified in only four of the 20 vigorous individuals amplifying a marker associated with *DW1*. The absence of the segregating band in these individuals indicated that NZraI14(250) and NZraAG02(500) were not associated with *DW1*, and suggested the presence of a second locus influencing the dwarfing ability of the 'M.9' rootstock. A paired t-test was performed (Minitab®) and confirmed that the DNA bands amplified by these two markers were significantly (P=0.055 and P=0.001) more often present in dwarfing and semi-dwarfing compared to vigorous individuals.

### 3.4.2 BSA second round

Of the 423 RAPD markers screened over the DNA bulks, 398 amplified DNA bands and 100 showed patterns of interest. Figure 3.1 illustrates the banding profile for a RAPD marker tested during the second BSA round and segregating among bulks. The 850 bp DNA band is clearly amplified in bulks 1 (dwarfing), 2 (semi-dwarfing), 3 (semi-dwarfing) and 6 (dwarfing) while it is absent from all the other bulks, indicating that it might be linked to *DW1*.

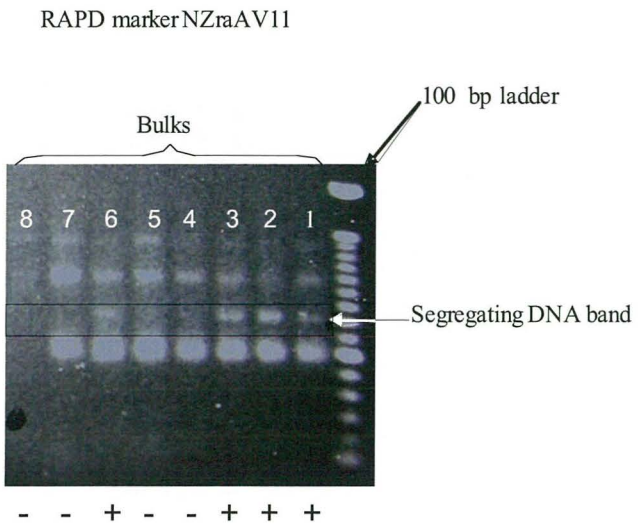



**Figure 3.1. Segregation of RAPD marker NZraAV11.** The 850 bp DNA band segregates among the eight DNA bulks set up for the second BSA round. Refer to Table 3.2 for bulks composition.

Each of these markers was screened over 46 individuals and their association to a particular phenotype was evaluated. Following the screening of markers showing the most interesting pattern (such as NZraAV11(850)) over the whole population, nine extra markers were identified for the *DW1* locus (Table 3.4.) and three were identified for a putative second dwarfing locus (*DW2*) (NZraAZ13(400), NZraAQ13(300), and NZraAU15(900)). The segregation pattern of NZraAZ13(400) was similar to the marker NZraAG02(500) identified during the first BSA round.

**Table 3.4. Markers linked to *DW1* identified after the second BSA round.** The segregating band size and the percentage of recombination are indicated for each marker. Percentage of recombination was calculated according to the supposed location of *DW1* from Rusholme (2004). The RAPD markers are prefixed with 'NZra' followed by the Operon primer code.

| RAPD primer | Segregating band size (bp) [+] | Percentage of recombination (%) |
|---|---|---|
| NZraAI06 | 1800 | 19.3 |
| NZraAJ03 | 1700 | 6.3 |
| NZraAJ14 | 1800 | 28.5 |
| NZraAF07 | 500 | 39.1 |
| NZraAV11 | 850 | 14.3 |
| NZraAJ13 | 1600 | 21.2 |
| NZraAR15 | 900 | 32.7 |
| NZraAQ06 | 850 | 31.8 |
| NZraAR10 | 1200 | 25.9 |

[+] All the markers originated from the 'M.9' parent.

Figure 3.2 illustrates the RAPD marker NZraAV11, linked to *DW1*, identified during the second BSA round. The 850 bp band amplified on 'M.9' and indicated by an arrow is clearly segregating between the dwarfing and vigorous individuals screened.

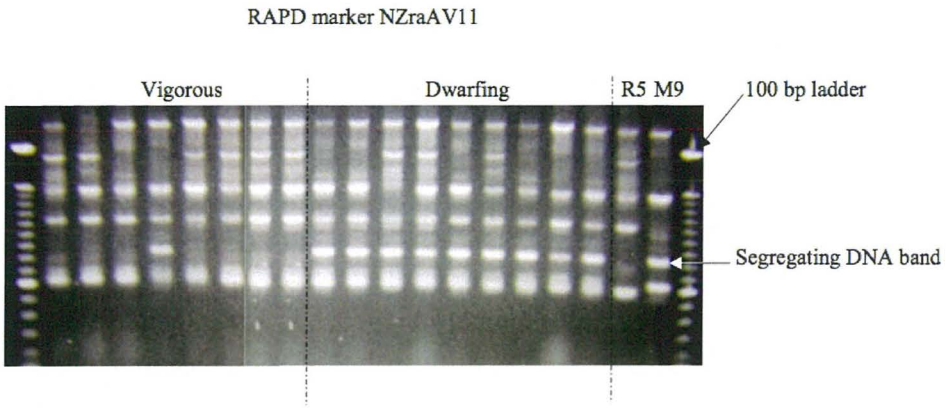



**Figure 3.2. Gel picture representing the screening of NZraAV11 over individuals of the 'M.9' × 'R5' population.** Dwarfing (right) and vigorous (left) individuals, as well as the parents of the population ('M.9' and 'R5') have been included in this screen. The segregating 850 bp DNA band is amplified from 'M.9' and absent in 'R5'.

In total, 759 RAPD primers were screened over the different DNA bulks (first and second BSA round) and 660 successfully amplified DNA bands. Each of these RAPD primers amplified between one and sixteen DNA bands, with an average of eight bands. Of these, 141 were screened over a subset of 46 individuals and 42 over the whole population.

### 3.4.3 Mapping

Out of the 759 genetic markers screened over DNA bulks, 14 new RAPD markers appeared to be linked to the visual classification of the dwarfing phenotype performed by tree physiologist Stuart Tustin. These new markers all originated from 'M.9'. Chi-square analysis of the genotypes at these marker loci identified that the loci detected by the RAPD markers NZraI12 ($\chi^2$=7.9), NZraAI06 ($\chi^2$=7.3) and NZraAJ13 ($\chi^2$=11.1) had an excess of 'M.9' alleles (possibly due to the incorrect scoring of the segregating band), and so were removed from the analysis. The marker NZraAB07 was also removed from the analysis because the segregating band was extremely close to another amplified DNA band, making it difficult to score. Attempts were made to improve the quality of scoring by reducing, then increasing the electrophoresis time (1h and 3h30mn respectively, at 110 volts), but no

improvement was observed. The location on the 'M.9' genetic map of the remaining markers is presented in figure 3.3.

The location of the *DW1* locus was determined using all the individuals from the population, including the vigorous individuals amplifying markers associated with *DW1*. The availability of flanking markers on both sides of *DW1* decreased the number of vigorous individuals with 'M.9' allele at the *DW1* locus from 20 to 12 (see Appendix II for the score of markers flanking *DW1*), indicating that these markers mapped closer to *DW1* than the markers identified previously by Rusholme *et al.* (2004).

Individuals classified as dwarfing had only alleles from the 'M.9' parent at the loci surrounding *DW1*. Fourteen of the 15 semi-dwarfing individuals had alleles from the dwarfing 'M.9' parent, whereas 12 of the individuals classified as intermediate had 'M.9' alleles in the region of the dwarfing locus. The remaining 10 intermediates had alleles from the vigorous 'R5' parent. None of the individuals classified as very vigorous amplified alleles from the 'M.9' parent at the *DW1* locus.

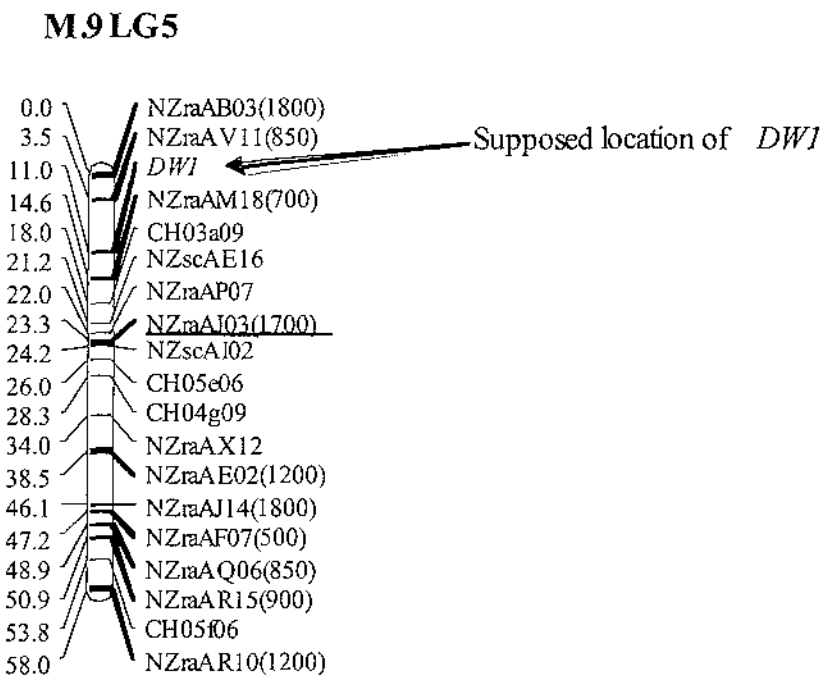
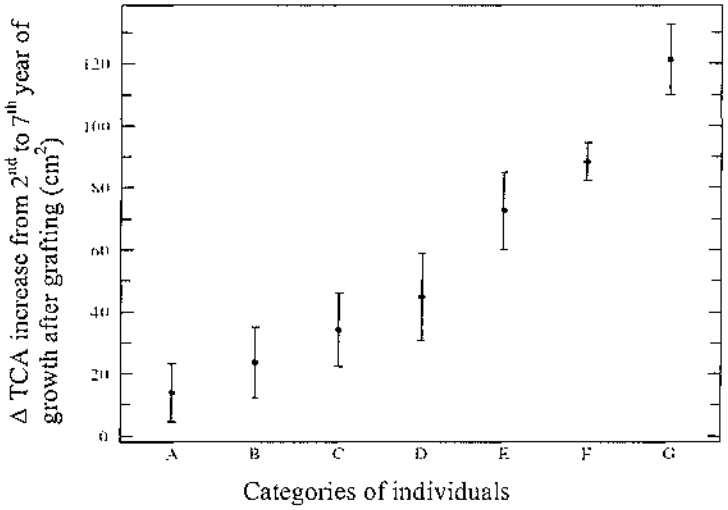



**Figure 3.3. Location of the *DW1* locus on 'M.9' LG5 after two rounds of BSA.** The 10 newly identified RAPD markers are underlined and prefixed with 'NZra' followed by the Operon primer code and the size of the segregating DNA band in bp is given in parentheses. The SCAR markers developed at HortResearch are prefixed with 'NZsc'. The CH prefix designates SSR markers from the literature. On the left hand side of the figure, genetic distances are shown in cM.

As shown in figure 3.4, the *DW1* locus has a major impact in determining the degree of dwarfing of an apple rootstock. One-way ANOVA test indicated that the mean TCA increase of dwarfing individuals (Figure 3.4, category A) from the 2$^{nd}$ to the 7$^{th}$ year of growth after grafting was significantly different from the mean TCA increase of semi-dwarfing individuals (P=0.002) (Figure 3.4, category B individuals). Semi-dwarfing individuals were significantly different from intermediates with 'M.9' alleles at *DW1* locus (P=0.012) (Figure 3.4, category C individuals). Individuals classified as intermediates and amplifying markers associated with *DW1* had a significantly smaller increase in TCA between 2$^{nd}$ and 7$^{th}$ year of growth after grafting (P=0.018) than intermediate individuals that did not amplify markers associated with *DW1* (Figure 3.4, category D individuals). The increase in TCA between intermediate individuals without 'M.9' alleles and vigorous with 'M.9' alleles was significantly different (P=0.002). Individuals classified as vigorous and amplifying markers associated with *DW1* (Figure 3.4, category E individuals) did not have a significant difference (P=0.143) in the increase of TCA compared with

vigorous individuals without 'M.9' alleles (Figure 3.4, category F individuals). However, their mean TCA increase tended to be smaller. Finally, the significant difference (P=0.000) exhibited by very vigorous individuals (Figure 3.4, category G individuals) compared to vigorous (F) individuals in their TCA increase justified the creation of the fifth phenotypic class observed by the breeder.




A: Dwarfing

B: Semi-dwarfing

C: Intermediate amplifying 'M.9' allele at *DW1* locus

D: Intermediate without 'M.9' allele at *DW1* locus

E: Vigorous amplifying 'M.9' allele at *DW1* locus

F: Vigorous without 'M.9' allele at *DW1* locus

G: Very vigorous

**Figure 3.4. TCA increase between 2001 and 2006 in cm² per class of individuals.** TCA measured on individuals derived from the first dwarfing population Rep 1 were used in this analysis. Bars represent 95% confidence intervals. Graph produced with the help of the statistic software developed by Kirkman (1996).

Figure 3.4 also shows that the vigorous individuals amplifying markers associated with *DW1* have not been miss-phenotyped because their TCA increase over the five years recorded is not significantly different from the TCA increase of vigorous individuals without 'M.9' alleles.

The five RAPD markers identified for the *DW2* all mapped together and were unlinked to the *DW1* locus. They covered a 25.3 cM portion of the 'M.9' genome (Figure 3.5). The location of DW2 in the 'M.9' genome is unknown because no transferable marker (such as SSR markers) has been mapped to this locus in this study.

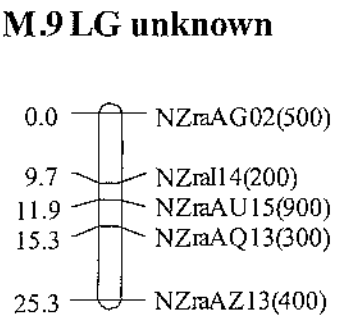



**Figure 3.5. Mapping of the markers linked to *DW2* on 'M.9'.** The five RAPD markers identified for this locus are prefixed with 'NZra' followed by the Operon primer code and the size of the segregating DNA band in bp (indicated in brackets). Genetic distances are shown in cM on the left side of the linkage group.

## 3.5 Discussion

In this study, 10 RAPD markers linked to the locus *DW1* were added to the LG5 of 'M.9', and five markers were identified for a putative second locus (*DW2*) influencing dwarfing. The location of *DW1* on the linkage group was re-estimated according to the latest marker data available, and this locus is now believed to be located between NZraAM18(700) and NZraAV11(850) (Figure 3.3). One of the major achievements of this study is the identification of markers on both sides of the dwarfing locus. Such flanking markers for *DW1* will enhance the quality of MAS in future apple rootstock breeding programmes.

We have also shown that in the population derived from the first 'M.9' × 'R5' cross, the *DW1* locus is required by a rootstock to have a dwarfing or semi-dwarfing effect on the scion which is grafted to it. However, the presence of markers associated with the *DW1* locus in 12 vigorous rootstocks indicates that other genes controlling *DW1* or influencing the dwarfing ability, such as *DW2* identified in this study, and possibly other QTL with minor effects, are also present in the genome.

### 3.5.1 BSA design

The underlying principle of bulked segregant analysis is the grouping of informative individuals together so that a particular genomic region can be studied against a randomized genetic background of unlinked loci (Michelmore *et al.* 1991). In this study, we used this method to detect genetic markers for two QTL influencing the dwarfing capacity of 'M.9' apple rootstock. This strategy is usually not regarded as a practical approach for either detection of QTL for quantitative traits which may be conditioned by several genes with small effects, or when the QTL is loosely linked to the marker (Shen *et al.* 2003). This is because the bulks are likely to be contaminated with alternative alleles if mischaracterization exists or recombination occurs (Wang and Paterson 1994). In our study, in the region around the *DW1* locus, no recombination was observed between the locus and the markers linked to it because the individuals composing the bulks were selected based on their phenotype and genotype (as identified previously by Rusholme *et al.* 2004). However, for markers linked to *DW2*, the bulks were contaminated by alleles different from the one associated withthis locus, and the polymorphism revealed by the markers in the region around it between the bulks was based on the difference in intensity of amplification of the DNA bands, indicating the relative difference in allele frequency (Figure 3.6).

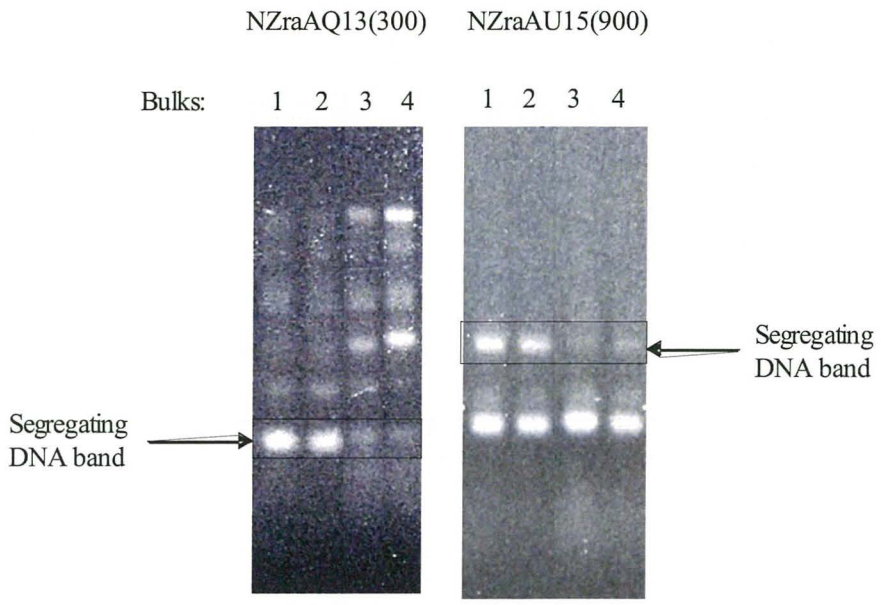



**Figure 3.6. Gel pictures representing the DNA bands amplified by two RAPD markers linked to *DW2* identified during the first BSA round.** These bands segregate among the DNA bulks: bulk 1 (dwarfing); bulk 2 (semi-dwarfing); bulk 3 (vigorous); bulk 4 (vigorous). For both markers (NZraAQ13(300) and NZraAU15(900)), the intensity of the segregating DNA band is stronger in bulks 1 and 2 compared to bulks 3 and 4. This difference of intensity indicates the relative difference in allele frequency.

Analysis of the genotype of individuals composing the bulks in the region of the second dwarfing locus identified showed that 10 out of 11 dwarfing and six out of eight semi-dwarfing individuals amplified a band linked to *DW2*, while three out of 12 vigorous individuals (BSA round 1, bulk 3) and six out of 12 vigorous individuals (BSA round 1, bulk 4) amplified the segregating DNA band linked to *DW2*.

For the second BSA round, the use of eight bulks allowed a more detailed comparison among the different phenotypic and genotypic classes composing the 'M.9' × 'R5' population. Analysis of so many bulks had never been attempted before in apple, most of the BSAs being performed using two types of bulks (resistant individuals versus susceptible individuals) (Markussen *et al*, 1995; Yang *et al.* 1997; Cheng *et al.* 1998; Gygax *et al.* 2004; James and Evans, 2004; Patocchi *et al.* 2004). However, using such a design, a failure during the PCR amplification of one bulk meant a direct loss of information for this bulk as it was not replicated (except bulks composed of DNA extracted from dwarfing individuals, bulks 1 and 6, Table 3.2). To avoid a loss of information, the option of duplicating all the bulks had been considered. However, it would have resulted in doubling the number of PCR

reactions to be performed for the same number of primers screened. One of the main factors contributing to a failure in PCR reactions using RAPD primers is a change in the PCR conditions, most often because of variations in the reaction mix (Karp *et al.* 1996). The use of an automated platform minimised this hazard and allowed for the screening of a high number of primers (759 in total). Automation played a crucial part in the success of this project, reducing by three to four fold the time and effort needed for setting up the PCR reactions and loading them onto agarose gels.

### 3.5.2 Identification of RAPD markers

The bulks constructed in round one yielded three RAPD markers linked to *DW1* [NZraAM18(700); NZraAB03(1800) and NZraAE02(1200)] (Table 3.3) and two markers linked to *DW2* [NZral14(250) and NZraAG02(500)]. The bulks constructed in round two permitted the identification of seven additional markers linked to *DW1* [NZraAJ03(1700); NZraAJ14(1800); NZraAF07(500); NZraAV11(850); NZraAR15(900); NZraAQ06(850) and NZraAR10(1200)] (Table 3.4) and three markers linked to *DW2* [NZraAZ13(400); NZraAQ13(300) and NZraAU15(900)]. In total, 10 new RAPD markers were identified for *DW1* and five markers were identified for *DW2*.

Compared to other studies on apple using BSA, the number of RAPD markers identified for the two dwarfing loci is significant. Gygax *et al.* (2004) identified three RAPD markers linked to a gene of resistance to apple scab (*Vbj*) (between 16.2% and 22.0% of recombination between the gene and the markers) for a total of 506 RAPD markers screened over the bulks. Markussen *et al.* (1995) identified seven markers linked to a gene of resistance to *Pl-1* for a total of 850 RAPD markers screened. Patocchi *et al.* (2004) identified 10 RAPD markers linked to another gene of resistance to apple scab (*Vr2*) for a total of 464 RAPD primers screened. However, the closest marker to the gene was found to be at a distance of 12 cM, compared to the 3.6 cM between *DW1* and NZraAM18(700) (Figure 3.3).

The higher number of markers identified in the second BSA round can be attributed to the higher number of RAPD markers screened over the bulks and the selected 46 individuals, and also to the more complex bulk design.

Despite the fact that RAPD markers are dominant and of low repeatability, their use in this study was a success. Associated with the use of a high throughput automated system, optimal conditions were consistently obtained for maximising the use of RAPD's potential. Using this technology, any region in a genome can be targeted

and quickly saturated with markers. Some of the RAPD markers used in BSA rounds one and two were the same, which may have limited the identification of new markers linked to the trait. Without purchasing extra RAPD markers, we could have increased the number of new markers by mixing different RAPD primers in the same reaction, as performed in Monna *et al.* (1995). The presence of another random primer in the reaction may increase the possibility of amplification of polymorphic fragments by dividing regions which are flanked by original primer but which are too long for PCR. Assuming a random distribution of loci detected as RAPD markers and sufficient polymorphism in the target region, screening more markers should identify more closely linked markers (Michelmore *et al.* 1991).

All the RAPD markers screened in this study were electrophoresed on 0.9% agarose gels. This low agarose concentration resulted for a number of markers in a poor discrimination among DNA bands, which may have resulted in discarding potential markers for dwarfing. Higher concentrations of agarose in electrophoresis gels, such as those employed in Yang *et al.* (1997) (1.3%) and Cheng *et al.* (1998) (2.0%), may have been more suitable in this study and more markers might have been identified.

### 3.5.3 Genetic mapping of *DW1*

The identification of linked markers flanking *DW1* and positioning this locus on the apple genome was the initial step in understanding the genetic control of dwarfing by the apple rootstock 'M.9'. The location of *DW1* on 'M.9' LG5 (Figure 3.3) was modified compared to its original location as identified by Rusholme *et al.* (2004) (Figure 1.5, Chapter 1), because of the integration of data obtained from semi-dwarfing and intermediate rootstocks. The best apparent position for *DW1* was determined by looking for the minimum number of recombination between the *DW1* locus and dwarfing and semi-dwarfing phenotype, and the minimum number of vigorous individuals amplifying markers associated with the apparent *DW1* locus. As a result of the most recent genotypic and phenotypic data, we believe that *DW1* is located in the 11.1 cM interval between the markers NZraAM18(700) and NZraAV11(850) (Figure 3.3; Appendix II), approximately 3.6 cM from NZraAM18(700).

However, the precise mapping of *DW1* relies on the accuracy of the phenotyping of the segregating progeny. Rousselle *et al.* (1974) found that the degree of resistance conferred by a major resistance gene can be modified by the action of minor or

modifier genes inherited from both parents. In addition, Gessler (1989) postulated that the differences in the degree of resistance among plants carrying a gene of resistance to apple scab (*Vf*) could be due to modifier genes. Depending on the combination of alleles present in the parents of a cross and the combination inherited, plants carrying the *Vf* gene may also show a susceptible phenotype. This hypothesis can also be applied to the dwarfing character because some individuals carrying the major gene *DW1* were consistently rated as intermediate or vigorous. Therefore the 12 intermediate and 12 vigorous plants carrying the *DW1* markers could be plants carrying the *DW1* gene and a combination of modifier alleles (such as *DW2*, identified in this study) that shifted the plants into an intermediate/vigorous class (Figure 3.7).

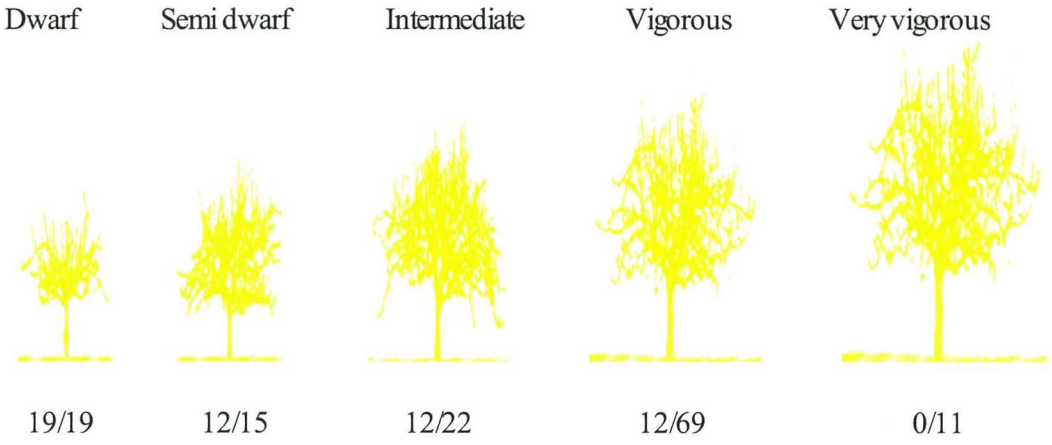



**Figure 3.7. A summary of phenotypic and genotypic classes**. The classes Dwarf, Semi dwarf, Intermediate, Vigorous and Very vigorous are written above the yellow trees illustrating their phenotype. Numbers given below the illustrated phenotypes are the number of individuals with markers associated with *DW1* over the total number of individuals in the class. Individuals from the Intermediate and Vigorous phenotypic classes that carry the *DW1* markers could also carry a combination of modifier alleles or QTL.

No genotypic difference was observed among the classes of phenotypically related individuals (i.e. dwarfing and semi-dwarfing; vigorous and very vigorous). The BSA did not detect genetic differences between dwarfing and semi-dwarfing individuals or between intermediate (without markers linked to *DW1*) and vigorous individuals. Such subtle differences between classes can possibly be attributed to small effect QTL, which explains why the BSA was not able to identify genetic markers for them.

The locus *DW1* has been shown to have a major effect on the degree of dwarfing conferred by apple rootstocks. Intermediate individuals amplifying markers associated with *DW1* were shown to have a smaller TCA increase than intermediate individuals without the *DW1* locus (Figure 3.4). This may indicate that even if *DW1* is not sufficient in itself to induce dwarfing, it still has the effect of reducing the vigour of non-dwarfed trees.

### 3.5.4. Genetic mapping of *DW2*

The location of the *DW2* locus in the genomic region of 'M.9' (Figure 3.5) could not be determined because of the more complex segregation pattern presented by the markers linked to it. In fact, unlike *DW1*, this locus may not be required to influence the dwarfing effect of rootstocks. Based on the marker data from NZraI14(250) and NZraAG02(500), sixteen of the 19 dwarfing individuals, and seven to eight semi-dwarfing individuals out of 15, amplify markers linked to this locus. However, these two markers could be located quite a few cM away from the locus, and markers covering a larger portion of the genome are needed to confirm this hypothesis. The location of *DW2* in the 'M.9' genome is not known because no SSR marker was screened in this study. The genome scanning approach (GSA), based upon the existence of a dense genetic map covering the whole genome and from which transferable genetic markers can be selected at regular intervals and screened over segregating DNA bulks, has been used previously by Rusholme *et al.* (in press) to identify microsatellite markers linked to the dwarfing locus *DW1*. However, knowing that we were going to construct genetic maps of 'M.9' and 'R5' using SSR markers in order to perform a QTL analysis of the dwarfing characteristic, we decided to not adopt this approach and to wait for the results of the genetic map construction (see Chapter 4).

The identification of close markers flanking *DW1* was the first step toward the identification of the *DW1* gene via positional cloning. The markers obtained by BSA are RAPD markers, and it is difficult to use them directly for the construction of physical maps. However, they can be converted easily and reliably to sequence tagged sites (STSs) or SCAR markers which are required for MAS and very useful for map based cloning (Monna *et al.* 1995). We envisaged the possibility of developing SCAR markers from the two RAPD markers flanking the *DW1* locus as well as from two markers linked to the *DW2* locus (NzraI14(250) and

NZraAG02(500)), to enable their use in MAS. However, knowing that we were going to construct the genetic maps of 'M.9' and 'R5' using SSR markers, we believed that more reliable markers would soon be available in the genomic region around *DW1*.

Map-based cloning of genes is possible now that techniques for cloning and manipulating large fragments of genomic DNA have been developed (Michelmore *et al.* 1992). One possible approach involves identifying overlapping genomic clones by chromosome walking. The principle of chromosome walking is that overlapping clones (from a genomic library) will hybridize to each other, allowing them to be assembled into a contiguous sequence (Yang *et al.* 1997). This can be used to isolate genes whose identity is unknown but whose genetic location is known. A prerequisite for chromosome walking is knowledge of the precise location of a gene on a genetic map. DNA markers tightly linked or flanking a target gene can provide a starting point (Yang *et al.* 1997). Following identification of a clone containing a linked marker, this clone is used to screen the genomic library (based on high-capacity vectors such as bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs)) by hybridization, which should reveal additional overlapping clones. These clones are then isolated and used in a second round of screening. The process is repeated until the clone containing the gene of interest is identified (Primrose *et al.* 2001). The last step is the sequencing of the clone and the identification of candidate genes. Identification of genetic markers closer to *DW1* as well as the use of an enlarged segregating population will be necessary to achieve this objective. QTL analysis of the dwarfing trait and other related characteristics will be necessary to identify genetic markers linked to other loci influencing dwarfing and understand the mechanisms controlling them. QTL analysis will also aid confirmation of the existence of *DW2* identified by BSA and measure its impact on the dwarfing effect. Such confirmation would attest to the power of this technique in identifying markers linked to major QTL, as previously shown by Quarrie *et al.* (1999).

The identification of additional QTL influencing dwarfing and other related traits will also provide apple rootstock breeders with a set of genetic markers to enable selection of rootstocks with desired dwarfing or semi-dwarfing ability early in the breeding process. The availability of such markers, together with the development of a strong understanding of the architectural model of dwarfed trees, will have the potential to halve the breeding time per generation and improve the breeding

efficiency by up to 75% via a reduction of land and research orchard resources (Tustin, personal communication). The combination of these factors would result in the development of a breeding programme capable of producing larger families in a shorter timeframe, thereby significantly accelerating the rate of breeding superior new rootstocks.

# 4- CONSTRUCTION OF A SATURATED GENETIC LINKAGE MAP FOR APPLE ROOTSTOCKS USING SSR MARKERS DEVELOPED FROM APPLE ESTS AND PYRUS

## 4.1 Abstract

Marker assisted selection (MAS) offers quick and reliable prediction of the phenotypes of young seedlings in large populations and thus opens new approaches to breeders of apple (*Malus* × *domestica* Borkh.). Genetic mapping and the development of framework maps in segregating populations enables the discovery of genetic markers linked to desired traits. Although framework genetic maps have been reported for apple scion cultivars (Hemmat *et al.* 1994, Conner *et al.* 1997, Maliepaard *et al.* 1998, Liebhard *et al.* 2002, Gianfranceschi *et al.* 1998 and Silfverberg-Dilworth *et al.* 2006) none have previously been constructed for apple rootstocks. We report the construction of framework genetic maps in a cross between 'M.9' and 'R5' apple rootstocks. The maps comprise 224 SSR (Simple Sequence Repeats) markers, 18 SCAR markers, 14 SNP markers and 42 RAPD markers. A new set of 47 polymorphic SSR markers was developed from apple EST sequences containing di-, tri- and tetra-nucleotide repeats and used for construction of this rootstock map. All 17 linkage groups have been identified and aligned to existing apple genetic maps using markers in common, and each linkage group carries a minimum of three markers. The maps span 1175.7 cM ('M.9') and 1086.7 cM ('R5'), which is comparable, in terms of coverage, with the latest apple cultivar genetic maps (Silfverberg-Dilworth *et al.* 2006). To improve the efficiency of mapping traits and markers to this framework map, we developed a bin mapping set (Vision *et al.* 2000). Applications of these new framework genetic maps include the elucidation of the genetic basis of architectural characters such as the dwarfing effect of the apple rootstock 'M.9', and the analysis of disease resistance traits such as fire blight (*Erwinia amylovora*) and apple scab (*Venturia inaequalis*), and the study of the resistance to insects, such as woolly apple aphid (*Eriosoma lanigerum*) conferred by 'R.5'. Markers for traits mapped in this population will be of direct use both to apple breeders for MAS, and for identification of causative genes by map-based cloning.

## 4.2 Introduction

The cultivated apple (*Malus* × *domestica* Borkh.) comes fourth in the global fruit market with over 59 million metric tonnes produced every year (FAO 2004), and can be considered as one of the most economically important temperate fruit crops. To maintain this position, the demand for new apple cultivars with desirable traits such as superior fruit quality, specific architectural and physiological characters and disease and pest resistances, needs to be addressed. However, direct selection for these traits cannot be carried out before several years of growth after grafting, resulting in a long generation time. If prior knowledge of linkage relationships between marker loci and these characteristics were available, undesirable individuals could be eliminated from progeny populations by using marker assisted selection (MAS). High quality genetic linkage maps that enable the study of whole genome structure, the localisation of genes of interest and permit the detection and analysis of quantitative traits loci (QTL) (Tanksley *et al.* 1989) are an essential tool for geneticists and breeders who want to use MAS. Several high density linkage maps have already been constructed for apple, including a genetic map constructed in a 'Fiesta' × 'Discovery' progeny (Liebhard *et al.* 2003); a map developed in a 'Prima' × 'Fiesta' progeny (Maliepaard et al. 1998b), and a map constructed in a 'Discovery' × TN10-8 progeny (Calenge *et al.* 2005) (see Table 1.1 for details). These, together with other less saturated maps such as a 'Telamon' × 'Braeburn' (Kenis and Keulemans 2005) have all been constructed in scion cultivars or selections. Despite the crucial role apple rootstocks have on tree architecture and physiology, fruit quality and disease resistance, no genetic map has been developed for them until now.

One feature of published apple maps is the presence of simple sequence repeat (SSR) markers. In apple as in other plants, SSR markers are abundant and generally uniformly distributed in the genome (Morgante *et al.* 2002), they are very polymorphic due to the high mutation rate affecting the number of repeats (Hemmat *et al.* 2003) and they are co-dominant. Over 300 SSR markers have already been developed and mapped in apple (Guilford *et al.* 1997; Gianfranceschi *et al.* 1998; Liebhard *et al.* 2002; Silfverberg-Dilworth *et al.* 2006), as well as in other species of the Rosaceae family, such as pear (*Pyrus communis*) (Yamamoto *et al.* 2002a-c, Yamamoto 2007; Oddou-Muratorio *et al.* 2001, Fernández- Fernández *et al.* 2006) and peach (*Prunus persica*) (Dirlewanger *et al.* 2002, Sosinski *et al.* 2000, Testolin *et al.* 2000).

Because they are based on sequence specific PCR amplification, SSR markers are transferable between genetic maps within the same species and can be used as orthologous markers between species. For instance, apple SSR markers were successfully used as DNA markers in pear (Yamamoto *et al.* 2001; van Dyk *et al.* 2005). However, very few SSR markers developed from pear have been mapped in apple (Silfverberg-Dilworth *et al.* 2006), and considering how closely related these species are, pear SSR markers represent a considerable potential source of markers that have not been exploited in apple genetics.

In the past few years, several sequencing projects have produced over 250,000 Expressed Sequence Tags (ESTs) for apple (Newcomb *et al.* 2006, Gardiner *et al.* 2007). SSR markers associated with these sequences are a large resource of markers that have remained largely unexploited. Together with the qualities common to all SSR markers, EST-SSR markers can be used to cross-reference genes among species for enhancing the resolution in comparative genomic studies and identifying conserved genomic regions among species and genera (Brown *et al.* 2001; Decroocq *et al.* 2003).

The investment needed to construct a genetic map can be considerable as it requires the analysis of hundreds of markers over a relatively large number of plants and adding new markers increases this development cost. In order to incorporate new markers onto an existing genetic map and reduce this mapping effort, a strategy named bin mapping, or selective mapping, was developed by Vision *et al.* 2000. This method is based on the selection of a specific set of highly informative seedlings chosen for the number and position of crossover sites. The development of bin mapping requires the construction of a dense genetic map (Vision *et al.* 2005). However, once a saturated map has been obtained, this method can be used to add new markers, such as it was done in several crops such as peach (Howad *et al.* 2005), wheat (Johnson *et al.* 2007), and melon (Monforte *et al.* 2007) to improve the efficiency (reduced genotyping cost and high mapping accuracy). Despite the advantages of efficiency and cost saving that this method of mapping conveys, to our knowledge it has not previously been reported in apple.

The purpose of this investigation was to generate genetic maps for the two apple rootstocks 'Malling 9' ('M.9') and 'Robusta 5' ('R5'), to locate the genomic region of the *DW2* locus identified by bulked segregant analysis, and to identify SSR markers closely linked to the locus *DW1*. Parental maps were constructed using published SSR markers, as well as newly developed SSR markers derived from apple

ESTs. By comparing these maps with other apple and pear maps previously published, homoeologous linkage groups were identified. Finally, a bin mapping set was developed to improve the efficiency of mapping new markers.

## 4.3 Materials and methods

### 4.3.1 Plant material and DNA extraction

Two hundred and sixteen F1 individuals obtained from an interspecific cross between the dwarfing rootstock 'Malling 9' ('M.9') (*Malus* × *domestica*) and the vigorous rootstock 'Robusta 5' ('R5') (*Malus robusta*) were used in this study. A first progeny of 146 individuals was developed in 1999 from a cross at the Hawke's Bay Research Centre of the Horticultural and Food Research Institute of New Zealand (HortResearch). Following this, a second progeny of 350 individuals from a repeat cross between the same parents in 2003 was developed and raised at the same location. Seedlings were grown on their own roots during the first year. The seedlings were then used as rootstocks to evaluate their dwarfing capacity (see Chapter 2, section 2.1 for details). Plants in the first progeny were grown as stool-beds. Leaf material was harvested from these stool-beds for the first progeny, and from the rootstock seedling before grafting for the second progeny. DNA was isolated as described in section 2.2.

### 4.3.2 Published SSR markers developed in apple

A total of 185 published SSR markers were tested for segregation across a set of six progeny plants chosen at random among the first dwarfing population Rep 1 (Table 2.1), and both parents. These included 76 'CH' SSR markers (Liebhard *et al.* 2002), 75 'Hi' SSR markers developed by the HiDRAS consortium (Silfverberg-Dilworth *et al.* 2006), 17 'GD' SSR markers with known or unknown map position (Hemmat *et al.* 2003), four 'MS' SSR markers (Liebhard *et al.* 2002), seven 'NZ' SSR markers (Guilford *et al.* 1997), five 'NZms' SSR markers (Chagné *et al.* 2007b; Bus *et al.* 2007) and the 'Col' SSR (Hemmat *et al.* 1997). 27 SSR markers developed from genomic libraries at HortResearch (Erik Rikkerink, personal communication) were also tested for segregation. In order to identify markers mapping in the region around *DW1*, all the SSR markers mapping on LG5 were screened on the 'M.9' × 'R5' population. All segregating markers were then screened over a set of DNA from 94 individuals. The SSR amplifications were performed as described by Gianfranceschi

*et al.* (1998) in a Hybaid MBS Satellite 0.5G Thermal Cycler (Thermo Electron, Waltham, MA).

### 4.3.3 SSR markers developed from apple ESTs

SSR motifs were detected in the HortResearch apple EST database using an automated bioinformatics tool as described by Newcomb *et al.* (2006). The selection criterion for primer design was made on the basis of the type and length of the repeated motif in the sequence. Tri- and tetranucleotide were favoured over dinucleotide repeats and the length of the SSR motif was over 12 bp. Primer pairs were designed for 130 SSR markers using Primer3 (Rozen and Skaletsky 2000). The theoretical melting temperature of the PCR primers was of approximately 60°C and an amplification product length was kept between 100 and 400 bp motifs. PCR amplifications were performed as described by Gianfranceschi *et al.* (1998) with cycling modifications: an initial denaturation step at 94°C for 2 min 30s was followed by four cycles of 94°C for 30s, 60°C for 1 min, 72°C for 1 min where the annealing temperatures was reduced by 1°C per cycle, and then followed by 30 cycles of 94°C for 30s, 55°C for 1 min, 72°C for 1 min and a final 5 min 72°C extension.

### 4.3.4 SSR markers from pear, peach, raspberry and cherry

A total of 117 SSR markers developed from pear (Yamamoto *et al.* 2002a-c, Yamamoto 2007; Oddou-Muratorio *et al.* 2001, Fernández- Fernández *et al.* 2006) as well as five SSR markers from peach (Dirlewanger *et al.* 2002), three SSR markers from red raspberry (*Rubus idaeus* subsp. *idaeus*) (Graham *et al.* 2004) and one SSR from cherry (*Prunus avium*) (Sosinski *et al.* 1999) were tested for segregation. PCR amplifications were performed as described above but with an annealing temperature of 55°C for the first of the four cycles.

### 4.3.5 SSR analysis

All SSR markers screened over the 'M.9' × 'R5' population were genotyped using a capillary electrophoresis system: the CePro 9600 $^{TM}$ (Combisep, Ames, Iowa). This system offers the advantage of being completely automated, has high throughput (96 samples run in 90 min) and does not require the use of any hazardous chemical (UV light is used to detect amplified DNA fragments). PCR products must be desalted in

96 well microplate devices UNIFILTER® 350µl (Whatman®, Clifton USA) using Sephadex™ G-75 Superfine (Amersham, Uppsala Sweden) prior to running on the CePro 9600 ™. The gel used in the capillaries is non-denaturating, enabling the detection of heteroduplexes that can deter (or help in some cases) the analysis of PCR products, and multiplexing is only possible when the amplification products differ in size. As this capillary electrophoresis system could only differentiate alleles which had a minimum length difference of two to three bases, an initial selection of tri- and tetra-nucleotide SSR type repeats was made.

### 4.3.6 SCAR, SNP and RAPD markers

A total of 82 SCAR (sequence characterised amplified region) and 21 SNP (single nucleotide polymorphism) markers (Gardiner *et al.* 2007, Gardiner *et al.* unpublished) were tested over DNA from the parents and a set of six progeny plants of the cross 'M.9' × 'R5'. Polymorphic markers were screened over 94 individuals. PCR reactions for SCAR and SNP markers were carried out in a 16.5 µl volume containing: 1.5 ng DNA, 18 mM Tris-HCl pH 8.4, 46 mM KCl, 0.1 µM of each dNTP, 1.3 mM $MgCl_2$, 0.08 µM each of forward and reverse primers and 0.44 unit of Platinum™ *Taq* DNA polymerase (Invitrogen). Amplifications were performed in a Hybaid MBS 0.5G PCR machine under the following conditions: an initial denaturation at 94°C for 2 min 45s followed by 40 cycles of 94°C for 55s, the temperature specified for each marker for 55s, 72°C for 1 min 39s and a final 10 min 72°C extension was included. The 42 polymorphic RAPD (Operon Technologies Inc.) markers had previously been screened over the 'M.9' × 'R5' population in the course of using bulked segregant analysis (BSA) to identify markers linked to dwarfing of a grafted scion (see Chapter 3). RAPD reactions were carried out as for SCAR markers and SNP markers except that 1% formamide was added to the PCR mix, and the annealing temperature was 37°C. All amplification products were electrophoresed in 0.9% agarose gels (USB Corporation, Cleveland, OH USA) with TAE buffer at 110 volts and stained with a solution of ethidium bromide (0.75 mg $L^{-1}$).

### 4.3.7 Map construction

Linkage analysis was performed using JoinMap version 3.0 (Van Ooijen and Voorrips 2001) with a LOD score of 4 for grouping. First, genetic maps were built

independently for each parent using informative markers and based on a first set of 94 individuals. Parental maps were aligned using fully informative markers and the linkage group numbering was assigned based on SSR markers in common with published maps (Maliepaard *et al.* 1998). Drawings of the linkage maps were generated with MapChart (Voorrips 2001).

### 4.3.8 Nomenclature of markers and linkage groups

SSR markers developed from the EST library during this project are prefixed with 'NZms' followed by the GeneBank accession number. Markers amplified by the same primer pair but mapping at different loci are qualified with an 'a' or 'b' suffix. The names of the SSR markers from the literature were not changed. The SCAR and SNP markers developed in HortResearch are prefixed with 'NZsc' and 'NZsn' respectively. The RAPD marker name starts with the Operon primer code followed by the indication of the fragment size in base pairs. Linkage groups are numbered in accordance with Maliepaard *et al.* (1998).

### 4.3.9 Development of a bin mapping set

The aim for the selection of the bin mapping set was to minimize the number of DNA samples to be genotyped while obtaining a maximum number of bins covering the whole genome. We also considered only the plants and co-dominant markers showing at least 80% of the data points, in order to minimize the number of missing data points. Marker data used for the construction of the genetic maps was sorted by linkage groups and ordered as shown on the maps (Figure 4.1). The linkage phase for each marker was determined using JoinMap and adjusted so that all markers within the same linkage group were in coupling phase. Following that, individuals with the most crossovers were selected. After comparison of the location of the crossovers between the remaining individuals for both parental maps, a subset of individuals was selected. This bin set was then tested to test the efficiency of the method. For this, a two-step procedure was employed: first a set of markers was screened over the bin mapping set and second, the same markers were screened over the 94 individuals used for the map construction and analysed using JoinMap v3.0. The location of the markers as found by the bin mapping method was compared to their actual location using JoinMap. The set of markers used for this test consisted of 27 SSR markers including 20 SSR from the literature, seven SSR developed from *Pyrus*, and four

newly developed SNP markers. The genotype for each marker was compared to the genotype of the bins by visual inspection.

## 4.4 Results

### 4.4.1. SSR polymorphism

The results of the SSR analysis of 'M.9' and 'R5' are given in table 4.1. Of the 468 primer pairs tested, 224 were polymorphic and were mapped on 'M.9' and/or 'R5'. The most polymorphic SSR markers in the 'M.9' × 'R5' population were the markers developed by Liebhard *et al.* (2002) (CH), followed by the markers developed by Silfverberg-Dilworth *et al.* (2006) (Hi), and the markers developed by Hemmat *et al.* (2003).

**Table 4.1. Summary of SSR polymorphism in the 'M.9' × 'R5' population.** The number of markers tested and the number of markers mapped is indicated according to the origin of the marker. The percentage of markers mapped is indicated in parentheses.

| SSR origin | | | Number of markers tested | Number of markers mapped |
|---|---|---|---|---|
| SSR from literature | | CH [a] | 76 | 56 (74%) |
| | | Hi [b] | 75 | 44 (59%) |
| | | GD [c] | 17 | 12 (70%) |
| | Apple | NZ [d] /NZms [j] | 12 | 5 (42%) |
| | | MS [a] | 4 | 1 (25%) |
| | | Col SSR [e] | 1 | 0 |
| | | Total | 182 | 118 (65%) |
| | Pear [f] | | 117 | 49 (42%) |
| | Peach [g] | | 5 | 0 |
| | Raspberry [h] | | 3 | 0 |
| | Cherry [i] | | 1 | 0 |
| SSR newly developed | EST-SSR | | 130 | 47 (36%) |
| | genomic SSR [k] | | 27 | 10 (37%) |
| TOTAL | | | 468 | 224 (48%) |

[a] Liebhard *et al.* (2002)
[b] Silfverberg-Dilworth *et al.* (2006)
[c] Hemmat *et al.* (2003)
[d] Guilford *et al.* (1997), Chagné *et al.* (2007)
[e] Hemmat *et al.* (1997)
[f] Yamamoto *et al.* (2002a-c and 2007), Oddou-Muratorio *et al.* (2001), Fernández- Fernández *et al.* 2006
[g] Dirlewanger *et al.* (2002)
[h] Graham *et al.* (2004)
[i] Sosinski *et al.* (1999)
[j] Bus *et al.* (2007); Chagné *et al.* (2007)
[k] E. Rikkerink, personal communication

### 4.4.2 Genetic mapping

On the basis of the initial screening over 94 individuals, 85 markers (77 SSR, 5 SCAR, 3 SNP and 1 RAPD) spanning the 'M.9' genome with an average distance

between them of 15 cM, and 85 markers (78 SSR, 4 SCAR and 3 SNP markers) spanning the 'R5' genome with an average distance between them of 14 cM were selected and screened over a second set of 122 individuals. During the construction of the final map, the markers that had not been screened over this second set of individuals were entered as missing data.

The final maps (Figure 4.1) span a total of 1175.7 cM ('M.9') and 1086.7 cM ('R5'). The average chromosome length of 'M.9' is 69.1 cM and 'R5' is 63.9 cM. The longest linkage group is LG15 for both 'M.9' and 'R5' (126.5 cM and 93.5 cM respectively). Each of the linkage groups contains at least 3 markers and 316 markers amplified from 298 primer pairs have been mapped. These 298 primer pairs include 56 'CH' SSR markers, 44 'Hi' SSR markers developed by the HiDRAS consortium, 12 'GD' SSR markers (including some previously unmapped), five NZ/NZms, one MS SSR, 10 genomic SSR markers developed by Erik Rikkerink, 47 EST-SSR markers developed from the apple EST database, 49 pear SSR, 18 SCAR, 14 SNP and 42 RAPD markers. The longest distances between two flanking SSR markers are on 'M.9' LG3 (36.3 cM) and 'R5' LG10 (31.5 cM). The maps of 'M.9' and 'R5' have 82 markers in common, including 76 SSR, three SCAR, one SNP and two RAPD markers. Table 4.2 gives a detailed analysis of each of the linkage groups. Few discrepancies with the literature were observed: (i) a second locus was amplified by CH01c06 and was mapped on the LG2 ('R5'); (ii) Hi07d08 (LG1 'Fiesta') was mapped on LG3 ('R5'); (iii) Hi01d06 (LG11 and LG16 'Fiesta'/'Discovery') was mapped on LG17 ('M.9'); (iv) Hi07d11 (LG11 and LG16 'Fiesta'/'Discovery') was mapped to LG15 ('R5'); (v) a second locus was amplified by Hi01c04 and was mapped 2.1 cM away from its first locus on LG5 ('R5'). A few inversions in the order of markers can be observed. These can be explained by the low number of individuals screened in our study and by potential genotyping errors.

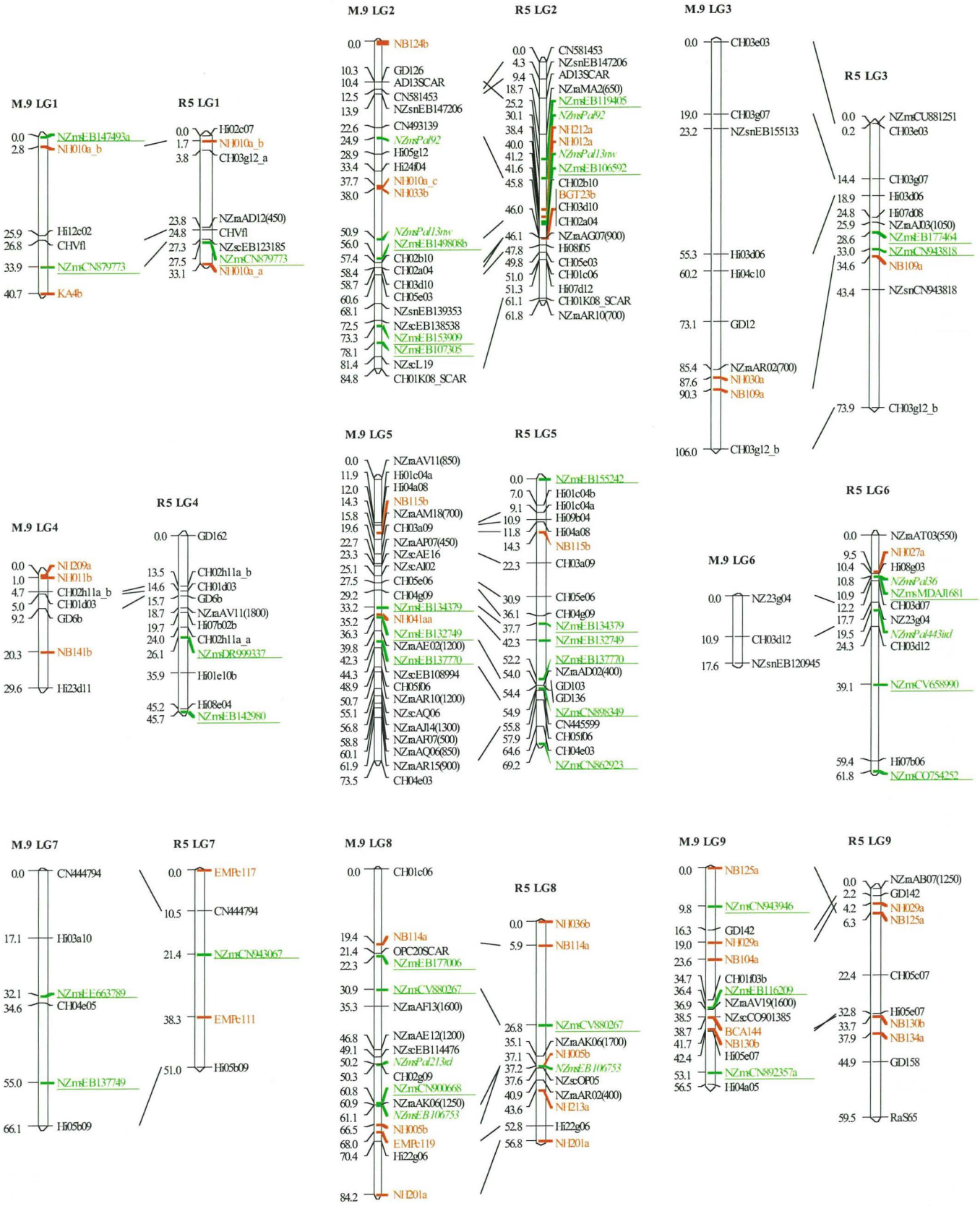



**Figure 4.1. Genetic map of 'Malling 9' ('M.9') and 'Robusta 5' ('R5').** Numbering of the linkage groups is according to Maliepaard *et al.* (1998). Newly developed SSR markers from the EST database are prefixed by NZms and are indicated in green and underlined. SNP, SCAR and RAPD markers developed at HortResearch are prefixed respectively by NZsn, NZsc and NZra. *Pyrus* SSR markers are indicated in red.

Figure 4.1. (continued)

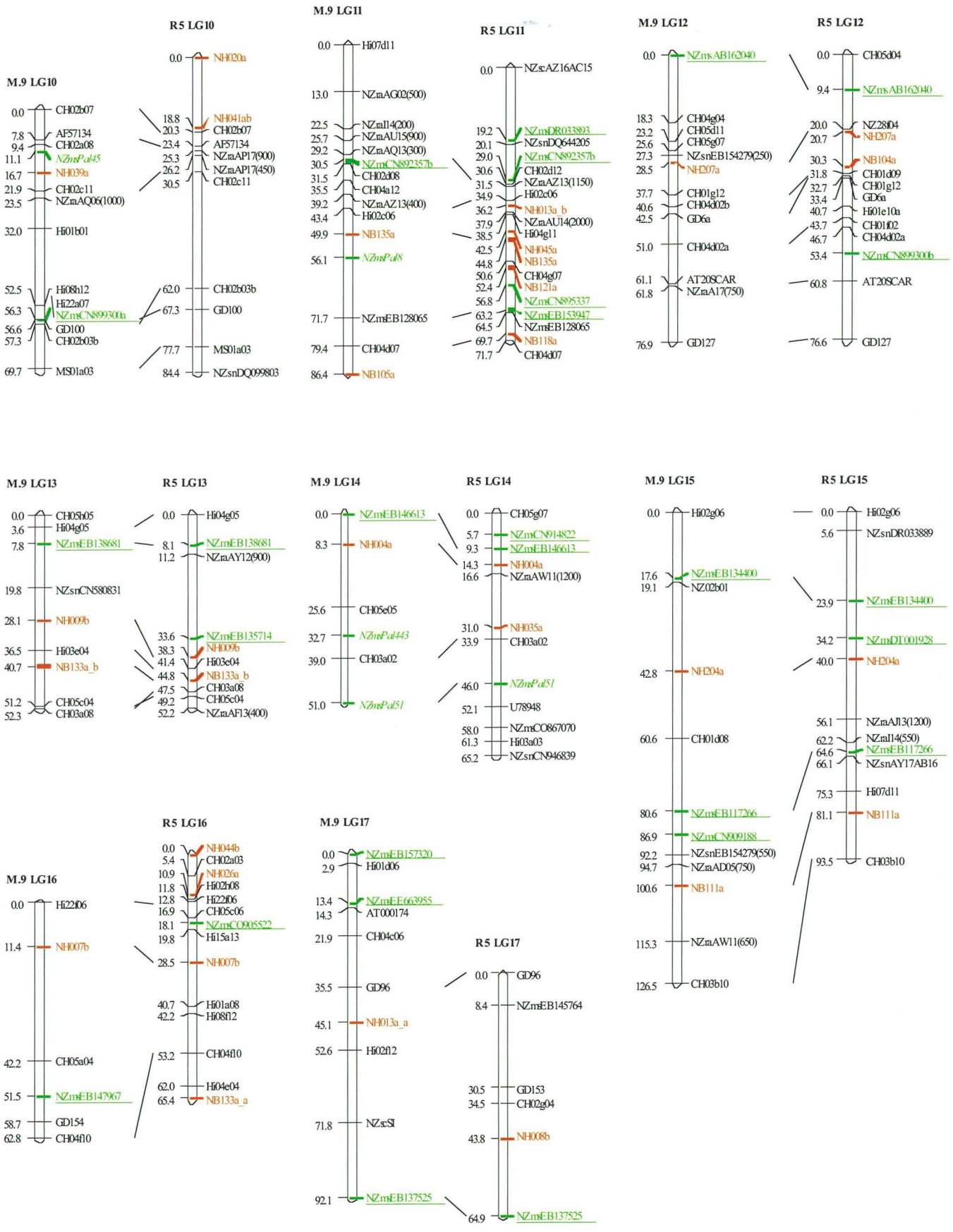


**Table 4.2. Detailed analysis of the 'M.9' × 'R5' map.** The number and type of marker, marker density, length and largest gap are indicated for each linkage group for both parents 'M.9' and 'R5'.

| *Marker type* | | *Linkage groups* | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *M*9 1 | *R*5 1 | *M*9 2 | *R*5 2 | *M*9 3 | *R*5 3 | *M*9 4 | *R*5 4 | *M*9 5 | *R*5 5 | *M*9 6 | *R*5 6 | *M*9 7 | *R*5 7 | *M*9 8 | *R*5 8 | *M*9 9 |
| SSR | Apple | 4 | 4 | 11 | 12 | 6 | 8 | 4 | 10 | 10 | 18 | 2 | 10 | 6 | 3 | 8 | 3 | 7 |
| | Pear | 2 | 2 | 3 | 3 | 2 | 1 | 3 | - | 2 | 1 | - | 1 | - | 2 | 4 | 5 | 5 |
| SCAR | | - | 1 | 4 | 2 | - | - | - | - | 4 | - | - | - | - | - | 2 | 1 | 1 |
| SNP | | - | 1 | 2 | 1 | 1 | 1 | - | - | - | - | 1 | - | - | - | - | - | - |
| RAPD | | - | 1 | - | 3 | 1 | 1 | - | 1 | 9 | 1 | - | 1 | - | - | 3 | 2 | 1 |
| **Total** | | **6** | **8** | **20** | **21** | **10** | **11** | **7** | **11** | **25** | **20** | **3** | **12** | **6** | **5** | **17** | **11** | **14** |
| Length in cM | | 40.7 | 33.1 | 84.8 | 61.8 | 106 | 73.9 | 29.6 | 45.7 | 72.5 | 69.2 | 17.6 | 61.8 | 66.1 | 51 | 84.2 | 56.8 | 56.5 |
| Marker density in markers/cM | | 0.15 | 0.24 | 0.24 | 0.34 | 0.09 | 0.16 | 0.24 | 0.24 | 0.34 | 0.29 | 0.17 | 0.19 | 0.09 | 0.1 | 0.2 | 0.19 | 0.25 |
| Largest gap | | 23.1 | 20 | 12.9 | 9.8 | 32.1 | 30.5 | 9.3 | 13.5 | 11.9 | 9.9 | 10.9 | 20.3 | 20.4 | 16.9 | 19.4 | 20.9 | 10.7 |

| **Linkage groups** | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *R*5 9 | *M*9 10 | *R*5 10 | *M*9 11 | *R*5 11 | *M*9 12 | *R*5 12 | *M*9 13 | *R*5 13 | *M*9 14 | *R*5 14 | *M*9 15 | *R*5 15 | *M*9 16 | *R*5 16 | *M*9 17 | *R*5 17 |
| 4 | 12 | 6 | 8 | 10 | 9 | 11 | 6 | 6 | 5 | 8 | 7 | 6 | 5 | 10 | 9 | 6 |
| 4 | 1 | 2 | 2 | 5 | 1 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 1 | 4 | 1 | 1 |
| - | - | - | - | 1 | 1 | 1 | - | - | - | - | - | - | - | - | 1 | - |
| - | - | 1 | - | 1 | 1 | - | 1 | - | - | 1 | 1 | 2 | - | - | - | - |
| 2 | 1 | 2 | 5 | 2 | 1 | - | - | 2 | - | 1 | 2 | 2 | - | - | - | - |
| **10** | **14** | **11** | **15** | **19** | **13** | **14** | **9** | **10** | **6** | **12** | **12** | **12** | **6** | **14** | **10** | **6** |
| 59.5 | 69.7 | 84.4 | 86.4 | 71.7 | 76.9 | 76.6 | 52.3 | 52.2 | 51 | 65.2 | 127 | 93.5 | 62.8 | 65.4 | 92.1 | 64.9 |
| 0.17 | 0.2 | 0.13 | 0.13 | 0.29 | 0.17 | 0.18 | 0.17 | 0.19 | 0.12 | 0.18 | 0.09 | 0.13 | 0.1 | 0.21 | 0.11 | 0.09 |
| 16.1 | 20.5 | 31.5 | 17.5 | 19.2 | 18.3 | 15.8 | 12 | 22.4 | 17.3 | 14.4 | 23.7 | 18.3 | 30.8 | 12.2 | 19.2 | 22.1 |

### 4.4.3 Mapping of the markers linked *DW2*

The five RAPD markers linked to *DW2* and identified during the bulked segregant analysis (Chapter 3) were mapped at the top of 'M.9' LG11 (Figure 4.1). This group of markers is linked to several other SSR markers of known location (Silfverberg-Dilworth *et al.* 2006) such as Hi07d11, CH02d08 and CH04a12.

### 4.4.4 Mapping of SSR markers linked to *DW1*

As a result of the screening of SSR markers on the 'M.9' × 'R5' population, eight additional SSR markers were mapped on the linkage group containing *DW1* on 'M.9' (Figure 4.1). These include three previously published apple SSR markers (Hi01c04, Hi04a08 and CH04e03), three apple EST-SSR markers developed during this project (NZmsEB134379, NZmsEB132749 and NZmsEB137770), and two SSR markers developed in pear (NB115b and NH041aa).

### 4.4.5 Efficiency of EST-SSR development

Of the 130 SSR markers developed from the apple EST database, 119 amplified a DNA product, and of these 47 segregated and were subsequently mapped in the 'M.9'x'R5' population. These SSR are described in table 4.3. Fourteen of these new EST-SSR markers are di-nucleotide type repeats, fourteen are tri-nucleotide type repeats, eight are tetra-nucleotide type repeats and eleven are compound SSR markers or composed of an association of repeat type. The repeat type of the SSR sequences affected the success of development of polymorphic markers. EST sequences containing a compound SSR, or having two different SSR markers in their sequence were the most polymorphic, and consequently had the highest success rate (50%). The di-nucleotide repeat type (mainly CT/TC and AG/GA repeats) had a success rate of 43% while 19% only of the tri-nucleotide repeat type segregated in the population and were mapped. Forty four percent of the SSR markers with tetra-nucleotide type repeats produced bands polymorphic between the parents and in the progeny, and were mapped.

**Table 4.3 Names, primers, SSR motif, origin of the EST, type of marker and map position of the new set of SSR markers developed**

| GeneBank nb | Forward primer sequence | Reverse primer sequence | Motif in EST database | Origin of EST | Type of marker | Map position | |
|---|---|---|---|---|---|---|---|
| | | | | | | Malling 9 | Robusta 5 |
| NZms CN879773 | CCCTCTGTTACTTTGACTCTTCTC | TGGTTTGGGTTGAAAATGGT | $(AG)_{11}$ | RG* | sloc+ | $M_9$1 | $R_5$1 |
| NZms EB147493a | ACACCCCCTCCCCAAACTT | GCACCCCTCTGATGTTGTG | $(CCCT)_3 + (CT)_{11}$ | RG | mloc± | $M_9$1 | |
| NZms EB149808 | CGAGCCATGCTGATGATTC | TGTCCATCGATCCTCCAATG | $(TC)_7(TATG)_8$ | RG | mloc | $M_9$2 | |
| NZms EB153909 | GTGGAGGGCACACACTCATA | AACAAAGAGGGAGGGAAGGA | $(GAG)_4 + (TCA)_4$ | M.9 | sloc | $M_9$2 | |
| NZms EB106592 | CTCCCACTACTAGCCAAACG | TTGGGATTTGAAGGACAGG | $(TCC)_8$ | RG | sloc | | $R_5$2 |
| NZms EB119405 | GCATGTCAAACCACTTGTCC | ATTCTCTCGCGGCAGTT | $(CGT)_6$ | RG | sloc | | $R_5$2 |
| NZms EB107305 | AACTTCCAAACCCCATCTCC | AGAGCAACCTCACCATCTTCA | $(TC)_7 + (TC)_9$ | RG | sloc | $M_9$2 | |
| NZms EB177464 | CTCCCACCCATCCCTCTAAG | GCTCCTCTTCCCCTCCTCAT | $(CT)_8 + (CT)_{12}$ | RG | sloc | | $R_5$3 |
| NZms CN943818 | CGGGAAGAGGAAATGTGATT | TGAACAGCTCATCGTCGGTA | $(ATG)_4$ | RG | sloc | | $R_5$3 |
| NZms DR999337 | ACAGATTGGGATCTCGTGCT | CTTAGCCTGTGTTGCACATTG | $(GAA)_5(GAT)_6$ | Red Delicious | sloc | | $R_5$4 |
| NZms EB142980 | CCAGTTGGTTATACAAATCGCAAAG | CCTGATCCTCAAAATTACAGCA | $(AAAG)_3$ | RG | sloc | | $R_5$4 |
| NZms EB155242 | CCCCATCATCAAATCAGAAAC | GGAGGTGGTGTTGTCATTCTC | $(TC)_9$ | Braeburn | sloc | | $R_5$5 |
| NZms EB134379 | GATGGGATTATTACATGCATAAGG | AGTGCGGCACGTAGTGTTTT | $(TTTA)_6$ | RG | sloc | $M_9$5 | $R_5$5 |
| NZms EB132749 | CTTTCCAGAGGAGGGCAGTT | GGTACACACTCTTTCCTCTTACATT | $(AAAT)_8$ | RG | sloc | $M_9$5 | $R_5$5 |
| NZms EB137770 | TTGCTGGATCTGAAAACTAGAGG | TCCATTTGCTCTCTCTTCCAA | $(CT)_{13} + (CTT)_6$ | RG | sloc | $M_9$5 | |
| NZms CN898349 | GAGTTGGCAGAAAGAAACCA | CTGGGTGAAGACGAGATGCT | $(TC)_{19} + (TTC)_6$ | M.9 | sloc | | $R_5$5 |
| NZms CN862923 | ATTTTCAAGTACCCCCAACC | CTTCTCCCTACAGCACAAGC | $(ATC)_7$ | RG | sloc | | $R_5$5 |
| NZms MDAJ1681 | CCTGAGGTTATTGACCCAAAA | CACTCAGTTGGAAAACCCTACA | $(CT)_{25}$ | Gene Bank | sloc | $M_9$6 | $R_5$6 |
| NZms EB132582 | AGGCCACATCATACCTCCAC | CACCTTTGTTGTCGTTGTCG | $(CAA)_6$ | RG | sloc | | $R_5$6 |
| NZms CO754252 | CTGCCCTCAAGGAGAATGTC | ACAGGTGCAGCAAAGGCTAT | $(AG)_9$ | Gene Bank | sloc | | $R_5$6 |
| NZms EE663789 | GGGGGACATAAACCTTACCC | TAGTGGGGGCGTAGAAATTG | $(AAC)_4$ | RG | sloc | $M_9$7 | |
| NZms EB137749 | ATCTCCTGCTGTGCTGGTCT | TCACCAAACACCAATCAACAA | $(GA)_8$ | RG | sloc | $M_9$7 | |
| NZms CN943067 | AAATGGGGCACGCTATTATG | TGTGCCATTCAAGAGCTCAA | $(TTC)_{14}$ | RG | sloc | | $R_5$7 |
| NZms EB177006 | AACCAATTCACCCGCCATA | CCTCTGCTGTGGCATGATT | $(CCA)_4$ | RG | sloc | $M_9$8 | |
| NZms CV880267 | TGACCAAAAGAAAAGCAGGAG | AAATTGGCTGGAGGAGCTG | $(CTT)_4$ | RG | sloc | $M_9$8 | $R_5$8 |
| NZms CN900668 | AGGCGTCAGCTGCTACTTTC | CTTTCCCACTTTCCTCACCA | $(AG)_{15}$ | Pacific Rose | sloc | $M_9$8 | |

| GeneBank nb | Forward primer sequence | Reverse primer sequence | Motif in EST database | Origin of EST | Type of marker | Map position | |
|---|---|---|---|---|---|---|---|
| | | | | | | Malling 9 | Robusta 5 |
| NZms EB116209 | AAAATCCCAATTCCAAAACC | TTGGAGCAGTGAAAGATTGG | $(AGA)_{10}$ | RG | sloc | *M*9 | |
| NZms CN943946 | GGCGTGTTAGGGATTCAAAC | TGGATCTCACCACAAAATGC | $(ATTA)_6$ | RG | sloc | *M*9 | |
| NZms CN892357 | AGGTGAAGGTGGCTCAAAGA | AATTGTACGGCTCGGAACAG | $(GA)_{13}$ | RG | mloc | *M*9-11 | |
| NZms CN899300 | ACGAGCGCTCTCTCATCAC | TACGGGATTTTGTTGGTGCT | $(TC)_{15} + (GCA)_5$ | M.9 | sloc | *M*10 | |
| NZms DR033893 | CACTTAGGGTGTATGGGTGTGA | TCATTTTGGGCAGGCACT | $(AGT)_6$ | RG | sloc | | *R*11 |
| NZms CN895337 | GGCACCAACACATCCAAAC | CAGGAAGAGAACCACCGTACA | $(CA)_6$ | RG | sloc | | *R*11 |
| NZms EB153947 | GGGAGAGTTAGGGGAAAAGG | ACTGAGGCCTGCAACATACC | $(ACC)_4$ | M.9 | sloc | | *R*11 |
| NZms AB052994 | TGCTATTAGCTCCTCCTGAATTG | TTGTTGTTGCAGTGAAGGAA | $(CT)_{17}$ | Gene Bank | sloc | *M*12 | *R*12 |
| NZms EB111793 | TTGAGGGCTGCTTTCCAG | GGAGACATACAAGATTTCCAATGAG | $(TC)_{16}$ | RG | sloc | *M*13 | *R*13 |
| NZms EB135714 | CAAAGCCAGAACAGAGAGACAG | TGTTGGGATTGGAGTGATGA | $(AGAC)_3$ | RG | mloc | | *R*13 |
| NZms EB146613 | AGAGTTCCGTTCCCCTCTCT | GTGGATTCGGAAATGCACTC | $(TTCC)_8$ | RG | sloc | *M*14 | *R*14 |
| NZms CN914822 | GACGATGATCAGGCCATTCT | TGTTCATGTCGGTGCTCAAT | $(ACA)_6 + (AGG)_4$ | Braeburn | sloc | | *R*14 |
| NZms EB134400 | TTCATCTTCACCGTCCTCCT | CCCCTCTTCTTATTGCTTCG | $(TGTT)_3$ | RG | mloc | *M*15 | *R*15 |
| NZms CN909188 | CCCTTCTTCTTTCACCACCA | TGAGGAGTTGCTGGGTGTCT | $(CCT)_6$ | Braeburn | sloc | *M*15 | |
| NZms EB117266 | CATCCATGATCATTCAAGACC | CGTGGCCCTCTATATTAAAGC | $(CA)_{11}$ | RG | sloc | | *R*15 |
| NZms CN878021 | TAATCATGAGCGGAGGAGGA | ATGATTGGAACAGCGAGAGG | $(AGC)_7$ | RG | sloc | | *R*15 |
| NZms CO905522 | CAGGGCACTGACAAAGACAG | AATTGGAGATTTGCGGTGTC | $(AG)_6$ | RG | sloc | *M*16 | *R*16 |
| NZms EB147967 | CTTTCTCTCTCTTCGCTCCAA | AGTGCCTTCGGATTCAACAG | $(TC)_7$ | RG | sloc | *M*16 | |
| NZms EB157320 | TGACGAACAACTCCAACAATG | AAGCAAGAACCACCCGAAG | $(GCAA)_3$ | RG | sloc | *M*17 | |
| NZmsEE663955 | CGGAGGCCGCTATAATTAGG | CCTGGAAAGAAAGTAAAAGGACAC | $(TA)_7(TGTA)_3$ | RG | mloc | *M*17 | |
| NZms EB137525 | TCTTTCGCTGGTGTCCTCTT | GTGCTGCTTGCTGTTGTTGT | $(TC)_6$ | RG | sloc | *M*17 | *R*17 |

* Royal Gala

+ sloc = single locus

± mloc = multi loci

### 4.4.6 SSR markers from pear, peach, raspberry and cherry

All 117 of the SSR markers developed from pear amplified a DNA product when tested on apple and 49 of them segregated and have been mapped on 'M.9' or 'R5', giving a success rate of 41.9%. Some pear SSR markers amplified more than one locus and the amplification products were generally longer than expected from results reported in pear. Of the SSR markers developed in the other species, one peach SSR (BPPCT014) as well as one raspberry SSR (RfE8) and one cherry SSR marker (PS12a02) amplified a PCR product. None of these were polymorphic in the 'M.9' × 'R5' population.

### 4.4.7 SCAR, SNP and RAPD markers

18 SCAR and 14 SNP markers segregated between the parents and in the progeny. All these markers were successfully mapped. All 42 RAPD markers previously screened were successfully mapped on the 'M.9' × 'R5' map.

### 4.4.8 Development and test of a bin mapping set

The best set found by visual inspection for both 'M.9' and 'R5' was composed of 14 individuals. This set detected a total of 113 bins for 'M.9' and 106 bins for 'R5' (Table 4.4), each of them identifying a single joint genotype. The maximum number of bins was found for 'M.9' on LG5 and 'R5' on LG11 with respectively 14 and 10 bins (Table 4.4). The average bin length was 10.4 cM for 'M.9' and 10.2 cM for 'R5', while the longest bin span was 31.5 cM (LG10 'R5'). Details of the bin mapping for 'M.9' and 'R5' are presented in Appendix III and Appendix IV, respectively.

**Table 4.4. Summary of the number of bins identified by the 14 selected individuals composing the bin mapping set**, for each linkage group (LG) and for both parents 'M.9' and 'R5'.

| LG | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 'M.9' | 3 | 10 | 7 | 4 | 14 | 3 | 5 | 6 | 6 | 6 | 9 | 8 | 5 | 5 | 10 | 5 | 7 | 113 |
| 'R5' | 4 | 6 | 5 | 4 | 8 | 6 | 5 | 7 | 7 | 6 | 10 | 8 | 5 | 6 | 9 | 3 | 7 | 108 |

The 31 SSR and SNP markers screened over the bin mapping set had been selected because of their polymorphism and co-dominance. The majority of the markers had a genotype that corresponded to the bins established. Seven markers had a genotype that did not correspond to any of the bins, however their location could be established by comparing adjacent bins and analysing the possibility of recombination between them. Finally, one SSR marker, whose score did not match exactly any of the pre-established bins, was predicted to map at one end of a LG which was not included in the genetic map. The 31 markers were then screened over 94 individuals of the population and their actual position in the genome was established. All the markers mapped close to their predicted location, including the SSR marker mapping at one end of a LG, with an average distance between the predicted location and the actual location of 8 cM. The shortest distance was 0.8 cM and the longest 15.3 cM.

## 4.5 Discussion

### 4.5.1 SSR polymorphism

SSR markers from the literature were the most polymorphic (Table 4.1) as 46.7% and 48.9% of them segregated in 'M.9' and 'R5', respectively. In total, 65.0% of SSR markers from the literature were mapped on 'M.9' and 'R5', indicating a sufficient degree of polymorphism to create genetic linkage maps for both cultivars.

### 4.5.2 Development of a saturated map for apple rootstocks 'M.9' and 'R5'

We achieved our goal of constructing the first saturated genetic maps for apple rootstocks 'M.9' and 'R5'. The maps contain 316 markers and span 1175.7 cM and 1086.7 cM for 'M.9' and 'R5' respectively. In addition, we report the generation and mapping of a new set of 47 apple SSR markers developed from EST sequences, the mapping of 49 SSR markers developed from pear, and the use of a new genotyping method for screening the SSR markers (Table 4.1).

### 4.5.3 Mapping of the markers linked to *DW2*

Strong linkages were observed between the RAPD markers identified for *DW2* and the SSR markers mapped on 'M.9' LG11 in this study. The RAPD marker NZraAG02(500) and the SSR CH04a12 were linked with a LOD=16.1. The RAPD

marker NZraI14(250) was linked to CH02d08 and CH04a12 with a LOD score of 15.5 and 16.9, respectively. These strong linkages, and the fact that Hi07d11, CH02d08 and CH04a12 have been mapped previously to LG11 in the reference map 'Fiesta' × 'Discovery' (Silfverberg-Dilworth *et al.* 2006), indicate that *DW2* is located at the top of this linkage group.

### 4.5.4 Mapping of SSR markers linked to *DW1*

Of the eight SSR markers mapped to 'M.9' LG5, two markers (Hi01c04 and Hi04a08) appeared to map in the genomic region containing the *DW1* locus (Figure 4.1). Both SSR markers map at the same location, which is 3.8 cM above NZraAM18(700) and 11.9 cM below NZraAV11(850) (Figure 4.1). This genomic region of 'M.9' LG5 corresponds to the region where we had mapped *DW1* previously (Chapter 3, Figure 3.4). Analysis of the results of these two markers showed that all the dwarfing and semi-dwarfing individuals amplified alleles from 'M.9' at this locus, indicating that these SSR markers were closer to *DW1* than the RAPD NZraAM18(700) and NZraAV11(850) identified in Chapter 3 (see Appendix V for details). The number of intermediate individuals with *DW1* (12) as well as the number of vigorous individuals amplifying 'M.9' alleles at the *DW1* locus (12) did not change compared to what we had found previously in Chapter 3. Because of the presence of recombination events between the SSR markers and NZraAV11(850) in vigorous (trees 90 and 93, Appendix V) and intermediate (trees 128 and 136, Appendix V) individuals, it is hard to determine if the *DW1* locus is present or absent in these rootstocks. However, because no recombination was identified between the SSR markers and the dwarfing and semi-dwarfing phenotype, we can presume that the score of Hi01c04 and Hi04a08 is more likely to give us a correct indication of the presence or absence of the *DW1* locus.

The SSR markers Hi01c04 and Hi04a08 will be ideal markers for MAS of future dwarfing apple rootstocks. The success of MAS with one marker is determined by the linkage distance of the marker to the gene of interest (Gardiner *et al.* 2007). With recombination rates of 1%, as it is the case between the *DW1* locus and the SSR markers Hi01c04 and Hi04a08, inaccurate selection becomes negligible (Gardiner *et al.* 2007). SSR markers are already used for MAS in several crops such as wheat (Zhou *et al.* 2003), soybean (Cregan *et al.* 1999) and rice (Ping *et al.* 2004). The SSR markers will also be useful for germplasm screening. Genetic markers, and

particularly SSR markers because of their transferability between cultivars, linked to a specific gene are efficient for the screening of germplasm for the distribution of that gene (Gardiner *et al.* 2007). SSR markers such as CHVf-1 (mapped on the 'M.9' × 'R5' map on LG1 (Figure 4.1)) and CHVf-2 have already been used for that purpose in apple to identify apple accessions carrying an allele of the resistance gene *Vf* (Vinatzer *et al.* 2004).

### 4.5.5 Development of a new set of EST based SSR markers

Silfverberg-Dilworth *et al.* (2006) established that the level of polymorphism of di-nucleotide repeat type SSR markers from EST sequences is higher than what we observed (61% compared to 36% (Table 4.1)). This can be explained by (i) the lower sensitivity of the capillary electrophoresis system we used to screen SSR markers and (ii) by the low selection criteria applied: some of the di- and tri-nucleotide SSR markers tested had as few as six repeated units.

In the set of SSR markers tested in our study, higher polymorphism was displayed by di- and tetra-nucleotide repeats compared to tri-nucleotide repeat. Di- and tetra-nucleotide repeats are usually found in higher frequency in untranslated regions (UTRs) of a gene (3' and 5') (Morgante *et al.* 2002), and it is very likely that the di- and tetra-nucleotide SSR markers mapped in this study originate from these UTRs. The eight tetra-nucleotide SSR markers developed during this project are the first of their kind to be mapped in apple. These SSR markers showed good polymorphism, as four of them amplified more than two different alleles. Tetra-nucleotide SSR markers could become markers of choice for fingerprinting studies in apple, as it is already the case in human DNA, because of their low 'stuttering' and larger size difference between alleles (Guarino *et al.* 2006). The screening of these newly developed SSR markers over a set of cultivars, such as the one used in Silfverberg-Dilworth *et al.* (2006) and containing the apple cultivars 'Fiesta', 'Discovery', 'Florina', Nova Easaygro', TN10-8, 'Durello di Forlì', 'Prima', 'Mondial Gala' and 'Fuji', will allow the estimation of their true level of polymorphism and determination of their allele size range. This screening will also enable selection of the most suitable markers for genotyping and genetic diversity studies.

Tri-nucleotide repeats are more frequent in coding regions of genes, which may be a result of mutation pressure and positive selection for specific single amino-acid stretches (Morgante *et al.* 2002). A mutation in a mono-, di- or tetra –nucleotide SSR

in the open reading frames (ORFs) would result in a frameshift that could change the translated protein structure and function. It is interesting to note that in our study, the tri-nucleotide types TTC/CTT/TCC displayed a lower polymorphism (12%) compared with the tri-nucleotide type AAC/CCA (57%).

The 47 new SSR markers developed from apple EST sequences are distributed throughout the whole genome (Figure 4.1). However, differences in the number of SSR markers mapped between LGs can be observed: six EST-SSR markers have been mapped to LG5 on 'M.9' and 'R5' while only one has been mapped to LG10. Small clusters of SSR markers selected as sequences coding for proteins believed to be involved in disease resistance can be observed: (i) NZmsEB153909 and NZmsEB107305 at the bottom of LG2; (ii) NZmsEB177464 and NZmsCN943818 on LG3, (iii) NZmsEB137770 and NZmsCN898349 on LG5.

### 4.5.6 Comparison of the 'M.9' × 'R5' genetic map with other apple maps

The length of the 'M.9' and 'R5' maps (1175.7 cM and 1086.7 cM respectively) is comparable with the genetic maps of 'Fiesta' (1145.3 cM) and 'Discovery' (1417.1 cM) (Silfverberg-Dilworth *et al.* 2006).
With the exception of LG1, LG4 and LG6 for 'M.9', and LG1 and LG8 for 'R5' (Figure 4.1), the coverage of the other linkage groups is comparable to the reference map 'Fiesta' × 'Discovery'. The absence of markers in these regions on the 'M.9' and 'R5' maps is the result of an absence of polymorphism at the marker loci. Very few SSR markers mapping to LG1 have been published in the literature. We report in this study the development of two new SSR markers on LG1: NZmsEB147493 and NZmsCN879773. LG6 on 'M.9' is the smallest group of all, being just over 17 cM long. Coverage of the 'M.9' LG4 and 'R5' LG8 is not complete and it can be estimated that a portion of 20 to 30 cM in length remains to be covered. In order to fill the gaps in our linkage map, monomorphic SSR markers could be sequenced to identify single nucleotide polymorphism between parental alleles. Using these sequences, SNP markers could be developed and mapped at the desired location.

The 'M.9' and 'R5' maps are mainly composed of SSR markers with relatively few markers of other types. Only the map 'Fiesta' × 'Discovery' (Silfverberg-Dilworth *et al.* 2006) contains more SSR markers (over 300). In total we have mapped 224 SSR

markers on 'M.9' and 'R5' compared with 62 SSR markers for 'Discovery' × 'TN10-8' (Calenge *et al.* 2004, 2005) and 20 SSR markers for 'Telamon' x 'Braeburn' (Kenis and Keulemans 2005). In addition the 'M.9' × 'R5' map is one of the most functional maps available, since it contains over 50 SSR markers and 12 SNP markers derived from EST sequences.

### 4.5.7 Duplication of SSR loci and establishment of homoeologous linkage groups

The mapping of some SSR markers to two different linkage groups may indicate homoeologous pairs of chromosomes in the apple genome. Markers mapping to LG5 have already been found to amplify a second locus on LG10 (Maliepaard *et al.* 1998). This association of linkage groups is confirmed by NH020a mapping on LG5 in pear and LG10 on 'R5' (Table 4.5). The SSR marker CH03g12 amplifies two loci mapping to the bottom of the LG3 and to the top of LG1 (Liebhard *et al.* 2002 and Figure 4.1); the association between LG3 and LG1 is also found with Hi07d08: 'Fiesta' LG1 (Silfverberg-Dilworth *et al.* 2006) and 'R5' LG3 (Figure 4.1). The possible homoeology between LG4 and LG12 (Liebhard *et al.* 2002) is supported in the 'M.9' × 'R5' population by the SSR GD6 (Figure 4.1).

We combined this information on multilocus SSR and RFLP markers (from Maliepaard *et al.* 1998; Liebhard *et al.* 2002; Silfverberg-Dilworth *et al.* 2006 and the 'M.9' × 'R5' maps) to draw up a map representing homoeologous parts of the apple genome (Figure 4.2). Knowledge of the structure of the apple genome will have implications for locating members of multi gene families.

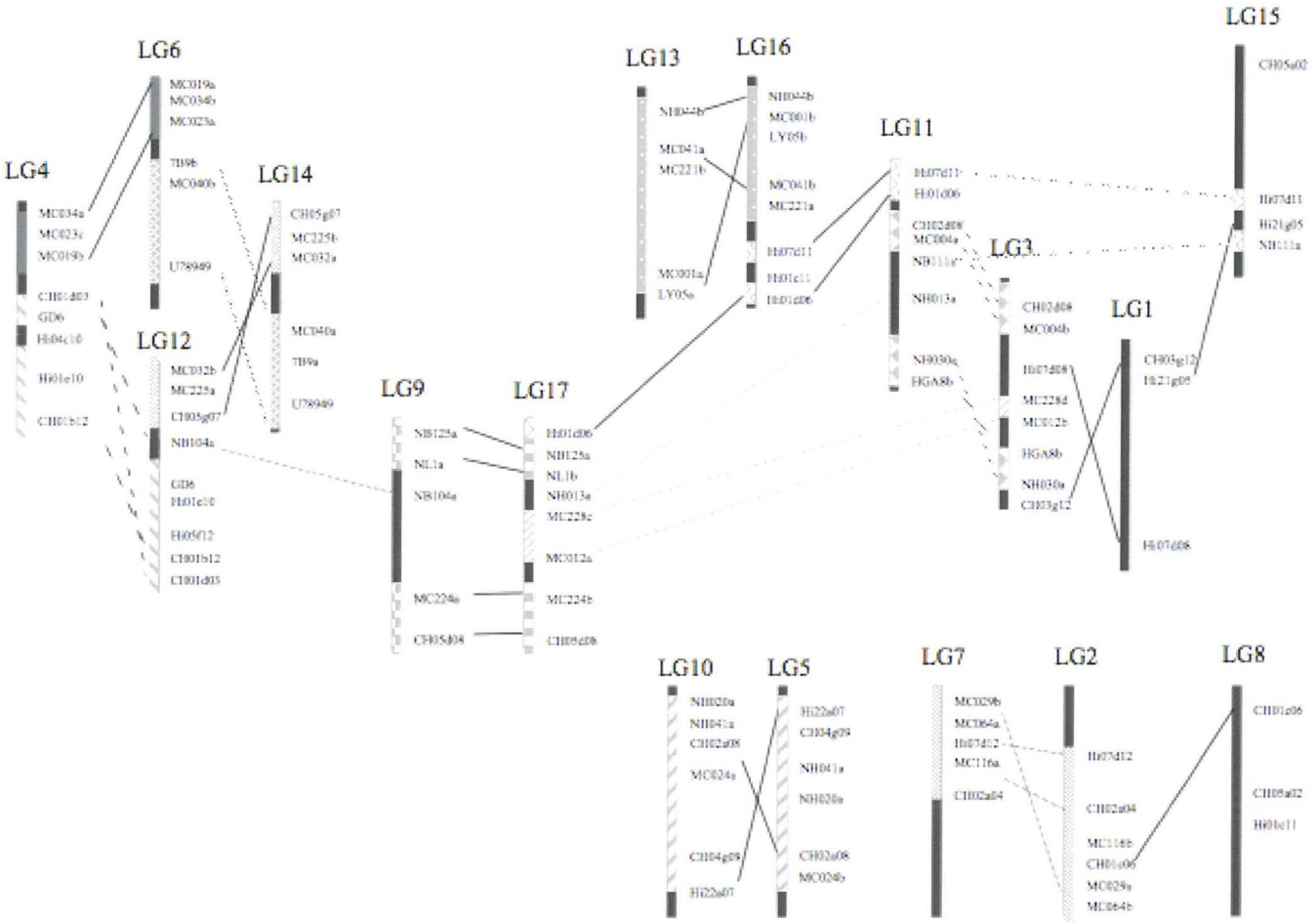



Figure 4.2. Map position of multi-locus SSR and RFLP markers reveal duplication within the apple genome. Map positions are estimated for all the markers. The homoeologous chromosome segments are indicated by different filling patterns of bar segments. The format of the lines linking the groups varies to allow for an easier reading of the map. Linkage groups are named and oriented according to Maliepaard *et al.* (1998).

### 4.5.8 Comparison with published pear genetic map

We were able to transfer SSR markers developed in pear to apple. Out of the 117 pear SSR markers tested, 49 were located on the apple genetic maps in this study, including 46 that had not been mapped in apple before. All the pear SSR markers mapped in this study were derived from genomic SSR markers. Mapping of this relatively large number of pear SSR markers on an apple map is a step towards the detailed study of synteny between these two species. In a study by Yamamoto *et al.* (2004a), 36 apple SSR markers out of the 84 tested were mapped onto the genetic maps of the pear cultivars 'Bartlett' and 'Housui', representing a mapping success rate of 43%. This is similar to the result found in our study where 42% of the pear markers tested were mapped on 'M.9' or 'R5' (Table 4.1). For the synteny between apple and pear, 41 of the 49 pear markers mapped to their expected location in apple (the eight SSR markers not mapping to their expected linkage group are shown in Table 4.5), which is comparable with the results of Yamamoto *et al.* (2004a) who found that only two of the 36 apple SSR markers were assigned to different linkage groups.

**Table 4.5. Pear SSR markers mapping in different linkage groups in apple.** SSR markers were mapped in the pear cultivars 'Bartlett', 'Housui' and 'La France', and in the apple rootstocks 'Malling 9' and 'Robusta 5'.

| | Apple (LG) | Pear (LG) |
|---|---|---|
| NB125a | 9 | 17 |
| NB111a | 15 | 11 |
| NH027a | 6 | 15 |
| NH041a | 5;10 | 7 |
| NH044b | 16 | 13 |
| NH045a | 11 | 10 |
| NH020a | 10 | 5 |
| NH013a | 17 | 1 |

With the exceptions of NH044b mapping on LG16 in apple and LG13 in pear, and NB125a mapping on LG9 in pear and LG17 in pear, the location of the loci amplified by the other SSR markers does not correspond to any of the generally accepted homoeologous regions within the apple genome (E van de Weg *in* Gardiner

*et al.* 2007, and section 4.5.7). These SSR markers may reveal, when confirmed, possible differences in the organisation of the pear genome compared to apple.
Pear SSR markers were mapped to all the linkage groups of the apple genome. The linkage groups with the most pear markers are LG2, LG8, LG9 and LG11 with six markers each, and the linkage group with the least pear markers is LG6 with one marker (Figure 4.1).

### 4.5.9 Perspective for comparative mapping using the 'M.9' × 'R5' map

This map utilizes a spread of SSR markers from different origins and offers possibilities for detailed comparative mapping with *Pyrus*. Broad scale alignment of pear and apple maps using mapping of 39 apple SSR markers to the pear reference map was reported by Yamomoto *et al.* (2004a) and this, with the present study will contribute to the development of a genome wide set of polymorphic and transferable SSR markers, that could be mapped on future pear and apple genetic maps. A larger set of transferable markers would also greatly contribute to the establishment of the microsynteny between apple and pear, as well as the detection of possible rearrangements between the original ancestral genomes indicated by mapping discrepancies in apple for 14% of pear markers (Table 4.5)

### 4.5.10 Contribution of the 'M.9' × 'R5' map to future mapping and to pipfruit breeding

This linkage map will become an essential tool for the mapping of simple major genes as well as for detection of more complex QTL in the near future, providing apple breeders with markers for these genes. Although the majority of the markers tested so far have been screened over 94 to 216 individuals, we plan to screen another set of 250 individuals originating from the same cross to enhance the power of our map for QTL detection. The diversity of segregating characters of economic importance displayed by the progeny increases considerably the significance of this genetic map. It will be possible to determine genetic loci for architectural characters such as the dwarfing effect of the apple rootstock 'M.9', as well as disease and insect resistances from 'R5', including fire blight (*Erwinia amylovora*), apple scab (*Venturia inaequalis*) and woolly apple aphid (*Eriosoma lanigerum*). The identification of molecular markers for each of these traits would significantly reduce

the time and resources required for the breeding of both apple rootstock and scion cultivars.

### 4.5.11 Efficiency of the use of the new capillary electrophoresis system

Although the CePro 9600™ capillary electrophoresis system (Combisep, Ames, Iowa) used in this experiment has a reduced resolution of DNA fragments compared to the traditional approach of screening SSR markers on ABI sequencer, 65% of previously published apple SSR markers mapped, compared to 62% polymorphism for the SSR markers mapped on the 'Telamon' × 'Braeburn' map (Kenis and Keulemans 2005). This relatively high percentage of SSR polymorphism might be explained by the fact that our cross is an interspecific cross. The rootstocks 'M.9' and 'R5' are derived from two different backgrounds, and are only distantly genetically related (Fazio and Baldo 2006), unlike the majority of the scion cultivars used in previous mapping studies. We conclude that the CePro 9600™ has proved to be an efficient tool for microsatellite screening, reducing the time involved in sample preparation and the costs associated with sample detection (i.e. fluorescent tags).

### 4.5.12 Development of a bin mapping set

The bin mapping approach has proven efficient when used in apple. We achieved our objective which was to minimize the effort involved in the mapping of new markers on a map by selecting few informative individuals (Appendices III and IV). We demonstrated that markers could be placed on the 'M.9' × 'R5' map with an average accuracy of 8 cM for thirty one markers, using only 14 individuals of the population instead of 94, representing an 85% reduction of the cost of genotyping. This makes the development of the bin mapping set one of the major achievements resulting from the construction of the genetic map. The incorporation of new markers can also potentially increase the number of bins, resulting in an increase of the accuracy of mapping. An area where bin mapping will be particularly efficient is the mapping of candidate genes (Howad *et al.* 2005), where a large number of candidates need to be screened for co-location with specific mapped phenotypic loci and only those co-locating needing to be mapped in detail over the whole population. Bin mapping in the highly polymorphic 'M.9' × 'R5' population will facilitate access by the scientific community to this reference mapping population, enabling the mapping of new markers or genes through the exchange of a limited number of DNA samples (14).

# 5- QTL ANALYSIS

## 5.1 Abstract

Although several studies have been conducted to investigate the genetic control of scion architecture in apple, (Conner *et al.* 1998, Segura *et al.* 2007, Kenis and Keulemans, 2007), none of them have focused on the genetic control of dwarfing, and other developmental characteristics, of the scion by the rootstock. In this study, we conducted a quantitative trait locus (QTL) analysis using two rootstock populations, derived from crosses between 'M.9' and 'R5', and for which the grafted scions ('Braeburn') segregated for the dwarfing characteristic. Significant correlations were found among the different traits measured, which included the increase in trunk cross-sectional area (TCA) over five years, the overall dwarfing phenotype, the number of flower clusters in trees two years old from grafting, the height of the main axis, the number of nodes and average internode length. Several genomic regions in 'M.9' and 'R5' were found to have a significant influence on each of these traits. The percentage of variation explained by these QTL ranged from 4.2% to 57.2%. A large number of traits had significant variation associated with the major dwarfing QTL, *DW1*, confirming the major influence of *DW1* on tree architecture. QTL stability over the years was estimated by comparing the location of putative QTL for traits measured in multiple years. The two genomic regions identified by bulked segregant analysis, *DW1* on 'M.9' LG5 and *DW2* on 'M.9' LG11, were constantly associated with the increase in TCA over five years of growth after grafting. The location of these QTL compared to QTL previously identified for tree architecture controlled by the scion, and the potential impact of the use of markers associated with the new QTL for dwarfing in future rootstock breeding programmes is discussed.

## 5.2 Introduction

An important goal in apple rootstock breeding is to develop genotypes that impart a dwarfing or semi-dwarfing habit to scions grafted to them, together with improved resistance to diseases, ease of propagation, good anchorage and positive effect on fruit maturation (Cummins and Aldwinckle, 1995). The dwarfing effect of the apple rootstock 'M.9' can be characterized using few measurements of the scion grafted

onto it, such as a reduced height and trunk cross-sectional area (TCA), a reduced number of nodes per shoots and a higher proportion of flowering shoots from the second year after grafting (Seleznyova *et al.* 2003; in press). Unfortunately, direct selection for these traits cannot be carried out before 3-5 years of growth after grafting. If prior knowledge of linkage relationships between marker loci and important tree characteristics were available, undesirable individuals could be eliminated from progeny populations with marker assisted selection, as early as when the seedlings develop their first few leaves. In apple, major genes and quantitative trait loci (QTL) have been reported for growth and development traits (see section 1.2.5). However, none of these studies have focussed on the effect of rootstock on scion development.

Genetic markers such as randomly amplified polymorphic DNA (RAPD), sequence characterized amplified region (SCAR) and simple sequence repeats (SSR) linked to a major QTL (*DW1*) and to another locus influencing dwarfing (*DW2*) have been developed and mapped to the top of LG5 and LG11 of 'M.9', respectively (Chapter 3 and Chapter 4). However, as discussed in Chapter 3, these loci are not sufficient to cause dwarfing, indicating the presence of other minor effect QTL in the genome influencing this phenotype.

QTL analysis is based on the principle of detecting associations between phenotype and the genotype of markers (Collard *et al.* 2005). QTL analysis of morphological traits is a process comprising a multitude of steps including (i) development of a segregating population, (ii) construction of a saturated genetic map of the cross with regularly spaced markers, (iii) phenotypic evaluation of the traits, which comprises an analysis of frequency distribution of the traits, an estimation of their heritability, and an analysis of correlation between the traits. The next steps are (iv) the actual QTL analysis of the traits using an appropriate statistical method, and (v) the interpretation of the results obtained and a comparison with QTL identified in previous studies. The last step (vi) consists in confirming the location and influence of QTL on the trait using independent populations.

I described in Chapter 2 (section 2.1) the development of two rootstock populations derived from crosses between 'M.9' and 'R5', as well as the construction of a saturated genetic map for these crosses (Chapter 4).

In this chapter, the objectives were to analyse the morphological traits measured on scions grafted onto the individuals derived from the 'M.9' × 'R5' crosses, and to estimate the locations and effects of QTL affecting scion development in apple

rootstocks. The results of the QTL analysis of the dwarfing effect and other related growth traits are presented. The stability over time and over repetitions, as well as the location of these QTL on the apple genome in comparison to QTL identified in previous studies are discussed.

## 5.3 Material and methods

### 5.3.1 Plant material and genetic map

History and details of the trees derived from the first'M.9' × 'R5' cross can be found in Chapter 2 (section 2.1).

Two parental framework maps were constructed using markers genotyped over a total of 216 individuals. The set of 216 individuals is composed of 135 individuals derived from the first 'M.9' × 'R5' cross, and 81 individuals derived from the second 'M.9' × 'R5' cross (see material and methods chapter).

As indicated in Chapter 4, 85 markers (77 SSR markers, 5 SCAR markers and 3 SNP markers and 1 RAPD) spanning the 'M.9' genome with an average distance between them of 15 cM, and 85 markers (78 SSR markers, 4 SCAR markers and 3 SNP markers) spanning the 'R5' genome with an average distance between them of 14 cM were used to construct the genetic maps.

Assignment of markers to map positions was performed using JoinMap® 3.0 (Van Ooijen and Voorrips 2001) and a LOD score of 4 was used for grouping. The framework maps were aligned with published maps using common markers, and marker order and distances were checked for discrepancies.

### 5.3.2 Measurements

As stated in Chapter 2, all measurements of the trees derived from the 'M.9' × 'R5' cross were taken by the tree physiologist Stuart Tustin.

Trees derived from the first 'M.9' × 'R5' cross:

For replicate one (Rep1) of this cross, 103 trees were assessed and QTL analysis was performed for the following traits: overall dwarfing phenotype (DW%), estimation of the number of flower clusters in trees two years old from grafting, and annual TCA increase from the second until the seventh year after grafting (Table 2.1).

For replicates two (Rep2) and three (Rep3) of this cross, 112 and 57 trees were assessed respectively. QTL analysis was performed for the following traits: increase

in TCA between fourth and fifth year after grafting, and increase in TCA between fifth and sixth year after grafting (Table 2.1).

The trees derived from the second 'M.9' × 'R5' cross were assessed for the following characteristics: TCA (second year after grafting); height, internode number, and average internode length (first year after grafting). All these measurements were taken on the main axis of the tree.

The table 5.1 summarizes all the traits studied for QTL analysis.

**Table 5.1. Summary of the phenotypic traits measured for the two populations derived from the 'M.9' × 'R5' cross.** The number of individuals is indicated for each trait and population.

| Traits | Number of individuals phenotyped | | | |
|---|---|---|---|---|
| | First 'M.9' × 'R5' population | | | Second 'M.9' × 'R5' population |
| | Rep1 | Rep2 | Rep3 | |
| DW% | 135 | - | - | - |
| Number of flower clusters | 103 | - | - | - |
| TCA 2nd year * | 103 | - | - | 81 |
| TCA 2nd→3rd * | 103 | - | - | - |
| TCA 3rd→4th * | 103 | - | - | - |
| TCA 4th→5th * | 103 | 112 | 57 | - |
| TCA 5th→6th * | 103 | 112 | 57 | - |
| TCA 6th→7th * | 103 | - | - | - |
| TCA 2nd→7th * | 103 | - | - | - |
| Height | | - | | 73 |
| Number of nodes | | - | | 73 |
| Average internode length | | - | | 73 |

* refers to years after grafting

The data obtained from the measure of the TCA during the second year after grafting were analysed separately for both populations.

### 5.3.3 Data analyses

Growth measurements were analysed in order to investigate the variation within the 'M.9' × 'R5' populations. To test the distribution of the different growth measurements, the normality of quantitative traits was evaluated using histograms

and the Kolmogorov-Smirnov (KS) test. Using the results obtained in Chapters 3 and Chapter 4, growth measurements were also analysed to compare the subpopulation of individuals amplifying markers linked to *DW1* and the subpopulation of individuals without the markers associated with *DW1*. The markers used to determine the presence of DW1 in the individuals were the SSR markers Hi01c04 and Hi04a08 (see section 4.5.3).

Broad-sense heritability was estimated for the TCA increase between the 4$^{th}$ and 6$^{th}$ year of growth after grafting. The 45 individuals present in all repetitions were used in this analysis. Two measures of broad sense heritability were performed: the first one involved only Rep2 and Rep3 as they were grafted the same year and therefore subjected to similar environmental conditions. The second measure was performed using all three repetitions.

Broad-sense heritability was calculated using the following formula:

$$H^2 = \frac{s^2_g}{s^2_p} = \frac{s^2_g}{s^2_g + s^2_e}$$

In this formula, $s^2_p$ is the phenotypic variation in a population, $s^2_g$ is the genetic variance and $s^2_e$ the environmental variance.

Correlation coefficients between growth traits and between repetitions were determined using scatter diagrams.

### 5.3.4 QTL analysis

The QTL analysis was performed independently for 'M.9' and 'R5'. The linkage phases of the markers were determined using JoinMap® 3.0 (all markers from the same linkage group must be in the same phase for the QTL analysis to be performed).

QTL analysis was performed for all growth traits using MapQTL® 5 Software (Van Ooijen 2004). QTL mapping was performed using simple interval mapping (SIM) together with the approximate multiple QTL model (MQM) analysis of MapQTL® 5 for non-ordinal traits (non-categorized traits). SIM and MQM mapping were chosen for the reason that they are robust when dealing with non-normally distributed traits. These methods assume that the trait residuals are normally distributed, not the trait itself. After fitting of the QTL, the residuals should behave according to the normal distribution (Van Ooijen, personal communication).

Selected markers were used as cofactors to absorb the effect of QTL, thereby increasing the power for mapping other segregating QTL. The number of QTL was inferred from the number of LOD peaks exceeding the significance threshold. Uncertainty of the map position was indicated by a 2-LOD support interval (Van Ooijen 1992). For traits evaluated over several years and replicates, such as TCA, each year and replicate was considered as a different environment, and therefore data were treated separately.

For the analysis of ordinal traits (DW% and number of flower clusters), the Kruskal-Wallis test was selected for the reason that this test does not have any underlying assumption regarding the distribution. For QTL identified by Kruskal-Wallis analysis, the effect of the marker with the highest significance level on the specific character was estimated by simple linear regression between the marker and the trait (Conner *et al.* 1998, Wang *et al.* 2000, Kenis and Keulemans, 2007).

### 5.3.5 Permutation test and selection of cofactors

The selected cofactors for MQM analysis were the markers which displayed a LOD score close or superior to the LOD score determined by the permutation test.

The permutation test was used to determine the significance threshold of the LOD score. Over a set of 1,000 iterations per LG, the frequency distribution of the maximum LOD score was determined. In each iteration, the quantitative trait data were permuted over the individuals while the marker data remained fixed. An estimate of the frequency distribution of the maximum LOD under the null-hypothesis (no QTL) was then obtained. A P-value of 0.05 was used to determine the significance threshold for each LG.

Several rounds of MQM mapping were performed and marker cofactors were added or dropped depending on whether they were above or below the threshold calculated for the LG they mapped to. Estimates of the phenotypic variance explained by a single QTL at its peak LOD position were obtained from the output of the QTL analysis software.

## 5.4 Results

### 5.4.1 Data analysis

5.4.1.1 Phenotypic data analysis

For individuals derived from the first 'M.9' × 'R5' population, the phenotypic analysis was carried out using data gathered from Rep1, as five years of growth after grafting were assessed in this replicate. The increase in TCA as a proportion of the previous year's TCA was calculated for each individual and for each year. These results were then grouped according to visual classification. The increase in tree vigour from dwarfing to very vigorous corresponded to an increase in the average gain of TCA for each group (Figure 5.1). Dwarfing and semi-dwarfing classes followed the same pattern over time with the largest increase in TCA occurring during the third year of growth after grafting (2001-2002). Intermediate individuals exhibited a constant TCA increase over the five years assessed of approximately twice the magnitude of dwarf individuals, with a peak in the fifth year of growth after grafting. Trees assessed as vigorous and very vigorous showed the largest increase in TCA in the last year assessed (2005-2006, 7$^{th}$ year of growth after grafting).

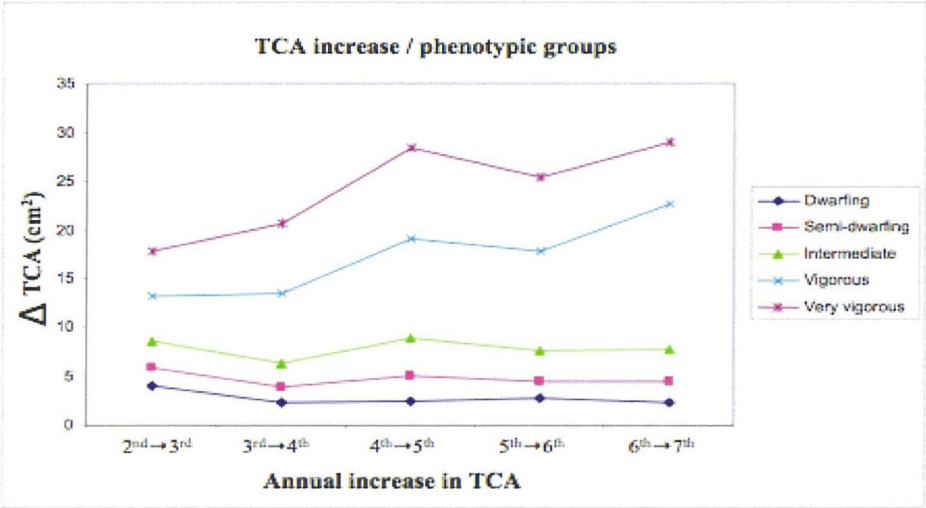



**Figure 5.1. Increase in the average gain of trunk cross-sectional area (TCA).** Increase is represented for each phenotypic group (indicated on the right side of the graph) for the data obtained from Replicate 1 of the first 'M.9' × 'R5' population. The TCA increase was measured each year from the second year until the fifth year after grafting.

### 5.4.1.2 Frequency distribution

Figure 5.2 represents the results of the architectural traits for both 'M.9' × 'R5' populations. All traits exhibited continuous variation in the progenies, which is typical for a quantitative or polygenic inheritance. A bimodal distribution of the data collected from all the individuals of the population can be observed for the different

increases in TCA, particularly between the 2nd and 3rd year of growth and between the 4th and 5th year. Such distributions often indicate the presence of a major QTL influencing the trait, in the present case most likely *DW1*, identified by BSA (Chapter 3).

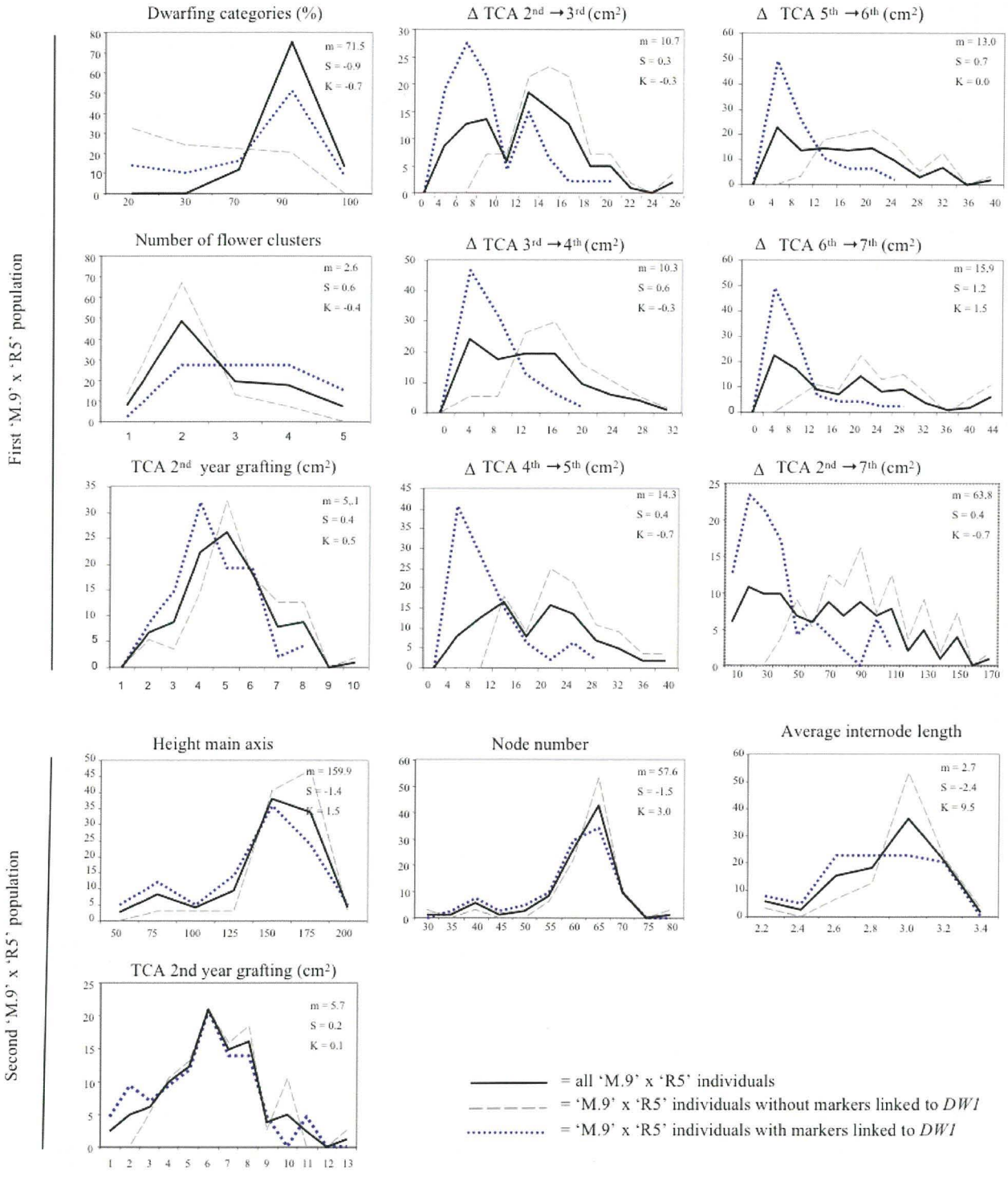



**Figure 5.2. Frequency distribution of the growth traits measured over individuals derived from both 'M.9' × 'R5' populations.** Measurements of all 'M.9' × 'R5' individuals (for each population) are represented by a solid line, and those from the subpopulation of 'M.9' × 'R5' individuals amplifying markers linked to *DW1* (dashed blue line), and without markers linked to *DW1* (dashed grey line). The *Y*-axis represents the percentage of individuals. The mean (m), skewness (S) and kurtosis (K) values of each distribution for the whole population are given in the upper right corner.

To determine whether the values of the phenotypic traits differed significantly between the subpopulation of individuals amplifying markers linked to *DW1* and the subpopulation of individuals without the markers associated with *DW1*, the data were subjected to a non parametric analysis of variance using the Kruskal-Wallis test (Table 5.2). The increase in TCA was significantly smaller for individuals amplifying markers associated with *DW1* than for individuals without markers linked to *DW1*. Significant differences were also observed between the two subpopulations for the height of the main axis and the average internode length. However, no difference was observed between the subpopulations for the number of nodes on the main axis, indicating that *DW1* may not have an effect on this growth trait. No difference was observed for the TCA 2$^{nd}$ year of growth (second 'M.9' × 'R5' population). These results can also be observed in figure 5.2.

**Table 5.2. Mean values of the growth measurements for the two subpopulations of the 'M.9' × 'R5' populations with different genotypes at the *DW1* locus.** The P value represents the probability that the two subpopulations medians are different. *DW1* + indicates the individuals amplifying markers (Hi01c04 and Hi04a08) associated with *DW1*, and *DW1* - indicates the individuals without markers associated with *DW1*

| Growth trait | Mean values | | |
|---|---|---|---|
| | *DW1* + | *DW1* - | P value |
| DW% [a] | 2.31 | 4.02 | 0.000 |
| Number of flower clusters [a] | 3.25 | 2.17 | 0.000 |
| TCA 2$^{nd}$ year (cm$^2$) [a] | 4.07 | 4.95 | 0.004 |
| TCA 2$^{nd}$→3$^{rd}$ (cm$^2$) [a] | 7.27 | 13.64 | 0.000 |
| TCA 3$^{rd}$→4$^{th}$ (cm$^2$) [a] | 5.60 | 14.22 | 0.000 |
| TCA 4$^{th}$→5$^{th}$ (cm$^2$) [a] | 7.17 | 20.32 | 0.000 |
| TCA 5$^{th}$→6$^{th}$ (cm$^2$) [a] | 6.41 | 18.52 | 0.000 |
| TCA 6$^{th}$→7$^{th}$ (cm$^2$) [a] | 5.94 | 23.24 | 0.000 |
| TCA 2$^{nd}$→7$^{th}$ (cm$^2$) [a] | 33.50 | 89.19 | 0.000 |
| Height (cm) [b] | 152.60 | 169.37 | 0.045 |
| Node number [b] | 56.49 | 58.97 | 0.308 |
| Average internode length (cm) [b] | 2.67 | 2.88 | 0.007 |
| TCA 2$^{nd}$ year (cm$^2$) [b] | 5.18 | 6.27 | 0.67 |

[a] Data from first 'M.9' × 'R5' population

[b] Data from second 'M.9' × 'R5' population

The KS test indicated that the majority of the growth measurements did not fit the normal or lognormal distribution (except for the TCA 2nd year after grafting, data not shown). This may be justified by the fact that the distribution curve of most traits showed a severe skewness and a bimodal distribution in some cases (Figure 5.2).

#### 5.4.1.3 Broad-sense heritability

High broad-sense heritability values were estimated for the TCA increase between the 4th and 6th year of growth after grafting using data measured in Rep2 and Rep3, and using all repetitions, with values of $H^2$=0.85 and $H^2$=0.82 respectively. The high heritability estimates for TCA increase indicated that the values for this trait were consistent over years, and that the genotype × year interaction was weak. This result indicates a strong genetic control of the increase in TCA.

#### 5.4.1.4 Analysis of correlation

##### *5.4.1.4.1 Correlation between TCA increase and phenotype*

Calculation of the gain in TCA over the entire recorded growth period for Rep1 (TCA in final recorded year as a proportion of TCA after second year of growth) and comparison with the most recent assigned phenotypic class demonstrated a strong correlation between the increase in TCA and assigned classes (Figure 5.3). ANOVA established that the total TCA increase among the different phenotypic groups was significantly different ($P<0.0001$).

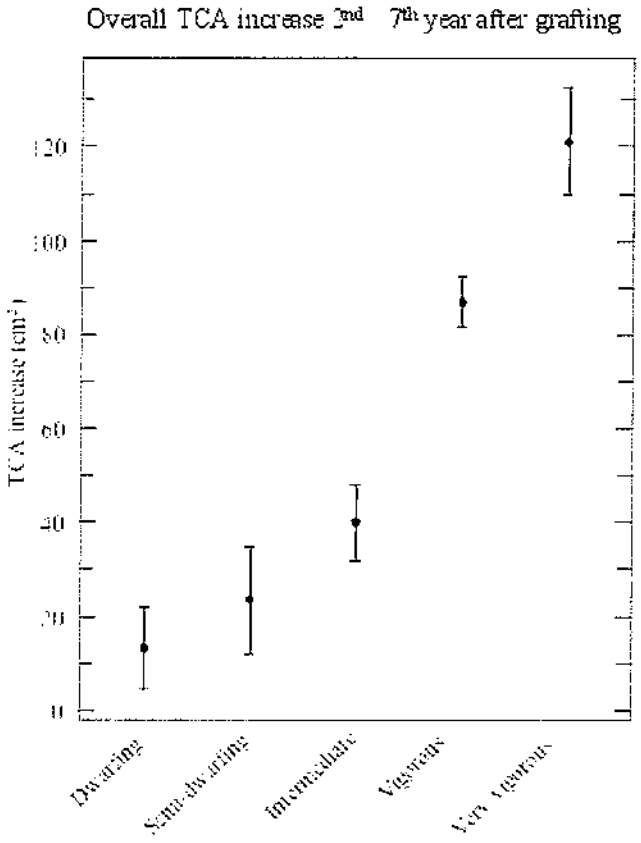



**Figure 5.3. Correlation between the average increase in trunk cross-sectional area (TCA) and vigour classes** (95% interval confidence are indicated by error bars for each class). The classes are indicated under the x axis of the graph. The average increase in TCA (in $cm^2$) is indicated on the y axis of the graph.

As could be expected, individuals classified as dwarfing demonstrated a smaller increase in TCA, while the largest TCA increase was observed in vigorous and very vigorous individuals. As observed previously in figure 5.3, individuals classified as semi-dwarfing were in the lower range. Individuals classed as intermediate can be seen in the middle/lower range of TCA increases.

*5.4.1.4.2 Correlation between traits*

To determine whether the phenotypic traits were correlated with each other, scatter diagrams were plotted and correlation coefficients (Tables 5.3 and 5.4) were calculated for each pair of traits. For TCA increases, only data collected from Rep1 was used in this analysis.

For traits measured on individuals derived from the first 'M.9' × 'R5' cross, strong correlations were observed among the various TCA increases from the 2nd to the 7th

year of growth after grafting (from 0.376 to 0.879; Table 5.3). Correlation among DW% and the various TCA increase over the five recorded years was also very strong, especially when considering the overall TCA increase between the 2nd and 7th year of growth ($R^2$=0.665; Table 5.3). These results confirm that the increase in TCA of the main axis is a good indicator of the degree of dwarfing induced by a rootstock (Rusholme Pilcher *et al.* in press).

**Table 5.3. Correlation coefficient among traits measured from the first 'M.9' × 'R5' population, Rep1**

| | DW% | Number flower clusters | TCA 2nd year | TCA 2nd→3rd | TCA 3rd→4th | TCA 4th→5th | TCA 5th→6th | TCA 6th→7th |
|---|---|---|---|---|---|---|---|---|
| DW% | - | - | - | - | - | - | | |
| Number flower clusters | 0.227 | - | - | - | - | - | | |
| TCA 2nd year | 0.200 | 0.003 | - | - | - | - | | |
| TCA 2nd→3rd | 0.648 | 0.083 | 0.581 | - | - | - | | |
| TCA 3rd→4th | 0.59 | 0.232 | 0.273 | 0.694 | - | - | | |
| TCA 4th→5th | 0.647 | 0.190 | 0.274 | 0.700 | 0.752 | - | | |
| TCA 5th→6th | 0.558 | 0.238 | 0.239 | 0.612 | 0.802 | 0.834 | | |
| TCA 6th→7th | 0.422 | 0.176 | 0.188 | 0.376 | 0.435 | 0.477 | 0.509 | |
| TCA 2nd→7th | 0.665 | 0.226 | 0.327 | 0.733 | 0.830 | 0.875 | 0.879 | 0.747 |

For traits measured on individuals derived from the second 'M.9' × 'R5' cross, a strong correlation was found between the height of the main axis and the number of nodes ($R^2$=0.673; Table 5.4). The number of nodes and the average internode length were not correlated ($R^2$=0.048; Table 5.4).

**Table 5.4. Coefficient correlation among traits measured from the second 'M.9' × 'R5' population**

| | Height | Node number | Average internode length |
|---|---|---|---|
| Height | - | - | - |
| Node number | 0.673 | - | - |
| Average internode length | 0.531 | 0.048 | - |
| TCA 2nd year | 0.449 | 0.348 | 0.194 |

*5.4.1.4.3 Correlation among repetitions*

To estimate the reproducibility of the data obtained in the first 'M.9' × 'R5' population, scatter diagrams were plotted and correlation coefficients were calculated

for each pair of repeated individuals (Figure 5.4). Only those individuals that were present in all three repetitions were taken into account in this analysis (45 individuals). The total TCA increase between the fourth and sixth year were used for all repetitions. For Rep1, this corresponds to the TCA increase between 2003 and 2005, and for Rep2 and Rep3, it corresponds to the TCA increase between 2004 and 2006.

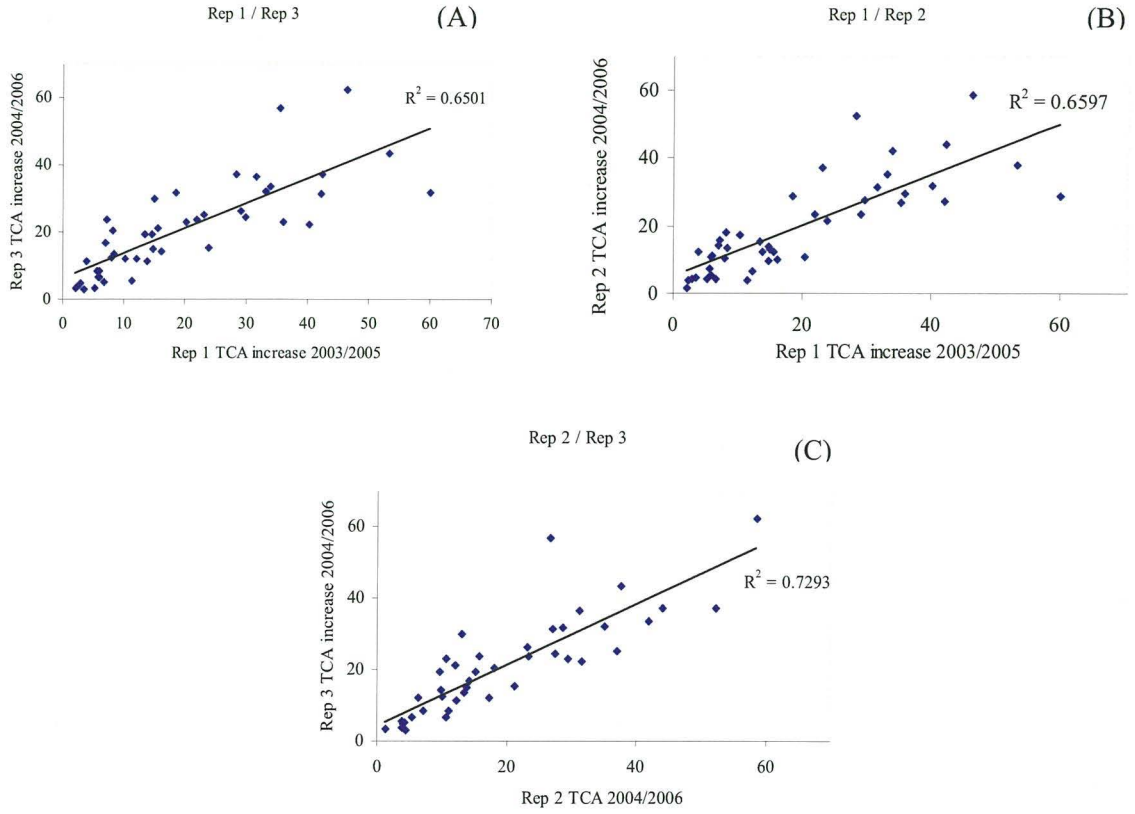



**Figure 5.4. Scatter diagrams of each pair of repeated individuals for their total TCA increase between the fourth and sixth year.** (A) represents the correlation between individuals of Rep 1 and Rep 3. (B) represents the correlation between individuals of Rep 1 and Rep 2. (C) represents the correlation between individuals of Rep 2 and Rep 3. Correlation coefficients are indicated in the top right corner.

As shown in figure 5.4, strong correlations were observed among the different repetitions. The strongest correlation occurred between Rep2 and Rep3 ($R^2 = 0.729$). The coefficient of correlation between Rep1 and Rep2, and between Rep1 and Rep3 were respectively 0.659 and 0.650. These strong correlations indicate a high level of reproducibility between the repetitions, which also confirms the high level of heritability of the character (see section 5.4.1.3).

5.4.1.5 Determination of LOD significance threshold

The LOD significance threshold estimated by permutation tests was determined for each LG and non-ordinal phenotypic trait for both maps. Results of this analysis for the first and second 'M.9' × 'R5' populations are presented in table 5.5 and table 5.6 respectively.

**5.4.2 QTL analysis**

QTL were identified for all traits studied in this analysis.

5.4.2.1 First 'M.9' × 'R5' population

Two QTL were identified for DW% on 'M.9'. The first one at the top of LG5 explained 50.2% (P<0.0001) of the phenotypic variation (Table 5.7, Figure 5.5), and the second QTL on LG11 explained 11.4% (P<0.0001) of the variation. This second QTL on 'M.9' was identified in the same genomic region where markers identified for *DW2* during BSA were located (Chapter 3).
On 'R5' two QTL affecting DW% were also identified on LG10 and LG12, explaining respectively 7.6% (P<0.005) and 5.4% (P<0.01) of the phenotypic variation.

Four QTL were found for TCA of the second year after grafting, using data from the first 'M.9' × 'R5' population Rep1. Two QTL were identified on 'M.9' on LG5 and LG11, explaining respectively 7.1% and 5.4% of the variation (Table 5.7). Two QTL were identified on 'R5' on LG6 and LG14 and explained respectively 5.3% and 7.4% of the variation. No QTL was identified for the TCA second year after grafting of the second 'M.9' × 'R5' population (Table 5.7).

For the different increases in TCA between the 2$^{nd}$ and 7$^{th}$ year of growth after grafting for Rep1, two QTL were always identified on 'M.9' LG5 in the region of *DW1*, and 'M.9' LG11, except for the TCA increase between the 2$^{nd}$ and 3$^{rd}$ year of growth after grafting where only the QTL on LG5 was identified. The first QTL on LG5 explained between 23.9% and 40.7% of the variation, the highest being for the total TCA increase between the 2$^{nd}$ and 7$^{th}$ year. The second QTL on LG11 explained between 4.2% (TCA 5$^{th}$→6$^{th}$ year) and 6.6% (TCA 3$^{rd}$→4$^{th}$ year) of the variation.

For repetitions two and three, the QTL located on LG5 was confirmed for both repetitions and both years (TCA $4^{th}\rightarrow5^{th}$ year and TCA $5^{th}\rightarrow6^{th}$ year). The QTL identified on LG11 was also detected for Rep2 TCA $5^{th}\rightarrow6^{th}$ year, and for Rep3 TCA $4^{th}\rightarrow5^{th}$ year (Table 5.7).

**Table 5.5. Results of the permutation test for the first 'M.9' × 'R5' population**. The table shows the LOD threshold for each of the non-ordinal traits measured on Rep1, Rep2 and Rep3, and for all the linkage groups

| LG | | First 'M.9' × 'R5' population | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Rep1 | | | | | | | Rep2 | | Rep3 | |
| | | TCA $2^{nd}\rightarrow3^{rd}$ | TCA $3^{rd}\rightarrow4^{th}$ | TCA $4^{th}\rightarrow5^{th}$ | TCA $5^{th}\rightarrow6^{th}$ | TCA $6^{th}\rightarrow7^{th}$ | TCA $2^{nd}\rightarrow$7th | TCA $2^{nd}$ year | TCA $4^{th}\rightarrow5^{th}$ | TCA $5^{th}\rightarrow6^{th}$ | TCA $4^{th}\rightarrow5^{th}$ | TCA $5^{th}\rightarrow6^{th}$ |
| 1 | M9 | 2.6 | 2.9 | 3.5 | 2.9 | 3.2 | 3.1 | 2.9 | 5.0 | 3.1 | 3.3 | 4.2 |
| | R5 | 2.7 | 2.8 | 3.2 | 2.6 | 2.7 | 2.2 | 3.0 | 3.4 | 2.6 | 2.7 | 3.3 |
| 2 | M9 | 1.5 | 1.6 | 1.5 | 1.4 | 1.4 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| | R5 | 1.4 | 1.5 | 1.4 | 1.5 | 1.6 | 1.6 | 1.4 | 1.3 | 1.2 | 1.6 | 1.3 |
| 3 | M9 | 1.6 | 1.6 | 1.6 | 1.5 | 1.7 | 1.6 | 1.5 | 1.6 | 1.6 | 1.7 | 1.5 |
| | R5 | 1.4 | 1.4 | 1.4 | 1.5 | 1.6 | 1.3 | 1.3 | 1.7 | 1.4 | 1.3 | 1.2 |
| 4 | M9 | 0.9 | 1.0 | 1.0 | 0.9 | 0.9 | 0.9 | 1.0 | 0.9 | 1.1 | 1.0 | 1.2 |
| | R5 | 1.4 | 1.4 | 1.2 | 1.3 | 1.3 | 1.2 | 1.3 | 1.3 | 1.3 | 1.4 | 1.2 |
| 5 | M9 | 1.5 | 1.7 | 1.6 | 1.6 | 1.6 | 1.5 | 1.7 | 1.7 | 1.6 | 1.6 | 1.6 |
| | R5 | 1.5 | 1.6 | 1.5 | 1.6 | 1.4 | 1.5 | 1.6 | 1.6 | 1.8 | 1.7 | 1.9 |
| 6 | M9 | 1.1 | 1.1 | 1.2 | 1.1 | 1.2 | 1.0 | 1.0 | 1.0 | 1.0 | 1.1 | 1.2 |
| | R5 | 1.4 | 1.5 | 1.5 | 1.4 | 1.6 | 1.4 | 1.4 | 1.4 | 1.4 | 1.5 | 1.4 |
| 7 | M9 | 1.5 | 1.6 | 1.6 | 1.6 | 1.5 | 1.6 | 1.4 | 1.4 | 1.5 | 1.6 | 1.6 |
| | R5 | 1.4 | 1.4 | 1.4 | 1.2 | 1.7 | 1.3 | 1.2 | 1.2 | 1.6 | 1.2 | 1.1 |
| 8 | M9 | 1.5 | 1.3 | 1.5 | 1.4 | 1.4 | 1.5 | 1.4 | 1.5 | 1.4 | 1.6 | 1.5 |
| | R5 | 2.7 | 3.2 | 3.9 | 3.4 | 4.2 | 3.1 | 2.5 | 5.7 | 3.1 | 3.4 | 4.5 |
| 9 | M9 | 1.5 | 1.4 | 1.4 | 1.5 | 1.5 | 1.4 | 1.4 | 1.5 | 1.5 | 1.4 | 1.5 |
| | R5 | 1.4 | 1.3 | 1.3 | 1.3 | 1.4 | 1.3 | 1.3 | 1.3 | 1.6 | 1.5 | 1.6 |
| 10 | M9 | 1.5 | 1.4 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.6 | 1.4 | 1.5 | 1.6 |
| | R5 | 1.4 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.3 | 1.8 | 1.5 | 1.5 | 1.4 |
| 11 | M9 | 1.7 | 1.6 | 1.7 | 1.6 | 1.7 | 1.7 | 1.5 | 1.7 | 1.6 | 1.6 | 1.7 |
| | R5 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.3 | 1.4 | 1.3 | 1.1 |
| 12 | M9 | 1.5 | 1.5 | 1.4 | 1.5 | 1.5 | 1.4 | 1.5 | 1.4 | 1.5 | 1.4 | 1.5 |
| | R5 | 1.6 | 1.5 | 1.6 | 1.5 | 1.6 | 1.4 | 1.5 | 1.7 | 1.6 | 1.3 | 1.6 |
| 13 | M9 | 1.4 | 1.4 | 1.4 | 1.2 | 1.4 | 1.5 | 1.4 | 1.4 | 1.4 | 1.5 | 1.5 |
| | R5 | 1.4 | 1.4 | 1.3 | 1.2 | 1.3 | 1.3 | 1.2 | 1.0 | 1.2 | 1.3 | 1.2 |
| 14 | M9 | 1.4 | 1.4 | 1.4 | 1.3 | 1.4 | 1.3 | 1.4 | 1.4 | 1.5 | 1.4 | 1.5 |
| | R5 | 1.6 | 1.5 | 1.5 | 1.5 | 1.7 | 1.6 | 1.5 | 1.7 | 1.5 | 1.4 | 1.7 |
| 15 | M9 | 1.6 | 1.7 | 1.6 | 1.7 | 1.6 | 1.6 | 1.5 | 1.7 | 1.6 | 1.7 | 1.7 |
| | R5 | 1.6 | 1.6 | 1.6 | 1.6 | 1.7 | 1.6 | 1.5 | 1.7 | 1.5 | 1.4 | 2.1 |
| 16 | M9 | 1.4 | 1.5 | 1.5 | 1.5 | 1.4 | 1.6 | 1.5 | 1.5 | 1.4 | 1.5 | 1.5 |
| | R5 | 1.5 | 1.7 | 1.5 | 1.7 | 1.6 | 1.4 | 1.5 | 1.7 | 1.9 | 1.5 | 1.3 |
| 17 | M9 | 1.7 | 1.6 | 1.7 | 1.6 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.9 | 1.7 |
| | R5 | 1.5 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.2 | 1.5 | 1.1 | 1.6 |

**Table 5.6. Results of the permutation test for the second 'M.9' × 'R5' population.**

| LG | | Second 'M.9' × 'R5' population | | |
|---|---|---|---|---|
| | | Height | Node number | Average internode length |
| 1 | M9 | 7.7 | 8.3 | 7.2 |
| | R5 | 6 | 7.0 | 7.4 |
| 2 | M9 | 1.4 | 1.4 | 1.3 |
| | R5 | 1.4 | 1.4 | 1.3 |
| 3 | M9 | 1.6 | 1.5 | 1.3 |
| | R5 | 1.4 | 1.5 | 1.3 |
| 4 | M9 | 0.9 | 0.9 | 0.8 |
| | R5 | 1.3 | 1.3 | 1.2 |
| 5 | M9 | 1.6 | 1.5 | 1.4 |
| | R5 | 1.5 | 1.5 | 1.5 |
| 6 | M9 | 1.1 | 1.1 | 1.0 |
| | R5 | 1.4 | 1.3 | 1.4 |
| 7 | M9 | 1.5 | 1.5 | 1.4 |
| | R5 | 1.4 | 1.3 | 1.1 |
| 8 | M9 | 1.5 | 1.3 | 1.4 |
| | R5 | 7.9 | 7.6 | 3.1 |
| 9 | M9 | 1.5 | 1.3 | 1.2 |
| | R5 | 1.3 | 1.3 | 1.2 |
| 10 | M9 | 1.4 | 1.5 | 1.4 |
| | R5 | 1.4 | 1.4 | 1.3 |
| 11 | M9 | 1.5 | 1.5 | 1.3 |
| | R5 | 1.4 | 1.4 | 1.3 |
| 12 | M9 | 1.5 | 1.4 | 1.3 |
| | R5 | 1.6 | 1.5 | 1.4 |
| 13 | M9 | 1.4 | 1.4 | 1.2 |
| | R5 | 1.3 | 1.3 | 1.2 |
| 14 | M9 | 1.4 | 1.4 | 1.3 |
| | R5 | 1.5 | 1.5 | 1.3 |
| 15 | M9 | 1.6 | 1.6 | 1.5 |
| | R5 | 1.5 | 1.6 | 1.4 |
| 16 | M9 | 1.5 | 1.6 | 1.4 |
| | R5 | 1.6 | 1.6 | 1.4 |
| 17 | M9 | 1.6 | 1.6 | 1.5 |
| | R5 | 1.4 | 1.4 | 1.3 |

Four QTL were detected for the estimation of the number of flower clusters in trees two years old from grafting. Three QTL were located on the 'M.9' map. The first one was identified at the top of LG5 and explained 21.1% ($P<0.0001$) of the variation. The second QTL was identified on LG11 and explained 16.5% ($P<0.0001$) of the variation. The third QTL on 'M.9' detected for the number of flower clusters was identified on LG9 and explained 6.8% ($P<0.01$) of the variation. One QTL was identified on 'R5' LG6 and explained 9.0% ($P<0.005$) of the variation (Table 5.7).

#### 5.4.2.2 Second 'M.9' × 'R5' population

Three QTL were identified for the height of the main axis after the first year of growth. Two of these were located on 'M.9' on LG11 and LG15 and explained 17.8% (LOD=3.8) and 17.3% (LOD=3.1) of the variation respectively. The third QTL was detected on 'R5' LG6 and explained 8.8% (LOD=1.6) of the variation (Table 5.8).

Four QTL were identified for the number of nodes on the main axis for the first year of growth after grafting. Three of these QTL were identified on 'M.9' on LG8

(15.8%; LOD=2.2), LG11 (11.3%; LOD=2.4) and LG16 (12.5%; LOD=2.6), and one QTL was found on 'R5' LG6 explaining 13.7% of the variation (Table 5.8).

**Table 5.7. QTL associated with growth characteristics of 'Braeburn' apple scions grafted on the segregating population derived from the first 'M.9' × 'R5' cross,** listed by linkage group. For each QTL detected with the multiple QTL model (MQM) the LOD score is given by the number before "/". For each QTL detected with the Kruskal-Wallis test the significance level (indicated by asterisks: ***P < 0.01; ****P < 0.005, *******P < 0.0001) is given. The percentage of variance explained by each QTL is indicated by the number after "/".

| Populations | Trait | Map | LG1 | LG2 | LG3 | LG4 | LG5 | LG6 | LG7 | LG8 | LG9 | LG10 | LG11 | LG12 | LG13 | LG14 | LG15 | LG16 | LG17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | DW% | M.9 | | | | | *******/50.2 | | | | | | *******/11.4 | | | | | | |
| | | R5 | | | | | | | | | | ****/7.6 | | ***/5.4 | | | | | |
| | Number flower clusters | M.9 | | | | | *******/21.1 | | | | ***/6.8 | | *******/16.5 | | | | | | |
| | | R5 | | | | | | ****/9.0 | | | | | | | | | | | |
| | TCA 2$^{nd}$ year | M.9 | | | | | 3.1/7.1 | | | | | | 2.4/5.4 | | | | | | |
| | | R5 | | | | | | 1.8/5.3 | | | | | | | | 2.0/7.4 | | | |
| | TCA 2$^{nd}$→3$^{rd}$ | M.9 | | | | | 11.3/36.3 | | | | | | | | | | | | |
| | | R5 | | | | | | | | | | | | | | | | | |
| First population Rep1 | TCA 3$^{rd}$→4$^{th}$ | M.9 | | | | | 11.5/34.4 | | | | | | 2.7/6.6 | | | | | | |
| | | R5 | | | | | | | | | | | | | | | | | |
| | TCA 4$^{th}$→5$^{th}$ | M.9 | | | | | 13.3/39.9 | | | | | | 2.5/5.7 | | | | | | |
| | | R5 | | | | | | | | | | | | | | | | | |
| | TCA 5$^{th}$→6$^{th}$ | M.9 | | | | | 12.8/38.9 | | | | | | 1.6/4.2 | | | | | | |
| | | R5 | | | | | | | | | | | | | | | | | |
| | TCA 6$^{th}$→7$^{th}$ | M.9 | | | | | 8.2/23.9 | | | | 2.4/6.2 | | 2.0/5.2 | | | | | | |
| | | R5 | | | | | | | | | | | | | | | | | |
| | TCA 2$^{nd}$→7$^{th}$ | M.9 | | | | | 14.2/40.7 | | | | | | 2.9/6.3 | | | | | | |
| | | R5 | | | | | | | | | | 1.9/8.0 | | | | | | | |
| First population Rep2 | TCA 4$^{th}$→5$^{th}$ | M.9 | | | | | 21.6/57.2 | | | | | | | | | | | | |
| | | R5 | | | | | | | | | | 1.8/7.1 | | | | | | | |
| | TCA 5$^{th}$→6$^{th}$ | M.9 | | | | | 12.4/39.8 | | | | | | 2.2/11.7 | | | | | | |
| | | R5 | | | | | | | | | | 2.2/8.7 | | | | | | | |
| First population Rep3 | TCA 4$^{th}$→5$^{th}$ | M.9 | | | | | 9.8/56.5 | | | | | | 1.6/10.1 | | | | | | |
| | | R5 | | | | | | | | | | 1.7/14.9 | | | | | | | |
| | TCA 5$^{th}$→6$^{th}$ | M.9 | | | | | 9.8/57.1 | | | | | | | | | | | | |
| | | R5 | | | | | | | | | | | | | | | | | |

**Table 5.8. QTL associated with growth characteristics of 'Braeburn' apple scions grafted on the segregating population derived from the second 'M.9' × 'R5' cross**, listed per linkage group. For each QTL detected with the multiple QTL model (MQM) the LOD score is given by the number before "/". The percentage of variance explained by each QTL is indicated by the number after "/".

| Populations | Trait | Map | LG1 | LG2 | LG3 | LG4 | LG5 | LG6 | LG7 | LG8 | LG9 | LG10 | LG11 | LG12 | LG13 | LG14 | LG15 | LG16 | LG17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Height | M.9 | | | | | | | | | | | 3.8/17.8 | | | | 3.1/17.3 | | |
| | | R5 | | | | | | 1.6/8.8 | | | | | | | | | | | |
| Second | Node number | M.9 | | | | | | | | 2.2/15.8 | | | 2.4/11.3 | | | | | 2.6/12.5 | |
| population | | R5 | | | | | | 2.6/13.7 | | | | | | | | | | | |
| | Average internode length | M.9 | | | 3.3/14 | | 3.1/13 | | | | | | 1.8/7.5 | | | | 2.0/8.3 | | |
| | | R5 | | | | | | | | | | | | 1.6/11.4 | | | | | |

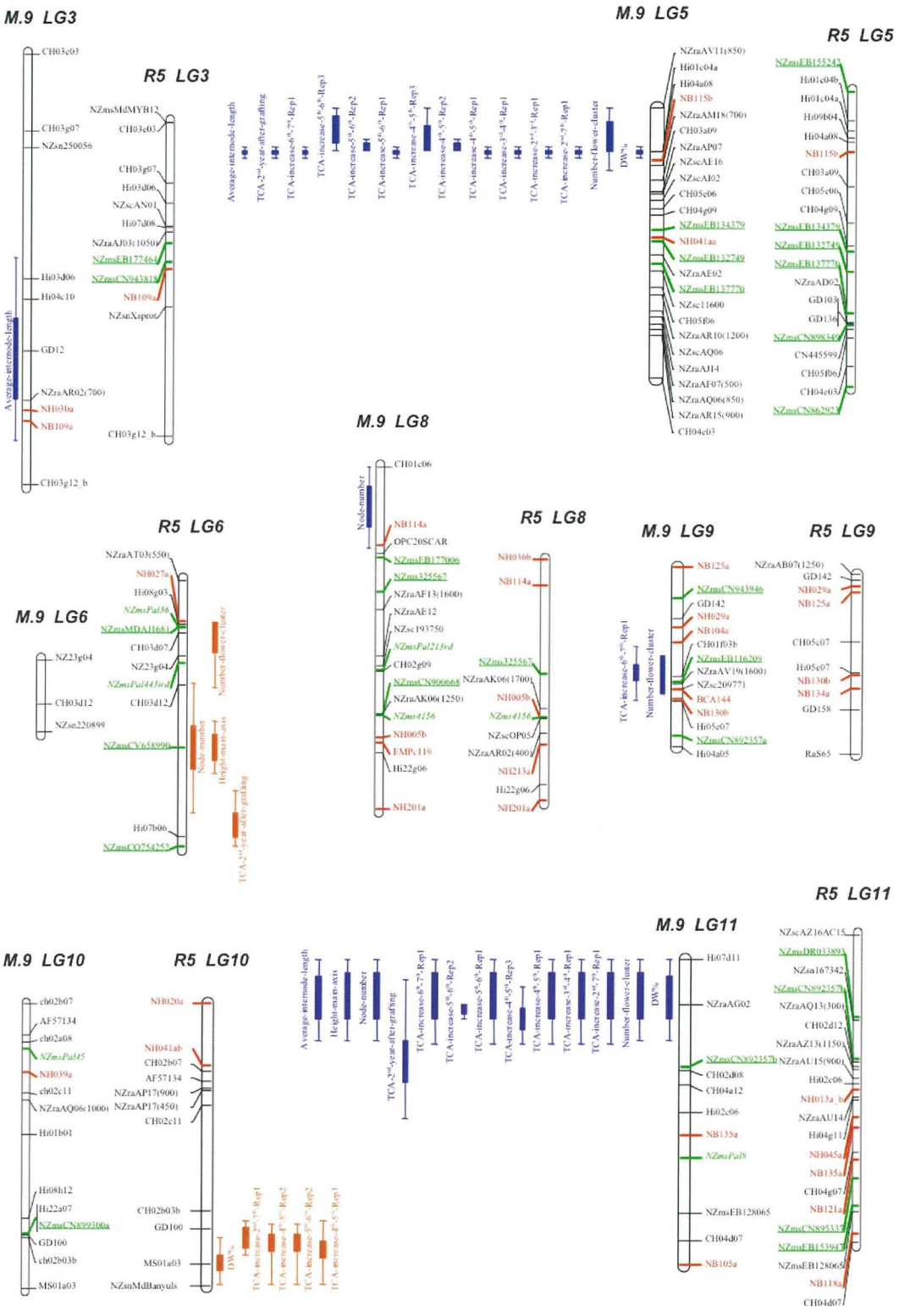
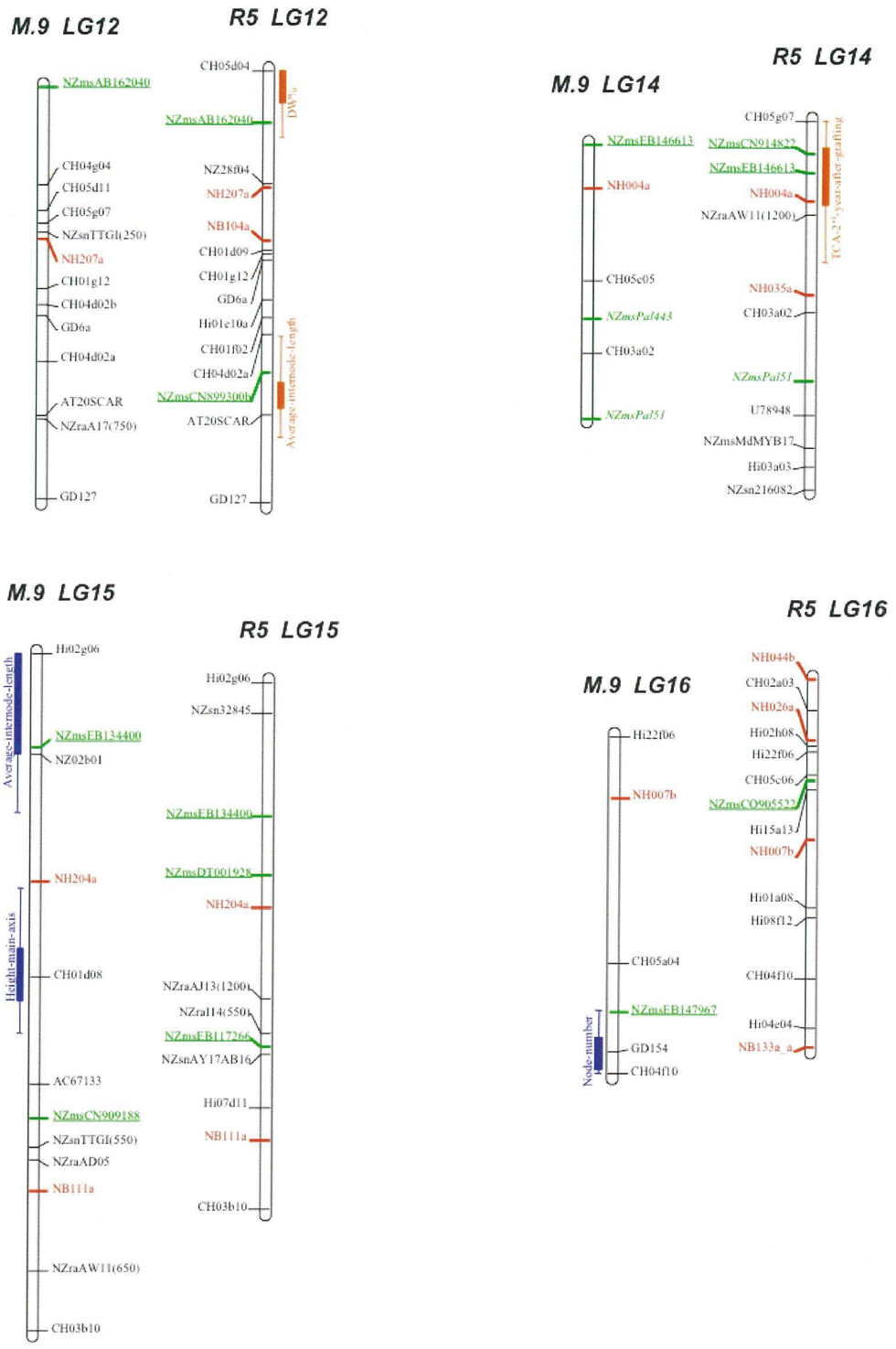



**Figure.5.5. Representation of QTL for all growth traits on the linkage groups (LG) of 'M.9' and 'R5'.** The solid part of the bars of the QTL symbols indicates the most likely position of the QTL, and the lines represent the confidence interval. The QTL identified for 'M.9' are in blue and located on the left side of the LGs, and the QTL identified for 'R5' are in orange and located on the right side of the LGs.

Figure 5.5 continue…




Five QTL influencing the average internode length were identified. Four QTL were located on 'M.9'. One QTL was identified in the genomic region around *DW1* on LG5 and explained 13.0% (LOD=3.1) of the variation. A second QTL was found in the genomic region of *DW2* on LG11 and explained 7.5% (LOD=1.8) of the variation. The remaining two QTL located on 'M.9' were identified on LG3 and LG15 and explained 14.0% (LOD=3.3) and 8.3% (LOD=2.0) of the variation,

respectively. One QTL was located on 'R5' LG12 and explained 11.4% (LOD=1.6) of the variation.

Thirty five out of the 46 QTL identified in this analysis were found in only four locations. Fourteen QTL were detected on 'M.9' at the top of LG5 near Hi01c04, 13 QTL were located on 'M.9' at the top of LG11, 5 QTL were identified at the bottom of 'R5' LG10, and a small clusters of 3 QTL were detected on 'R5' at the bottom of LG6. For LG11, QTL were detected for all traits studied except for TCA increase $2^{nd}\rightarrow3^{rd}$ Rep1, TCA increase $4^{th}\rightarrow5^{th}$ Rep2, and TCA increase $5^{th}\rightarrow6^{th}$ Rep3, where the apparent QTL peaks did not reach the LOD threshold.
The percentage of phenotypic variance explained by the QTL detected ranged from 4.2% (for TCA increase $5^{th}\rightarrow6^{th}$ Rep1 on 'M.9' LG11) to 57.2% (for TCA increase $4^{th}\rightarrow5^{th}$ Rep2 on LG5 of 'M.9'). The QTL located on 'M.9' LG5 and controlling the TCA increase as well as DW%, can all be considered as major QTL because they influence over 20% of the phenotypic variation.

Following the recommendations for standard QTL nomenclature established by the Genome Database for Rosaceae (GDR), the major QTL influencing dwarfing and other related traits on LG5 will be deposited under the name: q*Md*-Dw5.2$^{m}$. This abbreviation stands for:
q = quantitative trait
*Md* = *Malus domestica*
Dw = Dwarfing
5 = location of the QTL on LG5
2 = $2^{nd}$ trait reported on this chromosome
m = the same QTL has been identified in multiple years
All the other detected QTL, including those on the linkage map of 'R5' are moderate QTL.

## 5.5 Discussion

### 5.5.1 Trait variation

The variation among the individuals of both 'M.9' × 'R5' populations was large for all traits: overall dwarfing phenotype, TCA increase, number of flower clusters first year after grafting, height and number of nodes on the main axis. The dwarfing and

semi-dwarfing individuals have, on average, more flower clusters in the second year after grafting (first 'M.9' × 'R5' population) than intermediate and vigorous individuals (data not shown). Some of the trees derived from the second 'M.9' × 'R5' cross even bore fruit in their second year of growth after grafting (Figure 5.6).


**Figure 5.6. 'Braeburn' scion grafted on a rootstock derived from the second 'M.9' × 'R5' cross and producing fruit in the second year after grafting.**

Individuals with a reduced TCA in the second year after grafting have fewer nodes and a shorter main axis from the first year of growth after grafting.

This study highlighted that the measure of TCA 20 cm above the graft union was a reliable indicator of the degree of dwarfing exerted by a rootstock on a scion at all stages of its development (from the second to the seventh year of growth after grafting). The relatively high heritability value estimated for TCA (between $H^2$=0.82 and $H^2$=0.85) also showed that the dwarfing characteristic was a highly heritable character. However, heritability estimates are specific to the population and environment analysed (Souza *et al.* 1998).

Data distribution suggested the existence of a major QTL involved in the regulation of scion development (Figure 5.2). The analysis of data for some traits measured on the two subpopulations showed significant differences between the subpopulation of individuals amplifying markers linked to *DW1* and the subpopulation of individuals without the markers associated with *DW1*. These phenotypic differences between

subpopulations suggest that the *DW1* QTL is partially involved in regulating some of the main axis traits. The question is whether this suggestion can be confirmed by the results of the QTL analysis.

### 5.5.2 Genetic analysis of traits

As expected from the results of the BSA (Chapter 3) and data distributions (Figure 5.2), the genomic region located on the LG5 of 'M.9' (*DW1*) has a major influence on the overall dwarfing phenotype induced by the rootstock. This genomic region also has an important influence on other traits such as the number of flower clusters in the second year after grafting, the TCA increase and the average internode length of the main axis (Table 5.7 and Table 5.8). The percentage of phenotypic variance explained by these QTL varied from 7.1% (LOD=3.1) (for TCA 2$^{nd}$ year after grafting first 'M.9' × 'R5' cross, Rep1) to 57.2% (LOD=21.6) (for TCA 4$^{th}$→5$^{th}$ year after grafting, 'M.9' × 'R5' cross, Rep2), and therefore indicate for some traits a minor influence of *DW1*, while for other traits a major *DW1* influence is proven. These results corroborate the graphical presentation in figure 5.2 where more distinct curves for both subpopulations can be observed as the percentage of phenotypic variance explained by the *DW1* QTL is larger. The results also indicate that the *DW1* QTL exercises a relatively constant effect on the TCA increase.

In addition to *DW1*, the second genomic region *DW2* identified by BSA (Chapter 3) on 'M.9' LG11 was shown to moderately influence the majority of the traits studied. The percentage of phenotypic variance explained by these QTL varied from 4.2% (LOD=1.6) (TCA 5$^{th}$→6$^{th}$ year after grafting first 'M.9' × 'R5' cross, Rep1) to 16.5% (P<0.0001) (number of flower clusters first year after grafting) (Table 5.7 and Table 5.8).

QTL analysis revealed the existence of QTL on 'M.9' for most traits on LG5 and LG11 (Table 5.7, Table 5.8 and Figure 5.5). Correlated traits often have QTL that map to similar genomic locations (Paterson *et al.* 1991; Xiao *et al.* 1995). This phenomenon of QTL clustering can be due to either a single QTL with pleiotropic effects, or to a tight linkage between the QTL. A small population size associated with the rarity of crossovers between tightly linked genes mean that differentiating between pleiotropy and QTL clustering is difficult. However, because each of these QTL influences the overall dwarfing phenotype, pleiotropy seems the most likely explanation.

Many traits measured in the progenies were correlated with each other (Table 5.3 and

Table 5.4) and many QTL clustered on the genetic map (Figure 5.5). For example strong correlations were identified between the number of nodes and the height of the main axis (Table 5.4). Only a single QTL was associated with both these traits ('M.9' LG11, Table 5.8). It is probable that this is the result of a single QTL with pleiotropic effect on both the number of nodes and the height of the main axis, rather than separated linked genes. Other regions were only associated with the number of nodes ('M.9' LG8; 'M.9' LG16 and 'R5' LG6) or the height of the main axis ('R5' LG6) (Table 5.8 and Figure 5.5). This observation shows that some QTL act in a general manner, increasing the overall scion vigour, while others are more specific with significant effects only on separate traits.

For all traits measured from the main axis, QTL located on linkage groups other than LG5 and LG11 were identified. The percentage of phenotypic variance explained by these additional QTL ranged from 5.3% to 15.8% (Table 5.7 and Table 5.8). These QTL confirm the hypothesis formulated in the Chapter 3 that growth traits associated with the dwarfing phenotype are not completely determined by the QTL *DW1* and *DW2*.

The relatively low LOD scores (between 1.6 and 3.8 (Table 5.7 and Table 5.8)) and percentage of variation explained by each of the QTL (except *DW1* on LG5) detected in this study may be a consequence of the very low number of individuals for which phenotypic data were available, especially for Rep3 of the first 'M.9' × 'R5' population, and the second 'M.9' × 'R5' population (Table 5.1). Genotyping of the whole second 'M.9' × 'R5' cross will be necessary to validate the QTL detected in this study. Phenotyping the individuals over the coming growing seasons will also be important as it will allow us to differentiate QTL showing a continual expression during plant growth from QTL influencing the plant at only one stage of its development.

### 5.5.3 QTL stability

The most valuable QTL for a breeder are those that show a consistent positive expression in multiple environments and over multiple seasons (Conner *et al.* 1998). QTL stability was evaluated by comparing the location of putative QTL for the TCA increase over multiple years and repetitions. For Rep1, QTL identified for this trait were consistently detected in each of the five years analysed, except for the QTL on LG11 for the increase in TCA between the second and third year of growth after

grafting (Table 5.7). In Rep2 grafted a year later and indeed subjected to different growing conditions, QTL located on LG5 and LG11 were also identified (Table 5.7). Although Rep3 comprised a much lower number of individuals (57 individuals), the analysis detected reproducibly across season two QTL on LG5 and LG11 respectively. These results indicate the robustness of the QTL detected.

Variations in QTL detected in different years can be the result of chance fluctuations in data sets, QTL × environment interactions, or developmentally regulated QTL. Developmentally regulated QTL for tree development have been reported in maritime pine (Plomion *et al.* 1996). In our experiment, the QTL for TCA increase on 'M.9' LG11 was not detected between the second and third year of growth after grafting, but was detected at a later stage of the plant growth (Table 5.7), suggesting that maturation might have induced a shift of the genetic control of TCA increase. However, this result could simply be due to a phenotyping error. Extending the phenotyping to all the individuals derived from the second 'M.9' × 'R5' cross will be essential to confirm this hypothesis.

### 5.5.4 Comparison with QTL influencing apple tree architecture detected in previous studies

Earlier studies on apple tree architecture have identified QTL and clusters of QTL affecting various characters of trees planted on their own roots or grafted to clonal rootstocks (Chapter 1, section 1.2.5). The originality in our study is that the scion architecture was only affected by the rootstock onto which it was grafted. Although the comparison between these investigations and the one presented in this study seems difficult to interpret, some similarities could be observed.

A QTL influencing the average internode length has been mapped at the bottom of LG3 in the 'M.9' × 'R5' population (Figure 5.5). This region of the LG3 is the same where Liebhard *et al.* (2003a) had previously located a QTL influencing stem diameter and height increment of the main axis. The top of LG11, LG14 and LG15 had also been found by the same authors to contain QTL associated with stem diameter, approximately at the position where QTL for TCA increase and average internode length have been detected in the 'M.9' × 'R5' population (Figure 5.5). A QTL influencing the sylleptic branch length of trees grown on their own roots had been identified at the bottom of LG12 (Kenis and Keulemans 2007), which corresponds to the region where we have identified a QTL influencing the average internode length ('R5' LG12) (Figure 5.5). A QTL influencing blooming date was

located on LG6 by Segura *et al.* (2007). The location of this QTL corresponds to the clusters identified on 'R5' LG6 (Figure 5.5). Finally, a QTL influencing the increase in TCA between the 6$^{th}$ and 7$^{th}$ year of growth after grafting, as well as a QTL influencing the number of flower clusters on 'M9' LG9 (Figure 5.5) corresponds in position approximately to a QTL influencing the number of axillary shoots (AS) identified by Segura *et al.* (2007).

Out of the 46 QTL identified, only five, influencing the overall dwarfing phenotype and TCA increases in Rep1, Rep2 and Rep3 were located on LG10 ('R5') (Table 5.7, Figure 5.5). This result may seem surprising since Kenis and Keulemans (2007) and Segura *et al.* (2007) had detected a major cluster of QTL around the *Col* gene at the bottom of this linkage group. Instead, the most important QTL cluster found in our analysis is located at the top of LG5 ('M.9'). We have already discussed in Chapter 1 (section 1.1.1.2) and Chapter 4 (section 4.5.7) that the apple genome contains homoeologous pairs of chromosomes. It has been shown in several studies that the top of LG5 is homoeologous to the bottom of LG10 (Liebhard *et al.* 2002; Maliepaard *et al.* 1998a; and Figure 4.2). This may indicate that the rootstock genes influencing the development of a grafted scion may be homoeologous to genes influencing the architecture of a tree on its own roots.

In order to understand better the development of dwarfed trees compared to vigorous trees, more data is needed on aerial morphogenesis. The phenotyping of the second 'M.9' × 'R5' cross for morphological traits such as the number and location of long shoots, the length and number of nodes of long shoots, the basic angle of branches, the number of flower clusters and the date of budbreak, will be essential to achieve this goal. The collection and analysis of such data would allow for a detailed comparison of tree architecture development as influenced by the scion (as in Segura *et al.* 2007) and the rootstock. Furthermore, the effects of environmental conditions must be tested on a range of genotypes, as genotype × environment interactions may be detected. The dwarfing effect of 'M.9' has been well characterized under various growing conditions (Hirst 2001), and growing replicates of the 'M.9' × 'R5' populations under different environmental conditions may accentuate the effect of one or several QTL, or reveal the presence of new QTL. A different QTL study on a population derived from a cross between the very dwarfing rootstock 'Ottawa 3' (progeny of 'M.9') and 'R5' is already being conducted at the New York State

Agriculture Experiment Station, Geneva (NY, USA) (Fazio, personal communication). The identification of similar QTL in both populations would be a significant step towards the confirmation of the QTL already identified.

This study indicated genomic regions in the apple rootstocks 'M.9' and 'R5' involved in the variation of quantitative traits related to scion architecture and development. Some of these QTL, particularly those located on 'M.9' LG5 and 'M.9' LG11, were consistently identified over five years and three repetitions, indicating a high level of heritability and stability.

Analysis of the data also confirmed the long suspected relationship between the dwarfing capacity of a rootstock and its ability to induce precocious flowering of the scion (Visser 1970; Aldwinckle 1975; Fischer 1994; Seleznyova *et al.* in press). The QTL analysis identified two QTL located on 'M.9' LG5 and 'M.9' LG11, explaining 21.1% and 16.5% of the phenotypic variation respectively, and co-locating with the two main QTL for dwarfing (Figure 5.5). A third QTL located on 'M.9' LG9 and influencing the number of flower clusters (explaining 6.8% of the phenotypic variation) was also found to co-locate with the increase in TCA $6^{th} \rightarrow 7^{th}$ year after grafting Repl (Figure 5.5). A genomic region located at the top of 'R5' LG6 and explaining 9.0% of the phenotypic variation was also found to affect the number of flower clusters, and was located close to a cluster of QTL influencing the node number, the height of the main axis and the TCA increase (Figure 5.5). These results indicate that in apple rootstocks, dwarfing and flowering are two characters tightly linked, which provides valuable information for the selection of potential candidate genes involved in the control of these characters.

The outcomes of this investigation will have direct applications in selecting progeny derived from present and future 'M.9' × 'R5' populations, as well as from any other cross involving the 'M.9' rootstock or any of its progeny, through marker assisted selection. The presence of numerous SSR markers flanking the QTL will allow for a reliable screening of both rootstock accessions and progeny, derived from 'M.9', in order to determine the presence of QTL influencing dwarfing and other morphological traits. The development of marker assisted selection for dwarfing based on these markers will, as mentioned in Chapter 3, more than halve the time required to breed new dwarfing rootstocks.

The relatively small size of the apple genome, about 750 Mb (Arumanagathan and Earle, 1991), gives the possibility of positional cloning of a QTL. This is particularly

relevant for the major QTL influencing dwarfing and located on 'M.9' LG5 (*DW1*). We estimate the closest marker to the QTL peak is located between 0.5 and 1.0 cM away. A programme aimed at discovering the gene explaining this QTL via positional cloning using bacterial artificial chromosomes (BAC) is currently being developed. Another strategy, the candidate gene approach by way of microarray analysis, aimed at identifying genes influencing the dwarfing effect has also been tested (results of this investigation are presented in the next chapter of this thesis). Finally, the sequencing of the apple genome (cv. 'Golden Delicious') by the Istituto Agrario San Michele all' Adige in Italy, by the end of 2007, will greatly facilitate the identification of positional candidates explaining the QTL. The knowledge of the apple genome sequence will permit us to identify genomic regions using the SSR marker sequences mapping close to QTL, and identify genes potentially involved in the dwarfing effect. This approach could be very appropriate for QTL that have been accurately located and for which flanking markers have been developed, as it is the case for *DW1* on 'M.9' LG5.

The identification of such genes, and particularly *DW1*, would not only have major impacts on apple rootstock breeding programmes, but potentially on all fruit tree crops of the Rosaceae family for which dwarfing rootstocks have not yet been developed. *Agrobacterium*-based transformation techniques have been developed for many members of the Rosaceae family, including apple (James *et al.* 1989), peach (Scorza and Hammerschlag 1992), pear (Mourgues *et al.* 1996), plum (Scorza *et al.* 1994), apricot (Petri *et al.* 2004) and cherry (Dolgov 1999). The development of genetically modified dwarfing rootstocks for these fruit trees could have the potential to increase fruit production and quality.

# 6- LOOKING INTO DWARFING BY THE APPLE ROOTSTOCK 'M.9' (*MALUS* × *DOMESTICA* BORKH.): THE CANDIDATE GENE APPROACH

## 6.1 Abstract

The identification of genes involved in the control of scion dwarfing by apple rootstocks would greatly assist breeders in creating new rootstock cultivars with desired dwarfing ability. Genomic regions influencing dwarfing have already been detected, based on a mapping study in populations derived from crosses between 'M.9' and 'R5' (see Chapter 3 and Chapter 5). The aim of our investigation was to associate these QTL with structural genes selected from microarray analysis. From a list consisting of the top 100 genes most differently expressed between dwarfing and vigorous rootstocks, twenty cDNA were selected as candidate genes (CG) since their nucleotide sequences were highly similar to those of genes involved in various pathways potentially responsible for the dwarfing effect. The CGs were amplified from both parental lines of the population using specific primers. Polymorphisms between parental alleles were used to develop single nucleotide polymorphism (SNP) or simple sequence repeat (SSR) markers that were mapped on the 'M.9' × 'R5' map using a bin mapping set. Twelve CGs amplifying 16 loci were mapped to 11 linkage groups in the apple genome. However no CG was mapped in the genomic region around the major dwarfing QTL *DW1*. This preliminary result may be the first step toward the identification of the genes involved in the dwarfing effect of the 'M.9' apple rootstock.

## 6.2 Introduction

The identification of the gene(s) influencing the dwarfing effect of the apple rootstock 'M.9' would be a major step towards the understanding of this phenomenon. Understanding of the biology and genetics controlling this trait would provide breeders with molecular markers located directly on the gene, and would allow the identification of alleles of the gene(s) with potential varying effects.

One of the possible strategies that can be adopted to identify the gene(s) influencing dwarfing is the candidate gene (CG) approach.

The CG approach consists of three successive steps. First, CGs are proposed based

on molecular or physiological studies. Second, a molecular polymorphism must be identified to localise the CGs on a genetic map. Third, if map co-segregation is found, complementary experiments must be conducted to confirm the actual involvement of the CG in the trait variation (Pflieger *et al.* 2001).

This approach has already been successful in identifying genes controlling sucrose accumulation (Klann *et al.* 1996) and fruit ripening (Yen *et al.* 1995) in tomato fruit, carotenoid accumulation in red pepper (Huh *et al.* 2001), flowering time in almond (Silva *et al.* 2005), and red flesh colour in apple (Chagné *et al.* 2007b).

CGs are sequenced genes of known or presumed function that are related to the traits of interest and which could correspond to major loci or QTL (Silva *et al.* 2005). The majority of putative CGs for agronomic species have been previously identified in the model plant *Arabidopsis thaliana*, for which many genes have been characterized and found to play important roles in growth and development (The Arabidopsis Information Resource (TAIR)). The diversity and the number of genes involved in these processes is very substantial. As illustrated in Chapter 1, a wide range of hypotheses has been proposed to explain the dwarfing effect of the apple rootstock 'M.9'. Testing all the genes potentially involved in the different pathways would be expensive and time consuming. Therefore, the question to be addressed is how to choose with some degree of confidence which kind of genes should be candidates for the dwarfing effect caused by the apple rootstock 'M.9'.

cDNA and oligonucleotide microarray technology hold great promise for identifying CGs as already shown in strawberry (Aharoni *et al.* 2000) and tomato (Giovanonni 2000). A microarray platform developed for apple, and containing 55,000 unique DNA sequences, has already been used to identify genes differentially expressed in scions grafted to different rootstocks (Jensen *et al.* 2006). Within HortResearch, an oligonucleotide microarray chip has also been developed for apple (Newcomb *et al.* 2006) and has already been successfully tested to identify genes controlling aroma production in apple fruit development (Schaffer *et al.* 2007).

Once a potential CG has been identified, the working hypothesis assumes that the CG plays a role in the expression of the phenotype if the trait and the allelic polymorphism co-segregate in the mapping population (Pflieger *et al.* 2001). A strategy for mapping CGs consists of discovering single nucleotide polymorphisms (SNP markers) or insertions/deletions (indels) within their sequence among parents of a mapping population.

Development of SNP markers is ideal for the mapping of CGs. SNP markers are

abundant in the genome (see section 1.2.1.3) and a wide variety of methods exist to detect polymorphism. One of the most common is allele-specific PCR amplification (Newton *et al.* 1989). The efficacy of this method was demonstrated by Délye *et al.* (2002) in a study to identify herbicide resistant black-grass genotypes. Despite being low throughput, it can easily be applied as part of the candidate gene approach (Chagné *et al.* 2007a).

The aim of the investigation reported here was to understand the molecular basis underlying variations in the dwarfing effect exhibited by a selection of apple rootstocks derived from a cross between 'M.9' and 'R5'. For this purpose, RNA from bark and root tip tissues from rootstocks was extracted. These tissues were chosen because of their suspected involvement in the control of scion architecture and development (Chapter 1). Using microarrays to detect differentially expressed genes among dwarfing and vigorous rootstocks allowed for rapid identification of candidate genes conferring a dwarf habit. Markers were developed for these genes and mapped using the bin mapping set constructed from the 'M.9' × 'R5' population. The position of these CGs were compared with those of the QTL identified in Chapter 5 and the results discussed within this framework.

## 6.3 Materials and methods

### 6.3.1 Plant material and RNA extraction

Seventeen rootstocks derived from the first 'M.9' × 'R5' population were selected for this analysis. As shown in table 6.1, this set contained nine rootstocks selected for their dwarfing effect on the scion and the presence of markers linked to *DW1* on both sides of this locus (Hi01c04 and NZraAV11(850)). The other eight rootstocks were selected for their vigour and the absence of markers associated with *DW1*.

**Table 6.1. Rootstock individuals from the 'M.9' × 'R5' population selected for microarray analysis.** Tree numbers in this table are the same as shown in appendices I, II and V. + indicates the presence markers linked to *DW1* and – indicates the absence markers linked to *DW1*.

| Tree number | Phenotype | *DW1* | |
|---|---|---|---|
| | | Hi01c04 | NZraAV11(850) |
| 3 | Dwarfing | + | + |
| 4 | Dwarfing | + | + |
| 5 | Dwarfing | + | + |
| 7 | Dwarfing | + | + |
| 8 | Dwarfing | + | + |
| 12 | Dwarfing | + | + |
| 13 | Dwarfing | + | + |
| 14 | Dwarfing | + | + |
| 16 | Dwarfing | + | + |
| 106 | Very Vigorous | - | - |
| 108 | Very Vigorous | - | - |
| 109 | Very Vigorous | - | - |
| 110 | Very Vigorous | - | - |
| 111 | Very Vigorous | - | - |
| 112 | Very Vigorous | - | - |
| 113 | Very Vigorous | - | - |
| 115 | Very Vigorous | - | - |

Braeburn scions were grafted onto these rootstocks and trees were grown in sawdust in a nursery for two years in Havelock North (New Zealand) (work done by Stuart Tustin). In spring 2005, bark (Figure 6.1.) and root tips tissues from the rootstocks were harvested and snap frozen in liquid nitrogen before storage at -80°C. Tissues collected as part of the bark were the epidermis cortex (parenchyma), fibres (sclereids), phloem (sieve tubes and companion cells) and part of the cambium (which separates the phloem from the xylem). Total RNA isolation was performed following the Chang *et al.* (1993) method. RNA was cleaned using RNAeasy cleanup kit (Qiagen) according to the manufacturer's protocol. Following quality check, reverse transcription of the RNA was performed as described in Schaffer *et al.* (2007). RNA extraction, clean up and reverse transcription was performed at the HortResearch Mt Albert Research Centre (Auckland) by Bebecca Bishop.

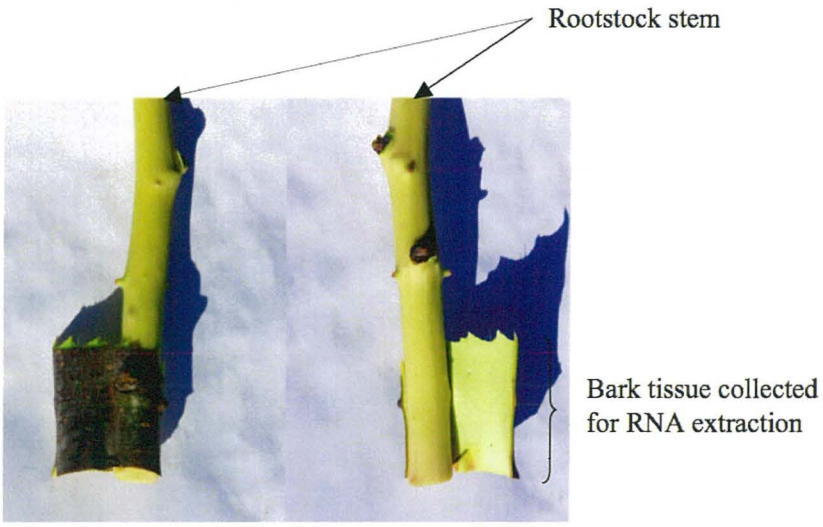



**Figure 6.1. Bark tissue from rootstocks collected for RNA extraction.** The bark tissue was pealed off the rootstock stem and snap frozen in liquid nitrogen before RNA extraction

### 6.3.2 Microarray analysis

To measure global gene expression patterns, an apple microarray developed at HortResearch Mt Albert and containing 15,723 45 -55 mer oligonucleotides was used (Newcomb *et al.* 2006). These oligonucleotides were designed from 'Royal Gala' EST sequences, and have a constant melting temperature.

Oligos were printed on epoxy slides (MWG) using a Biorobotic II robot. The microarray slides in this experiment were derived from three different printing. Each print can be identified by its serial number: BE, BM and BQ (Figures 6.2 and 6.3).

Each biological sample was repeated in a dye swap experiment and each array was hybridized with sheared genomic DNA from 'Royal Gala' in one channel to allow direct comparison between arrays.

Cy3 and Cy5 labelled cDNA and gDNA were mixed and hybridizations were performed in a Lucidea Hybridization machine (GE Healthcare) according to a protocol set up by Schaffer *et al.* (2007). Following a washing step, the microarrays were air dried and scanned using a Genepix 4000B scanner (Axon). Spots were aligned using Genepix 4 software (Axon). This microarray analysis was performed by Robert Schaffer at HortResearch Mt Albert (Auckland).

### 6.3.3 Data processing

Spots were aligned automatically and checked individually. Spots that did not fit the

quality requirement were flagged and were not taken into account. Analysis was done in R and S-plus 6.1 (Insightful, Washington) under the supervision of Robert Schaffer and Marcus Davy (Hortresearch Mt Albert, Auckland). Microarrays were normalised in R and S-Plus using modules from the BioConductor limma package (Smyth and Speed, 2003) and an in house normalization protocol where all RNA and DNA channels were normalized with global mean normalization, combined into two files (containing all gDNA and all cDNA channels), and the distribution of intensities were normalized using quantile normalization (Bioconductor). A ratio (M) of the Cy3 and Cy5 values for each slide was calculated.

Sample: Slide 1: cDNA (Cy5) versus gDNA (Cy3) → ratio
Slide 2: cDNA (Cy3) versus gDNA (Cy5) → ratio

For each spot the intensity of hybridization was quantified: $0 <$ intensity $< 256$. The intensity of the background was also measured and the intensity of the spot was adjusted. The M values of the dye swap were then smoothed with loess smoothing to remove dye bias. An absolute value for each spot was achieved by multiplying each ratio with the median gDNA value for that spot. Data processing was performed by Robert Schaffer and his team at the HortResearch Mt Albert Research Centre.

Figures 6.2 and 6.3 show the design of the experiment. Each treatment is compared to the genomic DNA. The presence of gDNA as an internal standard makes it possible to compare the samples among each other.

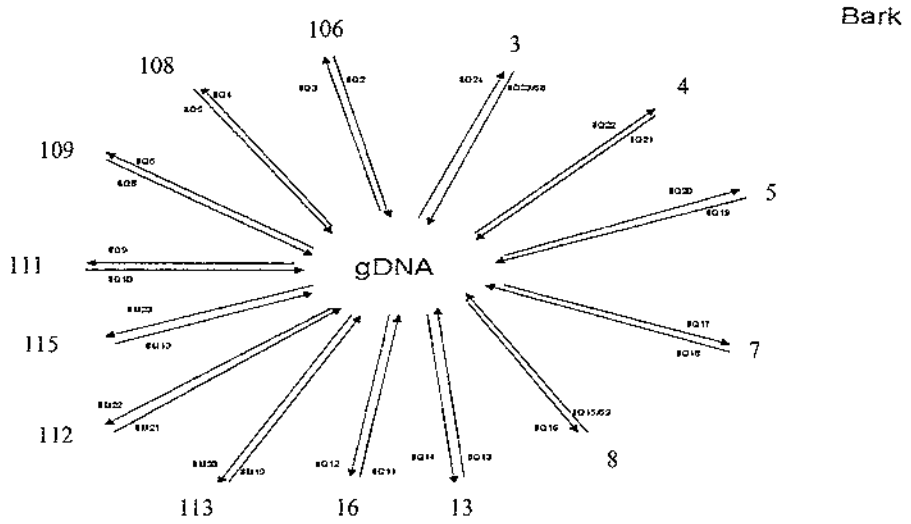



**Figure 6.2. Experimental design for RNA extracted from rootstock bark tissues.** The numbers on the outside of the figure represent the individual tree number. BQ and BM followed by a number are the serial numbers of the microarray print used for each individual. The double arrow indicates that each sample was compared to gDNA in a dye swap experiment.

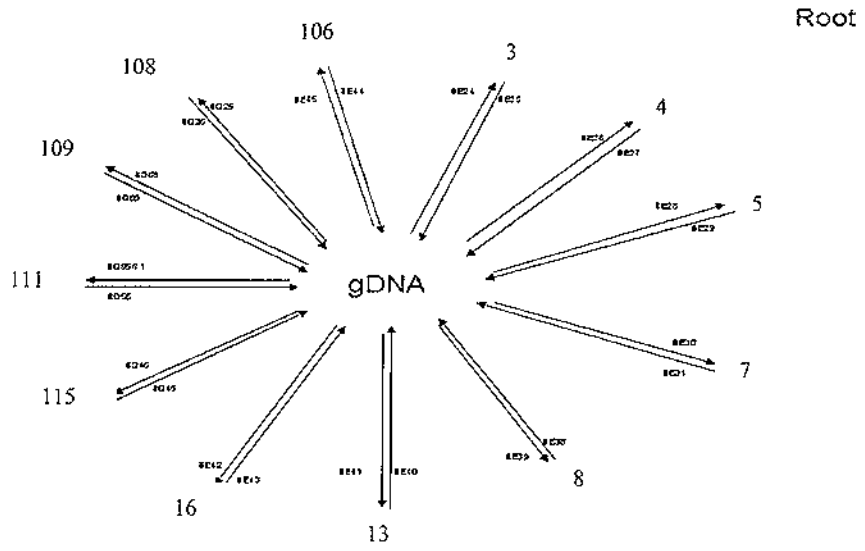



**Figure 6.3. Experimental design for RNA extracted from rootstock root tissues.** The numbers on the outside of the figure represent the individual tree number. BQ and BM followed by a number are the serial numbers of the microarray print used for each individual. The double arrow indicates that each sample was compared to gDNA in a dye swap experiment.

Because of the presence of gDNA as an internal standard, the experimental design can be further simplified. Microarray data from samples collected from rootstocks providing a dwarfing effect can be bulked together, as for microarray data from

samples collected from vigorous rootstocks. The final experimental design is represented in figure 6.4. This design allowed us to compare dwarfing versus vigorous rootstocks, and as a consequence individuals with *DW1* versus individuals without *DW1*, via gDNA. This also permitted us to consider dwarfing (or vigorous) samples as replicates of each others.

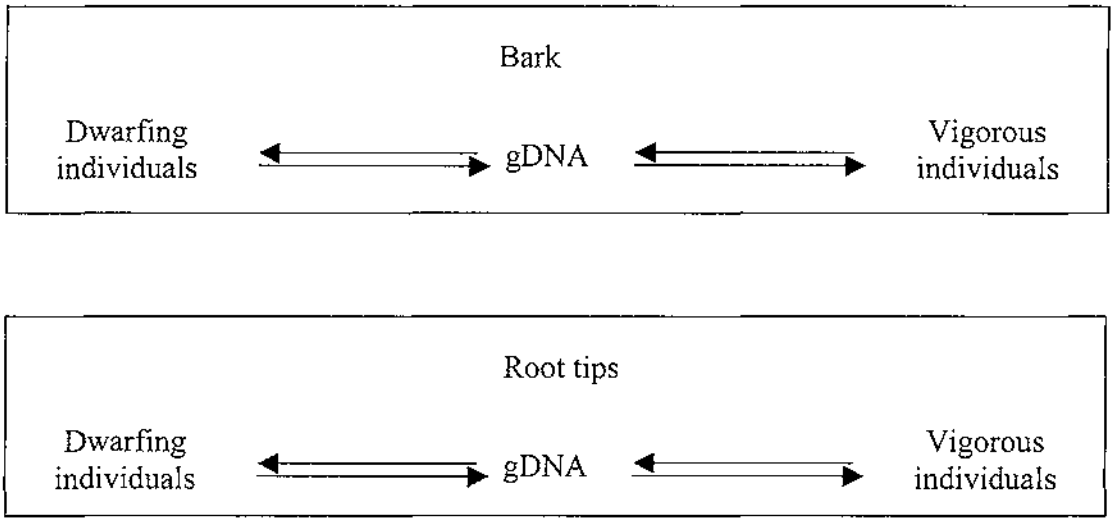



**Figure 6.4. Final experimental design for microarray analysis.** Microarray data collected from samples derived from dwarfing rootstocks have been bulked on one side, and microarray data collected from samples derived from vigorous individuals have been bulked on the other side. Gene expression can be compared between dwarfing and vigorous via gDNA.

Analysis of variance (ANOVA) was used to identify genes that changed their expression in the bulk composed of dwarfing individuals versus the bulk made of vigorous individuals. The number of significantly differentially expressed genes was examined using a 0.05 and 0.01 threshold using a non-adaptive False Discovery Rate (FDR) control (Benjamini and Hochberg, 1995).

This method of selecting differentially expressed genes has a 75-80% qPCR (quantitative PCR) success rate (Schaffer *et al.* 2007). Therefore, qPCR was not performed to confirm the validity of the differentially expressed genes.

### 6.3.4 Candidate gene selection

From a list composed of the 100 genes most differentially expressed, I selected 20 genes for their potential involvement in the dwarfing phenomenon. This selection

was performed with the help of a panel of experts as well as the use of databases such as TAIR and TIGR (The Institute for Genomic Research).

### 6.3.5 Identification of polymorphism between parental lines

CG sequences were analysed using a bioinformatics platform developed at HortResearch by Richard Newcomb and Ross Crowhurst. Detection of SSR markers was undertaken using the PERL program (Crowhurst, unpublished) within BioPipe that identified tandem repetition of sequence words in target sequences (Newcomb *et al.* 2006).

When a SSR was present in the sequence of the gene, primers were designed on both sides of the repeated fragment with the program Primer3 (Rozen and Skaletsky 2000). All primers were intended to have a similar theoretical melting temperature of approximately 60°C. The predicted size of the amplification products were comprised between 100 and 400 bp. Primers were synthesized by Invitrogen (Invitrogen New Zealand Limited, Auckland). PCR amplifications were performed as described in Chapter 4 (section 4.3.3). PCR products were genotyped using the CePro 9600™ (Combisep, Ames, Iowa).

When no SSR was detected in the sequence, PCR primers were designed to amplify the whole DNA sequence from both parents. When the whole DNA sequence was too long to be amplified with only one pair of primers, two sets of primer pairs were designed. These markers were tested over a range of annealing temperatures until a single DNA band was amplified from both 'M.9' and 'R5'. PCR reactions were performed as described in Chapter 4 (section 4.3.6). The resulting amplification product was run on a 0.9% agarose gel and stained in an ethidium bromide solution. When polymorphism was detected between the parents, the markers were tested for segregation.

When no polymorphism was observed, the DNA fragments amplified from both parents were sequenced with the aim to identify SNP markers and/or indels. Two methods were used for this purpose.

The first one consisted in cloning the DNA fragments using the TOPO TA cloning kit (Invitrogen). For the ligation step, PCR products were inserted into the PCR®2.1 – TOPO® vector (Invitrogen). Transformations were carried out in the chemically competent bacteria cells DH5α™-T1$^{R}$ (Invitrogen) following the manufacturer's instructions. Transformed bacteria were then plated out on solid 2×Ty medium

containing 0.2% glucose, 0.1mg $ml^{-1}$ ampicillin, 2µg $ml^{-1}$ Xgal and 16µg $ml^{-1}$ IPTG. After an overnight incubation step at 37°C, colonies were picked and DNA was amplified using M13 universal primer (sequence in Appendix VI). Following desalting in 96 well microplate devices UNIFILTER® 350µl (Whatman®, Clifton USA) using Sephadex™ G-75 Superfine (Amersham, Uppsala Sweden) and quantitation, DNA fragments were sent for sequencing to the Allan Wilson Centre, at Massey University Palmerston North or Albany campus.

The second method consisted in sequencing DNA fragments directly from the PCR product. Fragments were desalted and quantified prior to sequencing.

Sequences were aligned and analysed with Sequencher™ (Gene Codes Corporation). A BLASTn search was conducted to confirm homology between the amplified fragment and the desired sequence from the database.

Following the identification of a single nucleotide polymorphism between the parental alleles, SNP markers were designed with the help of Primer3.

### 6.3.6 SNP marker development

Markers were designed following the allele-specific PCR amplification method (Newton *et al.* 1989). In this method, one of the PCR primers defining a sequence-tagged site (STS) was designed to preferentially amplify one of the parental alleles. A mismatched base was added close to the SNP site, three nucleotides upstream from the 3'-terminus of the primer, in order to enhance the preferential amplification of one of the alleles (Rust *et al.* 1993). The PCR fragments were subsequently separated on agarose electrophoresis gels (0.9%) and stained in ethidium bromide.

### 6.3.7 Mapping

All the markers were screened over the bin mapping set developed from the 'M.9' × 'R5' saturated map (Chapter 4). The assignment of markers screened over the bin mapping set to linkage groups was performed visually. Some markers were subsequently screened over a sample of 94 individuals of the 'M.9' × 'R5' population to obtain a more accurate map location. On the map, SSR markers developed from the CGs are prefixed with 'NZms' followed by the GeneBank accession number. Markers amplified by the same primer pair but mapping at different loci are qualified with an 'a', 'b', 'c', 'd' or 'e' suffix. The SCAR and SNP markers developed are prefixed with 'NZsc' and 'NZsn' respectively.

## 6.4 Results

### 6.4.1 Genes differentially expressed among dwarfing and vigorous rootstocks

Microarrays were used to examine differences in gene expression among dwarfing and vigorous rootstocks derived from the first 'M.9' × 'R5' population. Because of time constraints, RNA from the dwarfing individual's tree number 98 and 137, as well as from the vigorous individual tree number 46 was not extracted. Comparison among the microarrays revealed a significant difference between the quality of the microarray slides derived from different printing. Slides derived from the BQ printing could not be analysed together with the slides BE and BM. Therefore the results from the microarrays for RNA extracted from root tip tissues could not be interpreted. As a consequence, only the RNA extracted from bark tissue was analysed, and seven dwarfing versus seven vigorous individuals were used to examine differences in gene expression.

Comparative expression analysis of tissue from dwarfing and vigorous bark tissue revealed 7772 genes with either gene expression enhanced or reduced using a P-value of $< 0.05$. Of the 7772 putative differentially expressed genes, 3539 gene showed higher expression and 4233 showed a reduction in the dwarfing rootstocks. At a P-value of $< 0.01$, 5487 genes were identified, 2380 were up regulated and 3107 were down regulated. Because of the high number of differentially expressed genes, we limited our search for the best CGs to the top 100 genes (Appendix VII). In this list of the top 100 genes, the expression level differed at P = 7.16E-6 for the most differentially expressed gene, and at P = 2.13E-4 for the least differentially expressed gene. Eighty genes showed higher expression in the dwarfing rootstocks, and 20 displayed an expression reduction.

### 6.4.2 Candidate genes selected for mapping

The CGs selected for dwarfing are presented in table 6.2. The P value, representing the probability of a gene to be differentially expressed between dwarfing and vigorous rootstocks, and the putative function of each gene are also presented in this table.

### 6.4.3 Amplification of CGs in the parental lines

For the nine CGs containing SSR markers in their sequence, SSR markers were developed and their primer sequences are listed in table 6.3.

For nine of the remaining 11 CGs, only a single product was amplified from genomic DNA of both parents. For CN908240, a single product was amplified for each parent but a length polymorphism was detected. This length polymorphism was used to map the CG (primer sequence in Table 6.3). This marker can be considered as a SCAR marker. For EE663938, several bands were amplified and the number of bands could not be reduced when PCR conditions were modified.

PCR products amplified from 'M.9' and 'R5' were cloned and sequenced for CN942650, CN927697, CN904514, EG631344, CN919641, EB112737, CN916259, EE663938, CN887354, and EB109549. After BLAST (BLASTn (nucleotide) versus *Malus* EST database) analyses, all the sequences amplified revealed homology to the genes they were supposed to code for, except for the three products amplified from the primers designed from EG631344, EB109549 and EE663938 for which none of the bands matched the expected sequence. These three CGs were not investigated further. PCR fragments amplified from genomic DNA by primers designed from CN942650 were longer than expected. Alignment with cDNA sequence indicated the presence of two introns of 745 and 89 bp in the genomic fragment.

**Table 6.2. Potential CGs for dwarfing differentially expressed between dwarfing and vigorous rootstocks**. Each candidate gene is represented by its GeneBank accession number. A P value representing probability of a gene to be differentially expressed between dwarfing and vigorous rootstocks is indicated. The putative function of each gene, and the presence of a SSR in the contig containing the EST, was automatically provided by the HortResearch BioPipe sequence annotation pipeline (a cluster annotation system written in PERL (Crowhurst, unpublished data) and utilizing a relational database (MySQL; http://www.mysql.com) (Newcomb *et al.* 2006).

| GeneBank accession number | P value | Putative function and comments from the HortResearch bioinformatic database | SSR |
|---|---|---|---|
| CN942650 | 7.16E-06 | ABI3-interacting protein, aspartyl protease family protein, family member of FRI-related genes, required for winter-annual habit. | - |
| CN927697 | 5.25E-05 | F-box family protein. | - |
| CN904514 | 5.25E-05 | Molecular function unknown. | - |
| EG631344 | 6.44E-05 | Rac GTP binding protein involved in polar growth control, related to growth, highly expressed in root hair zone. | - |
| CN887354 | 6.69E-05 | MIP family protein, involved in water channel activity, transport and root development. | - |
| CN908240 | 8.21E-05 | Homeobox related protein, similar to homeobox transcription factor Hox7. | - |
| EB112737 | 8.46E-05 | Embryonic flower 2, vernalization 2 protein (VRN2), mediates vernalization and Encodes a nuclear-localized zinc finger protein, in wild-type Arabidopsis, vernalization results in the stable reduction of the levels of the floral repressor FLC, highly expressed in root tips and root hair zone. | - |
| EE663938 | 8.46E-05 | Arabidopsis related ATPase, 35S:RNAi plants have reduced stature. | - |
| EB141306 | 8.46E-05 | Similar to zinc finger protein, very high expression in root tips. | $(GAC)_4$+$(AGA)_4$+ $(GAA)_4$ |
| CN945982 | 8.65E-05 | Transcription Factor, regulation of transcription, basic helix-loop-helix (bHLH) family protein. | $(CT)_{16}$ in contig[+] |
| EG631304 | 9.49E-05 | Transcription Factor, regulation of transcription, basic helix-loop-helix (bHLH) family protein. | $(CT)_{30}$ |
| CN868092 | 1.03E-04 | Transcription Factor, bHLH transcription factor PTF1 related cluster. | $(AAC)_4$ in contig |
| CN919641 | 1.08E-04 | EF2 (elongation factor 2), translation factor activity. | - |
| CN935411 | 1.16E-04 | Can influence gene expression, fringe encodes an extracellular protein that regulates Notch signalling. | $(CTC)_5$ |
| EB109549 | 1.69E-04 | Transducin family protein, mutations augments sensitivity to cytokinin, ethylene, ABA and auxin, high expression in xylem and cork. | - |
| CN891102 | 1.85E-04 | SBP-box family of putative plant transcription factors, homology to transcription factor that binds to AP1 promoter and can induce early flowering. | $(TGC)_4$+$(CT)_{13}$+ $(CT)_6$+$(TCCC)_3$ in contig |
| EB137861 | 1.91E-04 | Zinc finger (C3HC4-type RING finger) family protein. | $(AT)_{31}$ |
| CN916259 | 1.95E-04 | Member of Arabidopsis BAG proteins, accelerates vegetative to reproductive phase transition, found in 'M.9' root tips. | - |
| EB137941 | 2.09E-04 | Transcription factor activity, zinc ion binding, regulation of transcription. | $(CT)_{13}$ |
| CN890397 | 2.13E-04 | Zinc related protein, regulation of transcription. | $(ACG)_4$ |

[+] a contig is a set of overlapping DNA segments (ESTs in our case) derived from a single genetic source.

### 6.4.4 Detection of polymorphisms within the CGs

Sequence analyses of the genomic fragments amplified from both parental lines revealed that most of the polymorphisms involved base substitutions (SNP markers). For four of the CGs (CN904514, EB112737, CN919641 and CN916259) it was possible to identify SNP markers in the sequences obtained from the cloned DNA fragments, thereby enabling the development of SNP markers. Primer sequences for these SNP markers are listed in table 6.3.

Analyses of cloned genomic DNA amplified by primers developed from CN942650, CN927697 and CN887354 did not reveal polymorphism between parental alleles. New primers were designed for CN942650 and CN927697 to amplify part of the gene that was not included within the forward and reverse primers of the first primer set. After direct sequencing from these PCR products, polymorphisms were detected and SNP markers were developed (Table 6.3). An example of SNP detection from direct sequencing of PCR product is presented in figure 6.5.

The whole cDNA sequence had already been amplified and sequenced for CN887354. Therefore we could not investigate this CG any further.

Following polymorphism identification between parental alleles, SNP markers were developed. When two SNP markers were present near the end of the primer sequence, no mismatched base was added.

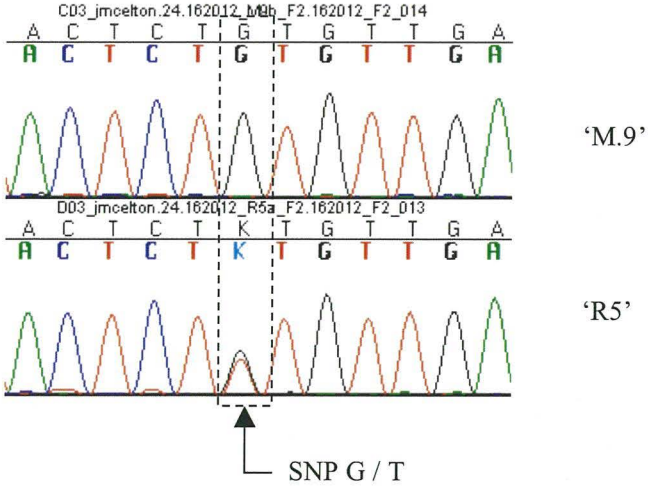



**Figure 6.5. Chromatogram of a PCR product amplified from 'M.9' and 'R5'.** The different bases in the sequence are represented by different colours (green=A; blue=C; black=G and red=T). The chromatogram of 'R5' presents two peaks of different colours (black and red) at the same position. This superposition of coloured peaks indicates that 'R5' is heterozygous at this position (G/T) while 'M.9' is homozygous (G/G) (Sequencher™ version 4.2, Gene Codes Corporation).

**Table 6.3. Primer pair sequences designed for dwarfing CGs**. The type of marker, fragment size and annealing temperatures for each candidate gene is given.

| Accession number | Specific primer sequence[a] | Marker type | Fragment size (bp) | T (°C)[b] |
|---|---|---|---|---|
| CN942650 | $F_3$ :CAGTAGTGATTGCTACTCTT | SNP | 218 | Ta 58 |
| | $R_3$: GCGGATTATTCCCAAATGTC | | | |
| CN927697 | $F_3$: CAAGTCCTGGCGTCGTTTGT | SNP | 163 | Ta 68 |
| | $R_3$: CTTGAGCACGACCTGGTTTC | | | |
| CN904514 | F: CTCTGCTCTTTAGTCCTCCAAA | SNP | 850 | Ta 55 |
| | $R_2$: ATGGGAGCACAGAGAACGTC | | | |
| CN908240 | F: TCAATCTGCCCAAACAGG | SCAR | 1500 | Ta 55 |
| | R: CAAATGCACCAGGAGGAA | | | |
| EB112737 | $F_2$: TTTATACTTTCTTGTCACTG | SNP | 124 | Ta 41 |
| | $R_2$: CAACACAAGTGGGTATGCTGA | | | |
| EB141306 | F: TCTCTCAGATTACTCATGGCGAAG | SSR | < 300 | TD 60-55 |
| | R: AATGAGCTTGAAGGAGCCGTAG | | | |
| CN945982 | F: CAAATGCCCCAGAACACCTA | SSR | < 300 | TD 60-55 |
| | R: CAATTGGGCAAGTGGGTAGA | | | |
| EG631304 | F: TCGTCTCGTCTTGACTTGCTT | SSR | < 300 | TD 60-55 |
| | R: TCTGGACAGAGAGCTTTTGAA | | | |
| CN868092 | F: GCAAGGCAAATTGCACTAGAA | SSR | < 300 | TD 60-55 |
| | R: CCACCCGATCCCTATAAACC | | | |
| CN919641 | $F_2$: ATCCTTTTTCTGAAGCTTAG | SNP | 182 | Ta 44 |
| | R:GGAAGAAAGGTGTAGCTGGTATGA | | | |
| CN935411 | F: GAGCCACCCCTAAACTCCTC | SSR | < 300 | TD 60-55 |
| | R: GGTTTTGAGCTGGAGCATCT | | | |
| CN891102 | F:CTGCTGCATTATTTTGTACAATCAC | SSR | < 300 | TD 60-55 |
| | R: CCCATTTCCATTATTGTCCTCTAC | | | |
| EB137861 | F: GGAAGTGTTGCGGTTTCAGT | SSR | < 300 | TD 60-55 |
| | R: AGACGTTTGATTCTTTTACATT | | | |
| CN916259 | $F_2$: CAATCCACTCCAGTGCAGCT | SNP | 165 | Ta 56 |
| | $R_2$: GTTTCCGGCTTCCATGAACT | | | |
| EB137941 | F: CCTTTTGGTCCTCTCTCTCTCTC | SSR | < 300 | TD 60-55 |
| | R: AGAAGTGGTGGTGAGGGTTG | | | |
| CN890397 | F: TCGCGGACTCCCTCAACC | SSR | < 300 | TD 60-55 |
| | R: GGTGGGCTTGGGAGCTGA | | | |

[a] Bases indicated in **Bold** are SNP markers detected in the sequence. Underlined bases are mismatched.

[b] Ta = annealing temperature; TD = Touch Down

### 6.4.5 Assignment of CGs to linkage groups

Twelve CGs amplifying 16 loci (five loci were amplified and mapped for one SSR primer pair) were assigned to 11 linkage groups of the 'M.9' × 'R5' map, using the bin mapping set (Chapter 4). The location of these markers is shown on figure 6.6. CN916259 mapped to LG1, CN927697 mapped to LG4, EB141306 mapped to LG6, CN908240 mapped to LG10, CN942650 mapped to LG11, CN945982 and CN891102 mapped both to LG12. CN904514, CN868092 and CN935411 mapped to LG15, and EB137861 mapped to LG16. EB137941 amplified multiple loci including five that were mapped to LG2, LG5, LG11, LG12 and LG14.

CN908240 appeared to be located on a non-previously mapped piece of the 'M.9' LG10. The location of this CG was confirmed when screened over 94 individuals of the 'M.9' × 'R5' population (Figure 6.6).

Markers developed for four CGs (EB112737, EG631304, CN919641 and CN890397) did not reveal any polymorphism between the parents and in the progeny and therefore were not mapped.

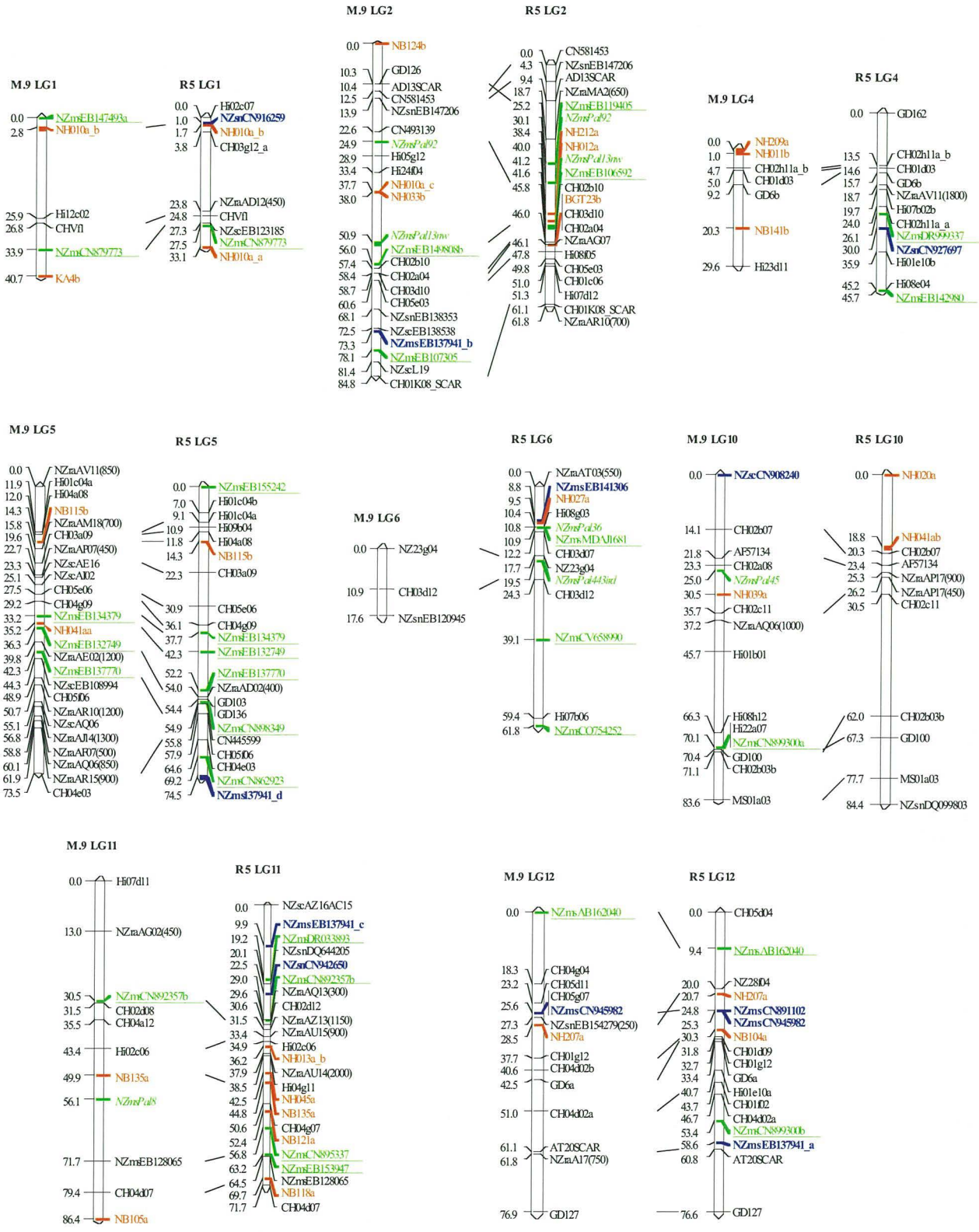
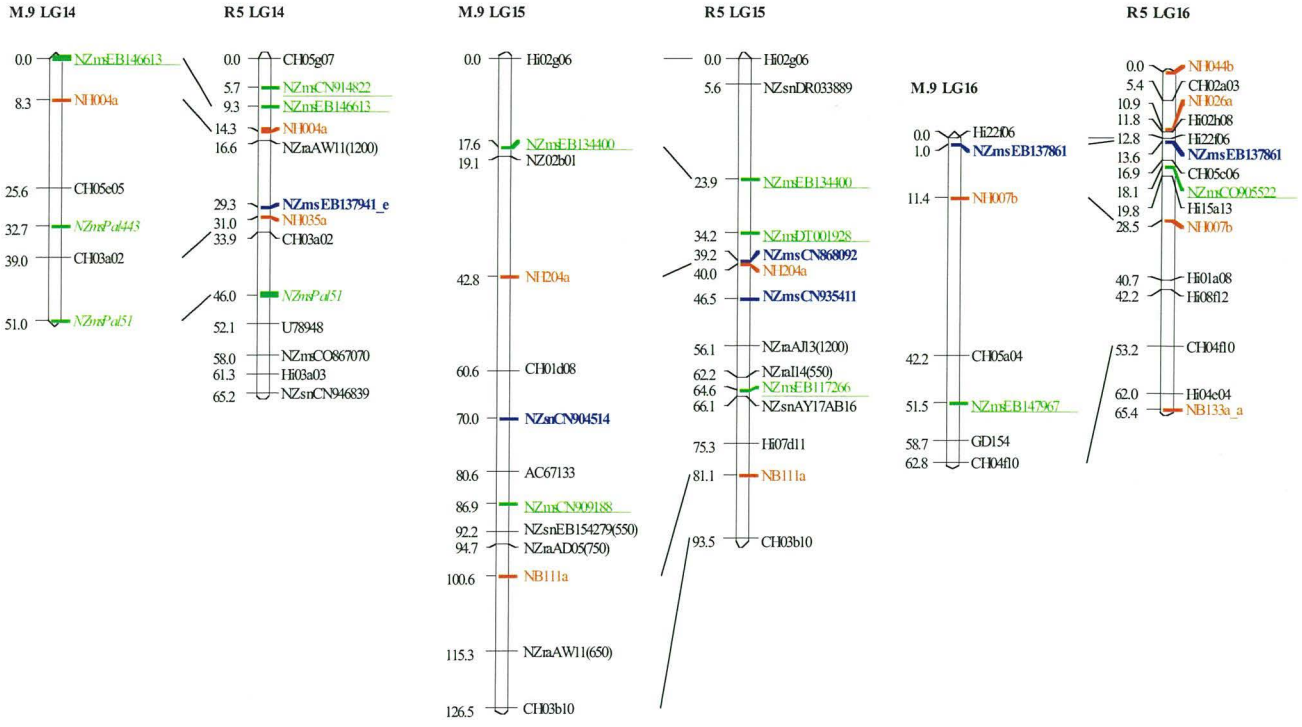



**Figure 6.6. Linkage groups of the 'M.9' × 'R5' genetic map with the position of dwarfing candidate genes** (indicated in bold and blue). Numbering of the linkage groups is according to Maliepaard *et al.* (1998). Pear SSR markers markers are indicated in red and SSR markers developed from the EST database during the genetic map construction are underlined and in green.

Figure 6.6. (continued)




The mapping of the CGs increased the coverage of the genetic maps of 'M.9' and 'R5' by 17.6 cM and 5.3 cM respectively. The genetic map of 'M.9' now covers 1193.3 cM, and the genetic map of 'R5' covers 1092 cM.

## 6.5 Discussion

The CG approach is a powerful strategy for identifying agronomically important genes controlling qualitative traits. However, it also has some limitations for application to plant genetics. Insufficient knowledge about the physiology of the trait can restrict the choice of functional CGs, which is particularly true for the dwarfing effect of the apple rootstock 'M.9' for which many hypotheses have been proposed to explain this phenomenon (see Chapter 1). The strategy we decided to use to reduce the number of potential CGs was to perform a microarray analysis.

### 6.5.1 Use of microarray to detect dwarfing CGs

Ideally a microarray slide should represent all the genes expressed in a given organism at the different stages of its development. The microarray developed at

HortResearch contains 15,102 non redundant *Malus* sequences, in comparison to the *Populus trichocarpa* genome which regroups more than 45,000 putative genes (Tuskan *et al.* 2006), and the *Arabidopsis thaliana* genome over 25,000 putative genes (The Arabidopsis Genome Initiative 2000). The majority of EST sequences from which the oligos are derived were derived from fruit tissues at different developmental stages. As a consequence, this microarray may not have been the most appropriate for the detection of genes involved in tree architecture and development. A microarray platform consisting of more *Malus* sequences from a more diverse library of tissues and treatments, such as the one developed by Jensen *et al.* (2006), or the platform currently developed for functional genomic studies in the Rosaceae, and containing over 40,000 unigene *Malus* oligo microarray (http://www.ars.usda.gov/research/projects) may have been more suitable for this analysis.

In order to detect genes differentially expressed, other methods are currently being developed, such as the use of the 454 Life Sciences Genome Sequencer 20 for RNA sequencing (http://www.prnewswire.com/). These technologies do not require prior knowledge of cDNA sequences, and are based on the sequencing of total RNA. Hence, the more copies of a RNA present in a sample, the more sequences of this particular RNA will be obtained, and differences between RNA expression profiles can be evaluated.

In our experiment, microarray slides derived from three different printings were used to identify differentially expressed genes. Each set of printing is unique and behaves differently under the same experimental conditions (Schaffer, personal communication). For this reason, slides derived from the BQ printing could not be analysed together with the slides from the BE and BM printing. As a consequence, the microarray analysis of mRNA extracted from root tissues could not be interpreted. We recommend in future studies the selection of a uniform set of microarray slides.

### 6.5.2 Candidate genes selected for mapping

From the list of the 100 genes most differentially expressed (out of the 7772 genes differentially expressed), 20 (Table 6.2) were selected for their potential involvement in the dwarfing effect. Among these 20 CGs, 10 are transcription factors (TF) or zinc finger proteins. These proteins have the ability to bind DNA upstream or downstream

to the gene they regulate and promote or repress the transcription of these genes. They control when and where genes, and proteins encoded by those genes, are expressed (Brivanlou and Darnell 2002). These TF were considered as potential candidates for their ability to switch on or off genes that might be involved in the dwarfing effect. CN919641 was selected because of its homology to a translation factor EF2. Translation factors are non-ribosomal proteins involved in translation initiation, elongation or termination (http://www.pantherdb.org). Such proteins, like TFs, can potentially affect the expression of a protein involved in plant development. CN935411 is a protein that has been identified in *Arabidopsis* as being part of the Notch signaling pathway. Notch proteins are located part inside and part outside of a cell membrane. When binding to the exterior part of a cell membrane, they release a portion of the interior part which then makes its way to the nucleus to alter gene expression (Oswald *et al.* 2001). The Notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes. This gene was selected as a dwarfing CG because of its involvement in gene regulation. CN942650 was the most significantly differentially expressed gene. It is a member of the FRI (FRIGIDA) related genes. In *Arabidopsis*, the requirement for vernalization is mostly controlled by the synergistic interaction of two dominant genes, FRI and FLOWERING LOCUS C (FLC) (Michaels and Amasino, 1999). FRI encodes a plant-specific coiled-coil domain-containing a protein required for the up-regulation of FLC, which produces a MADS domain-containing a transcription factor that acts as a strong floral repressor (Michaels *et al.* 2004) via the repression of LFY (LEAFY). A loss-of-function mutation in a FRI related gene could lead to a down-regulation of FLC, with the consequence of an up-regulation of LFY, leading to early flowering. As the genomic region around *DW1* was also found to have an influence on flowering (see Chapter 5), CN942650 was considered as a good CG for dwarfing. For the same reason CN916259 was chosen as a CG for its homology to a gene involved in the acceleration in the transition between vegetative and reproductive phase, which is typical of the effect of the 'M.9' apple rootstock.

CN927697 is part of the F-box family proteins. F-box proteins have been associated with cellular functions such as signal transduction and regulation of the cell cycle (Craig and Tyers 1999). For these reasons, and its low P value, this gene was selected as a potential CG for dwarfing. EG631344 was chosen because of its

involvement in plant growth control, and because it was highly expressed in *Arabidopsis* roots (TAIR database, Genevestigator Gene Atlas). CN887354 was selected because of its homology to a gene involved in water channel activity and transport. As described in Chapter 1 (section 1.1.3.3.2), the dwarfing effect of some rootstocks may be due to a smaller root system (Webster 1995). As this gene is also involved in the control of root development, it was considered as a potential CG. EE663938 is homologous to an *Arabidopsis* related ATPase. Huanca-Mamani *et al* (2005) demonstrated that degradation of mRNA of members of this gene family by RNA interference (RNAi) led to the observation of a reduced stature of *Arabidopsis* plants. For this reason, this gene was selected as a potential dwarfing CG. EB109549 exhibits homologies with the transducin family protein in Arabidopsis. Mutations in this gene have shown to enhance sensitivity to cytokinins, ethylene, ABA and auxins. The presence of such a mutated gene in the apple rootstock genome could well be a factor contributing to the dwarfing effect.

Finally, despite having no known molecular function, CN904514 was chosen as a potential CG for dwarfing because it appeared twice in the top 100 list of genes. Two oligos were present for this gene on the microarray slide.

### 6.5.3 Most differentially expressed genes

The result of the microarray analysis for mRNA extracted from rootstock bark tissues showed that 7772 genes were significantly differentially expressed. For practical reasons, all these genes could not be considered as potential candidate genes for dwarfing and mapped on the 'M.9' × 'R5' genetic map. The choice of selecting the best CG from the first 100 genes most differentially expressed may have lead to the non consideration of more important CGs. Genes influencing the dwarfing effect may not be highly differentially expressed between dwarfing and vigorous rootstocks. This idea is supported by the study of Seleznyova *et al.* (2003) who found that dwarfed trees were the result of a cumulative effect over time and that a small shift in the number of nodes per shoot (which characterizes the dwarfing effect of 'M.9'), and thus in the gene(s) controlling it, can have a dramatic effect on tree development.

It is interesting to note that none of the 100 genes most significantly differentially expressed were related to the production or transport of plant hormones. The presence of such genes might have been expected since the majority of studies on

dwarfing apple rootstocks have proposed an imbalance in hormone production and transport as the cause of dwarfing. In contrast, three genes involved in flowering were identified as differentially expressed. These include CN942650 (FRI related gene, activates FLC), EB112737 (VRN2 protein, mediates vernalization), and CN891102 (homolog to a transcription factor binding APETALA1 (AP1) promoter). The location of these genes in the flower development pathways of *Arabidopsis thaliana* is presented in figure 6.7.

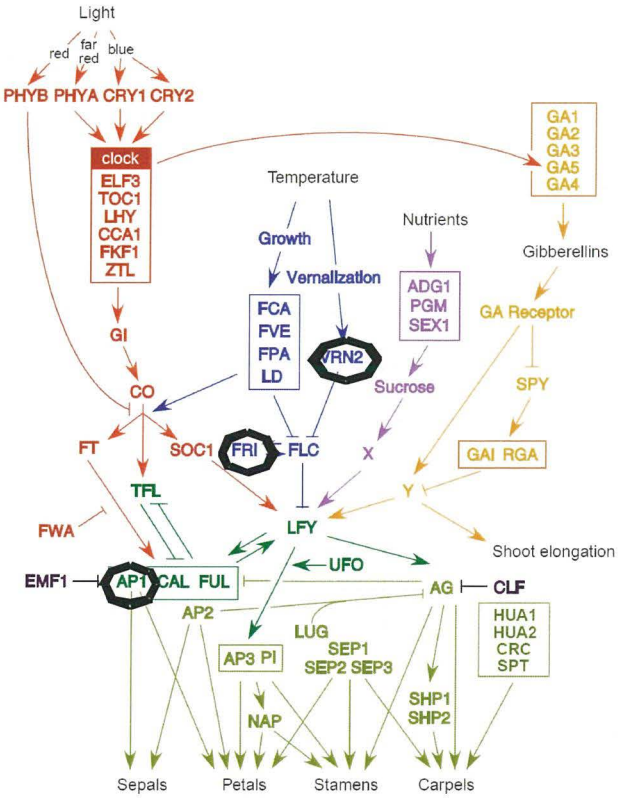



**Figure 6.7. Flower development pathways in *Arabidopsis thaliana*** (Blázquez, 2000). The genes identified by microarray analysis as significantly differentially expressed between dwarfing and vigorous rootstocks (FRI (FRIGIDA), VRN2 (vernalization 2 protein) and AP1 (APETALA1)) are indicated in a black circle.

Early flowering of the scion is related to its degree of dwarfing, and a QTL for the number of flower clusters on the main axis the second year after grafting has been located at the same position as the main dwarfing QTL *DW1* (see Chapter 5). However these genes may have been expressed as a consequence of the dwarfing effect, and may not be directly controlling it.

### 6.5.4 Detection of polymorphisms

The method for SNP genotyping used in our experiment is vulnerable to false negative effects, as PCR failures cannot be reliably distinguished from genuine primer-binding discrimination. More reliable methods, such as enzymatic cleavage, allele-specific oligonucleotides or sequencing (Chagné *et al.* 2007a), could be used in the future to confirm the location of CGs, particularly those located near QTL. These methods could also be applied to detect polymorphism in CGs that could not be mapped in this study (EB112737 and CN919641).

The BLASTn results of the PCR products obtained from three primer pairs designed from EG631344, EB109549 and EE663938 did not correspond to what we expected for these three CGs. To amplify the expected portion of gene, we should in the future re-design PCR primers and/or use more stringent PCR conditions.

### 6.5.5 Co-location with QTL related to the dwarfing effect

With respect to the QTL identified in Chapter 5, six associations were observed, two in LG11, one in LG12 and three in LG15 (Figure 6.8).

Two loci amplified by the SSR marker derived from EB137941 were found to co-locate with (i) a major cluster influencing many traits related to dwarfing on LG11 including the increase in trunk circumference area (TCA), the number of flower clusters the first year after grafting, and the height, node number and average internode length of the main axis (see QTL analysis), and (ii) a QTL influencing the average internode length on LG12. This CG codes for a transcription factor that might regulate a gene or group of genes influencing various traits. The multitude of loci amplified by this SSR marker indicates that this transcription factor is replicated throughout the apple genome, or is part of a large group of homologous genes that might have an influence on the tree architecture and development. A second CG, CN942650, first one on the list of the most differentially expressed genes and member of the FRI gene family which are required for the up-regulation of FLC, was also found to be located at the extreme limit of the interval of confidence of this major cluster of QTL on LG11. The role of such a gene in the control of characters associated with dwarfing can be envisaged. Since the number of flower clusters produced the first year after grafting is related to dwarfing (see Chapter 5), it might be possible that a precocious flowering could influence the development of a tree by favouring floral development over vegetative development.

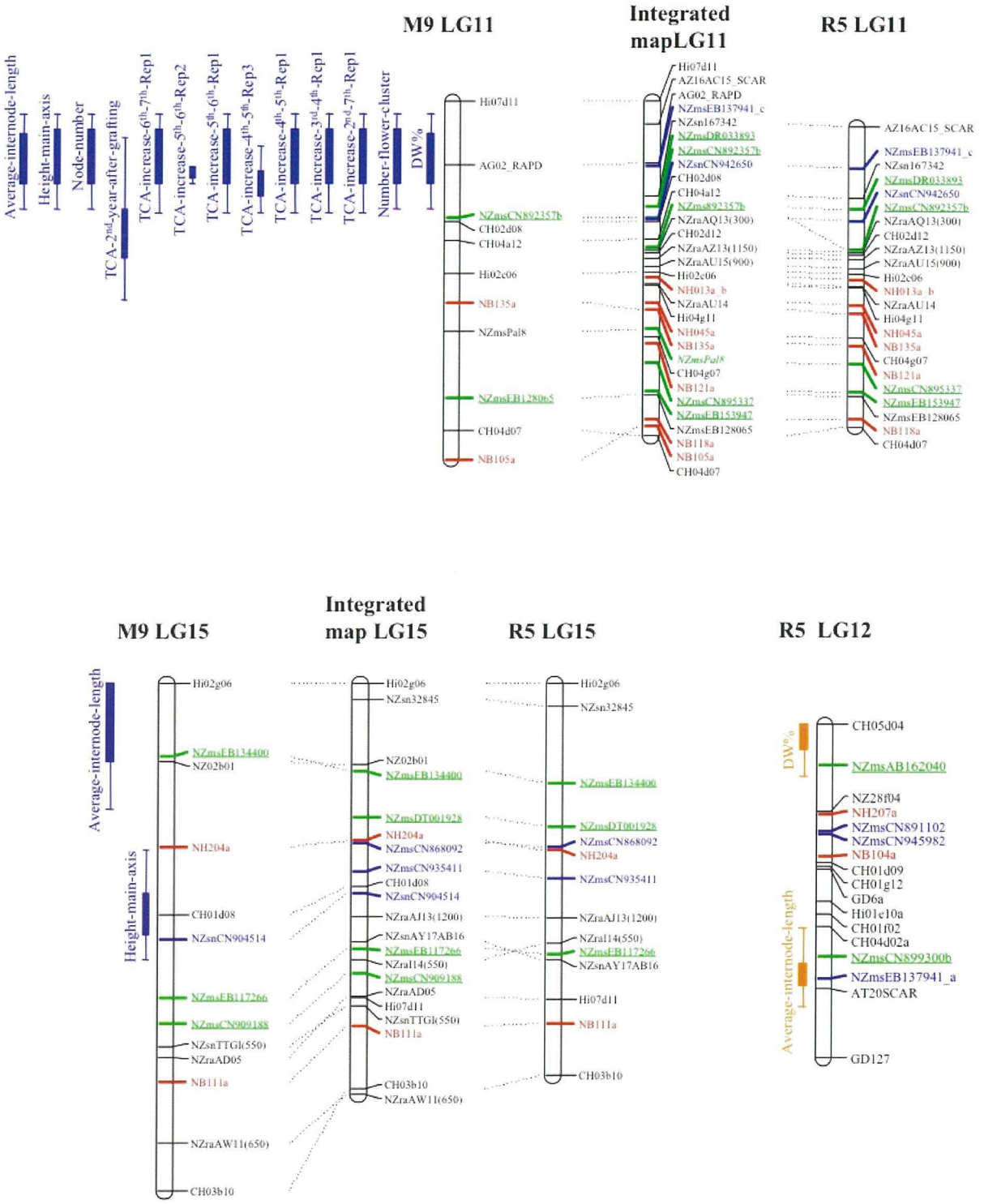



**Figure 6.8. Co-location between candidate genes and QTL on the linkage groups of 'M.9' and 'R5'.** Candidate genes are indicated in blue on the right hand side of the linkage groups. The accession number of each candidate gene is preceded by 'NZms' or 'NZsn' depending on the type of molecular marker developed, SSR and SNP respectively. QTL are indicated as bars (blue for 'M.9' and orange for 'R5') on the left hand side of the linkage groups.

The co-location of the CGs CN868092, CN935411 and CN904514 with a QTL influencing the height of the main axis the first year of growth after grafting on LG15 also seemed significant. These three CGs were all located within the interval of confidence of the QTL. The CG CN904514 seems particularly interesting since it is located at only 8 cM from the highest LOD for the QTL. Despite having an unknown molecular function, this gene may still be interesting to investigate as its function may only be understood in the context of grafting. The two other CGs are involved in gene expression regulation. As hypothesised previously, they may play a role in regulating a gene or group of genes influencing tree development.

The co-localisation of a CG with a QTL does not provide a strong enough basis upon which to draw a conclusion with respect to the involvement of this CG in the phenotypic variation (Pflieger *et al.* 2001). Furthermore, the more CGs that are mapped, the more co-segregation CG / QTL can occur by chance only (Leister *et al.* 1996). The putative polymorphic CG may be in linkage disequilibrium with the actual polymorphism responsible for the variation in the trait (non-random association of two genes). From a different standpoint, it is not possible to eliminate completely a CG on the basis that it does not co-segregate with a QTL, particularly when the QTL analysis was performed using a limited number of individuals as it is the case in our study (see Chapter 5) and CGs could co-segregate with minor QTL not identified by QTL analysis.

To validate a CG, association mapping studies can be performed. Association mapping involves the use of unstructured populations that are both phenotypically and genotypically characterized to detect statistical associations between genetic polymorphism and heritable trait variation (Oraguzie and Wilcox 2007). The condition for detection of such associations is the non-random association of causative trait polymorphism (i.e. candidate gene) with the observed polymorphism. The advantages of such studies are that they provide high QTL resolution with the power to incorporate a wide range of alleles. Physiological analyses, involving the measurement of CG expression using qPCR and at the protein level, can also provide evidence for, or against, the role of the CGs.

We report here the map location of 16 loci amplified from 12 CGs identified by microarray analysis as being differentially expressed among dwarfing and very vigorous individuals derived from the first 'M.9' × 'R5' cross.

Our CG approach focussed primarily on the identification of the *DW1* gene. Using the same individuals and the same data obtained from the microarray slides, we could re-organise the experimental design based on the score of genetic markers at the various QTL locations. This approach may allow us to identify additional CGs associated with dwarfing related traits. In future experiments, RNA extracted from individuals composing the bin mapping set could be used for the microarray analysis. Expression of particular cDNA could be scored for each individual and cDNA expressions segregating in a mendelian fashion could then be mapped using the bin mapping set. This would potentially allow for the identification of regions in the apple genome controlling the expression of genes (Kirst *et al.* 2005).

This microarray experiment is the first one ever performed for apple rootstocks, and represents the first data collected on gene expression for the dwarfing characteristic. Before that, the only information available to plant breeders and geneticists were physiological data. Our experiment represents the first step towards the understanding of the dwarfing phenomenon, and many more CGs from this microarray analysis and from the literature remain to be screened.

# 7- PEDIGREE ANALYSIS OF A MAJOR QTL CONTRIBUTING TO DWARFING IN APPLE ROOTSTOCK

## 7.1 Abstract

Apple rootstocks *(Malus domestica* Borkh and *Malus* spp.) have been selected for centuries for their ability to control tree size as well as other traits such as disease, pest and cold resistance. At the beginning of the 20th century, all the apple rootstocks grown in Western Europe were grouped and classified 'M.1' to 'M.25' at East Malling (UK) according to their dwarfing capacity (Webster and Wertheim, 2003). Since then, most of the apple rootstock varieties bred throughout the world have used parents from this series, particularly the dwarfing 'M.9' selection. SSR markers linked to a major QTL influencing dwarfing (*DW1*) were identified and mapped in a population from the cross 'M.9' (dwarfing) × 'Robusta 5' (vigorous) (Chapter 5). Two of these SSR markers mapping about 0.5 cM away from the dwarfing QTL *DW1* were screened over 58 rootstock accessions that confer a range of effects on scion growth.

The majority of the dwarf and semi-dwarf rootstock accessions screened carried the locus *DW1*. The tight linkage of both SSR markers to the *DW1* locus indicates that these markers should be highly effective in identifying dwarfing rootstocks. The results of this analysis indicate that there may be only one genetic source of dwarfing in apple rootstocks.

## 7.2 Introduction

The dwarfing phenomenon, which is characterised by the ability of a rootstock to reduce the vegetative growth of the scion grafted onto it, has been known and exploited by horticulturalists for hundreds of years (Webster 2004). The use of dwarfing rootstocks results in mature trees that are significantly smaller than trees grown on their own roots.

It has been reported that the dwarfing effect of 'Malling 9' ('M.9') is mainly due to a reduced number of internodes per growth unit which results in fewer new extension growth units in the successive annual cycle (Seleznyova *et al.* 2003), and, as shown in Chapter 5, to a reduction in the average internode length. The combination of both

effects would lead to a reduced number of smaller extension growth units produced from axillary buds in the next cycle, resulting in the expression of the dwarfing phenotype over time.

A wide range of hypotheses has been proposed to explain this effect, from the production and translocation of hormones, to the altered anatomy of the graft union (Hartmann and Kester 1990; Simon and Chu 1984), but none of the data obtained support a single hypothesis. Little is known either of the genetic control of the dwarfing effect, though it has been reported to be polygenically determined (Tydemann 1933).

To build on the work of Rusholme *et al.* (2004) and Chapter 3 of this thesis, in which bulked segregant analysis (BSA) was successfully used over a population derived from a cross between the dwarfing rootstock 'M.9' and the non-dwarfing rootstock 'Robusta 5' to identify a major gene linked to dwarfing located on LG05, a genetic map was constructed using an enlarged population from the same cross (Chapter 4) to perform a QTL analysis of the character (Chapter 5). A major locus designated as *DW1*, previously identified by BSA, and explaining over 50% of the dwarfing effect was confirmed via QTL analysis. Simple Sequence Repeat (SSR) markers were subsequently mapped within 0.5 cM of the putative location of *DW1*. Our detection of this locus in some vigorous individuals from the 'M.9' × 'R5' population indicates there are one or more additional genes that affect the expression of *DW1*.

In 1912, at the East Malling Research Station (United Kingdom), the standardized lines of well known vegetatively propagated rootstocks in Europe, some several centuries old, were gathered together and assessed. Between 1913 and 1935, 25 clones of rootstocks were identified and designated 'M.1' to 'M.25' (Manhart 1995). Apple rootstock breeding programmes initiated at John Innes Institute and East Malling and later in Sweden, Czechoslovakia, Romania, Germany, the USSR, Poland, the USA and Japan (Menendez *et al.* 1986) used these initial East Malling selections to develop the rootstocks used today in the apple industries throughout the world.

In this investigation, we genotyped accessions of rootstock related or unrelated to 'M.9', with SSR markers linked closely to the dwarfing locus *DW1*. This enabled us to evaluate both the linkage between the SSR markers and the *DW1* locus, and to assess whether there could be potential genetic sources of dwarfing in apple rootstock other than 'M.9'.

## 7.3 Materials and Methods

### 7.3.1 Plant materials

Fifty eight rootstock accessions (*Malus* spp.) representing rootstock varieties from major apple growing regions in the world were used in this study. Leaves were collected from accessions in the HortResearch germplasm collection in Havelock North (New Zealand) and in the USDA-ARS collection in Geneva, NY, USA. The rootstocks were classified according to their dwarfing effect in accordance with the literature and inhouse HortResearch professional expertise (Stuart Tustin).

### 7.3.2 DNA isolation and PCR amplification

Total genomic DNA was extracted from leaves according to Gardiner *et al.* (1996). The isolated DNA was quantified using 0.9% agarose gels in 1×TAE buffer with 50 ng of lambda phage DNA as a standard. Electrophoresis runs were for one hour at 70 Volts, and the DNA was stained with ethidium bromide (0.5 $\mu g\ ml^{-1}$).

The SSR markers Hi01c04 and Hi04a08, developed by Silfverberg-Dilworth *et al.* (2006) and located at the top of LG05, were amplified from all 58 rootstock accessions. Both SSR markers map at the same location on the 'M.9' map (Figure 4.1). The M13 sequence TGTAAAACGACGGCCAGT was added to the 5' end of the forward primer to enable the use of Schuelke's (2000) economical approach to fluorescent labelling. PCR reactions were carried out in a total volume of 15 µl with 20 mM TrisHCl; 0.2 mM dNTPs, 2 mM $Mg^{2+}$; 0.01 mM of forward primer, 0.15 mM of reverse primer; 0.15mM M13 labelled primer (fluorescent dye FAM for Hi01c04 and VIC for Hi04a08 (Applied Biosystems, Foster City, CA, USA)); 0.25 Unit of Platinum™ *Taq* DNA polymerase (Invitrogen) and 1 ng of template DNA.

PCR amplification was carried out in a Hybaid MBS Satellite 0.5G Thermal Cycler with the same amplification programme for both SSR reactions: an initial denaturation at 94°C for 2 min 30s followed by four cycles of 94°C for 30s, 60°C for 1 min, 72°C for 1 min with the annealing temperatures being reduced by 1°C per cycle; these four cycles were followed by 30 cycles of 94°C for 30s, 55°C for 1 min, 72°C for 1 min with a final 5 min 72°C extension.

One and a half microliters of each differently labelled amplification product were mixed together with 1 µl $H_20$ and 2 µl of ET-900 Rox internal size standard (Pharmacia Amersham, Freiburg, Germany) and denatured for 3 minutes at 94°C.

The reactions were loaded on an ABI 377 automatic sequencer (PE Applied Biosystems). The size of the bands was calculated based on the internal standard using GeneScan (version 2.0) software (PE Applied Biosystems).

The RAPD marker NZraAV11(850) located at the top of LG05 on 'M.9' and mapping on the other side of the locus *DW1* with respect to the SSR markers (Figure 4.1) was also screened over DNA from the rootstock accessions. PCR reactions were carried out in a 15 µl volume containing: 1.5 ng DNA, 18 mM Tris-HCl pH 8.4, 46 mM KCl, 0.1 µM of each dNTP, 1.3 mM $MgCl_2$, 0.08 µM of primer and 0.44 Units of Platinum™ *Taq* DNA polymerase (Invitrogen). RAPD amplifications were performed in a Hybaid MBS Satellite 0.5G Thermal Cycler under the following conditions: an initial denaturation at 94°C for 2 min 45s followed by 40 cycles of 94°C for 55s, 37°C for 55s, 72°C for 1 min 39s with a final 10-min 72°C extension. Amplification products were electrophoresed in 0.9% agarose gels (USB Corporation, Cleveland, OH USA) with TAE buffer at 110 Volts and stained with ethidium bromide (0.5 µg $ml^{-1}$).

## 7.4 Results and Discussion

The two SSR markers each exhibited one or two discrete DNA fragments for most of the rootstock accessions tested. Amplified fragments from SSR Hi01c04 ranged in size from 112 bp to 124 bp, and from SSR Hi04a08 fragments were from 220 bp to 250 bp. Table 7.1 shows the size (in bp) of the alleles amplified by the two SSR markers from 'M.9'and 'R5' DNA.

**Table 7.1. Allele sizes (in bp) of the SSR markers Hi01c04 and Hi04a08 amplified from the apple rootstocks 'M.9' and 'R5'.** The alleles associated with the dwarfing locus *DW1* are indicated in italic and bold.

| | Hi01c04 | | ***Hi04a08*** | |
|---|---|---|---|---|
| 'M.9' | 116 | ***122*** | 229 | ***231*** |
| 'R5' | 120 | 124 | 227 | 237 |

**Table 7.2. Rootstock accession, parentage, phenotype and size of alleles amplified by SSR markers Hi01c04 and Hi04a08**

| Accession | Parentage | Phenotype | Allele size (bp) | |
|---|---|---|---|---|
| | | | Hi01c04 | Hi04a08 |
| 'M.27' | 'M.9'x'M.13' | Very dwarf | *122** | *231** |
| 'M.20' | ? | Very dwarf | 120;*122* | *231*;- |
| 'Mac 9' | 'M.9' OP | Dwarf | 116;123 | 229;234 |
| 'CG41' | 'M.27'x'R5' | Dwarf | 120;*122* | *231*;237 |
| 'CG007' | 'Ottawa 3'x'R5' | Dwarf | *122*;124 | *231*;237 |
| 'CG11' | 'M.26'x'R5' | Dwarf | *122*;124 | *231*;237 |
| 'GenevaZ10' | 'M.9'x ? | Dwarf | 115;*122* | *231*;244 |
| 'M.9' | ? | Dwarf | 116;*122* | *231*;229 |
| 'JM7' | 'M.9'x'Marubakaido' | Dwarf | 120;*122* | *231*;- |
| 'Vineland 1' | 'Kerr'x'M.9' | Dwarf | 116;120 | 232;- |
| 'Bud9' | 'M.8'x'Red Standard' | Dwarf | 118;*122* | *231*;236 |
| 'M.8' | ? | Dwarf | 120;*122* | *231*;- |
| 'J-TE-E' | ? | Dwarf | 120;*122* | *231*;- |
| 'P.2' | 'M.9'x'Antonovka' | Dwarf | 120;*122* | *231** |
| 'CG179' | 'Ottawa 3'x'R5' | Dwarf / Semi-dwarf | *122*;124 | *231*;237 |
| 'Ottawa 3' | 'M.9'x'Robin' | Dwarf / Semi-dwarf | *122*;123 | *231*;235 |
| 'M.26' | 'M.16'x'M.9' | Semi-dwarf | *122*;- | *231*;236 |
| 'CG202' | 'M.27'x'R5' | Semi-dwarf | *122*;124 | *231*;237 |
| 'CG935' | 'Ottawa 3'x'R5' | Semi-dwarf | *122*;- | *231*;237 |
| 'CG210' | 'Ottawa 3'x'R5' | Semi-dwarf | *122*;- | *231*;237 |
| 'P.1' | 'M.4'x'Antonovka' | Semi-dwarf | 120;*122* | *231*;- |
| 'M.4' | ? | Semi-dwarf / Intermediate | 120;123 | 234;250 |
| 'M.3' | ? | Semi-dwarf / Intermediate | 120;123 | 234;- |
| 'Bud 57396' | 'M.8'x'Bud 9' | Intermediate | *122*;- | *231*;- |
| 'CG 6210' | 'Ottawa 3'x'R5' | Intermediate | 116;*122* | *231*;237 |
| 'JM 6' | 'M.9'x'Marubakaido' | Intermediate | 120;*122* | *231*;234 |
| 'AR 86-1-25' | 'M.27'x'MM 106' | Intermediate | 120;*122* | *231*;232 |
| 'P.16' | ? | Intermediate | *122*;- | *231*;233 |
| 'M.6' | ? | Intermediate | 120;- | 238 |
| 'Bemali' | 'Mank's Codlin'x'M.4' | Intermediate | 117;123 | *231*;234 |
| 'AR 10-3-2' | 'M.27'x'MM 106' | Intermediate | 120;*122* | *231*;232 |
| 'MM.106' | 'Northern Spy'x'M.1' | Intermediate | 120* | 232* |
| 'MM.102' | 'Northern Spy'x'M.1' | Intermediate | 120* | 232* |
| 'Northern Spy' | ? | Intermediate / Vigorous | 120;- | 232;236 |
| 'MM.111' | 'Northern Spy'x'M.793' | Intermediate / Vigorous | 120* | 232* |
| 'MM.108' | 'Northern Spy'x'M.15' | Vigorous | 120* | - |
| 'MM.104' | 'Northern Spy'x'M.2' | Vigorous | 120* | 236* |
| 'MM.105' | 'Northern Spy'x'M.2' | Vigorous | 120* | 232;236 |
| 'Merton 793' | 'Northern Spy'x'M.2' | Vigorous | 120* | 232* |
| 'M.2' | ? | Vigorous | 120;- | 232;236 |
| 'M.25' | ? | Vigorous | 115;120 | - |
| 'Aotea 106' | ? | Vigorous | 120;*122* | 233;236 |
| 'Red Standard' | ? | Vigorous | - | *231*;236 |
| 'M.11' | ? | Vigorous | 120;- | 232;- |
| '750363-013' | 'Ottawa 3'x ? | Vigorous | 120;*122* | *231*;- |

Table 2 continued

| Accession | Parentage | Phenotype | Allele size (bp) | |
|---|---|---|---|---|
| | | | Hi01c04 | Hi04a08 |
| 'M.1' | ? | Vigorous | 120;- | 232;236 |
| 'Antonovka' | ? | Vigorous | 120;- | ***231***;237 |
| 'Robin' | ? | Vigorous | 113;123 | 235;- |
| 'Kerr' | ? | Vigorous | 114;120 | 232;- |
| 'M.13' | ? | Very Vigorous | ***122***;- | ***231***;- |
| 'M.10' | ? | Very Vigorous | ***122***;124 | ***231***;235 |
| 'M.12' | ? | Very Vigorous | 120;***122*** | ***231***;- |
| 'M.15' | ? | Very Vigorous | 120;***122*** | ***231***;- |
| 'MM.113' | 'Northern Spy'x'M.12' | Very Vigorous | 120* | 232;- |
| 'MM.109' | 'Northern Spy'x'M.2' | Very Vigorous | 120* | - |
| 'R5' | ? | Very Vigorous | 120;124 | 234;236 |
| 'Mo84a' | ? | Very Vigorous | 120;***122*** | 234;240 |
| 'M.16' | ? | Very Vigorous | 120;***122*** | ***231***;236 |

* = homozygous individual for this allele; - = uncategorized allele; Bold and Italic type indicate an allele of the same size as the allele linked to the dwarfing locus in 'M.9'.

A clear allele profile was obtained for the majority of the accessions screened. For some accessions, such as 'Bud 57396' and 'M.13', analysis of the results was more difficult and only one allele could be clearly identified.

The size range of the alleles amplified by the marker Hi04a08 in this study is consistent with the study of Silfverberg-Dilworth (2006). However a consequent difference in the size range of the alleles amplified by Hi01c04 is observed. Silfverberg-Dilworth (2006) reported allele size ranging between 214 bp and 232 bp compared to the range 113 bp to 124 bp in this study (Table 7.2). The difference in allele sizes might be due to differences in selection between scion and rootstock cultivars.

For the very dwarf, dwarf and semi-dwarf rootstocks, whether or not related to 'M.9', the 122bp allele amplified by Hi01c04 and the 231bp allele amplified by Hi04a08 are both always present, with the exception of 'Mac 9' and 'Vineland 1' (Table 7.2), indicating a tight linkage between these two markers as well as between the markers and the *DW1* locus.

The validity of each stated parentage was assessed when possible, based on the transmission of SSR alleles from parents to offspring. In total, 29 clones from controlled crosses had both parents in the study. The parentage of all those accessions was confirmed with the exception of 'P1'. The genotype of this individual

can not be derived theoretically from its presumed parent 'M.4'. 'P1' is a semi-dwarf rootstock and amplifies alleles associated with *DW1* for both SSR markers, this result suggests that 'P1' may have 'M.9' or one of its progeny as one parent. On the other hand, the cultivar 'Mac 9' did not amplify any alleles of the same size as 'M.9' for the SSR Hi01c04. Because 'Mac 9' is issued from an open pollinated 'M.9', this result indicates that we may have collected an incorrect 'Mac 9' sample or that the results obtained by the ABI 377 automatic sequencer were misinterpreted. Additional 'Mac 9' samples will be collected for screening in the future to confirm the results.

To test for a possible recombination between the SSR loci and the *DW1* locus in 'Mac 9' and 'Vineland 1', the RAPD marker NZraAV11(850) (Chapter 3) was screened. Both accessions amplified a 850bp band shown to be linked to *DW1*. It is therefore probable that a recombination occurred between the SSR loci and *DW1* locus. Both these accessions will be very valuable for the development of future markers linked to *DW1*, as they will allow us to determine whether new markers are closer to the dwarfing locus than the existing SSR loci.

The very dwarf rootstock 'M.27' showed an interesting banding profile for both SSR markers. This accession is derived from a cross between the dwarf rootstock 'M.9' and the very vigorous rootstock 'M.13'. Both these accessions amplified alleles associated with *DW1*. For each of the SSR markers tested, the 'M.27' accession amplified one clear DNA band, suggesting that it may be homozygous for the allele associated with *DW1*. Homozygosity for *DW1* in 'M.27' may explain the very dwarfing effect this rootstock has on a scion.

The dwarf accessions 'M.20', 'M.8' and 'J-TE-E' are not known to be related to 'M.9' but all amplified alleles associated with dwarfing. This result indicates that there might be only one allele of the *DW1* locus influencing the dwarfing ability of apple rootstocks, derived from different sources, or that they are actually originally derived from 'M.9'. We cannot exclude the possibility of allelic homoplasy, i.e. sequence variation due to base substitution that does not cause allelic size change, observed in the SSR loci. The sequencing of these alleles will be necessary to confirm this hypothesis.

The accessions 'M.3' and 'M.4' are commonly described as semi-dwarf or intermediate. However it is important to remember that over the decades, and especially since the introduction of 'M.9' as the reference dwarfing rootstock, description of the effect of 'M.3' and 'M.4' has evolved from semi-dwarfing to intermediate (Tustin, personal communication). They are not known to be related to 'M.9' and do not amplify SSR alleles associated with *DW1*. However their ability to reduce the growth of a scion indicates that they might carry a different allele of the *DW1* locus, or that they have in their genome other QTL influencing the dwarfing effect. Further investigations are needed to determine the mechanism by which 'M.3' and 'M.4' influence the growth of the scions grafted onto them.

Half of the rootstock accessions classified as intermediate amplified alleles associated with *DW1*. This proportion is consistent with the results previously found in the 'M.9' × 'R5' population where about half of the individuals classified as intermediate carried markers associated with *DW1* (Chapter 3). The majority of the intermediate accessions amplifying the markers associated with *DW1* are derived from 'M.9' or one of its progeny.

As might be expected, the majority of the vigorous accessions did not amplify alleles associated with *DW1*. 'Aotea 106', 'Red Standard', 'Antonovka' and 'Mo84a' amplified alleles of the same size as 'M.9' for one SSR marker only. This may indicate that these individuals do not possess the *DW1* locus conferring the dwarfing ability.

However, six vigorous and very vigorous accessions out of 25, amplified alleles associated with *DW1* for both SSR markers. As seen in the 'M.9' × 'R5' population, the presence of the *DW1* locus is not sufficient in itself to induce and regulate dwarfing. One or more additional genes affect the expression of *DW1* (see Chapter 3 and Chapter 5). It is therefore not completely surprising to find some vigorous individuals with the *DW1* locus. The accession '750363-013' is derived from a cross with 'Ottawa 3', which itself has 'M.9' as a parent. The presence of *DW1* in '750363-013' is then not unexpected. Concerning 'M.10', 'M.12', 'M.13', 'M.15' and 'M.16', they all originate from the initial East Malling selection, as do the dwarfing rootstocks 'M.9', 'M.8' and 'M.20'. This result may indicate a relationship between the rootstocks collected together at East Malling. These clonal apple

rootstocks had been selected from seedling populations and growers and nurserymen would have exchanged the best rootstocks, resulting in a narrow genetic base.

The results of our investigation confirm the crucial role played by the locus *DW1* in the establishment of a dwarfed scion by the rootstock. The results also indicate that there may be a unique genetic source of dwarfing in the apple rootstocks commercially used throughout the world. All of the very dwarf, dwarf and semi-dwarf rootstocks screened, whether related or not to 'M.9', amplified markers linked to the major dwarfing QTL *DW1*. This may seem surprising since it is believed that as horticulture developed, new forms of dwarfing rootstocks arose spontaneously and independently in Germany, France, The Netherlands and England (Hatton 1916). One possibility is that R. Wellington, who undertook the study of apple rootstocks at East Malling in 1912, may not have collected all the dwarfing rootstocks available at that time. Hatton (1916) reported that the collections received at East Malling represented the majority, but no all, of rootstocks available to nurserymen in Europe at that time. For example, *P. malus praecox* (also known as the praecox of Pallas) was not included in the East Malling trial, despite being considered as the most dwarfing of all the Paradise stocks (Bunyard, 1920). Therefore, additional important sources of dwarfing might have been ignored. Other forgotten sources of dwarfing might thus still be found across Europe.

The outcomes of this study will have direct applications in selecting parents for apple rootstock breeding, as SSR markers Hi01c04 and Hi04a08 can be reliably used to determine the presence of the major dwarfing QTL *DW1* in rootstock accessions from germplasm collections internationally. Seedlings in progenies from such parents that do not possess the relevant alleles for the two markers can be culled as being extremely unlikely to dwarf scions. This will more than halve the cost for phenotyping of progeny in rootstock breeding programmes (Tustin, personal communication). Further studies will be necessary to identify markers linked to other dwarfing QTL that modify the dwarfing phenotype controlled by *DW1*. Such markers will enable further refinement in the selection of breeding parents, and also identification of progeny that possess a predictable degree of dwarfing influence.

# 8- CONCLUSIONS AND PERSPECTIVES

Many of the most important horticultural attributes of the apple tree as a biotic unit are substantially influenced by dwarfing rootstocks, including vigour, blossom initiation, fruit set, and phenology (Cummins and Aldwinckle 1995). The most important goal in apple rootstock breeding is to develop genotypes that impart a dwarfing or semi-dwarfing habit to the grafted scions. In addition, only a very limited number of dwarfing and semi-dwarfing apple rootstocks are available to apple growers, and these rootstocks are not always particularly suited to the growing conditions encountered in the various apple growing regions of the world. This makes it necessary for breeders to develop new rootstocks incorporating a combination of useful characters, apart from dwarfing, that makes them appropriate for use over a range of climates, and resistant to various pest and diseases. Knowledge of linkage relationships between marker loci and the dwarfing characteristic would significantly reduce the breeding time per generation and improve the breeding efficiency. In this work we have used multiple approaches including bulked segregant analysis (BSA), quantitative trait loci (QTL) analysis, and the candidate gene (CG) approach, to identify markers and genes associated with the dwarfing effect of the apple rootstock 'M.9' on grafted scions. The results obtained throughout the different stages of this thesis have contributed to, and later relied on, the development of a genetic map for apple rootstocks.

## 8.1 Apple rootstock genetic maps

The genetic maps constructed for 'M.9' and 'R5' were the first saturated maps developed for apple rootstocks (Chapter 4). These maps were constructed using new SSR markers developed from apple ESTs and made use of the majority of the *Pyrus* SSR markers published so far.

The associated development of a new set of 47 EST-SSR markers (Table 4.2) will provide geneticists with an extended choice of polymorphic markers for the development of genetic maps in apple. Because of the attributes associated with EST-SSR markers, such as improved scorability and fewer stutter bands (Leigh *et al.* 2003), these EST-SSR markers could also be an advantageous source of transferable orthologous markers between species of the Rosaceae family, which are necessary tools for comparative genome mapping studies. Until now, these studies have been

limited by the low number of transferable markers (only 24 RFLPs and six isozymes from *Prunus* have been mapped on a published apple map (Maliepaard *et al.* 1998)) and very little information is available on the homology between *Malus* and *Prunus*. Furthermore, the presence in this set of eight tetra-nucleotide SSR markers will also contribute to future fingerprinting studies in apple because of their low stuttering and larger relative size difference between alleles, as it is already the case for tetra-nucleotide SSR markers in human studies (Guarino *et al.* 2006).

Because of their large number of SSR markers, the 'M.9' and 'R5' parental maps can be aligned with the majority of the apple maps previously published (see section 4.5.6), permitting direct comparisons of the location of major genes and QTL. The mapping of *Pyrus* SSR markers was also a major step toward the complete alignment of the apple and pear genetic maps, necessary for the comparison of their genome structure. Preliminary results suggest a good, if not complete, colinearity between both genomes (see section 4.5.8), which confirmed the low level of divergence between the two genera observed by Evans and Campbell (2002). Nevertheless, this genome synteny is surprising considering the fact that the *Maloideae* group extends back to the Middle Eocene (Eocene epoch: 33.9 to 55.8 million years ago) (Wolfe and Wehr 1988). Knowledge gained from this work as well as from a previous study by Yamamoto *et al.* (2004a) may lead to consideration of the genomes of apple and pear as a unique genetic system, thus allowing an accelerated transfer of knowledge from one genus to the other. This may facilitate future identification of dwarfing genes in pear rootstocks with comparable effect on scion architecture and development. Another outcome of the genetic map was the development of the first bin mapping set for apple (see section 4.4.8). This will ensure that the 'M.9' × 'R5' map will be used to its maximum capacity for mapping new markers and genes by scientists from HortResearch and other institutes across the world.

The number of SSR markers mapped onto the genetic maps of 'M.9' and 'R5' has probably reached a sufficient level as 233 SSR markers have been mapped in total (including SSR markers developed from dwarfing candidate genes), representing a density of one SSR per 7.6 cM and 5.9 cM for 'M.9' and 'R5', respectively. Considering the current SSR marker density, one must now ask if it is necessary to add new markers of this type. The development of large apple EST libraries and the identification of SNP markers within these EST sequences provide an almost

limitless number of SNP markers. Newcomb *et al.* (2006) recently published a set of 151,687 apple EST sequences. Out of the 17,460 contigs generated, 18,408 bi-allelic SNP markers were identified and confirmed by more than one sequence. SNP markers were found every 706 bp of transcribed DNA. This high abundance of SNP markers was found despite the fact that ESTs from 'Royal Gala' accounted for 78.9% of all the ESTs sequenced. The addition of ESTs from other cultivars should increase the instances of SNP markers in the apple EST data. In addition to their high frequency in the genome, SNP markers are also easier to use than SSR markers and many high-throughput screening methods have been developed to genotype them (e.g. multiplex PCR-based genotyping and microarray-based methods (Syvänen 2005)). Beside their use for the construction of high density linkage maps, SNP markers can be applied to a wide range of purposes, and most importantly the mapping of candidate genes to test their association with QTL, as demonstrated in this thesis. Combined with high throughput screening methods, these markers will considerably increase the level of saturation of the genetic maps in the future.

High-throughput screening methods require the use of laboratory automation, which has already been implemented at HortResearch. Automation played a critical role in this project, from the set up of PCR reactions in 96 or 384 well plates, to the loading of amplification products in agarose gels. As mentioned previously in Chapter 1, automation reduced by three to four fold the time and effort needed to achieve the same results by hand. Thanks to automation, 759 RAPD markers were screened over DNA bulks, and 316 markers were screened over 94 to 216 individuals for the construction of the 'M.9' × 'R5' genetic map, representing over 70,000 PCR reactions! At HortResearch, automation is not only limited to the set up of PCR, but it has recently been extended to the extraction of DNA from leaf tissues. The automated DNA extraction system, developed by Cook (Cook and Gardiner 2004), eliminates the time consuming process of DNA extraction by hand, which is a limiting factor for marker assisted selection.

In addition to segregating for the dwarfing characteristic, the 'M.9' × 'R5' population also segregates for resistance to woolly apple aphid (*Eriosoma lanigerum* Hausm.) and fire blight (*Erwinia amylovora* Burrill). Both resistance genes originate from the parent 'R5' (Aldwinckle and Beer 1979). The new genetic maps have

already been used to locate the major resistance gene *Er2* (Bus *et al.* 2007) and identify a major fire blight resistance QTL (Celton, unpublished). The integration of all these markers for resistance genes, together with the markers developed for dwarfing, will allow rootstock breeders to significantly increase their breeding capacity and efficiency, which will ultimately result in the development of elite rootstock cultivars with a dwarfing capacity as well as resistance to fire blight and woolly apple aphid.

## 8.2 Bulked segregant analysis

Bulked segregant analysis using RAPD markers proved to be a very efficient method for the identification of markers linked to QTL influencing dwarfing (Chapter 3). In total, 19 RAPD markers (including four previously identified by Rusholme *et al.* 2004) were mapped to two QTL influencing the dwarfing effect of 'M.9', which were both confirmed by QTL analysis. The dwarfing QTL *DW1* was accurately located in the interval between NZraAM18(700) and NZraAV11(850) on the linkage group 5 (LG5) of 'M.9' (Figure 3.4). Results from the BSA indicated that all the rootstocks classified as dwarfing amplified markers associated with *DW1*, from which we concluded that the *DW1* locus was necessary to induce dwarfing. However, we observed that 12 of the 81 rootstocks classified as vigorous also amplified markers associated with *DW1* (see section 3.5.3), which led us to conclude that *DW1* was probably under the influence of other modifier alleles capable of altering the expression of the dwarfing phenotype. This assumption was corroborated by the fact that most of the semi-dwarfing individuals (12 out of 15) and half of the intermediate individuals also amplified markers associated with *DW1* (see section 3.5.3), meaning that other minor QTL, of which only one could be identified by BSA, influenced the dwarfing phenotype.

The results obtained by BSA show that this strategy is efficient for gene mapping because it allows a substantial saving in time (it took less than six months to perform two rounds of BSA and screen 759 RAPD markers over the bulks) and money, compared to comprehensive genotyping such as QTL analysis for which the construction of the genetic map required one and a half years. This study also showed that in a population with precise phenotypic characterisation, it is possible to use BSA to detect QTL with major and moderate effects, as both QTL identified by BSA were confirmed by QTL analysis. Using a combination of phenotypic and

genotypic results, we were able to set the target interval (region around *DW1*) very precisely. The BSA method enables us to localize molecular markers anywhere in the genome without limiting the number of markers to be mapped, and a significant sized segregating population will have multiple recombinants in a genomic region, resulting in a precise selection of the width of the target region (Monna *et al.* 1995). BSA can also be used to fill gaps in a linkage map, which can be very valuable in species such as apple, where few transferable markers have been identified for some linkage groups (i.e. LG1 and LG7) (Silfverberg-Dilworth *et al.* 2006). Each bulk needs to be homozygous for each non-recombinant genotype for the interval, and recombinants need to be excluded from the analysis. Similarly, BSA could be used sequentially for neighbouring regions to define the genetic end of a linkage group. When new markers are identified that are distal to the original terminal marker, BSA would be repeated until no more distal markers are identified and the genetic end of the LG is reached. It is the equivalent of genetic walking along the chromosome. For the 'M.9' × 'R5' genetic map, this method could be employed to identify genetic markers at the bottom of 'M.9' LG6 as well as at the top of 'R5' LG17 (Figure 4.1). With the use of optimal PCR conditions and a high screening capacity, the RAPD markers were exploited to their full potential. The RAPD markers that were not linked to either of the two dwarfing loci proved to be very valuable during the construction of the genetic map, often allowing the linking of SSR markers and parts of fragmented linkage groups.

## 8.3 QTL analysis

The QTL analysis confirmed the presence of the two QTL identified by BSA. Furthermore, it identified additional QTL influencing the dwarfing trait and estimated the percentage of variation explained by each of these QTL (Table 5.7). However, one question remains to be raised: do the QTL identified correspond to one locus or to a chromosomal region with a cluster of genes, each with a relatively small genetic effect? The QTL identified as *DW1* spanned a very narrow portion of the 'M.9' LG5 apple rootstock genome (Figure 5.5), indicating that this QTL might be under the influence of a single gene with pleiotrophic effects. Markers for this QTL were amplified from all the dwarfing and semi-dwarfing individuals of the 'M.9' × 'R5' populations, as well as from all the dwarfing and semi-dwarfing rootstocks accessions screened in the pedigree analysis (Table 7.2). Considering

these results, it might be more appropriate to call *DW1* a major gene rather than a major QTL. In apple, several resistance genes such as *Vf* and *Pl-1* are considered to be major dominant genes despite being influenced by minor genes modifying their activity (Gessler 1989; Dunemann *et al.* 2004). In fact, *Vf* by itself does not induce a complete resistance to the pathogen *Venturia inaequalis* (Cke.) Wint., but confers a resistance of three (partial sporulation) on a scale from zero to five (Durel, personal communication). A second gene identified as *Vfh* is necessary to induce complete resistance to the pathogen. *Pl-1* which confers resistance to powdery mildew (*Podosphaera leucotricha* (Ell. *et* Ev.) Salm.) is also dependant on another precursor gene designated as "C/c" (Dunemann *et al.* 2004). Without this precursor gene, individuals with only *Pl-1* are susceptible to powdery mildew. A similar observation can be made for *DW1*, which does not induce a dwarfing effect for some rootstocks (12 rootstocks rated as vigorous amplified markers associated with *DW1*, see section 3.5.3). Thus, it may be more appropriate to classify *DW1* as a major gene that is required to the expression of the dwarfing (or semi-dwarfing) phenotype, but whose expression is dependant on other minor QTL.

In maize, Beavis *et al.* (1991) hypothesized the possibility of coincidence between major genes determined by classical genetic analyses and QTL estimated using molecular markers and statistical analyses. Yano *et al.* (1997) also indicated that some of the QTL detected for the heading date in rice coincided with known major genes based on a comparison of their map locations. As we can see from the literature, confusion exists over the definition of major genes and major QTL, which might be based on the type of analysis performed to identify them.

## 8.4 Identification of *DW1* and possible impact on Rosaceae fruit tree crops

The identification of genes responsible for dwarfing will be a major subject of research in the near future. Despite the current insufficient knowledge on the physiology of this trait, further candidate genes will be screened over the 'M.9' × 'R5' population in an effort to identify the genes responsible for the dwarfing effect of the apple rootstock 'M.9'. These will include additional candidates from the microarray analysis, as well as those that will be identified from bacterial artificial chromosomes (BACs) linked to markers associated with *DW1*, and

information from an international apple genome sequencing effort. Additional candidate genes will be selected and tested on the basis of their function in plant model species. However, one further question should be posed here: based on the results of the QTL analysis and on the confirmation of the relationship between the dwarfing capacity of a rootstock and its ability to induce precocious flowering of the scion, could the dwarfing effect of 'M.9' be a consequence of the early flowering of the scion? In other words, should we be looking for genes influencing flowering rather than genes potentially involved in a dwarfing effect? This hypothesis is supported by Seleznyova *et al.* (2003; in press) who concluded that early transition to flowering accelerated the progression of axillary shoots to less vigorous forms, and that dwarfing rootstocks and interstocks caused an accelerated developmental aging of apple trees into a mature state.

In the quest for the nucleotide sequence responsible for dwarfing, another question arises: if the mutation responsible for the dwarfing phenotype was not located in a gene, but in a regulatory region, such as a promoter, or enhancer or intron, would we be able to detect it? To identify a mutation in the regulatory region of a gene, we must not restrict our search to candidate genes only. This means that positional cloning may be the most appropriate approach to identify the cause of dwarfing. The principle behind positional cloning resides in the fact that recombination and physical distance are directly related. The number of plants required to ensure the mapping of any mutation to a single BAC or BAC contig is at least 1000 (in maize) (Bortiri *et al.* 2006). Such an extended population is required to ensure the detection of the recombinants that refine the position of the target gene in the genome. As the number of individuals increases, it is important to identify additional markers to narrow the mapping interval. BAC contigs can provide a source of markers. Once the mapping interval is small enough so that it spans a few BACs, these BACs can be sequenced and candidate genes, or candidate mutations, identified.

However, it is hard to make generalizations about what kind of changes will have phenotypic consequences. Nonsense polymorphisms (nucleotide mutation that changes a nucleotide triplet into a stop codon) and deletion polymorphisms make a candidate gene more likely than anonymous mutations (i.e. SNP with an unknown functional effect (Khlestkina and Salina 2006)). Amino-acid changes as well as expression level changes may also be important in providing functional variation (Borevitz and Chory 2004). Mutation in the binding site of microRNAs (miRNAs),

which function as post-transcriptional regulator of target genes, can also influence the expression of one or more gene (Allen *et al.* 2004) and are thus potential candidates for dwarfing.

Once candidate genes have been identified and shown to co-segregate with a QTL, they will need to be tested functionally. Transgenic approaches could be used to directly introduce the candidate genes into a vigorous rootstock. However, would this be sufficient to observe a dwarfing effect? As observed in the 'M.9' × 'R5' populations, some vigorous rootstocks amplify markers linked to *DW1*, indicating that *DW1* is influenced by other minor QTL. The choice of an appropriate vigorous rootstock would then be crucial in order to observe a change in the phenotype. The selection of such a rootstock could be based on the presence of markers for other QTL related to dwarfing, particularly the second QTL identified by BSA. Since it is not clear yet which genetic background is necessary for the expression of the dwarfing phenotype, another possibility would be to introduce an RNAi (RNA interference), complementary to the RNA produced from *DW1*, into a dwarfing rootstock. This RNAi would induce homology-dependant degradation of a messenger RNA (Montgomery *et al.* 1998), likely resulting in the development of a non-dwarfed scion.

Another way to confirm a QTL gene is to use gene replacement, which has been demonstrated successfully in rice (Terada *et al.* 2002). Gene replacement can be used to specifically substitute alleles at the QTL locus while maintaining the correct genomic context, as was performed in *Drosophila* (Greenberg *et al.* 2003).

The identification of *DW1* will have a major impact on apple rootstock breeding. It will provide breeders with molecular markers located directly on the gene, and will make possible the identification of alleles of the gene(s) with potential varying effects. It will also provide breeders with the opportunity of developing dwarfing rootstocks by introducing the dwarfing genes (*DW1* and others) into trees of other members of the Rosaceae family such as pear, peach, cherry and apricot, via *Agrobacterium*-mediated transformation methods that have already been developed for these crops (respectively in Mourgues *et al.* 1996; Scorza *et al.* 1990; Song and Sink 2006; Machado *et al.* 1994).

Assuming that the use of dwarfing rootstocks for other members of the Rosaceae family has the same benefits as for apple, the impact on production systems would be

very significant. The production of these crops would become much more efficient and would enable crops which are environmentally vulnerable, such as pears and cherries, to be more readily protected against birds and wind damage. Operating efficiency of orchards would also be enhanced economically and costs per kilogram of production would be reduced. Finally, fruit quality might also be improved.

The identification of new rootstock accessions displaying dwarfing characteristics is another likely objective for apple rootstock breeding. As shown by the pedigree analysis, all commercially available rootstock accessions rely on a unique genetic source of dwarfing. The identification of other sources of dwarfing would expand the panel of architectural characteristics available to apple growers. New sources of dwarfing might be found in the numerous germplasm collections of apple throughout the world. The detection of accessions with potential dwarfing abilities could be achieved by identifying phenotypic characteristics of a tree on its own roots that would indicate a dwarfing effect when used as a rootstock (i.e. precocity of flowering). A second possibility would be to use all the available accessions as rootstocks and record their effect on grafted scions. Such studies would permit the identification of alleles of *DW1* or even unrelated dwarfing genes acting in a completely different way. The introduction of such genes into rootstock breeding programmes, along with other characters such as pathogen resistance, would have the effect of diversifying the characteristics displayed by today's rootstocks to better suit the needs of apple growers.

The research presented in this thesis has provided a foundation for the establishment of a robust marker assisted selection system for dwarfing apple rootstocks based on reliable markers associated with QTL influencing the dwarfing phenotype and other related architectural characteristics. The precise location of the major dwarfing QTL *DW1* on the linkage group 5 of the apple genome has also provided the basis for future cloning of this QTL via positional cloning. To this end, work is already in progress to identify additional markers closer to *DW1*. The knowledge of the gene(s) responsible for the dwarfing effect would close the discussion that has animated the world of apple physiology for the past 100 years and open the way for the transfer of the causative gene(s) to other fruit tree crops members of the Rosaceae, thus revolutionizing their production systems.

# APPENDIX I

Score of the markers issued from the bulked segregant analysis mapping in the genomic region of the *DW2* locus. The markers are in the same order as shown in Figure 3.6.

| Phenotype | Tree number | NZraAG02(500) | NZral14(200) |
|---|---|---|---|
| dwarfing | 1 | + | 0 |
| dwarfing [a] | 2 | - | - |
| dwarfing | 3 | + | + |
| dwarfing | 4 | + | + |
| dwarfing [a] | 5 | - | - |
| dwarfing | 6 | + | + |
| dwarfing | 7 | + | + |
| dwarfing | 8 | + | + |
| dwarfing | 9 | + | + |
| dwarfing | 10 | + | + |
| dwarfing | 11 | + | + |
| dwarfing | 12 | + | + |
| dwarfing | 13 | + | + |
| dwarfing | 14 | + | + |
| dwarfing | 15 | + | + |
| dwarfing | 16 | + | + |
| dwarfing [a] | 17 | - | - |
| dwarfing | 18 | + | + |
| dwarfing | 19 | + | + |
| semi-dwarfing [a] | 20 | - | - |
| semi-dwarfing [a] | 21 | - | - |
| semi-dwarfing | 22 | + | + |
| semi-dwarfing | 23 | + | + |
| semi-dwarfing | 24 | - | 0 |
| semi-dwarfing | 25 | + | + |
| semi-dwarfing | 26 | + | + |
| semi-dwarfing | 27 | - | + |
| semi-dwarfing [a] | 28 | - | - |
| semi-dwarfing [a] | 29 | - | - |
| semi-dwarfing | 30 | + | + |
| semi-dwarfing | 31 | + | + |
| semi-dwarfing [a] | 32 | - | - |
| semi-dwarfing [a] | 33 | - | - |
| semi-dwarfing [a] | 34 | - | - |
| vigorous | 35 | - | - |
| vigorous | 36 | - | - |
| vigorous | 37 | + | + |
| vigorous | 38 | - | - |
| vigorous | 39 | - | - |
| vigorous | 40 | + | + |
| vigorous | 41 | + | + |
| vigorous | 42 | - | + |
| vigorous | 43 | + | + |
| vigorous | 44 | + | + |

Appendix I continued...

| Phenotype | Tree number | NZraAG02(500) | NZraI14(200) |
|---|---|---|---|
| vigorous | 45 | - | - |
| vigorous | 46 | + | + |
| vigorous | 47 | - | - |
| vigorous | 48 | - | - |
| vigorous | 49 | - | - |
| vigorous | 50 | - | - |
| vigorous | 51 | - | - |
| vigorous | 52 | + | + |
| vigorous | 53 | - | - |
| vigorous | 54 | + | + |
| vigorous | 55 | + | + |
| vigorous | 56 | + | + |
| vigorous | 57 | - | - |
| vigorous | 58 | - | - |
| vigorous | 59 | - | - |
| vigorous | 60 | - | - |
| vigorous | 61 | - | - |
| vigorous | 62 | - | - |
| vigorous | 63 | + | + |
| vigorous | 64 | + | + |
| vigorous | 65 | - | + |
| vigorous | 66 | - | - |
| vigorous | 67 | - | - |
| vigorous | 68 | - | + |
| vigorous | 69 | - | + |
| vigorous | 70 | - | - |
| vigorous | 71 | - | + |
| vigorous | 72 | - | - |
| vigorous | 73 | + | + |
| vigorous | 74 | + | - |
| vigorous | 75 | - | - |
| vigorous | 76 | + | + |
| vigorous | 77 | - | - |
| vigorous | 78 | - | - |
| vigorous | 79 | + | + |
| vigorous | 80 | - | - |
| vigorous | 81 | - | - |
| vigorous | 82 | - | - |
| vigorous | 83 | + | - |
| vigorous | 84 | + | + |
| vigorous | 85 | - | + |
| vigorous | 86 | + | + |
| vigorous | 87 | + | + |
| vigorous | 88 | - | - |
| vigorous | 89 | - | + |
| vigorous | 90 | - | - |
| vigorous | 91 | + | + |
| vigorous | 92 | - | - |
| vigorous | 93 | - | - |
| vigorous | 94 | - | - |
| vigorous | 95 | - | - |
| vigorous | 96 | - | - |
| vigorous | 97 | - | - |

Appendix I continued...

| Phenotype | Tree number | NZraAG02(500) | NZral14(200) |
|---|---|---|---|
| vigorous | 98 | - | - |
| vigorous | 99 | - | - |
| vigorous | 100 | - | - |
| vigorous | 101 | - | - |
| vigorous | 102 | - | - |
| vigorous | 103 | - | - |
| vigorous | 104 | - | - |
| very vigorous | 105 | + | + |
| very vigorous | 106 | - | - |
| very vigorous | 107 | - | - |
| very vigorous | 108 | - | - |
| very vigorous | 109 | + | + |
| very vigorous | 110 | - | + |
| very vigorous | 111 | - | - |
| very vigorous | 112 | - | - |
| very vigorous | 113 | - | - |
| very vigorous | 114 | - | - |
| very vigorous | 115 | - | - |
| intermediate | 116 | + | 0 |
| intermediate | 117 | + | + |
| intermediate | 118 | - | 0 |
| intermediate | 119 | - | + |
| intermediate | 120 | - | - |
| intermediate | 121 | + | + |
| intermediate | 122 | + | + |
| intermediate | 123 | - | - |
| intermediate | 124 | + | - |
| intermediate | 125 | - | - |
| intermediate | 126 | + | + |
| intermediate | 127 | + | - |
| intermediate | 128 | - | - |
| intermediate | 129 | - | - |
| intermediate | 130 | - | - |
| intermediate | 131 | - | - |
| intermediate | 132 | + | + |
| intermediate | 133 | + | + |
| intermediate | 134 | - | - |
| intermediate | 135 | + | + |
| intermediate | 136 | + | + |
| intermediate | 137 | + | + |

+ indicates the presence of the marker associated with *DW2*

- indicates the absence of the marker associated with *DW2*

0 indicates missing data

[a] dwarfing and semi-dwarfing individuals without markers associated with *DW2*

# APPENDIX II

Score of the markers issued from the bulked segregant analysis and flanking the *DW1* locus. The markers are in the same order as shown in Figure 3.4.

| Phenotype | Tree number | NZscAI02 | NZraAP07 | NZscAE16 | CH03a09 | NZraAM18 (700) | *DW1* | NZraAV11 (850) | NZraAB03 (1800) |
|---|---|---|---|---|---|---|---|---|---|
| dwarfing | 1 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 2 | - | - | - | - | + | *DW1* | + | + |
| dwarfing | 3 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 4 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 5 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 6 | + | + | + | + | + | *DW1* | - | + |
| dwarfing | 7 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 8 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 9 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 10 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 11 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 12 | + | + | + | + | + | *DW1* | + | - |
| dwarfing | 13 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 14 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 15 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 16 | + | + | + | + | + | *DW1* | + | 0 |
| dwarfing | 17 | + | + | + | + | + | *DW1* | 0 | + |
| dwarfing | 18 | + | + | + | + | + | *DW1* | + | + |
| dwarfing | 19 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing | 20 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing | 21 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing | 22 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing | 23 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing | 24 | + | + | + | + | - | *DW1* | + | + |
| semi-dwarfing | 25 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing | 26 | - | - | - | + | + | *DW1* | + | + |
| semi-dwarfing | 27 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing | 28 | - | + | + | + | + | *DW1* | + | 0 |
| semi-dwarfing | 29 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing | 30 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing | 31 | - | - | - | 0 | + | *DW1* | + | + |
| semi-dwarfing | 32 | + | + | + | + | + | *DW1* | + | + |
| semi-dwarfing[a] | 33 | - | - | - | - | - | *DW1* | - | + |
| semi-dwarfing | 34 | - | - | - | - | + | *DW1* | + | + |
| vigorous | 35 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 36 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 37 | - | - | - | - | - | *DW1* | - | + |
| vigorous | 38 | - | - | - | - | - | *DW1* | + | - |
| vigorous | 39 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 40 | - | - | - | - | - | *DW1* | + | - |
| vigorous | 41 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 42 | - | - | - | - | - | *DW1* | - | + |
| vigorous | 43 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 44 | + | + | - | - | - | *DW1* | - | - |
| vigorous | 45 | - | - | - | - | - | *DW1* | - | 0 |

Appendix II continued…

| Phenotype | Tree number | NZscAI02 | NZraAP07 | NZscAE16 | CH03a09 | NZraAM18 (700) | *DW1* | NZraAV11 (850) | NZraAB03 (1800) |
|---|---|---|---|---|---|---|---|---|---|
| vigorous | 46 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 47 | - | - | - | - | - | *DW1* | - | + |
| vigorous | 48 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 49 | - | - | - | - | - | *DW1* | + | - |
| vigorous | 50 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 51 | - | - | - | - | - | *DW1* | + | - |
| vigorous | 52 | - | - | - | - | - | *DW1* | - | 0 |
| vigorous | 53 | - | - | - | - | - | *DW1* | - | 0 |
| vigorous | 54 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 55 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 56 | + | - | - | - | - | *DW1* | - | 0 |
| vigorous | 57 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 58 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 59 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 60 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 61 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 62 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 63 | - | - | - | 0 | - | *DW1* | - | - |
| vigorous | 64 | - | - | - | - | - | *DW1* | - | + |
| vigorous | 65 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 66 | - | - | - | - | - | *DW1* | + | - |
| vigorous | 67 | - | - | - | 0 | - | *DW1* | - | + |
| vigorous | 68 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 69 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 70 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 71 | - | - | - | - | - | *DW1* | - | 0 |
| vigorous | 72 | - | - | - | - | - | *DW1* | - | 0 |
| vigorous | 73 | - | - | - | - | - | *DW1* | - | + |
| vigorous | 74 | - | - | - | - | -- | *DW1* | - | 0 |
| vigorous | 75 | - | - | + | - | - | *DW1* | - | + |
| vigorous | 76 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 77 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 78 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 79 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 80 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 81 | - | - | - | - | - | *DW1* | - | + |
| vigorous | 82 | - | - | - | - | - | *DW1* | - | + |
| vigorous | 83 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 84 | - | - | - | - | - | *DW1* | - | - |
| vigorous | 85 | + | + | + | - | - | *DW1* | - | + |
| vigorous | 86 | + | + | + | - | - | *DW1* | - | + |
| vigorous | 87 | + | + | + | - | - | *DW1* | - | + |
| vigorous | 88 | + | + | + | + | - | *DW1* | - | - |
| vigorous | 89 | + | + | + | + | - | *DW1* | - | - |
| vigorous | 90 | + | + | + | + | + | *DW1* | - | + |
| vigorous | 91 | + | + | + | + | + | *DW1* | - | + |
| vigorous | 92 | + | + | + | + | + | *DW1* | - | 0 |
| vigorous | 93 | + | + | + | + | + | *DW1* | - | + |
| vigorous [b] | 94 | + | + | + | + | + | *DW1* | + | + |
| vigorous [b] | 95 | + | + | + | + | + | *DW1* | + | 0 |
| vigorous [b] | 96 | + | + | + | + | + | *DW1* | + | + |
| vigorous [b] | 97 | + | + | + | + | + | *DW1* | + | + |

Appendix II continued...

| Phenotype | Tree number | NZscAI02 | NZraAP07 | NZscAE16 | CH03a09 | NZraAM18 (700) | *DW1* | NZraAV11 (850) | NZraAB03 (1800) |
|---|---|---|---|---|---|---|---|---|---|
| vigorous [b] | 98 | + | + | + | + | + | *DW1* | + | 0 |
| vigorous [b] | 99 | + | + | + | + | + | *DW1* | + | 0 |
| vigorous [b] | 100 | + | + | + | + | + | *DW1* | + | + |
| vigorous [b] | 101 | + | + | + | + | + | *DW1* | + | + |
| vigorous [b] | 102 | + | + | + | + | + | *DW1* | + | + |
| vigorous [b] | 103 | + | + | + | + | + | *DW1* | + | + |
| vigorous [b] | 104 | + | + | + | + | + | *DW1* | + | + |
| very vigorous | 105 | - | - | - | - | - | *DW1* | - | - |
| very vigorous | 106 | - | - | - | - | - | *DW1* | - | - |
| very vigorous | 107 | - | - | - | - | - | *DW1* | - | - |
| very vigorous | 108 | - | - | - | - | - | *DW1* | - | - |
| very vigorous | 109 | - | - | - | - | - | *DW1* | - | - |
| very vigorous | 110 | - | - | - | - | - | *DW1* | - | + |
| very vigorous | 111 | - | - | - | - | - | *DW1* | - | - |
| very vigorous | 112 | - | - | - | - | - | *DW1* | - | + |
| very vigorous | 113 | - | - | - | - | - | *DW1* | - | - |
| very vigorous | 114 | - | - | - | - | - | *DW1* | - | - |
| very vigorous | 115 | - | - | - | - | - | *DW1* | - | - |
| intermediate [c] | 116 | + | + | + | + | + | *DW1* | + | + |
| intermediate [c] | 117 | + | + | + | + | + | *DW1* | + | + |
| intermediate [c] | 118 | + | + | + | + | + | *DW1* | + | + |
| intermediate [c] | 119 | + | + | + | 0 | + | *DW1* | + | 0 |
| intermediate [c] | 120 | - | - | - | - | + | *DW1* | + | + |
| intermediate [c] | 121 | + | + | + | 0 | + | *DW1* | + | 0 |
| intermediate [c] | 122 | 0 | + | + | + | + | *DW1* | + | + |
| intermediate [c] | 123 | + | + | + | + | + | *DW1* | + | + |
| intermediate [c] | 124 | + | + | + | + | - | *DW1* | + | - |
| intermediate [c] | 125 | + | + | + | + | + | *DW1* | + | + |
| intermediate [c] | 126 | + | + | + | + | + | *DW1* | + | + |
| intermediate [c] | 127 | + | + | + | + | + | *DW1* | + | + |
| intermediate | 128 | - | - | - | - | - | *DW1* | + | + |
| intermediate | 129 | - | - | - | - | - | *DW1* | - | + |
| intermediate | 130 | + | - | - | - | - | *DW1* | - | + |
| intermediate | 131 | - | - | - | - | - | *DW1* | - | 0 |
| intermediate | 132 | - | - | - | - | - | *DW1* | - | + |
| intermediate | 133 | - | - | - | - | - | *DW1* | - | + |
| intermediate | 134 | - | - | - | - | - | *DW1* | - | - |
| intermediate | 135 | - | - | - | - | - | *DW1* | - | - |
| intermediate | 136 | - | - | - | - | - | *DW1* | + | + |
| intermediate | 137 | - | - | - | - | - | *DW1* | - | - |

+ indicates the presence of the marker associated with *DW1*

- indicates the absence of the marker associated with *DW1*

0 indicates missing data

[a] semi-dwarfing individuals without markers associated with *DW1*. Two hypotheses are possible to interpret this result:

- The semi-dwarfing individual may have been misclassified: it may be a small intermediate individual rather than a semi-dwarfing
- There may be a double recombination between the markers NZraAM18(700) and NZraAV11(850)

[b] vigorous individuals amplifying markers associated with *DW1* on both sides of the locus.

[c] intermediate individuals amplifying markers associated with *DW1* on both sides of the locus.

# APPENDIX III

Bin mapping set composed of 14 individuals and developed from markers screened on 'M.9'. Markers on each LG are organised in the same order as shown in Figure 4.1.

1 = presence of the marker; 0 = absence of the marker; x = no data

| LG | position | marker | Individuals* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| | 1 | NZmsEB147493a | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| | 2 | Hi12c02 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| 1 | 3 | CHVf1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| | 4 | NZmsCN879773 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| | 5 | KA4b | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| | 1 | CN581453 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| | 2 | NZmsPal92 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 |
| | 3 | CN493139 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 |
| | 4 | GD126 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 |
| | 5 | Hi05g12 | x | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| | 6 | NH010a_c | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 |
| | 7 | NZmsPal13nw | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 8 | NZmsEB149808b | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| 2 | 9 | CH02b10 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 10 | CH02a04 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 11 | CH03d10 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 12 | CH05e03 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 13 | NZsnEB139353 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| | 14 | NZscEB138538 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 15 | NZmsEB153909 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 16 | NZmsEB107305 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 17 | CH01K08_SCAR | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 |
| | 1 | CH03e03 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| | 2 | CH03g07 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| | 3 | NZsnEB155133 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| | 4 | Hi04c10 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| 3 | 5 | GD12 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| | 6 | NZraAR02(700) | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| | 7 | NH030a | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| | 8 | NB109a | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| | 9 | CH03g12_b | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 |
| | 1 | NH209a | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| | 2 | NH011b | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| 4 | 3 | NB141b | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | x | 0 | 0 | 1 | 1 | 0 |
| | 4 | CH01d03 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 5 | Hi23d11 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | x | 1 | 0 | 0 | 0 | 0 |

Appendix III continued…

| LG | position | marker | Individuals* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 5 | 1 | NZraAV11(850) | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | 2 | Hi01c04a | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | 3 | Hi04a08 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | x | 0 | 0 | 0 | 1 | 0 |
| | 4 | NZraAM18(700) | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | 5 | CH03a09 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| | 6 | NZscAE16 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| | 7 | CH05e06 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| | 8 | CH04g09 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| | 9 | NZmsEB134379 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 |
| | 10 | NH041aa | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | 11 | NZmsEB132749 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 |
| | 12 | NZraAE02(1200) | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| | 13 | NZmsEB137770 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| | 14 | NZscEB108994 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 15 | CH05f06 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| | 16 | NZraAR10(1200) | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| | 17 | NZscAQ06 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| | 18 | NZraAJ14(1300) | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| | 19 | NZraAQ06(850) | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| | 20 | NZraAR15(900) | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| | 21 | CH04e03 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| 6 | 1 | NZ23g04 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| | 2 | CH03d12 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 3 | NZsnEB120945 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 7 | 1 | CN444794 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | x | x | x | x | x |
| | 2 | Hi03a10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| | 3 | NZmsEE663789 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 4 | CH04e05 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 5 | NZmsEB137749 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 6 | Hi05b09 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| 8 | 1 | CH01c06 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 2 | OPC20SCAR | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 3 | NZmsEB177006 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 4 | NZraAF13(1600) | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 5 | NZraAE12(1200) | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 6 | NZscEB114476 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 7 | NZmsPal213rd | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 8 | CH02g09 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 9 | NZmsCN900668 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 10 | NZraAK06(1250) | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 11 | Hi22g06 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 12 | NH201a | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |

Appendix III continued...

| LG | position | marker | Individuals* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 9 | 1 | NB125a | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 |
| | 2 | NZmsCN943946 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 |
| | 3 | GD142 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 |
| | 4 | NH029a | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| | 5 | NB104a | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| | 6 | CH01f03b | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| | 7 | NZmsEB116209 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| | 8 | NZraAV19(1600) | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| | 9 | NZscCO901385 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| | 10 | Hi05e07 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| | 11 | NZmsCN892357a | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 12 | Hi04a05 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| 10 | 1 | CH02b07 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 2 | AF57134 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 3 | CH02a08 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 4 | NZmsPal45 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 5 | CH02c11 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| | 6 | NZraAQ06(1000) | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| | 7 | Hi01b01 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 |
| | 8 | Hi08h12 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| | 9 | Hi22a07 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| | 10 | NZmsCN899300a | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| | 11 | GD100 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| | 12 | CH02b03b | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| | 13 | MS01a03 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| 11 | 1 | Hi07d11 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| | 2 | NZraAG02(500) | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 3 | NZmsCN892357b | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 4 | CH02d08 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 5 | CH04a12 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| | 6 | Hi02c06 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| | 7 | NB135a | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 |
| | 8 | Pal_8 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 |
| | 9 | NZmsEB128065 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | 10 | CH04d07_a | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | 11 | NB105a | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| 12 | 1 | NZmsAB162040 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | x | 0 | 0 |
| | 2 | CH04g04 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | x | 0 | 0 |
| | 3 | CH05d11 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 4 | CH05g07 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 5 | NZsnEB154279(250) | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| | 6 | NH207a | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | x | 1 | 0 | 0 |
| | 7 | CH01g12 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 8 | CH04d02b | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 9 | GD6a | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| | 10 | NZraA17(750) | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 |
| | 11 | GD127 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |

Appendix III continued

| LG | position | marker | Individuals* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 13 | 1 | CH05h05 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 2 | Hi04g05 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| | 3 | NZmsEB138681 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 4 | NZsnCN580831 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 5 | NH009b | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | 6 | CH05c04 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 7 | CH03a08 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 1 | NZmsEB146613 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| | 2 | NH004a | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| | 3 | CH05e05 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| | 4 | NZmsPal443 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| | 5 | CH03a02 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| | 6 | NZmsPal51 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| 15 | 1 | Hi02g06 | 0 | 1 | 0 | 0 | x | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | 2 | NZmsEB134400 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | 3 | NZ02b01 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| | 4 | NH204a | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| | 5 | CH01d08 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 6 | NZmsEB117266 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 |
| | 7 | NZmsCN909188 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 |
| | 8 | NZsnEB154279(550) | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| | 9 | NZraAD05(750) | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| | 10 | NB111a | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| | 11 | NZraAW11(650) | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 12 | CH03b10 | x | 0 | 1 | 1 | x | 1 | 0 | 0 | 1 | x | 0 | 0 | 1 | 1 |
| 16 | 1 | Hi22f06 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 |
| | 2 | NH007b | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 |
| | 3 | CH05a04 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| | 4 | NZmsEB147967 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| | 5 | GD154 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| | 6 | CH04f10 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| 17 | 1 | NZmsEB157320 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| | 2 | Hi01d06 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| | 3 | NZmsEE663955 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| | 4 | AT000174 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| | 5 | CH04c06 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| | 6 | GD96 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| | 7 | NH013a_a | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| | 8 | Hi02f12 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| | 9 | NZscSI | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| | 10 | NZmsEB137525 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |

* Individual numbers are different from tree numbers:

Individual 1=tree 4
Individual 2=tree 7
Individual 3=tree13
Individual 4=tree 18
Individual 5=tree 28
Individual 6=tree 37
Individual 7=tree 57
Individual 8=tree 59
Individual 9=tree 61
Individual 10=tree 80
Individual 11=tree 82
Individual 12=tree 83
Individual 13= tree 94
Individual 14=tree 88

# APPENDIX IV

Bin mapping set composed of 14 individuals and developed from markers screened on 'R5'. Markers on each LG are organised in the same order as shown in Figure 4.1. 1 = presence of the marker; 0 = absence of the marker

| LG | position | marker | individuals | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 1 | 1 | Hi02c07 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| | 2 | ch03g12_a | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| | 3 | NZraAD12(450) | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 |
| | 4 | CHVf1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 |
| | 5 | NZscEB123185 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| | 6 | NZmsCN879773 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| | 7 | NH010a_a | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| 2 | 1 | CN581493 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 |
| | 2 | NZsnEB147206 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 |
| | 3 | AD13SCAR | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 |
| | 4 | NZraMA2(650) | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| | 5 | NZmsEB119405 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| | 6 | NZmsPal92 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| | 7 | NH212a | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 8 | NH012a | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 9 | NZmsPal13nw | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 10 | ch02b10 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 11 | BGT23b | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 12 | ch03d10 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 13 | ch02a04 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 14 | NZraAG07(900) | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 15 | Hi08f05 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 16 | ch05e03 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 17 | ch01c06 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 18 | Hi07d12 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 19 | ch01K08_SCAR | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 |
| | 20 | NZraAR10(700) | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 |
| 3 | 1 | NZmsCU881251 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 2 | ch03e03 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 3 | ch03g07 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| | 4 | Hi03d06 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| | 5 | Hi07d08 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| | 6 | NZraAJ03(1050) | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| | 7 | NZmaEB177464 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| | 8 | NZmsCN943818 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| | 9 | NB109a | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| | 10 | NZsnCN943818 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| | 11 | ch03g12_b | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| 4 | 1 | GD162 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 2 | ch01d03 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 3 | GD6b | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 4 | NZraAV11(1800) | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 5 | Hi07b02b | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 6 | NZmsDR999337 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 7 | Hi08e04 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 8 | NZmsEB142980 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |

Appendix IV continued...

| LG | position | marker | individuals | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 5 | 1 | NZmsEB155242 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| | 2 | Hi01c04b | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| | 3 | Hi01c04a | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| | 4 | Hi09b04 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| | 5 | Hi04a08 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| | 6 | NB115b | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| | 7 | ch03a09 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 8 | ch05e06 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 9 | ch04g09 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 10 | NZmsEB134379 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 11 | NZmsEB132749 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 12 | NZmsEB137770 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 13 | NZraAD02(400) | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 14 | GD103 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 15 | GD136 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 16 | NZmsCN898349 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 17 | CN445599 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 18 | ch05f06 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 19 | ch04e03 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| | 20 | NZmsCN862923 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| 6 | 1 | NZraAT03(550) | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 2 | NH027a | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| | 3 | Hi08g03 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 4 | NZmsPal36 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 5 | NZmsMDAJ1681 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 6 | ch03d07 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 7 | NZ23g04 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 8 | NZmsPal443ird | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 9 | ch03d12 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 10 | NZmsCV658990 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 11 | Hi07b06 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 12 | NZmsCO754252 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| 7 | 1 | EMPc117 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 2 | CN444794 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 3 | NZmsCN943067 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 |
| | 4 | EMPc111 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 |
| | 5 | Hi05b09 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 |
| 8 | 1 | NH036b | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| | 2 | NB114a | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| | 3 | NZmsCV880267 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| | 4 | NH005b | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 |
| | 5 | NZscOP05 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 |
| | 6 | NZraAR02(400) | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 7 | NH213a | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | 8 | NH201a | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| 9 | 1 | NZraAB07(1250) | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 2 | GD142 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 3 | NH029a | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 4 | NB125a | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 5 | ch05c07 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 6 | Hi05e07 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 7 | NB130b | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 8 | NB134a | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 9 | GD158 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 10 | RaS65 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |

Appendix IV continued…

| LG | position | marker | individuals | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| | 1 | ch02b07 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| | 2 | AF57134 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| | 3 | ch02c11 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| 10 | 4 | ch02b03b | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 |
| | 5 | GD100 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 |
| | 6 | MS01a03 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 7 | NZsnDQ099803 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 |
| | 1 | NZscAZ16AC15 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| | 2 | NZmsDR033893 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| | 3 | NZsnDQ644205 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| | 4 | NZmsCN892357b | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| | 5 | ch02d12 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| | 6 | NZraAZ13(1150) | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| | 7 | Hi02c06 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 |
| | 8 | NZraAU14(2000) | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| 11 | 9 | Hi04g11 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 10 | NH045a | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 11 | NB135a | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 12 | ch04g07 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 13 | NB121a | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 14 | NZmsCN895337 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 15 | NZmsEB153947 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 16 | NZmsEB128065 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 17 | NB118a | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 1 | ch05d04 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| | 2 | NZmsAB162040 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| | 3 | NZ28f04 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| | 4 | NH207a | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| | 5 | NB104a | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 |
| | 6 | ch01d09 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| 12 | 7 | ch01g12 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 8 | GD6a | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 9 | Hi01e10a | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 10 | ch01f02 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 11 | NZmsCN899300b | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 12 | AT20SCAR | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 13 | GD127 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| | 1 | Hi04g05 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| | 2 | NZmsEB138681 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 3 | NZraAY12(900) | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 4 | NZmsEB135714 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| 13 | 5 | NH009b | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 6 | ch03a08 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 7 | ch05c04 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | 8 | NZraAF13(400) | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |

Appendix IV continued...

| LG | position | marker | individuals | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 14 | 1 | ch05g07 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| | 2 | NZmsCN914822 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| | 3 | NZmsEB146613 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| | 4 | NH004a | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| | 5 | NZraAW11(1200) | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| | 6 | NH035a | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| | 7 | ch03a02 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| | 8 | NZmsPal51 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 9 | U78948 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 10 | NZmsCO867070 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 11 | Hi03a03 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | 12 | NZsnCN946839 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| 15 | 1 | Hi02g06 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| | 2 | NZsnDR033889 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 |
| | 3 | NZmsEB134400 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 |
| | 4 | NZmsDT001928 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| | 5 | NH204a | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| | 6 | NZraAJ13(1200) | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| | 7 | NZral14(550) | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 |
| | 8 | NZmsEB117266 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 |
| | 9 | NZsnAY17AB16 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 |
| | 10 | Hi07d11 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| | 11 | NB111a | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| | 12 | ch03b10 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 1 | NH044b | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 2 | ch02a03 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 3 | NH026a | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 4 | Hi02h08 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 5 | Hi22f06 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 6 | NZmsCO905522 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| | 7 | Hi15a13 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| | 8 | NH007b | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| | 9 | Hi01a08 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| | 10 | Hi08f12 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| | 11 | ch04f10 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| | 12 | Hi04e04 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| 17 | 1 | GD96 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 2 | NZmsEB145764 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| | 3 | GD153 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 4 | ch02g04 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| | 5 | NH008b | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 6 | NZmsEB137525 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |

# APPENDIX V

Score of the markers identified during the bulked segregant analysis and the genetic map construction, and flanking the *DW1* locus. The markers are in the same order as shown in Figure 4.1.

| Phenotype | Tree number | CH03a09 | NZraAM18(700) | Hi04a08 | Hi01c04a | *DW1* | NZraAV11(850) |
|---|---|---|---|---|---|---|---|
| dwarfing | 1 | + | + | + | + | *DW1* | + |
| dwarfing | 2 | - | + | + | + | *DW1* | + |
| dwarfing | 3 | + | + | + | + | *DW1* | + |
| dwarfing | 4 | + | + | + | + | *DW1* | + |
| dwarfing | 5 | + | + | + | + | *DW1* | + |
| dwarfing | 6 | + | + | + | + | *DW1* | - |
| dwarfing | 7 | + | + | + | + | *DW1* | + |
| dwarfing | 8 | + | + | + | + | *DW1* | + |
| dwarfing | 9 | + | + | + | + | *DW1* | + |
| dwarfing | 10 | + | + | + | + | *DW1* | + |
| dwarfing | 11 | + | + | + | + | *DW1* | + |
| dwarfing | 12 | + | + | + | + | *DW1* | + |
| dwarfing | 13 | + | + | + | + | *DW1* | + |
| dwarfing | 14 | + | + | + | + | *DW1* | + |
| dwarfing | 15 | + | + | + | + | *DW1* | + |
| dwarfing | 16 | + | + | + | + | *DW1* | + |
| dwarfing | 17 | + | + | + | + | *DW1* | 0 |
| dwarfing | 18 | + | + | + | + | *DW1* | + |
| dwarfing | 19 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 20 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 21 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 22 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 23 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 24 | + | - | + | + | *DW1* | + |
| semi-dwarfing | 25 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 26 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 27 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 28 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 29 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 30 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 31 | 0 | + | + | + | *DW1* | + |
| semi-dwarfing | 32 | + | + | + | + | *DW1* | + |
| semi-dwarfing | 33 | - | - | 0 | + | *DW1* | - |
| semi-dwarfing | 34 | - | + | + | + | *DW1* | + |
| vigorous | 35 | - | - | 0 | - | *DW1* | - |
| vigorous | 36 | - | - | - | - | *DW1* | - |
| vigorous | 37 | - | - | - | - | *DW1* | - |
| vigorous | 38 | - | - | - | 0 | *DW1* | + |
| vigorous | 39 | - | - | - | - | *DW1* | - |
| vigorous | 40 | - | - | - | - | *DW1* | + |
| vigorous | 41 | - | - | - | - | *DW1* | - |
| vigorous | 42 | - | - | - | - | *DW1* | - |
| vigorous | 43 | - | - | - | - | *DW1* | - |
| vigorous | 44 | - | - | - | - | *DW1* | - |

Appendix V continued...

| Phenotype | Tree number | CH03a09 | NZraAM18(700) | Hi04a08 | Hi01c04a | *DW1* | NZraAV11(850) |
|---|---|---|---|---|---|---|---|
| vigorous | 45 | - | - | - | - | *DW1* | - |
| vigorous | 46 | - | - | - | - | *DW1* | - |
| vigorous | 47 | - | - | - | - | *DW1* | - |
| vigorous | 48 | - | - | 0 | - | *DW1* | - |
| vigorous | 49 | - | - | - | - | *DW1* | + |
| vigorous | 50 | - | - | - | - | *DW1* | - |
| vigorous | 51 | - | - | - | - | *DW1* | + |
| vigorous | 52 | - | - | - | - | *DW1* | - |
| vigorous | 53 | - | - | - | - | *DW1* | - |
| vigorous | 54 | - | - | - | - | *DW1* | - |
| vigorous | 55 | - | - | - | - | *DW1* | - |
| vigorous | 56 | - | - | - | - | *DW1* | - |
| vigorous | 57 | - | - | - | - | *DW1* | - |
| vigorous | 58 | - | - | - | - | *DW1* | - |
| vigorous | 59 | - | - | - | - | *DW1* | - |
| vigorous | 60 | - | - | - | - | *DW1* | - |
| vigorous | 61 | - | - | 0 | - | *DW1* | - |
| vigorous | 62 | - | - | - | - | *DW1* | - |
| vigorous | 63 | 0 | - | - | - | *DW1* | - |
| vigorous | 64 | - | - | - | - | *DW1* | - |
| vigorous | 65 | - | - | - | - | *DW1* | - |
| vigorous | 66 | - | - | - | - | *DW1* | + |
| vigorous | 67 | 0 | - | - | - | *DW1* | - |
| vigorous | 68 | - | - | - | - | *DW1* | - |
| vigorous | 69 | - | - | - | - | *DW1* | - |
| vigorous | 70 | - | - | - | - | *DW1* | - |
| vigorous | 71 | - | - | - | - | *DW1* | - |
| vigorous | 72 | - | - | - | - | *DW1* | - |
| vigorous | 73 | - | - | - | - | *DW1* | - |
| vigorous | 74 | - | 0 | 0 | - | *DW1* | - |
| vigorous | 75 | - | - | - | - | *DW1* | - |
| vigorous | 76 | - | - | - | - | *DW1* | - |
| vigorous | 77 | - | - | - | - | *DW1* | - |
| vigorous | 78 | - | - | - | - | *DW1* | - |
| vigorous | 79 | - | - | - | - | *DW1* | - |
| vigorous | 80 | - | - | - | - | *DW1* | - |
| vigorous | 81 | - | - | - | - | *DW1* | - |
| vigorous | 82 | - | - | - | - | *DW1* | - |
| vigorous | 83 | - | - | - | - | *DW1* | - |
| vigorous | 84 | - | - | - | - | *DW1* | - |
| vigorous | 85 | - | - | - | - | *DW1* | - |
| vigorous | 86 | - | - | - | - | *DW1* | - |
| vigorous | 87 | - | - | - | - | *DW1* | - |
| vigorous | 88 | + | - | - | - | *DW1* | - |
| vigorous | 89 | + | - | - | - | *DW1* | - |
| vigorous | 90 | + | + | + | + | *DW1* | - |
| vigorous | 91 | + | + | - | - | *DW1* | - |
| vigorous | 92 | + | + | - | - | *DW1* | - |
| vigorous | 93 | + | + | + | + | *DW1* | - |
| vigorous[a] | 94 | + | + | + | + | *DW1* | + |
| vigorous[a] | 95 | + | + | + | + | *DW1* | + |
| vigorous[a] | 96 | + | + | + | + | *DW1* | + |
| vigorous[a] | 97 | + | + | + | + | *DW1* | + |

Appendix V continued...

| Phenotype | Tree number | CH03a09 | NZraAM18(700) | Hi04a08 | Hi01c04a | *DW1* | NZraAV11(850) |
|---|---|---|---|---|---|---|---|
| vigorous[a] | 98 | + | + | + | + | *DW1* | + |
| vigorous[a] | 99 | + | + | + | + | *DW1* | + |
| vigorous[a] | 100 | + | + | + | + | *DW1* | + |
| vigorous[a] | 101 | + | + | + | + | *DW1* | + |
| vigorous[a] | 102 | + | + | + | + | *DW1* | + |
| vigorous[a] | 103 | + | + | + | + | *DW1* | + |
| vigorous[a] | 104 | + | + | + | + | *DW1* | + |
| very vigorous | 105 | - | - | - | - | *DW1* | - |
| very vigorous | 106 | - | - | - | - | *DW1* | - |
| very vigorous | 107 | - | - | - | - | *DW1* | - |
| very vigorous | 108 | - | - | - | - | *DW1* | - |
| very vigorous | 109 | - | - | - | - | *DW1* | - |
| very vigorous | 110 | - | - | - | - | *DW1* | - |
| very vigorous | 111 | - | - | - | - | *DW1* | - |
| very vigorous | 112 | - | - | - | - | *DW1* | - |
| very vigorous | 113 | - | - | - | - | *DW1* | - |
| very vigorous | 114 | - | - | - | - | *DW1* | - |
| very vigorous | 115 | - | - | - | - | *DW1* | - |
| intermediate[b] | 116 | + | 0 | + | + | *DW1* | + |
| intermediate[b] | 117 | + | + | + | + | *DW1* | + |
| intermediate[b] | 118 | + | 0 | + | + | *DW1* | + |
| intermediate[b] | 119 | 0 | + | + | + | *DW1* | 0 |
| intermediate[b] | 120 | - | + | + | + | *DW1* | 0 |
| intermediate[b] | 121 | 0 | 0 | + | + | *DW1* | + |
| intermediate[b] | 122 | + | + | + | + | *DW1* | + |
| intermediate[b] | 123 | + | + | + | + | *DW1* | + |
| intermediate[b] | 124 | + | -- | + | + | *DW1* | + |
| intermediate[b] | 125 | + | + | + | + | *DW1* | + |
| intermediate[b] | 126 | + | + | + | + | *DW1* | + |
| intermediate[b] | 127 | + | + | + | + | *DW1* | + |
| intermediate | 128 | - | - | - | - | *DW1* | + |
| intermediate | 129 | - | - | - | - | *DW1* | - |
| intermediate | 130 | - | 0 | - | - | *DW1* | - |
| intermediate | 131 | - | - | - | - | *DW1* | - |
| intermediate | 132 | - | - | - | - | *DW1* | - |
| intermediate | 133 | - | - | - | - | *DW1* | - |
| intermediate | 134 | - | - | - | - | *DW1* | - |
| intermediate | 135 | - | - | - | - | *DW1* | - |
| intermediate | 136 | - | - | - | - | *DW1* | + |
| intermediate | 137 | - | - | - | - | *DW1* | - |

+ indicates the presence of the marker associated with *DW1*

- indicates the absence of the marker associated with *DW1*

0 indicates missing data

[a] vigorous individuals amplifying markers associated with *DW1* on both sides of the locus.

[b] intermediate individuals amplifying markers associated with *DW1* on both sides of the locus.

# APPENDIX VI

Primer sequences of the M13 Universal primer:

| | |
|---|---|
| M13 Forward: | GTT TTC CCA GTC ACG AC |
| M13 Reverse: | CAG GGA ACA GCT ATG AC |

# APPENDIX VII

List of the top 100 genes most differentially expressed among the bark tissues of dwarfing and vigorous rootstocks derived from the 'M.9' × 'R5'.

| GeneBank accession number | GeneBank accession number | GeneBank accession number | GeneBank accession number |
|---|---|---|---|
| CN942650 | CN887354 | CN942476 | EB109549 |
| CN870910 | CN898554 | CN882947 | CN883313 |
| CN935725 | CN918277 | CN859470 | CN918822 |
| CN930677 | CN882949 | CN893561 | CN899835 |
| CN927687 | CN939813 | EB134390 | EB146881 |
| CN927697 | CN908240 | CN917032 | EB123262 |
| CN860451 | CN892057 | CN919641 | CN903494 |
| CN866532 | EB112737 | CN930354 | CN891102 |
| CN889403 | CN896182 | CN930516 | CN911327 |
| CN897943 | EE663938 | CN914028 | EB153005 |
| CN900730 | EB141306 | EB176287 | EB128042 |
| EB122799 | CN872626 | CN894651 | CN904514 |
| EB156300 | EB152581 | CN935411 | EB121684 |
| CN915884 | CN861143 | EB150279 | EB128036 |
| CN904514 | CN938667 | EB142351 | EB128659 |
| CN899846 | CN945982 | EB110379 | EB137861 |
| CN873894 | CN930289 | CN902006 | EB123778 |
| CN887518 | CN945826 | CN946828 | CN916259 |
| CN889088 | EG631304 | EB121698 | EB139243 |
| CN894411 | CN860207 | CN883860 | CN898763 |
| EG631344 | CN894181 | CN898457 | EB137941 |
| EB146826 | CN902525 | EB112431 | EE663634 |
| CN916137 | CN863539 | CN899807 | CN945369 |
| EB152683 | CN868092 | CN879439 | CN890397 |
| CN870205 | CN890846 | CN901386 | CN882949 |

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