The genetic determinants of vigour control and precocity by pear *(Pyrus communis L.)* rootstocks

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ABSTRACT

The growth habits of fruit trees have a significant influence on their efficiency in commercial production. Small tree stature allows high-density plantings and therefore more efficient land use, while minimising the length of the trees' juvenile period means the orchard becomes profitable sooner. Vigour control and precocity are therefore two of the most important traits in commercial pear production.

Dwarfing rootstocks are used to reduce the size of the scion and enable precocious flowering in many perennial cultivars. Currently there is a lack of *Pyrus* rootstocks that are vigour-controlling and precocity-inducing. The development of such a rootstock is a major focus in pear rootstock breeding. Marker assisted selection (MAS) could help to shorten this time consuming and costly breeding process.

The aim of this PhD project was to examine the genetic determinants of vigour control and early flowering conferred to a scion by *Pyrus* rootstocks. A segregating population of 421 F1 seedlings from a *P. communis* 'Old Home' x 'Louise Bonne de Jersey' (OHxLBJ) cross was grafted with clonal 'Doyenne du Comice' scions and used as the core experimental material for this project. High-density genetic maps were constructed for pear using two different high throughput genotyping tools, the Infinium® II 9K apple/pear SNP array and the genotyping-by-sequencing (GBS) approach. QTLs influencing expression of scion vigour and precocity were detected on linkage groups (LG)5 and LG6 of OH and LG6 of LBJ. The LG5 QTL was found in the same genomic region as the dwarfing (*DW1*) QTL identified in the 'M9' apple rootstock. The alignment of the QTL loci of apple and pear showed a high synteny between both loci and may help to identify candidate genes in both genera.

The ease of vegetative propagation, a crucial trait for rootstock breeding, was assessed in rooting experiments and small effect QTLs were identified. These results will help to understand the genetic control of vegetative propagation in pear, and may assist in developing markers for MAS for this complex trait.

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LIST OF ABBREVIATIONS

ABA: Abscisic acid

ADT: Assay design tool

AFLP: Amplified fragment length polymorphism

ANOVA: Analysis of variance

AP1: APETALA 1

AR: Adventitious rooting

ATC: *CENTRORADIALIUS*

BAC: Bacterial artificial chromosome

BFT: *BROTHER OF FT*

BLUE: Best linear unbiased estimator

BLUP: Best linear unbiased prediction

bp: Base pair

cM: centiMorgan

cDNA: Complementary deoxyribonucleic acid

CIM: Combining interval mapping

CO: *CONSTANS*

CTAB: Cetyltrimethyl ammonium bromide

DNA: Deoxyribonucleic acid

dNTP: deoxy-nucleotide triphosphate

ddNTP; Dideoxynucleotide triphosphates

Dw1: *Dwarfing locus 1*

Dw2: *Dwarfing locus 2*

FEM: Fondazione Edmund Mach

FT: FLOWERING LOCUS

FUL: FRUITFUL

GBS: Genotyping-by-sequencing

GxE: Genome by environment interactions

GS: Genomic selection

IAA: Indole-3-acetic acid

IBA: Indole-3-butyric acid

IM: Interval mapping

INRA-IRTA: Institut National de Recherche Agronomique

IPSC: International Peach SNP Consortium

IRSC: International RosBREED SNP Consortium

kbp: Kilo base pairs

LBJ: 'Louise Bonne de Jersey'

LD: Linkage disequilibrium

LFY: LEAFY

LG: Linkage group

LNG: Length of the new main axis growth

LOD: Logarithm of odds

M9: 'Malling 9'

M27: 'Malling 27'

MAF: Minor allele frequency

MAB: Marker assisted breeding

MAS: Marker assisted selection

Mbp: Mega base pairs

MFT: MOTHER OF FT

MQM: Multiple QTL mapping

NCBI: National Centre for Biotechnology Information

NGS: Next generation sequencing

OH: 'Old Home'

OHxF: 'Old Home' x 'Farmingdale'

PCR: Polymerase chain reaction

P: Parent

Pc: *Pyrus commuinis*

Pb: *Pyrus bretschneideri*

PFR: Plant and Food Research

QC: ‘Quince C’

qPCR: quantitative polymerase chain reaction

QTL: Quantitative trait locus

RACE: rapid amplification cDNA ends

RAPD: Random amplified polymorphic DNA

Rf: Recombination frequency

RFLP: Restriction fragment length polymorphism

RNA: Ribonucleic acid

RT-PCR: Real time-polymerase chain reaction

SCAR: Sequence characterized amplified regions

SNP: Single nucleotide polymorphism

SOC1: SUPRESSOR OF OVER-EXPRESSION OF CONSTANS 1

SSR: Simple sequence repeat

TCA: Trunk cross-sectional area

TFL1: TERMINAL FLOWER1

TSF: TWIN SISTER OF FT

1 INTRODUCTION

1.1 Taxonomy and origin of the genus *Pyrus*

Pear (*Pyrus* spp.) belongs to the Rosaceae family and the sub-family *Pyreae* (Potter *et al.*, 2007a). All *Pyrus* species are diploid and have seventeen pairs of chromosomes. Their strong gametophytic self-incompatibility makes them self-sterile and therefore highly heterozygous (Yamamoto and Chevreau, 2009). Pear is known to be interfertile between species leading to a wide diversity within the genus (Itai, 2007; Hancock *et al.*, 2008). *Pyrus* most likely arose during the Tertiary period in west China and spread over temperate Asia, Europe and northern Africa through speciation and dispersal (Bell, 1991; Yamamoto and Chevreau, 2009). According to Bell *et al.* (1996) there are twenty-three primary species divided into four groups: European, Circum-Mediterranean, Mid-Asian and East Asian species. The main European species is *P. communis* cultivated commercially in Europe, North America, South Africa and Australia. In northern China *P. bretschneideri* Rehd. and *P. ussuriensis* Max. are the main species grown for production, and *P. pyrifolia* (Burm) Nakai is the main crop species in southern and central China, Japan and Korea (Itai, 2007; Wu *et al.*, 2013).

1.2 Pear cultivation and economy

1.2.1 World pear production

Pear has been cultivated for more than 2000 years, and is therefore one of the oldest domesticated fruit crops in the world (Lombard and Westwood, 1987). Today, pear is one of the most important temperate fruit crops, after grape and apple (Yamamoto and Chevreau, 2009), with production of approximately 21.3 million tonnes per year. The world's top pear producer is China (9.3M tonnes), followed far behind by Italy (0.8M tonnes), the United States of America (0.8M tonnes), Spain (0.6M tonnes) and Argentina (0.5M tonnes) (FAO©, 2015).

The world's production is based on three main species: *P. communis* L. (European pear), *P. bretschneideri* Rehd. (Chinese pear) and *P. pyrifolia* Nakai (Japanese pear). As shown in Figure 1 the worldwide production of Asian (Japanese and Chinese) pear has increased rapidly over the last sixteen years, whereas the production of European pear remains almost static.

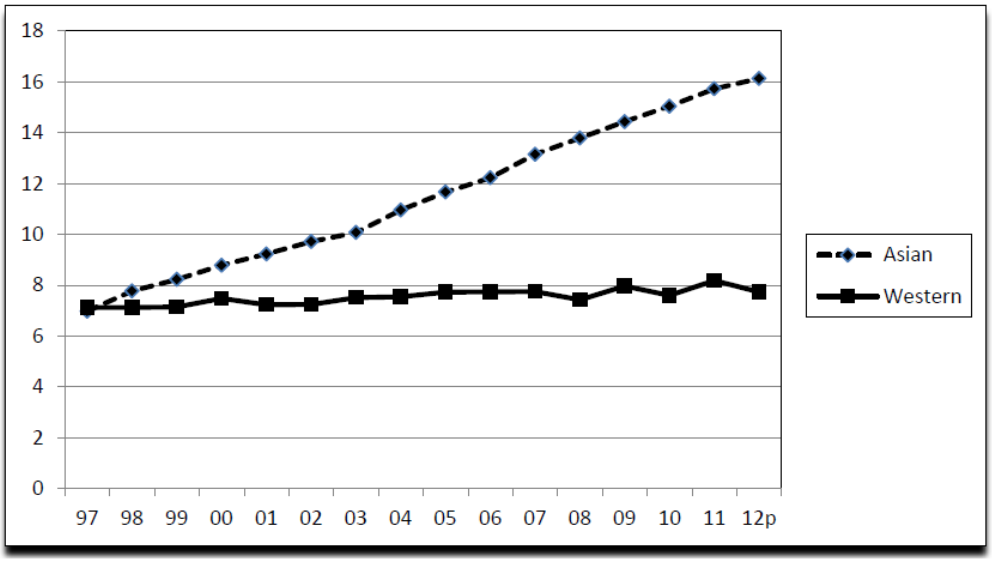

Figure 1: World estimated pear production in million metric tonnes from 1997-2012 (Belrose, 2013)

1.2.2 Pear production in New Zealand

New Zealand plays a minor role in the worldwide pear industry. New Zealand's pear exports (~5,000 tonnes/year) are minor compared to other fruits such as kiwifruit (~370,000 tonnes/year) and apple (~290,000 tonnes/year) (FAO©, 2015). Accordingly the pear production area is small and is constantly decreasing. Cultivars for pear export are mainly niche products such as 'Taylor's Gold', a mutation from 'Doyenne du Comice' discovered in New Zealand (Belrose, 2013). In order to increase the range of niche products, research is carried out in the PREVAR Ltd. funded Plant & Food Research (PFR) breeding programme to develop new varieties by crossing Asian and European pear.

New interspecific cultivars promise to have a novel taste, high in sweetness and juiciness as well as an appealing appearance. PREVAR Ltd. commercialized a western and Asian cross called Papple™ in 2013 and gained a high interest in international markets (Belrose, 2013). More recently PREVAR Ltd. announced a new trademark called Piqa® (www.piqafruit.co.nz) for novel interspecific hybrids of European, Chinese and Japanese pear from the PFR breeding programme. The first released cultivar out of this Piqa® trademark was branded PIQA®BOO® and is commercially grown in New Zealand and Australia. While promising, profitability of these new cultivars needs to be tested over several years of commercial production.

Unlike apple, pear shows several cultivation issues that make commercial production less profitable for growers. Pear trees grow vigorously, have a long juvenile period, and often a lower yield than apple. One of the major problems for pear cultivation is the lack of rootstocks, suitable for different site conditions, conferring disease resistance, vigour control and precocity to a wide range of commercially used pear cultivars (Webster, 1998, 2003).

1.3 Pear rootstocks

Rootstocks have been used for more than 2000 years in temperate fruit trees, mainly to overcome the difficulties in propagation of cultivars (Webster, 2003). The benefits of rootstocks for pear cultivation today are pest and disease resistance, cold hardiness and adaptation to site conditions, as well as good fruit size and quality with a consistently high yield (Jayawickrama *et al.*, 1991; Webster, 2003, 2004; Hancock *et al.*, 2008). Vigour controlling rootstocks are used to reduce the size of the scion in many woody perennial cultivars. Small tree stature allows high-density plantings and therefore more efficient land use. In addition the time, labour, and costs for tree maintenance can be reduced by using dwarf and compact trees.

Precocity-inducing rootstocks shorten the long juvenile phase in pear in which trees grow vegetatively but do not flower (Bell and Zimmermann, 1990; Itai, 2007). Earlier flowering means faster fruit production and therefore faster establishment of the orchard. As pear (*Pyrus* spp.) cultivars are mostly vigorous and have a long juvenile period with a small variability within breeding populations, a dwarfing rootstock is an important tool for commercial production.

1.3.1 Characteristics of dwarfing rootstocks

Dwarfing rootstocks reduce the size of the scion and induce precocious flowering. One example of a dwarfing rootstock is the 'Malling 9' ('M9') apple rootstock which is widely used for apple commercial production. Apple scions grafted onto 'M9' rootstocks show a significant size reduction and reduced time to flowering compared to the same cultivar on its own roots. As apple and pear are closely related genera (Potter *et al.*, 2007b), and their genomes exhibit a high degree of synteny (co-localization of genomic regions in both species preserved from their ancestor) (Yamamoto *et al.*, 2004; Celton *et al.*, 2009a; Celton *et al.*, 2009b; Chagné *et al.*, 2014), it is likely that the same mechanisms are responsible for the control of scion growth and precocity conferred by rootstocks. Nevertheless, there is currently no pear rootstock available that is able to induce dwarfing comparable to the dwarfing 'M9' rootstock in apple (Webster, 2003).

1.3.2 Quince, a semi-dwarfing rootstock for pear

The most effective dwarfing rootstock used for over one hundred years in commercial pear production is from a related species: *Cydonia oblonga* (quince). Currently, the pear industry relies on the use of quince rootstocks which confer pest and disease resistance to root lesion nematodes, powdery mildew (*Podosphaera leucotricha*), and crown gall (*Agrobacterium tumefaciens*), as well as improved tolerance to woolly pear aphid (*Eriosoma lanigerum*).

Quince rootstocks are easier to propagate vegetatively and exhibit improved size control and precocity when compared with *Pyrus* rootstocks. The rootstock cultivar 'Quince C' (QC) (developed by the Horticulture Research International-East Malling breeding program) shows the lowest vigour and highest productivity amongst all the commercially available rootstocks for pear (Massai *et al.*, 2008). However, it is not equivalent to apple 'M9' dwarfing rootstock, and would be classified as a semi-dwarfing rootstock in apple (Figure 2).

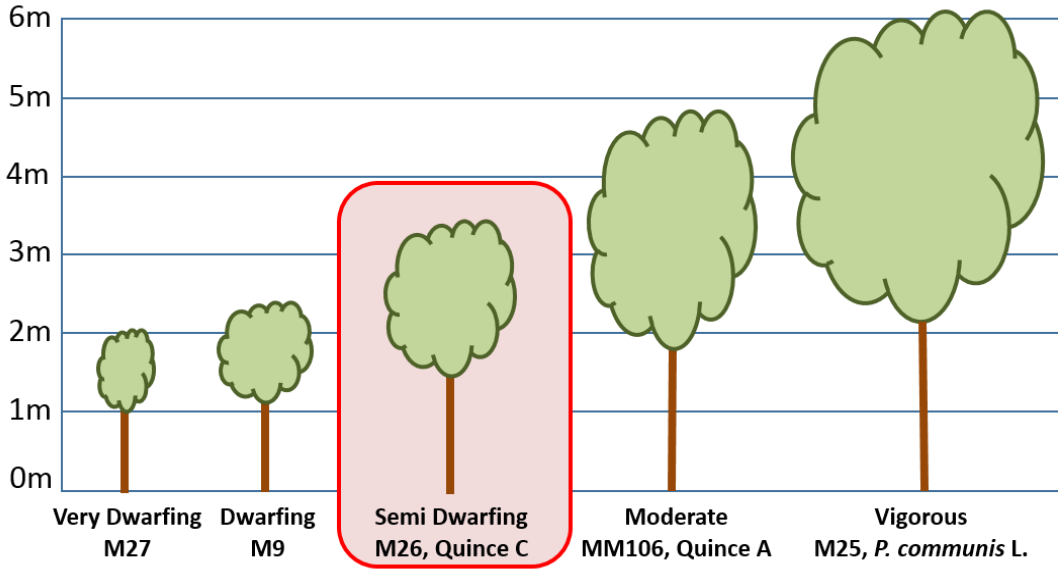

Figure 2: Range of rootstock induced vigour control in apple (M27, M9, M26, MM106, M25) and pear (Quince C, *P. communis* L.)

The disadvantage of using quince as a rootstock is its graft incompatibility with many pear cultivars, especially Asian pear (Ermel *et al.*, 1997; Webster, 1998). This is particularly an issue in New Zealand because, as mentioned above, novel pear cultivars are being developed from interspecific Asian x European pear crosses. The incompatibility reactions are cell necrosis and a gradual die-back of the trees at the graft union. This reaction gets worse in hot and dry conditions (Gur *et al.*, 1978; Alonso *et al.*, 2011).

Graft incompatibility is common in a range of species and the mechanisms behind the reaction have been studied in pea (Kawaguchi and Taji, 2005), grapevine (Gökbayrak *et al.*, 2007), and different *Prunus* species (Pedersen, 2006; Poëssel *et al.*, 2006). In pear Musacchi *et al.* (2002) found that the level of enzymatic activity differs between different rootstock/scion combinations in relation to graft compatibility and suggested to utilize the enzymatic activity as a measurement of the degree of graft-incompatibility in pear/quince combinations. Hudina *et al.* (2014) studied the involvement of phenolic compounds in graft incompatibility with respect to different rootstock scion combinations. They found that phenolics could be involved in the complex incompatibility process.

Using pear as a compatible interstock between the quince rootstock and the scion is a costly but effective solution under some growing conditions. In addition, quince is susceptible to winter freezing, sensitive to high soil pH (Webster, 1998), and susceptible to lime-induced chlorosis (Alonso *et al.*, 2011). Therefore, a replacement is required for quince rootstocks in pear production.

1.3.3 Vigorous *Pyrus* rootstocks

Due to the disadvantages of using quince as a rootstock, some growers rely on *Pyrus* rootstocks, which are more winter hardy, disease resistant and drought tolerant (Hancock *et al.*, 2008) than quince. However, *Pyrus* rootstocks are mostly vigorous and have poor precocity. Furthermore, many pear rootstocks exhibit problems with vegetative propagation and produce undesirable root suckers (Webster, 1998).

1.3.3.1 Vegetative propagation

Vegetative propagation is an important tool used to multiply uniform rootstocks without losing the desired traits through outcrossing in sexual reproduction. Vegetative propagation is possible because plants are able to regenerate new roots out of plant tissue other than the root apical meristem (adventitious roots) (Hartmann *et al.*, 1990).

While some plants are easy to propagate via hardwood cuttings, a fast and cheap propagation method used in commercial nurseries for fruit tree propagation (Webster, 1995b), pear cuttings often show difficulties in growing adventitious roots (Webster, 1998). When breeding novel pear rootstocks, it is essential that they are easy to propagate vegetatively. Identification of the genetic mechanism involved in rooting ability would speed up breeding of pear rootstocks which are easy to propagate.

1.3.3.2 Root suckering

Root suckers are shoots that grow out of the roots. While root suckering can occur after wounding (Fraser *et al.*, 2004), some tree species produce suckers spontaneously out of preformed root buds (Del Tredici, 2001). Root suckering was found to be inhibited by auxin (Farmer, 1962) and disturbance of the auxin flow from the shoots to the roots increases root suckering (Wan *et al.*, 2006). Inducing root suckering through wounding of the stem is a method used for vegetative propagation in many forest trees. However, root suckers in fruit orchards are unwanted as they compete with the trees for space, water and nutrients. Removing root suckers is laborious work and cut surfaces can lead to disease entrance.

1.3.4 Breeding new pear rootsock varieties

A review of existing rootstocks used for pear production indicates there is a need for breeding new pear rootstocks. The ideal pear rootstock would have the following characteristics (Webster, 2002):

- Precocity inducing
- Promote regular cropping with good fruit size and quality and high yield
- Vigour controlling
- Resistant to major pests and diseases
- Compatible with most pear cultivars, including new interspecific cultivars
- Easy and inexpensive to propagate vegetatively
- No root suckering
- Suitable for a range of environmental conditions

To date, breeders have focused on developing new dwarfing quince and *Pyrus* rootstocks for pear production, which would be suitable for various environmental conditions and have good propagation ability and disease resistance (Webster *et al.*, 2000; Jacob, 2002; Asín *et al.*, 2011).

One breeding programme used a cross between *P. communis* 'Old Home' and 'Farmingdale' (OHxF) (Lombard and Westwood, 1987; Jacob, 2002; Wertheim, 2002). Interestingly, a recent fingerprinting study revealed that the most likely male parent is actually 'Williams Bon Chrétien' (syn. 'Bartlett') rather than 'Farmingdale' (Postman *et al.*, 2013). Nevertheless, the OHxF progeny produced individuals conferring some size reduction to grafted scions as well as improved precocity. Although some promising dwarfing rootstocks were identified, the dwarfing effect within the progeny was significantly influenced by environmental conditions and differs among rootstock/scion combinations (Wertheim, 2002; Alonso *et al.*, 2011).

Jacob (1998) crossed the European pear cultivars 'Old Home' and 'Louis Bonne de Jersey' (OHxLBJ) and an individual named 'Pyrodwarf' was selected for its low vigour and high precocity combined with high yield.

‘Pyrodwarf’ showed good winter hardiness, no suckering, and no lime induced chlorosis when growing in high pH soil (Jacob, 2002). However, the low vigour of ‘Pyrodwarf’ was not confirmed by Lewko *et al.* (2007) indicating a high influence of environmental conditions. Furthermore, ‘Pyrodwarf’ showed high propensity for suckering in a study by Elkins *et al.* (2011). The Horticultural Research International-East Malling breeding program tested new quince clones as well as pear clones (Webster *et al.*, 2000). Although some promising vigour-reducing quince rootstock clones were developed, their graft compatibility with different cultivars remains to be tested. More recently, the Institut National de Recherche Agronomique (INRA-IRTA) Pear Rootstock Breeding Program tested seedlings form a cross between the INRA pear rootstock ‘Pyriam’ (open pollination of ‘Old Home’) and four Mediterranean species for tolerance to lime-induced chlorosis combined with low vigour. They obtained 150 hybrids tolerant to chlorosis that exhibited a significant reduction in vigour compared to the quince rootstock control ‘BA 29’ (Asín *et al.*, 2011). While promising, these findings need to be validated in different environmental conditions and over longer time periods.

The problem of breeding new rootstock cultivars with traditional breeding methods is that the overall breeding process can take 30 to 40 years (Webster, 2003), as the compatibility of a rootstock with a specific scion, as well as the induction of vigour and precocity can only be revealed after many years of field studies with different scion cultivars, environmental conditions and training methods (Yamamoto and Chevreau, 2009). To shorten this long process it is important to develop an understanding of the mechanism involved in tree development and reveal the genetic background of vigour control and precocity.

1.3.5 Marker assisted selection for apple rootstock breeding at PFR

Molecular markers linked to desired or undesired phenotypic traits can be screened over the seedlings of a breeding population and unwanted plants can be culled before reaching maturity and becoming expensive in terms of labour and space. This method is known as marker assisted selection (MAS) or marker assisted breeding (MAB). At PFR MAS has been successfully used for more than ten years to breed better cultivars faster. More recently PFR breeders have started to use MAS as a tool for apple rootstock breeding to select for traits that are difficult and time consuming to phenotype such as resistance to fire blight (*Erwinia amylovora*) and woolly apple aphid (*Eriosoma lanigerum*), as well as dwarfing of the grafted scion.

1.4 Rootstock effect on scion growth

Improved knowledge of architectural controls in plants enables the early identification of characteristics associated with vigour control and precocity (Kenis and Keulemans, 2007; Bai *et al.*, 2012). The identification of traits in scions linked to dwarfing by rootstocks in the first few years of growth will help breeders to select elite genotypes using standardised measurements, and therefore save time and resources used in phenotyping. Choosing the right measurements for phenotyping is a prerequisite for the genetic mapping of a complex trait such as vigour control.

Architectural analysis can help to understand the process of tree growth, and helps to classify tree structures by separating the genetically controlled growth process from the environmental influence (Seleznyova *et al.*, 2008).

1.4.1 Architectural analysis

In order to describe the architecture of a tree and compare tree growth, researchers break down the structure of a tree to a basic element, a metamer. It consists of a node, its leaves, axillary buds, and an internode (White, 1979). A group of metamers growing in the same season without interruption is called a growth unit.

Growth units may be identified by a ring of bud scale, bud scale scars and/or a zone of short internodes indicating the period of rest (Barthélémy and Caraglio, 2007). Growth units which grow in different growth cycles in the same growing season build annual shoots. Shoots can be either sylleptic or proleptic, growing without or after a period of bud rest, respectively (Hallé *et al.*, 1978). Due to their length, shoots can be classified as 'spurs', 'extension shoots', or 'continuous shoots'. Spurs are short shoots (<2.5cm) that do not grow long after bud burst (Figure 3) (Seleznyova *et al.*, 2003; Seleznyova *et al.*, 2008). Shoots that grow more than 2.5cm can be 'extension shoots' whose final length is limited by the number of preformed organs, or 'continuous shoots', also known as 'water shoots'. Continuous shoots develop by the elongation of preformed and neoformed organs, the latter resulting from apical growth (Costes *et al.*, 2006). Floral buds can either grow on the terminal position of shoots or spurs, or in an axillary position.

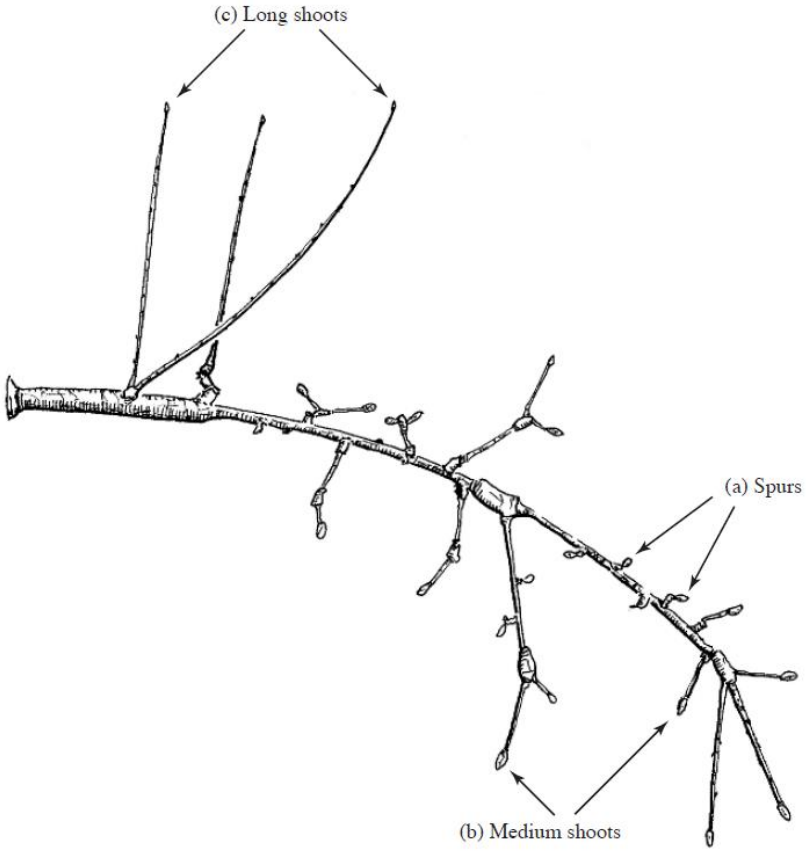

Figure 3: Example of shoot types in an apple branch. (a) Spurs, (b) medium shoots (extension shoots), and (c) long shoots (continuous shoots) (Costes *et al.*, 2006)

This division of the tree structure into different units helps to describe the architecture of a tree precisely during its growth period, taking into account parameters such as the number of nodes and the length and type of branches. As phenotypic measurements are time consuming and can be difficult, especially in large populations, understanding the structure of a tree can help to identify the traits of interest using fewer and more precise measurements (Costes *et al.*, 2004b).

1.4.2 Known architectural interactions between rootstocks and scions

Architectural studies in different fruit tree species have revealed the influence of dwarfing rootstocks on scion growth. In peach, a member of the Rosaceae family, Weibel *et al.* (2003) found that rootstocks affect the shoot length by influencing the length of the internodes, with dwarfing rootstocks having shorter internodes. In comparison Seleznyova *et al.* (2003) studied the differences in branching using dwarfing and non-dwarfing rootstock/interstock combinations in apple. They found that rootstocks influence the length of shoots by influencing the number of nodes and therefore indirectly the length of the internodes. This study suggests that dwarfing rootstocks influence the length of branches by reducing the number of neoformed nodes, which in turn influences successive growth in the following season (Seleznyova *et al.*, 2003).

The length of the vegetative period in apple was found to be reduced by dwarfing 'M9' rootstocks (Costes and Garcia-Villanueva, 2007). Scions grafted onto 'M9' first flowered in the second year of growth after grafting, while the same scion on a more vigorous rootstock first flowered in the third year. Scions on 'M9' developed a higher number of floral buds than on other rootstocks and reduced the total number of shoots, with a higher proportion of medium shoots and spurs than long shoots. The dwarfing effect of rootstocks may be correlated with the number and proportion of floral (sympodial) and vegetative (monopodial) annual shoots in apple (Seleznyova *et al.*, 2007; Seleznyova *et al.*, 2008). Both types of annual shoots develop from buds formed during the previous growing season. The number of preformed nodes in vegetative buds (approx. 9- 12) is higher than in floral buds (approx. 5- 6) (Rivals, 1965). For that reason, floral annual shoots grow less vigorously than vegetative annual shoots. The proportion of annual shoot types therefore influences the growth of a tree after flowering. Dwarfing apple rootstocks were shown to induce a higher proportion of sympodial annual shoots and fewer monopodial annual shoots, resulting in less vigorous trees.

In conclusion, apple rootstocks influence the number of flowers which indirectly affects the vigour of the shoot growth (Seleznyova *et al.*, 2007; Seleznyova *et al.*, 2008). Furthermore, dwarfing rootstocks may increase the number of axillary flowers, and influence their position on the stem (Seleznyova *et al.*, 2004). However, the number of axillary flowers on branches has been shown not to be directly influenced by the rootstock, but indirectly by influencing the number of nodes during the previous year (Hirst and Ferree, 1995; Costes *et al.*, 2001; Seleznyova *et al.*, 2003). All of the cited studies indicate that the effect of a dwarfing rootstock is cumulative and superimposed over years, making an early selection of dwarfing rootstocks difficult (Costes and Garcia-Villanueva, 2007).

1.4.3 Architectural studies in pear

Although studies on the architecture of pear cultivars are still far behind apple, their number is increasing. Du Plooy *et al.* (2002) and Cook and Du Plooy (2005) studied the branching habits of seven pear cultivars in South Africa, and could identify four groups of different branching habit by examining length and position of one-year-old shoots from two-year-old upright branches. Lauri *et al.* (2002) studied the growth of four pear cultivars in young and mature trees. The results of these studies leads to the suggestion that sylleptic branching in pear may influence the total number of flowers, and hence that sylleptic branching in young trees could be used as an indicator for precocity (Lauri *et al.*, 2002). Similar results were obtained by Costes *et al.* (2004b), who studied the correlation between branching in the vegetative period of tree growth, with early flowering in several cultivars that have different branching habits. They found a difference in sylleptic branching between the first and second year of growth influences precocity, with vigorous growth in the first year (strong sylleptic branching) and a reduced growth in the second year leading to early flowering (Costes *et al.*, 2004a). However, these studies concentrated on the scion cultivar, although the choice of rootstock is known to be most important in reducing the vigour of the scion in fruit trees (Lockard and Schneider, 1981).

Jacyna (2004) and Milošević and Milošević (2010) studied the effect of cultivar, rootstock and the combination of rootstock and cultivar on the height, trunk diameter, and the number of long and short sylleptic shoots. Neither study could find an influence of the rootstocks on the tree height and concluded that the height of a tree is more likely to be influenced by the scion. However, both studies phenotyped only the first and second year of growth after grafting and the rootstock effect on the scion may become visible after a longer growth period. The effect on the stem diameter was found to be significant for both scion and rootstock in both studies. The number of branches was not found to be influenced by the rootstock, but the effect of the scion cultivar was found to be significant. The combination of rootstock and scion was not found to influence tree height, trunk diameter or the number of branches. (Jacyna, 2004; Milošević and Milošević, 2010). Watson *et al.* (2012) studied the effect of different rootstocks (QC, BA29 and P. *calleryana*) on pear tree growth in the first two years of growth and found that the rootstocks had no effect on the number of annual shoots. However they found that rootstocks affected the node neoformation significantly, which influences the branching and therefore the vigour in the following year. The same study found that the rootstock influences the number of floral buds per tree and the position of the floral buds (axillary or terminal). Axillary buds were only found on scions grafted onto QC (Watson *et al.*, 2012).

1.4.4 Physiological mechanisms of dwarfing

It is important to understand the mechanisms underlying vigour control and precocity to enable a more informed breeding of new rootstock cultivars. Most of the research on rootstock-induced precocity and vigour reduction has been conducted in apple. Such research has mainly focused on physiological mechanisms influenced by rootstocks, such as water and nutrient restriction, as well as hormonal involvement in dwarfing (Lockard and Schneider, 1981; Jones, 1984; Soumelidou *et al.*, 1994; Webster, 1995a; Atkinson *et al.*, 2003; Webster, 2004).

However, the mechanism for the influence of the rootstock on the vigour and precocity of the scion is still not fully understood and needs further investigation.

1.4.4.1 Water and nutrient restriction

The root systems of dwarfing rootstocks are usually smaller than the root system of more vigorous rootstocks. A smaller root system means less water and nutrient uptake from the soil, which may restrict the growth of the scion. Indeed, in apple it was shown that the hydraulic conductivity of the roots is lower in dwarfing rootstocks (Atkinson *et al.*, 2003). Yonemoto *et al.* (2004) found that, after grafting mandarins on size reducing rootstocks the scion branches have a lower sap flow rate and higher soluble solid content than the same scions grafted onto other rootstocks. A study in peach showed that the reduced stem extension growth of trees grafted onto semi-dwarfing rootstocks is related to a lower stem water potential compared to more vigorous rootstocks (Basile *et al.*, 2003). It is well known that a dwarfing rootstock cultivar such as ‘M9’ reduces the size of the scion to the same extent when used as an interstock and that the longer the interstock, the smaller the scion (Parry and Rogers, 1972). This indicates that not only the size of the root system, but also the anatomy of the stem and the graft union, may influence the sap flow rate and the mineral concentration in the scion.

1.4.4.2 Hormonal control of rootstock-induced dwarfing

Many studies have focused on the potential role of plant hormones in controlling the dwarfing effect of rootstocks. Most research has focused on the growth regulating hormone auxin and predominantly on the transport of the most abundant auxin, Indole-3- acetic acid (IAA). Soumelidou *et al.* (1994) found reduced auxin movement from the scion to the rootstock when using apple dwarfing rootstocks compared to non-dwarfing rootstocks. This observation was confirmed by Kamboj *et al.* (1997), who measured the uptake of radio labelled [3H]- IAA of stem segments from five apple rootstocks which differed in their dwarfing effect, and found a reduced uptake and transport in dwarfing rootstock stems compared to stems from non-dwarfing rootstocks. However, this is contrary to findings in *Citrus* (Lliso *et al.*, 2004) where application of hormones (including Auxin) did not result in a more vigorous scion growth on dwarfing rootstocks. The concentration of another plant hormone, abscisic acid (ABA) was higher in the leaves of dwarfing rootstock seedlings than in leaves of non-dwarfing rootstock seedlings in *Mango* (Murti and Upreti, 2003). A higher concentration of ABA was also found in the shoot bark of dwarfing apple rootstocks compared to other rootstocks (Kamboj *et al.*, 1999). An increased concentration of ABA might be responsible for a reduced auxin transport, as ABA is known to have a negative effect on auxin transport (Basler and McBride, 1977; Kamboj *et al.*, 1997).

1.4.4.3 Transcriptional regulation of dwarfing rootstocks

FLOWERING LOCUS (*FT)* has been shown to stimulate the transition to flowering in *Arabidopsis* plants in response to environmental inputs (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). In apple, homologues of the *Arabidopsis FT* gene (*MdFT1* and *MdFT2)* were shown to be upregulated in vascular tissue of dwarfing and precocity inducing apple rootstocks compared to vigorous rootstocks using RNA sequencing and Quantitative Real Time PCR (RT-PCR). These results indicate that the upregulation of flowering genes may be part of the dwarfing mechanism of apple rootstocks (Foster *et al.*, 2014). The same study also found some stress response genes were upregulated and concluded that stress might be a factor of the dwarfing effect.

1.5 Genetic mapping and QTL analysis in crop plants

Complex agronomical traits such as tree architectural traits may segregate as continuous quantitative traits and are controlled by many genes. The genomic regions where the responsible genes are located are termed quantitative trait loci (QTLs). High density genetic linkage maps are commonly used to study the structure of a plant genome and to localise the genetic control of traits of interest. They are a prerequisite for the identification of QTLs.

1.5.1 Genetic mapping

Genetic mapping is based on the principle that genes or markers segregate via chromosomal recombination (crossing over) during meiosis. Genes that are physically close are more likely to be transmitted together from the parents to the progeny than more distant genes. The frequency of recombinant genotypes in a segregating population allows calculation of the genetic distance between markers. The construction of a linkage map can be carried out using statistical mapping software such as JoinMap (van Ooijen and Voorrips, 2001) and is based on the logarithm of odds (LOD) score, which is the ratio of linkage to no linkage. The distance between two markers is calculated by the recombination frequencies (Rf) between genetic markers. A high LOD and a low Rf score indicate a tight linkage between two markers (Collard *et al.*, 2005). Linkage groups (LG) are built by grouping together linked markers which represent chromosomes. The more individuals are in the mapping population, the more accurate the calculation of the genetic distance between markers (Collard *et al.*, 2005). Different molecular markers have been used for the construction of genetic maps including isoenzymes, RFLPs, Randomly Amplified Polymorphic DNA (RAPDs), Sequence Characterized Amplified Regions (SCARs), Simple Sequence Repeats (SSRs) and Single Nucleotide Polymorphisms (SNPs). Only markers relevant to this study will be described in the following paragraphs, with the emphasis on SNP markers.

1.5.1.1 Simple sequence repeats

Microsatellites or SSRs are tandem repeats of short DNA sequences of one to six base pairs, usually repeated ten to twenty times such as ATATATATAT. They are widely scattered at many hundreds of loci, mostly in non-coding regions of the genome (Li *et al.*, 2002). In plants, the most widespread repeated unit is (AT)n followed by (AG)n and (TC)n (where n is the number of repeats) (Mohan *et al.*, 1997). SSR markers are highly reproducible and polymorphic, which makes them frequently transferable between species (Morgante and Olivieri, 1993). SSR polymorphism relies on the variation in the number of repeated sequences and therefore the length of the amplified fragments. SSRs are usually amplified by PCR and scored by allele size following resolution using electrophoresis (Stafne *et al.*, 2005)

1.5.1.2 Single nucleotide polymorphism markers

SNP markers are believed to be the most efficient tool for in-depth genetic studies as they are abundant and relatively stable in the genome (Kruglyak, 1997; Syvänen, 2001). SNPs are single base variations in DNA sequences and therefore useful tools to identify differences within individuals or populations. SNPs are often transitions due to the high frequency of C to T mutations, and they are mostly found in non-coding regions (Edwards *et al.*, 2007). Within coding regions, SNPs can be synonymous or non-synonymous, the latter resulting in an amino acid change.

Genome wide SNP detection in plant genomes can be performed rapidly and cost effectively with today's next generation sequencing (NGS) technology by re-sequencing multiple accessions of a determined species and aligning the sequences to a reference genome. Today's medium to high-throughput methods can then be used to genotype large populations with thousands of SNPs simultaneously. The Illumina Infinium® II array (Illumina Inc.) is a high-throughput SNP genotyping technology useful for the screening of large genetic populations with multiple markers. Genotyping-by-sequencing (GBS) is a method developed by Elshire *et al.* (2011) and combines the simultaneous identification and screening of novel markers.

GBS uses restriction enzyme digestion of genomic DNA to reduce the complexity of a genome. A barcoding system enables cost-effective genotyping of many individuals at once (Ward *et al.*, 2013). Both SNP arrays and GBS are powerful enough to construct high density genetic maps with evenly spaced markers across the genome with a marker distance of 10cM or closer, suitable for the detection of QTLs (Darvasi *et al.*, 1993).

1.5.2 QTL-analysis

The principle behind QTL analysis is the detection of an association between the phenotype and the genotype of a segregating population. The presence or absence of a particular locus in the genotype of a mapping population enables the classification of seedlings into different genotypic groups. A significant difference between groups with respect to the phenotype can then be determined (Collard *et al.*, 2005). Whether or not a particular marker is linked to a QTL controlling a particular trait depends on the presence of a significant difference ($P<0.05$) between the phenotypic means of the genotypic groups (Figure 4).

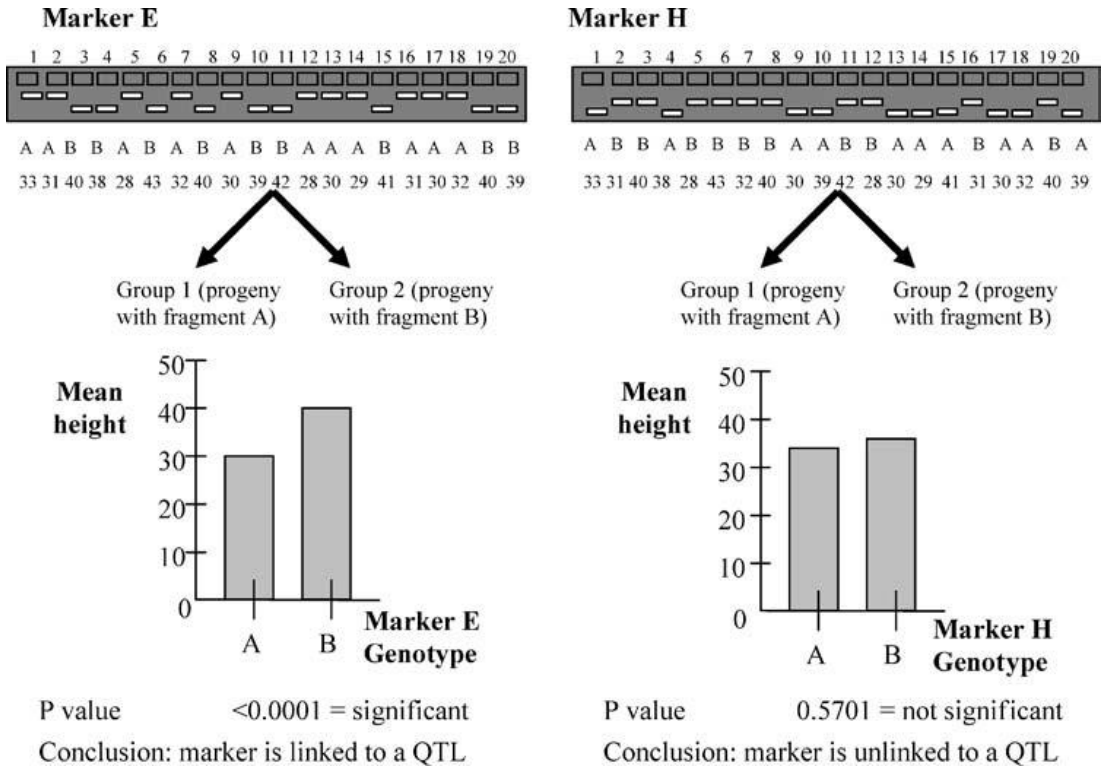

Figure 4: Basics of Quantitative Trait Loci (QTL)- analysis. Markers that are linked to a gene or QTL controlling a particular trait (e.g. plant height) will indicate significant differences when the mapping population is partitioned according to the genotype of the marker. Based on the results in this diagram, Marker E is linked to a QTL because there is a significant difference between means. Marker H is unlinked to a QTL because there is no significant difference between means (Collard *et al.*, 2005).

There are two limiting steps for QTL analysis: the number of individuals in the segregation population, and the availability of a dense genetic map. The latter can be overcome by the new high-throughput technologies described earlier. If the sample size is too small, small effect QTLs may fail to be detected and the size of a QTL that is identified might be overestimated (Beavis, 1998). This overestimation of QTLs is known as the Beavis effect. However, when the segregating population is reasonably large and a dense genetic map is available, QTL analysis can be carried out using software for composite interval mapping (CIM), combining interval mapping and linear regression such as MapQTL5.

The different steps of QTL- analysis are interval-mapping (IM), Multiple QTL Method (MQM) and permutation testing. If the data is not normally distributed, a Kruskal-Wallis test shows whether the samples originated from the same distribution. The IM identifies and locates preliminary QTLs. A QTL is significant when its LOD- score is higher than the genome wide threshold of 95% determined by the permutation test. The marker with the highest LOD- score is used as a cofactor in MQM. This enables detection of minor QTLs that might be hidden by a large effect QTL. QTL mapping has been used to analyse the genetic determinants of tree architecture in several genera. In pipfruit, most of the studies have concentrated on apple (Conner *et al.*, 1998; Liebhard *et al.*, 2003; Kenis and Keulemans, 2007; Segura *et al.*, 2009a).

1.5.3 Genetics of vigour control and precocity

Several studies on the identification of the genetic control of dwarfing apple rootstocks have been published in the past, however, the candidate genes are still unknown for most of the species under examination. Rusholme Pilcher *et al.* (2008) mapped the locus *Dwarfing 1* (*Dw1*) in dwarfing M9 rootstocks to LG5. This was the first mapped locus for the control of the dwarfing ability of an apple rootstock. However, some vigorous trees were shown to carry the *Dw1* allele indicating that dwarfing might be controlled by more than one locus (Rusholme Pilcher *et al.*, 2008). Indeed, a second dwarfing locus (*Dw2*) was detected in apple on LG11 and the dwarfing effect was strongest when both *Dw1* and *Dw2* were present (Celton, 2007; Fazio *et al.*, 2014; Foster *et al.*, 2015). Figure 5 shows the average trunk cross-sectional area (TCA) of seven year old apple trees from a segregating population developed by crossing ‘M9’ and ‘Robuster 5’ for the different genotypic classes. An intermediate dwarfing effect can be observed when individuals carry the *Dw1* locus only. No dwarfing effect was observed when neither was present. The *Dw1* locus is located on LG5, the same linkage group as a QTL controlling height increment and stem diameter detected in a ‘Fiesta’ x ‘Discovery’ mapping population by Liebhard *et al.* (2003). The QTL explained between 5% and 7% of the phenotypic variability of this population (Liebhard *et al.*, 2003).

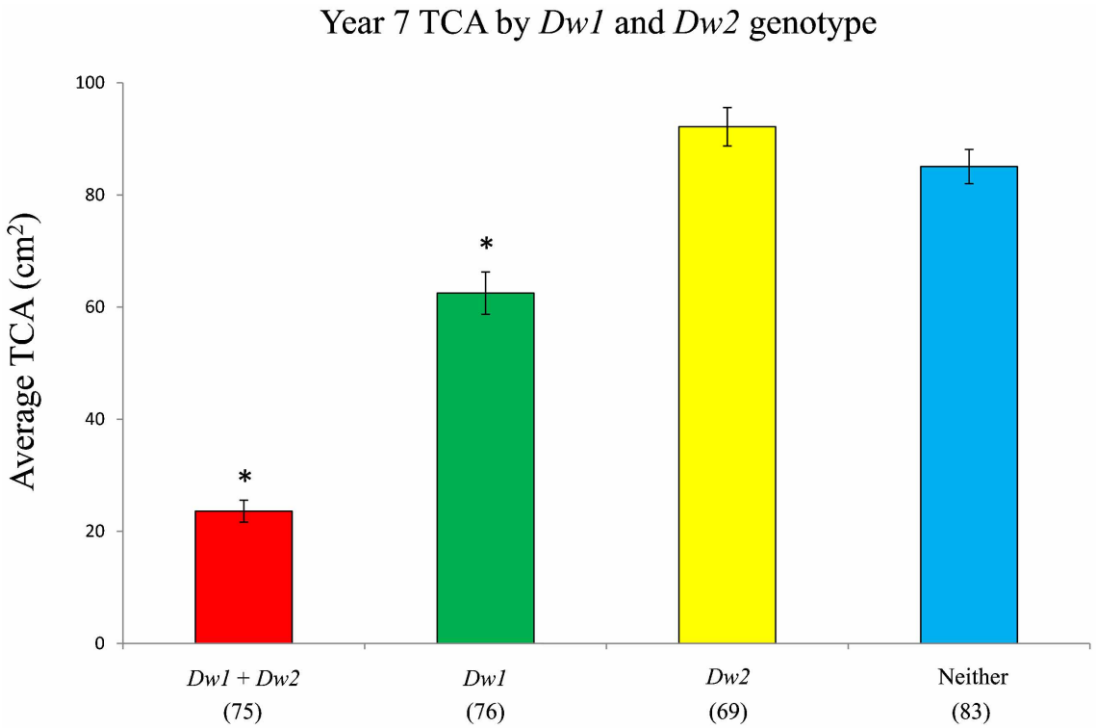

Figure 5: Average year 7 TCA of trees in each genotypic class. The number of individuals in each class is given in parentheses; error bars indicate standard error. Average TCAs were compared to the group with neither *Dw1* nor *Dw2* by ANOVA; asterisks indicate the means are significantly different with a P value of <0.001. Data is from 303 trees from the second population (Foster *et al.*, 2015).

Genetics of flowering traits have been studied in several tree crops. In apple, Liebhard *et al.* (2003) detected three QTLs controlling flowering time on LG7, 10 and 17. On LG8 and 15 they found a QTL associated with the number of flowers and two QTLs controlling the length of the juvenile phase on LG3 and 15. In Eucalyptus, Missiaggia *et al.* (2005) identified a major effect QTL controlling early flowering. Chen *et al.* (2007) reported a QTL controlling flower abundance (floral density) in avocado. A significant QTL associated with flowering time was detected on LG5 in apricot (Campoy *et al.*, 2010). Guitton *et al.* (2012) investigated QTLs controlling biennial bearing in apple and found QTLs related to the number of inflorescence on LG15.

1.6 Genetics and genomics of pear

The first genetic map constructed for pear was developed for *P. pyrifolia* with random amplified polymorphic DNA (RAPD) markers (Iketani *et al.*, 2001). Later, amplified fragment length polymorphism (AFLPs) and simple sequence repeat (SSRs) were used for mapping in an interspecific cross between 'Bartlett' (*P. communis*) and 'Hosui' (*P. pyrifolia*) (Yamamoto *et al.*, 2002; Yamamoto *et al.*, 2004). These were the first maps containing SSRs derived from apple and pear and were used to study the genome synteny between both genera. The transferability between apple and pear was confirmed in other studies in European pear (Dondini *et al.*, 2004; Celton *et al.*, 2009a) and an interspecific population between 'Mishirazi' (*P. pyrifolia* x *P. communis*) and 'Jinhua' (*P. bretschneideri*) (Lu *et al.*, 2010). Several studies have shown that SSR markers are transferable across apple and pear which led to the assumption of a high co-linearity between both species (Pierantoni *et al.*, 2004; Yamamoto *et al.*, 2007; Celton *et al.*, 2009b). The recently published genomes of apple (Velasco *et al.*, 2010), Chinese pear (Wu *et al.*, 2013) and European pear (Chagné *et al.*, 2014) revealed their high synteny. The extensively studied apple genome might therefore help to reveal the genetic background of the poorly studied pear genome used for the purpose of this study.

1.6.1 The Chinese and European pear genome sequence

The pear genome was first sequenced by Wu *et al.* (2013) who sequenced the Chinese cultivar 'Suli' (*P.bretschneideri*) using a BAC-by-BAC method and next generation sequencing technology. The Chinese pear genome sequence covers 97% (512 Mb) of the estimated genome size of 600 Mb with 53% repetitive sequence and 42812 protein-coding genes. Soon after, the draft genome sequence of European pear (*P.communis*) was published by Chagné *et al.* (2014) using second generation sequencing technology. The 'Bartlett' genome assembly covers 577 Mb with 43419 protein-coding genes of which 1219 proteins are only found in European pear when compared to other related genomes. These draft genome sequences are useful tools to speed genetic studies in pear and to identify genetic controls of important horticultural traits in both Asian and European pear cultivars.

1.6.2 QTL detection in pear

The number of QTLs detected in pear is increasing rapidly. Dondini *et al.* (2004) reported on QTL mapping, which involved fireblight (*Erwinia amylovora)* resistance, and Pierantoni *et al.* (2007a) and Won *et al.* (2014) detected QTLs for pear scab (*Venturia pyrina*) resistance in European pear. In Asian pear, studies identified QTLs for leaf morphology (Sun *et al.*, 2009) and fruit traits (Zhang *et al.*, 2012b). In interspecific cultivars QTLs were detected for postharvest disorders (Saeed *et al.*, 2014), pear scab (Won *et al.*, 2014) and pear psylla (Montanari *et al.*, 2015) resistance and fruit related traits such as fruit weight, size, colour, and juiciness (Wu *et al.*, 2014). No QTLs for architectural traits in pear have been published.

1.7 Thesis aim and objectives

The aim of the PhD project was to develop an understanding of the genetic determinants of vigour control and precocity by pear rootstocks utilising a segregating population of 405 seedlings from a *Pyrus communis* 'Old Home' x 'Louise Bonne de Jersey' (OHxLBJ) cross grafted with 'Doyenne du Comice' (Comice) as scion and planted in the Plant & Food Research orchards in Motueka, New Zealand in 2010. The parents were chosen, as previous breeding with the same cross resulted in a promising dwarfing pear rootstock 'Pyrodwarf' discovered in Geisenheim, Germany in 2002 (Jacob, 2002). 'Old Home' is often used as a parent in rootstock breeding because it is fire blight-, woolly pear aphid- and phytophthora- resistant as well as being tolerant to pear decline, high pH soils and drought. On top of that the frame develops strong wide-angled branches (Jacob, 2002; Wertheim, 2002). 'Old Home' was reported to be easy to propagate with hardwood cuttings. 'Louise Bonne de Jersey' is moderate in vigour, upright in habit, sturdy, easy to propagate, shows good precocity and is productive (Rogers and Beakbane, 1957). The ease of propagation with hardwood cuttings was also reported for 'Louise Bonne de Jersey' (Jacob, 2002). It is, however, susceptible to fireblight.

Using this population the following tasks were undertaken to accomplish the aim of this PhD:

- To identify the range of vigour control and precocity in the population, the trees were comprehensively phenotyped for scion growth and flowering in four successive years.
- The ease of vegetative propagation, a crucial trait for rootstock breeding, was assessed in rooting experiments and repeated three times.
- High density genetic maps were constructed using two different high throughput genotyping tools, the Infinium® II 9K apple/pear SNP array and the genotyping-by-sequencing (GBS) approach.

- The phenotypic data and the genetic maps were utilized for QTL- analysis to identify genetic regions controlling vigour control, precocity and vegetative propagation ability.
- QTL regions detected were compared to the apple genome sequence and genomic regions of interest were aligned to enable the identification of conserved regions involved in tree architecture, and also identification of candidate genes in pear based on apple.

Parts of the results presented in the next chapter were published in the paper: "Identification of *Pyrus* single nucleotide polymorphisms (SNPs) and evaluation for genetic mapping in European pear and interspecific *Pyrus* hybrids" in PlosONE in 2013 (Montanari *et al.*, 2013). The paper was written in collaboration with two other PhD students Sara Montanari and Munazza Saeed as well as a visiting scientist YoonKyeong Kim. We used the Infinium SNP array to genotype the European pear cross described in this study ('Old Home' x' Louise Bonne de Jersey') and four interspecific breeding families derived from Asian (*P. pyrifolia* Nakai and *P. bretschneideri* Rehd.) and European pear pedigrees. The constructed genetic maps were used to identify chromosomal regions associated with pest and disease resistance, orchard yield and fruit quality.

2 DEVELOPMENT OF HIGH DENSITY GENETIC MAPS USING THE SNP ARRAY AND GENOTYPING BY SEQUENCING APPROACH

2.1 Introduction

2.1.1 Next generation sequencing of complex plant genomes

Technology for the detection of DNA polymorphisms in genomes has developed rapidly in the last decade. Utilizing today's next generation sequencing (NGS) techniques, entire genomes of higher plants have been sequenced, including grape (Velasco *et al.*, 2007), apple (Velasco *et al.*, 2010), potato (Xu *et al.*, 2011), maize (Schnable *et al.*, 2012), tomato (Sato *et al.*, 2012), peach (Verde *et al.*, 2013), and most significantly for this study, Chinese (Wu *et al.*, 2013) and European pear (Chagné *et al.*, 2014). The genome size of European pear has been estimated to be 600Mb, based on flow cytometry (Arumuganathan and Earle, 1991). Using one lane of paired-end Illumina sequencing (200M reads x 150bp), a pear genome coverage of 50x might be expected. NGS can be used to detect polymorphisms within individuals or populations and to identify genetic loci associated with phenotypic variations.

2.1.2 Genotyping with the Infinium® II array

Recently developed high-throughput SNP genotyping technologies, such as the Illumina Infinium® II array (Illumina Inc.) can be used to genotype large populations with thousands of markers simultaneously. To develop an array, SNPs must first be detected in a small subset of individuals using re-sequencing with NGS. After validation, a chosen set of markers, reproducible in large numbers is used for construction of the array, which can then be screened over a set of individuals large enough for association or linkage analysis. The Illumina Infinium® array contains beads with attached oligonucleotides that are designed to target a DNA sequence immediately flanking the SNP. As shown in Figure 6, the DNA fragments, derived from enzymatic digestion, bind to the complimentary oligonucleotides. Fluorescently labelled dideoxynucleotides (ddNTP's) (terminating nucleotides), each labelled with a different dye colour, are attached to the 3' terminus of the oligonucleotide through single base extension by DNA polymerase (Syvänen, 1999). The fluorescence intensity of the single base extension product is detected by a fluorescence scanner and provides information about the allelic variation. The data obtained from the SNP array can be analysed using GenomeStudio software (GenomeStudio, Illumina Inc.).

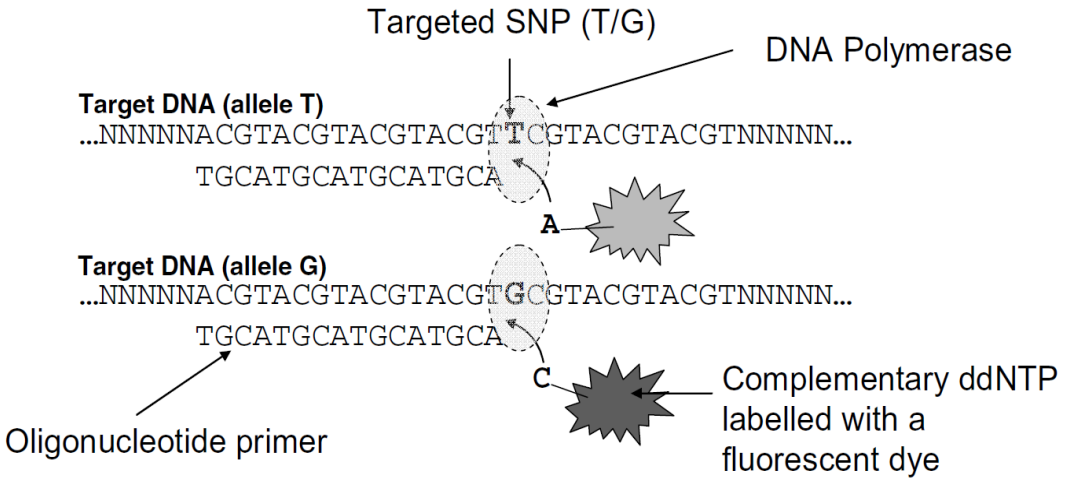

Figure 6: Single base primer extension. The oligonucleotide primer immediately flanking the SNP is extended using a DNA polymerase. Fluorescently labeled terminating nucleotides are incorporated, with a different dye colour for every nucleotide. (Chagné *et al.*, 2007)

Recent studies have successfully developed and applied SNP arrays in Rosaceae. The International RosBREED SNP consortium (IRSC) (www.rosbreed.org) developed an 8K apple Illumina Infinium® II SNP array (Chagné *et al.*, 2012) for the detection of allelic variation in apple germplasm. This array was enhanced by adding 1K of markers designed for pear resulting in the apple and pear Infinium® II 9K SNP array used in this study (Montanari *et al.*, 2013). IRSC also developed a 6K sweet cherry Illumina Infinium® II SNP array (Peace *et al.*, 2012) to genotype sweet and sour cherry germplasm. A 9K Illumina Infinium® II SNP array for peach was developed by the International Peach SNP Consortium (IPSC) to compare 709 accessions of peach cultivars, wild related *Prunus* species and interspecific hybrids (Verde *et al.*, 2012). For SNP detection they performed low depth genome re-sequencing of 56 peach accessions from the peach breeding germplasm. The peach SNP array was evaluated using 709 accessions and found to be highly successful in the genotyping of different peach cultivars (84.3% polymorphic SNPs) (Verde *et al.*, 2012).

The use of genetically diverse accessions for the development of the SNP arrays presented makes them applicable to a wide range of accessions in the same species or related species. While those SNP arrays could be used to create high density genetic maps, the number of robust, evenly distributed markers was not sufficient for genome-wide association studies (GWAS) or pedigree-based analysis (Chagné *et al.*, 2012; Peace *et al.*, 2012; Verde *et al.*, 2012). More recently, higher density SNP arrays have been constructed, such as the 20K Illumina Infinium® II SNP array for apple (Bianco *et al.*, 2014) and the 90K Axiom® SNP array for strawberry (Bassil *et al.*, 2015). These arrays promise to be useful for GWAS, pedigree-based analysis and genomic selection (GS).

2.1.3 Genotyping-by-sequencing

Genotyping-by-sequencing (GBS) is a NGS-based method developed by Elshire *et al.* (2011) that uses restriction enzymes digesting genomic DNA to reduce the complexity of a genome. GBS enables cost-effective genotyping of many individuals simultaneously by using a barcoding system which enables the pooling of samples for sequencing, and thereby reduces the cost. The protocol by Elshire *et al.* (2011) has been used in several plant species, for example in maize and switchgrass to characterize germplasm diversity (Lu *et al.*, 2013; Romay *et al.*, 2013). Schilling *et al.* (2014) used SNPs discovered by GBS for population studies in poplar. High-density genetic maps have been developed for *Triticum* (wheat) and *Hordeum* (barley) (Poland *et al.*, 2012), *Rubus* (Ward *et al.*, 2013), *Vitis* (Barba *et al.*, 2014) and *Malus* (Gardner *et al.*, 2014) using the GBS approach. Gardner *et al.* (2014) successfully employed GBS to verify a QTL for skin colour in a segregating apple population. They used the 'Golden Delicious' genome as a reference for SNP calling but discussed that additional SNPs could be detected and mapped by including non-anchoring genome sequences.

For the present study, a combined approach, utilizing both the apple and pear Infinium® II 9K SNP array (Montanari *et al.*, 2013) and the GBS method were employed to construct high density genetic maps for pear.

2.2 Material and Methods

2.2.1 Plant material

A segregating rootstock population of 421 F1 seedlings from a cross between the European pear (*Pyrus communis* L.) cultivars 'Old Home' (OH) and 'Louise Bonne de Jersey' (LBJ) was employed for the detection of polymorphic SNPs for genetic map construction. The rootstocks were planted out into the Plant & Food Research orchard in Motueka, New Zealand and grafted with 'Doyenne du Comice' (*Pyrus communis* L.) for architectural studies. The shoots removed from the OHxLBJ seedlings prior to this were grafted as scions onto *Pyrus calleryana* seedling rootstocks to provide leaf material for DNA extraction as well as phenotyping for rooting ability. A subset of 297 individuals and both parents were screened with the Illumina Infinium® II 9K SNP array. A further sixty individuals that were not genotyped with the SNP array were genotyped using the GBS approach.

2.2.2 Genotyping using the Illumina Infinium® II 9K SNP array

2.2.2.1 DNA extraction and SNP array screening

DNA was extracted using the a cetyltrimethyl ammonium bromide (CTAB) extraction method (Doyle and Doyle, 1987), followed by purification with NucleoSpin columns (Macherey-Nagel GmbH & Co. KG). DNA quantifications were carried out using the NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific Inc.). The DNA was sent to AgResearch Invermay Agricultural Centre for SNP genotyping with the Infinium® II 9K apple and pear SNP array. This array comprised 1096 pear SNPs added to the IRSC apple Infinium ® II 8K SNP array (Chagné *et al.*, 2012).

The three European cultivars, ‘Bartlett’, ‘Old Home’ and ‘Louise Bonne de Jersey’ were chosen for SNP detection using low coverage re-sequencing. Each accession was sequenced using one lane of Illumina GA II with 75 cycles per read and small insert paired-end sequencing, as described in Chagné *et al.* (2012). The detection and selection of SNPs for the array is described in Montanari *et al.* (2013). In brief, a *de novo* assembly of ‘Bartlett’ was developed. Contigs of 600bp or larger were used as a reference genome set for mapping the sequence data from OH and LBJ. SNPs were detected using *SoapSNP*, and quality parameters were applied for SNP filtering. The sequences of 2559 *Malus* MYB transcription factors were used as candidate genes and mapped to the ‘Bartlett’ genome to define their location within pear. Only SNPs located within these candidate gene sequences were included in the SNP array. The Illumina Infinium® assay design tool (ADT) was used on the detected SNPs with a threshold of 0.7. These pear SNPs were synthesized as probes and located on the same array as the IRSC apple Infinium® II 8 K array. The genomic DNA of the parents OH and LBJ and a subset of their progeny (297 F1 individuals) were screened over the SNP array following the Infinium® HD Assay Ultra protocol (Illumina Inc., San Diego, USA) for amplification and hybridisation, and scanned with the Illumina HiScan.

2.2.2.2 Data analysis

The Illumina GenomeStudio v 1.0 software Genotyping Module was used for data analysis. The software enables the clustering of the genotypes (AA/AB/BB), detected by the fluorescent scanner, for each SNP in normalized graphs (Figure 7).

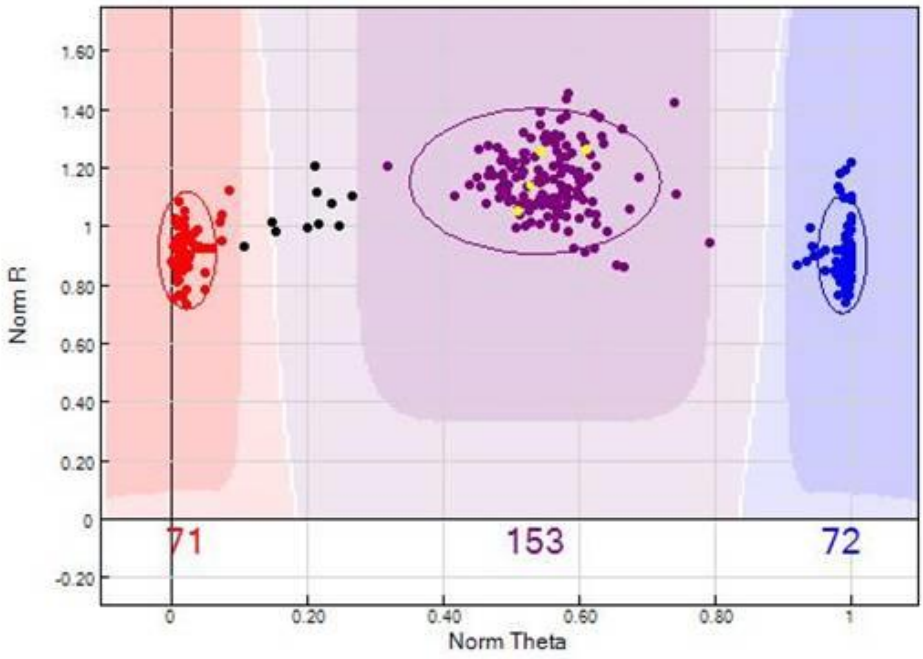

Figure 7: Heterozygous SNP with a segregation ratio 1:2:1 in the normalized GenomeStudio graph. Two replicates of the parents 'Old Home' and 'Louise Bonne de Jersey' are indicated in yellow; by default the red cluster shows AA, the blue BB and the purple AB genotypes. 'No Calls' are shown in black

Quality parameters were applied to ensure the detection of good quality SNPs and good quality DNA for each individual. The GenCall threshold is a quality score between 0 and 1 based on the distance of a sample from the midpoint of the cluster. The smaller the GenCall score, the further the sample location from the centre. A small GenCall score may indicate poor quality DNA of the sample. A GenCall threshold of 0.15 was set as a quality parameter of the individuals in this study and samples below this threshold were not retained for further analysis.

The SNPs were filtered using a GenTrain score ≥0.5, minor allelic frequency (MAF) ≥0.15 and call rate >80% to gain good quality SNPs. The GenTrain score (0-1) is a clustering algorithm that calculates the relative distance of each cluster and the cluster shape and therefore the quality of the clustering for each SNP. A low GenTrain score means poor cluster separation and is shown in Figure 8, where the AB and BB clusters are too close together to be considered reliable. The MAF (0-1) evaluates the number of calls per cluster and can help to identify incorrect clustering on the basis of the frequency of the least frequent allele. For example, a MAF smaller than 0.1 indicates an homozygous SNP called incorrectly as heterozygous.

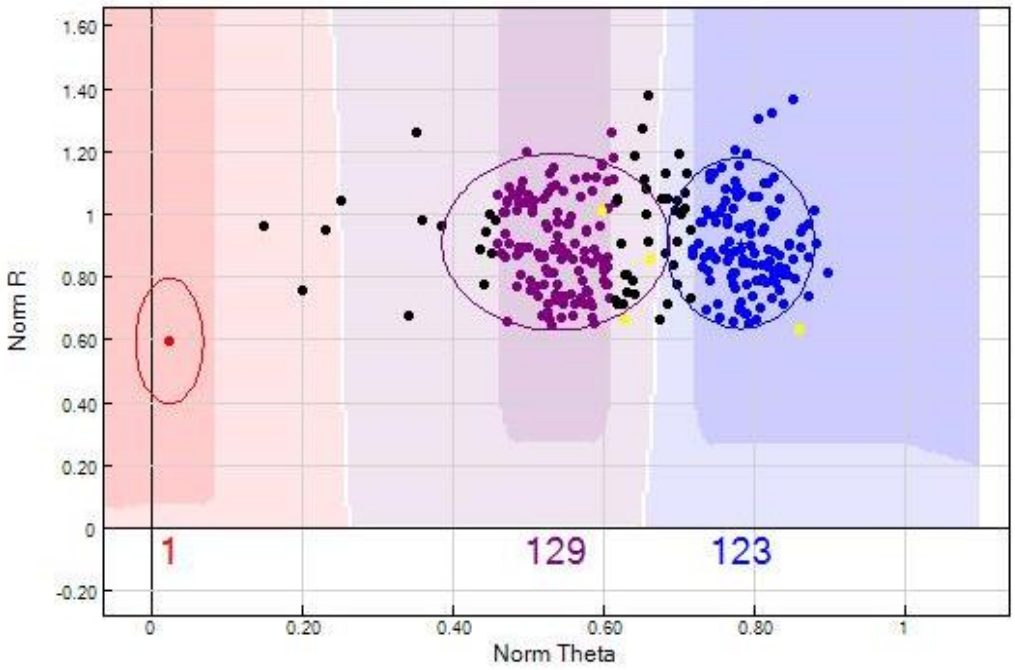

Figure 8: GenomeStudio graph with GenTrain score: 0.44, MAF: 0.26 and GenCall: 0.21. Two replicates of the parents 'Old Home' and 'Louise Bonne de Jersey' are indicated in yellow

2.2.2.3 Genetic map construction

High quality SNPs, heterozygous in one (ABxAA; AAxAB) or both parents (ABxAB), were used for the construction of parental linkage maps for both OH and LBJ, employing Joinmap v3.0 (van Ooijen and Voorrips, 2001) software. The two parental maps were built independently following the double pseudo-testcross strategy (Grattapaglia and Sederoff, 1994). A LOD score of 5 or higher was used for grouping and the genetic maps were calculated using the Kosambi function. MapChart v2.2 (Voorrips (Voorrips, 2002), 2002) was used to align LGs of the parental maps using the ABxAB markers. Linkage groups were assigned to the map by aligning OH and LBJ with parental maps of 'Moonglow' and PEAR1 (Montanari *et al.*, 2013). These maps were constructed using the same SNP array and contain SSR markers derived from apple.

2.2.3 Genotyping using genotyping-by-sequencing

2.2.3.1 DNA extraction and library preparation

DNA was extracted from leaves of 60 seedlings using the CTAB method (Doyle and Doyle, 1987) modified by Chris Kirk (PFR, 2014). DNA quality and quantity was evaluated with the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies Corporation). GBS libraries were prepared following an in-house protocol (Elena Hilario, PFR,Version 2.0, May 2013), which is a modified version of the protocol developed by Elshire *et al.* (2011). The protocol differs from Elshire *et al.* (2011) as follows:

- One microgram of DNA was used for the restriction digestion
- Adapter annealing was done following the protocol of Ko *et al.* (2003)
- The adapter ligation step was performed following digestion without drying out the DNA/ adaptor mixture
- High fidelity enzyme (AccuPrime Taq DNA polymerase High Fidelity, Life Technologies) was used for amplification
- The libraries were amplified and cleaned up prior to pooling

The restriction enzyme for digestion was *Bam*HI. The GBS adapters were designed by Deena Bioinformatics (http://www.deenabio.com/). The 60 samples were pooled and sent to Macrogen Inc. in Korea for DNA sequencing where they were sequenced on two lanes of Illumina HiSeq2000.

2.2.3.2 Processing of raw sequence data

The raw data analysis was done using the GBS pipeline TASSEL 5.0.2 & 3.0.158 (Glaubitz *et al.*, 2014). Figure 9 illustrates the different steps necessary for SNP discovery in a GBS pipeline. Using the barcode system, sequences were assigned to each of the 60 samples (A). Only unique sequence tags of each sample were kept when present at least five times in all samples (B). Bowtie 2.2.5 (Langmead and Salzberg, 2012) was used for the alignment of the GBS tags to the reference 'Bartlett' 1.0 genome assembly (*P. communis*) (Chagné *et al.*, 2014) and 'Suli' (*P. bretschneideri*) (Wu *et al.*, 2013) (C). A matrix (TOPM) containing the presence and absence of each tag for each sample was prepared and used for SNP calling. SNPs were called following the aligning of tags that mapped together at the same physical location on the reference genome (E).

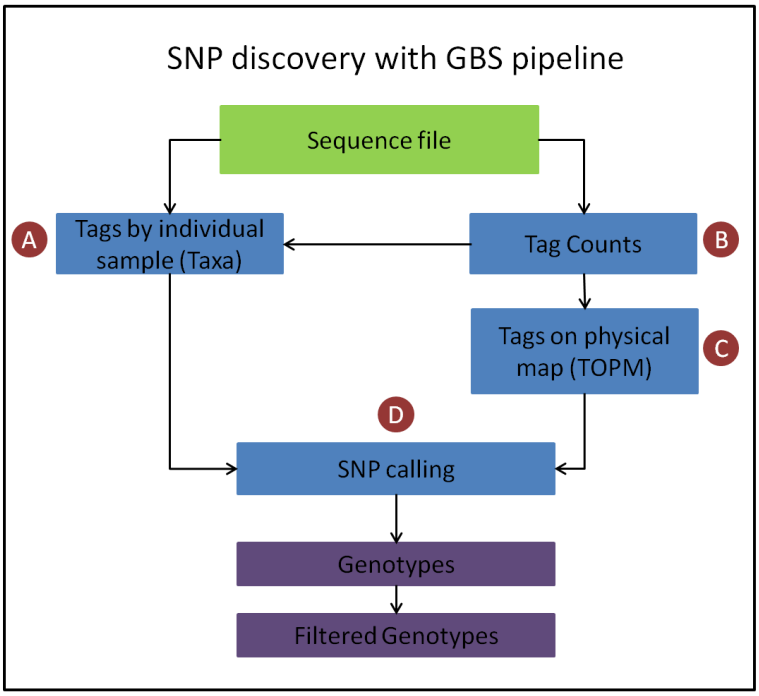

Figure 9: Schematic diagram of the genotyping-by-sequencing pipeline for SNP discovery

2.2.3.3 Map construction

SNP filtering was conducted in Microsoft Excel. All SNPs with missing data for more than ten individuals were removed from the analysis. Markers were considered of good quality when they segregated in a Mendelian manner: ABxAA (1:1 ratio), ABxAB (1:2:1 ratio) A0xA0 (1:3 ratio). Genetic maps were constructed with R-studio (R Core Development Team, 2013) using the package Onemap version 2.0-4. A LOD score of 5 or higher was used for grouping and the genetic maps were calculated using the Kosambi function. Maps were drawn and aligned using MapChart v2.2 (Voorrips, 2002).

2.2.4 Consensus map construction

To assign linkage groups to the GBS map, a consensus map was constructed combining it with the SNP array map. In order to do this, SNPs had to be converted into scaffolds according to their location, to gain European scaffolds in common between both maps. The consensus map was constructed in R-Studio using the package LPmerge (Endelman and Plomion, 2014).

2.3 Results

2.3.1 Genotyping with the SNP array

Out of the successful 1096 pear and 7692 apple bead types on the IRSC Infinium® II array (Illumina Inc.), 713 markers were polymorphic for the OHxLBJ population, including 54% (597) of the newly developed pear SNPs and 1.5% (115) of the apple SNPs. The remaining markers were either monomorphic (365) or had poor quality clustering (134). Genetic maps were constructed for both parents (Supplementary material 1). The OH map consists of 17 LGs containing 399 SNPs (341 pear, 58 apple) spanning 913cM. The LBJ map consists of 16 LGs containing 446 SNPs (382 pear, 64 apple) and spans 1044cM. Both parental maps have 135 markers in common that segregated ABxAB (Table1). The OHxLBJ consensus map was aligned to the apple 'Golden Delicious' genome (Velasco *et al.*, 2010) using 367 orthologous markers (Figure 10).

Table 1: Number of apple and pear SNPs, derived from the apple and pear Illumina Infinium® II 9K SNP array, mapped for parents 'Old Home' and 'Louise Bonne de Jersey'

Parent	Marker segregation	Pear SNPs	Apple SNPs	Total
'Old Home'	ABxAA	213	49	262
	ABxAB	128	9	137
'Louise Bonne de Jersey'	ABxAA	256	55	311
	ABxAB	126	9	135
Common	ABxAB	126	9	135

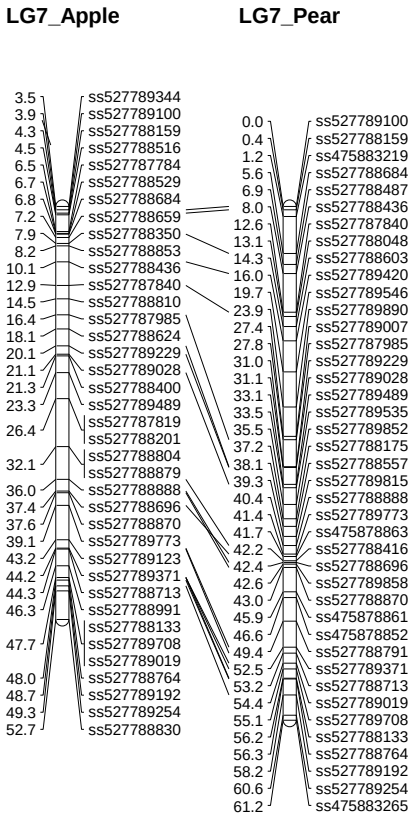

Figure 10: Alignment of LG7 of the OHxLBJ consensus map (LG7_Pear), constructed using markers from apple and pear Illumina Infinium® II 9K SNP array, with chromosome 7 of the 'Golden Delicious' genome (in base pair) (LG7_Apple) (Velasco *et al.*, 2010). Lines show the markers in common.

2.3.2 SNP genotyping with the GBS approach

A genetic map was constructed for both parents OH and LBJ with *P. communis* (Pc) and *P. bretschneideri* (Pb) SNPs (map not shown). The map consists of 9479 Pc SNPs located on 2682 'Bartlett' assembly scaffolds and 4954 Pb SNPs located on 1278 assembly scaffolds. The number of SNPs and scaffolds for each LG for both assemblies is shown in Table 2.

Table 2: Number of mapped GBS Scaffolds, SNPs, for the combined maps of both parents 'Old Home' (OH) and 'Louise Bonne de Jersey' (LBJ). The numbers of *P. communis* (*Pc*) and *P. bretschneideri (Pb*) SNPs and scaffolds are displayed separately.

LG	No. *Pc* scaffolds	No. *Pb* scaffolds	Size (Mb) scaffolds *Pc*	Total No. Markers	*Pc* SNPs	*Pb* SNPs
LG1	143	78	11443610	782	450	332
LG2	126	54	12082288	557	355	202
LG3	218	93	15738696	1245	822	423
LG4	174	104	13669014	938	616	322
LG5	180	54	11340565	841	618	223
LG6	164	92	14022176	1011	597	414
LG7	155	69	15959814	855	559	296
LG8	148	70	11036628	787	505	282
LG9	82	42	13918451	352	230	122
LG10	221	95	10601328	1183	816	367
LG11	207	84	9255348	929	640	289
LG12	154	88	19053599	883	603	280
LG13	134	68	20481853	705	458	247
LG14	94	49	17239952	593	387	206
LG15	204	99	12998555	1146	725	421
LG16	145	76	12997321	740	501	239
LG17	133	63	11735377	886	597	289
total	2682	1278	233574575	14433	9479	4954

2.3.3 Consensus map construction

A consensus map was then constructed comprising the scaffolds of both the SNP chip and the GBS map. This map consists of 1983 unique *P. communis* scaffolds and 5*34 unique P. bretschneideri* scaffolds. However, many markers mapped to the same position. When only one marker per cM position was considered, 882 scaffolds remained on the consensus map spanning 2011.95cM, with an average of 2.3cM distance between markers (Supplementary material 2). The information on linkage group assignment from the SNP array map enabled the assignment of LGs on the GBS pear genetic map in this study.

2.3.4 Anchoring of the 'Bartlett' genome to the consensus map.

When 'Bartlett' genome scaffolds were anchored to the consensus map, 98% of the scaffolds greater than 200kbp were anchored to the genetic map, while only 0.6% scaffolds smaller than 50kbp were anchored (Table 3). In total 40.46% of the 'Bartlett' genome was anchored to the constructed GBS and array consensus map.

Table 3: Number and size of *P. communis* scaffolds from the 'Bartlett' genome anchored to the consensus map of 'Old Home' and 'Louise Bonne de Jersey'.

	12Mbp-500kbp	500kbp-200kbp	200kbp-100kbp	100kbp-50kbp	50kbp-10kbp	<10kbp
No. Scaffolds	46	450	766	935	2720	137166
No. anchored on map	45	388	447	323	205	575
% anchored on map	98	86	58	35	7.5	0.4

2.4 Discussion

SNPs are highly efficient tools for comprehensive genetic studies (Yamamoto and Chevreau, 2009). At the beginning of this PhD project, the number of available SNPs in pear was marginal. More than 1000 SNPs from the re-sequencing of *P. communis* cultivars were included into the 8K apple Illumina Infinium® II SNP array, making them easily available for further studies. Here this tool was used to develop one of the first high-density SNP based genetic maps constructed for any species of pear. Genotyping-by-sequencing is currently a fast developing and cost-effective genotyping method, with potential to become the tool of choice for plant genetics and breeding. In this project the first GBS based genetic map was constructed for pear, improving the anchoring of the 'Bartlett' v1.0 genome to the 17 *Pyreae* LGs. Combining both methods resulted in a high density SNP based consensus map for pear with markers transferable between *P. communis, P. bretschneideri* and *Malus* and assigned linkage groups, anchored to the 'Bartlett' genome.

2.4.1 Marker development and genetic mapping using the SNP array

We used the apple and pear Infinium® II 9K SNP array for the genotyping of 297 individuals of a segregating European pear rootstock population. The high density of the constructed genetic maps will make them highly suitable for the detection of QTLs. Ideally, a map powerful enough for QTL detection consists of evenly spaced markers across the genome with a marker distance of 10cM or closer (Darvasi *et al.*, 1993). Both parental maps developed with the SNP array are suitable for QTL analysis, as they contain 399 markers and 446 markers for OH and LBJ respectively with an average of one marker every 2.3cM each. Of the 1096 pear SNPs used to construct the first pear genotyping array, 597 unique markers were polymorphic in the OHxLBJ segregating population and were demonstrated to be useful for construction of genetic maps for both parents. However, as sequence data from OH and LBJ were used to design the pear SNPs, a higher number of polymorphic SNPs might have been expected. The high number of monomorphic SNPs (365) was due to a high number of SNPs being homozygous in both parents, resulting in monomorphic SNPs in the progeny. SNPs were detected using a highly fragmented assembly genome, before the 'Bartlett' genome assembly v.1.0 became available, resulting in a low number of polymorphic SNPs. The number of SNPs showing poor clustering (134) might have been reduced with improved sample DNA quality.

The integration of apple SNPs with pear SNPs on the array enabled marker transfer from the genus *Malus* to *Pyrus.* We mapped 58 apple SNPs on the OH map and 64 on the LBJ map, demonstrating the presence of markers that are orthologous between apple and pear. Indeed, the alignment of the physical 'Golden Delicious' map with the OHxLBJ consensus map resulted in approximately 20 orthologous markers per LG. This shows the transferability of the selected SNPs between the two closely related genera *Malus* and *Pyrus*. The transferability of SNPs at lower taxonomic level, among plant species of the same genus has been reported previously in studies including *Vitis* (Vezzulli *et al.*, 2008), *Citrus* (Ollitrault *et al.*, 2012) and *Eucalyptus* (Grattapaglia *et al.*, 2011).

In this study more than 1000 pear SNPs, derived from re-sequencing three *P.communis* cultivars, were selected based on their location within candidate genes. This ensured their usefulness for marker-trait association and increases the possibility that flanking sequences are conserved between species and even genera.

2.4.2 GBS

While the SNP array has many advantages over lower throughput techniques such as SSR amplification by PCR, it requires SNP detection and validation in a small subset of samples prior to the array development. The risk thereby is the occurrence of ascertainment bias (non-random SNPs) with some regions over- or under- represented, decreasing the number of markers polymorphic in other crosses (Micheletti *et al.*, 2011). The GBS method, in contrast, identifies and screens novel markers simultaneously, and is more cost effective than the array based method (Ward *et al.*, 2013). Methylation sensitive enzymes are used to ensure the cutting occurs in coding regions and therefore enhances the usefulness of the marker for genetic association studies. The choice of the restriction enzyme determines the number of fragments and therefore sequencing depth. We used *Bam*HI, a 6-base cutter restriction enzyme with a GGATTC cut site that cuts less frequently than the 5-base cutter *Ape*KI used in many previous plant studies (Elshire *et al.*, 2011). We selected *Bam*HI on the basis of the criteria established by the GenePool group at the University of Edinburgh for RAD sequencing protocols (Davey, 2012). Although, using a 6-base cutter may reduce the number of missing values by cutting less frequently and thereby increasing the coverage per restriction enzyme, it also decreases the number of markers detected (Ward *et al.*, 2013; Gardner *et al.*, 2014). As pear is a highly heterozygous species we detected a large number of SNPs, even when using a less frequent cutter than others.
In this study, we increased the number of mapped markers nearly tenfold by using the GBS approach compared with using the SNP array.

The GBS analysis was performed by mapping sequence reads to both the 'Bartlett' v1.0 and Chinese genome assemblies. As we used a European pear segregating population for GBS, it was not surprising that we obtained more SNPs from the read mapping against 'Bartlett' than against 'Suli'.

Genotyping-by-sequencing increased both the number of markers on the genetic maps and the alignment of the pear genome to the maps. Indeed, we increased the percentage of 'Bartlett' v1.0 genome anchored to the 17 *Pyreae* LGs from around 30% (Chagné *et al.*, 2014) to more than 40%, however the percentage of anchoring remains low. Most scaffolds anchored to the GBS genetic map are greater than 200kbp, while only a few small scaffolds are anchored, indicating that adding still more SNPs to the genetic map will not increase the percentage of anchoring. Instead, longer scaffolds are needed to improve the genome anchoring to the existing maps. The low anchorage may limit the detection of positional candidate genes on the pear genome in this study. However, as apple and pear are closely related genera, positional candidate genes may be identified using the better studied, syntenic apple genome.

2.5 Conclusion

I developed high density genetic maps using both a SNP array and a GBS approach. This represents an important step for the discovery of chromosomal regions associated with commercially important horticultural traits, such as pest and disease resistance, orchard productivity, and fruit quality in pear production.

I have demonstrated the usefulness of the Infinium® II array for high throughput genotyping in a *P. communis* breeding population. The SNPs were shown to be transferable between the two closely related genera *Malus* and *Pyrus*. The map developed with GBS increased both the number of mapped markers and the percentage of the 'Bartlett' genome scaffolds aligned to the consensus map comprising both array and GBS data points.

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3 GENETIC CONTROL OF PEAR ROOTSTOCK-INDUCED DWARFING AND PRECOCITY IS LINKED TO A CHROMOSOMAL REGION SYNTENIC TO THE APPLE *DW1* LOCI

3.1 Abstract

We have identified quantitative trait loci (QTLs) controlling vigour and precocity in *Pyrus communis*, using a segregating population of 405 F1 *P. communis* seedlings from a cross between 'Old Home' and 'Louise Bonne de Jersey' (OHxLBJ). The seedlings were grafted as rootstocks with 'Doyenne du Comice' scions and comprehensively phenotyped over four growing seasons for traits related to tree architecture and flowering, in order to describe the growth of the scions. A high density single nucleotide polymorphism (SNP)-based genetic map comprising 597 polymorphic pear and 113 apple markers enabled the detection of QTLs influencing expression of scion vigour and precocity located on linkage groups (LG)5 and LG6 of 'Old Home'. The LG5 QTL maps to a position that is syntenic to the apple 'Malling 9' ('M9') *Dw1* locus at the upper end of LG5. An allele of a simple sequence repeats SSR associated with apple *Dw1* segregated with dwarfing and precocity in pear and was identified in other pear germplasm accessions. The orthology of the vigour-controlling LG5 QTL between apple and pear raises the possibility that the dwarfing locus *Dw1* arose before the divergence of apple and pear and that it might be present in other Rosaceae species.

3.2 Introduction

Commercial apple (*Malus* x *domestica* Borkh.) production relies on the use of dwarfing rootstocks to reduce scion vigour and promote early flowering in young trees (Lockard and Schneider, 1981; Webster, 2002). However, the closely related pear (*Pyrus communis* L.) lacks comparable dwarfing *Pyrus* rootstocks, which makes the cultivation of pear currently less profitable than apple. To develop a series of pear rootstocks, it is necessary first to develop an understanding of the mechanisms involved in vigour control and precocity in pear and the genetic determinants of the desired traits.

The physiology of rootstock-induced dwarfing in fruit trees is not fully understood and a number of mechanisms have been suggested to influence dwarfing in perennial fruit tree crops in general. Yonemoto *et al.* (2004) observed that mandarin scions grafted onto rootstocks had a lower sap flow rate and higher soluble solid content than non-grafted trees and Basile *et al.* (2003) found that the daily extension growth of shoots of a peach scion grafted on a semi-dwarfing rootstock was related to the dynamics of stem water potential. In citrus, Lliso *et al.* (2004) found significantly higher concentrations of carbohydrates in fruit and roots of trees on dwarfing rootstocks than on more vigorous ones, suggesting that dwarfing rootstocks promote heavier flowering and crop load and thereby reduce vegetative growth. In apple, research has focused on water and nutrient restriction at the graft union, as well as a reduction of auxin movement from the scion to the rootstock (Lockard and Schneider, 1981; Jones, 1984; Soumelidou *et al.*, 1994; Webster, 1995a; Atkinson *et al.*, 2003; van Hooijdonk *et al.*, 2010). Foster *et al.* (2014) observed that key flowering genes from the *Flowering Time* (*FT*) locus family were up-regulated in dwarfing rootstocks, which would promote flowering and reduce shoot extension growth. They also found several stress response genes were up-regulated and concluded that stress might be a factor in the dwarfing effect.

Recently, two major QTLs (*Dw1* and *Dw2*), which control most of the dwarfing effect conferred to the scion, have been identified in the apple rootstock 'Malling 9' ('M9') on LG5 and LG11 respectively, (Rusholme Pilcher *et al.*, 2008; Fazio *et al.*, 2014; Foster *et al.*, 2014; Foster *et al.*, 2015). This 'M9' dwarfing effect involves the reduction of the number and length of branches in the first year of growth after grafting and an increase in the proportion of floral buds (Seleznyova *et al.*, 2008; Foster *et al.*, 2014). However, in pear no QTL has been identified that controls tree productivity traits and no genetic analysis has been carried out on rootstocks, although several QTLs have been identified that control traits such as pest and disease resistance (Dondini *et al.*, 2004; Pierantoni *et al.*, 2007b; Won *et al.*, 2014), leaf morphology (Sun *et al.*, 2009), and fruit quality traits (Zhang *et al.*, 2012a; Saeed *et al.*, 2014; Wu *et al.*, 2014).

As pear and apple are closely related species within the Rosaceae family (Potter *et al.*, 2007b), and their genomes exhibit a high degree of synteny (Yamamoto *et al.*, 2004; Celton *et al.*, 2009a; Celton *et al.*, 2009b; Chagné *et al.*, 2014), we hypothesized that orthologous loci might occur in both pear and apple that are responsible for the control of scion growth conferred by rootstocks. In the present study, we tested this hypothesis using a segregating population of 405 seedlings from a *P. communis* 'Old Home' x 'Louise Bonne de Jersey' cross grafted with 'Doyenne du Comice' ('Comice') scions and phenotyped for precocity and scion growth (vigour). We present the results for a QTL analysis of these traits using a high density genetic map based on single nucleotide polymorphism (SNP) markers anchored to the 'Bartlett' v1.0 European pear genome assembly (Chagné *et al.*, 2014).

3.3 Materials and Methods

3.3.1 Segregating population

A cross was made between *Pyrus communis* L. 'Old Home' and 'Louise Bonne de Jersey' (OHxLBJ), resulting in a segregating population consisting of 421 F1 seedlings. The seedlings were grown in the glasshouse for three months and planted out into the Plant & Food Research orchard in Motueka, New Zealand (41°6'S; 172°58'E). After two months of acclimatisation, the seedlings were summer budded with 'Comice' (*Pyrus communis* L.). In the following spring when the trees were cut down to the bud, grafts from the shoots removed from the OHxLBJ seedlings were inserted onto *Pyrus calleryana* seedling rootstocks to provide leaf material for DNA extraction. As controls, fifty clonal *Cydonia oblonga* 'Quince C' (QC) rootstocks grafted with 'Comice' were systematically distributed throughout the orchard block to give some indication of the variation in growing conditions across the block. The trees were planted into three rows, each containing 157 trees, including the QC controls, with a spacing of 0.8m within the row and 3.3m between the rows. Of the original 421 seedlings, propagation of scions failed on 16 trees, leaving 405 for phenotyping. To avoid any horticultural influence on tree shape and vigour, the trees were neither pruned nor trained. Once the trees began to flower and crop, all fruit were removed from the trees each season after first drop to avoid biennial bearing, bending of the branches (to prevent increasing precocity) and a confounding effect of the crop on tree vigour. Drip irrigation, fertilisation and pest and disease control were carried out; woven plastic mat was laid down to repress weed growth.

3.3.2 Architectural measurements and inflorescence assessment

Scions were phenotyped for architectural traits for the first four years of growth after grafting (years 1- 4) (Table 1). Detailed architectural measurements were taken after growth cessation (June/July) in years 1-3, including trunk cross-sectional area (TCA) 20cm above the graft union; length of main axis (length taken for each new growing cycle); and number of branches and spurs (short shoots <2.5cm) (Table 1). Branches were classified as either sylleptic shoots, which extend in the same year they are initiated, or proleptic shoots, which extend after winter dormancy (Hallé *et al.*, 1978). In year 3, the tree canopies were visually categorised as being small, moderate or vigorous, using QC controls as models for moderate tree growth. An example for the three vigour classifications can be seen in Figure 1. The presence or absence of root suckers was recorded in the third year.

Table 4: Architectural measurements taken over the first four years of growth after grafting the OHxLBJ pear rootstock segregating population and Quince C (QC) controls with scions of 'Comice'. TCA: trunk cross-sectional area, spurs are short shoots (<2.5cm). The designation for the variables used for QTL analysis is indicated between brackets.

Trait	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**
Number of branches per tree (*Branches*)	x	x	x		
Total tree height (*Height*)	x	x	x	x	
Length of the new main axis growth (*LNG*)	x	x	x	x	
Number of inflorescence (*Inflorescence*)			x	x	x
Number of nodes per tree (*Nodes*)	x	x			
Number of spurs per tree (*Spurs*)	x	x	x		
TCA 20cm above graft unit (*TCAtrunk*)	x	x	x	x	
TCA of the rootstock (*TCAroot*)			x	x	
TCA secondary growth of the main axis (*TCAsec*)			x		
TCA tertiary growth of the main axis (*TCAtert*)			x		
Vigour classification (*Size*)			x		
Root suckering (*Suckers*)			x		

The first inflorescence assessment was done at the beginning of year 3 and repeated in the following two springs. No ‘Comice’ scions flowered either on the seedlings or on QC control rootstocks in year 2. In year 3, the total number of inflorescences was counted and their positions recorded; this was repeated in year 4. At the beginning of year 5, the proportions of inflorescence production were estimated according to the size of the tree, relative to the tree with the highest number of inflorescences. The trees were ranked into classes from 0-4, with 0=no flowers, 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100%.

Figure 11: Examples of the three different vigour classes of the 'Old Home' x 'Louise Bonne de Jersey' pear rootstocks grafted with 'Comice' in the second year of growth after grafting: 1) small, 2) moderate, 3) vigorous. The wires indicate the height of the trees, with the wire being 0.8, 1.3, 1.8, 2.3, 2.75 m from the ground, lowest to the top respectively.

3.3.3 Data analysis

Univariate mixed models were fitted to the data with row and a linear effect of tree position in the row as fixed effects, and genotype as the only random effect. Localized spatial trends were modelled by fitting first-order auto-correlations for tree positions (Gilmour *et al.*, 1997). The fixed effects were chosen based on an examination of the variograms when fitting the first-order auto-correlations to both row and tree position, and the auto-correlations to retain were based on likelihood ratio tests.

Having determined the optimal univariate model, it was then extended to bivariate models for every pairwise set of variates. These bivariate models allowed for separate fixed and spatial effects for each variate, and also a different genetic variance for each, as well as the genetic correlation. Predicted values from these bivariate analyses were used in the QTL analysis. Data from each year were analysed separately, in order to check whether the putative QTLs were stable across years. Residual plots were examined to check for outliers and assess the validity of the normality assumption. For all variates apart from *Branches_year2*, a square-root transformation was used to obtain a satisfactory approximation to normality. Basic statistical analysis was carried out using Minitab 16 Statistical Software (2010 Minitab Inc.). All further analysis were conducted using R 3.0.1 (R Core Development Team, 2013), and the mixed models were fitted using the asreml package version 3.0-1 (Butler *et al.*, 2007).

3.3.4 Genetic mapping and QTL analysis

DNA was extracted using a CTAB method (Doyle and Doyle, 1987), followed by purification with NucleoSpin® columns (Macherey-Nagel GmbH & Co. KG). DNA was quantified using a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific Inc.). SNP marker genotyping was performed using the apple and pear Infinium® II IRSC 9K SNP array (Chagné *et al.*, 2012; Montanari *et al.*, 2013) on 297 segregating individuals and both parents. Genomic DNA was amplified and hybridized to the apple and pear Infinium® II IRSC 9K SNP array following the Infinium® HD Assay Ultra protocol (Illumina Inc., San Diego, USA) and scanned with the Illumina HiScan. Data were analysed using Illumina's GenomeStudio v 1.0 software and genetic mapping carried out using JoinMap 3® (van Ooijen and Voorrips, 2001). A LOD score of 5 or higher was used for grouping and the genetic distance within the group was calculated using the Kosambi function. The linkage groups (LGs) were identified by aligning the parental maps of OH and LBJ to the map developed by Montanari *et al.* (2013), which contains apple and pear SSR markers from the 'Bartlett' consensus map of Celton *et al.* (2009b).

The map was drawn and aligned using MapChart v.2.2 (Voorrips, 2002). The parental genetic maps were used with raw and transformed phenotypic data of tree growth, precocity and suckering for QTL analysis employing MapQTL5 (van Ooijen, 2004). For normally distributed data, Interval Mapping (IM) followed by Multiple QTL Mapping (MQM) was performed and a permutation test (1000 permutations) was used to calculate the LOD threshold for QTL significance. ANOVA was used to calculate the percentage of the phenotypic variance explained by each QTL. When normalisation of the data failed, the Kruskal-Wallis test was used for QTL detection.

3.3.5 Identification of the dwarfing allelotype in a pear germplasm selection

The SSR marker Hi01c04, developed by Silfverberg-Dilworth *et al.* (2006) and identified as the proximal flanking marker for the *Dw1* region on LG5 of apple (Foster *et al.*, 2015) was screened over 96 individuals of the OHxLBJ population to determine the linkage phase between the QTL and the SSR alleles. PCR amplification was carried out using a modified version of the fluorescent M13 universal primer system (Schuelke, 2000) and a touchdown PCR programme with annealing temperature 60-55°C (94°C/2 min 45 s; 10 cycles: 94°C/55 s, 60°C/55s (-0.5°C per cycle); 72°C/1 min 30 s; 30 cycles: 94°C/55 s, 55°C/55 s, 72°C/1 min 30 s; 72°C/10 min). The fragments were separated using the ABI3500 sequencer, and their size analysed with GeneMarker® v 2.2.0 software (© SoftGenetics, LLC). The marker was then included in the OH map. The allele sizes were compared with those detected by screening the same SSR marker over 92 pear accessions from selections of germplasm from France, New Zealand, Germany and the USA, including OH and LBJ.

3.3.6 Finding orthologous loci in pear and apple

Apple and European pear regions were compared to identify orthologous genes using OrthoMcl2.0.3. (Li *et al.*, 2003). Synteny gene blocks were detected with OrthoCluster (Zeng *et al.*, 2008). *Pyrus* scaffolds were aligned to *Malus* scaffolds using the MUMmer 3.3 package (Kurtz *et al.*, 2004). Pear scaffolds were further filtered based on having at least two alignments, each alignment longer than 2kbp or total alignment length not shorter than 3kbp.

3.4 Results

3.4.1 Architectural measurements

Architectural measurements were taken on 'Comice' scions grafted on both the OHxLBJ segregating population and QC controls from the first to the fourth years of growth. The phenotypic variability of the raw data is illustrated in Table 5. A wide range of vigour was observed in the grafted scions as early as in the first year of growth. In total, 343 trees (89%) of the OHxLBJ population developed sylleptic shoots in year 1, of which 87 trees (25%) grew more than 10 sylleptic shoots.

After proleptic shoots developed in the second year of growth, a large variability was observed in the total number of branches, with a range of zero to 107 branches per tree. After the third year, third-order branches and spurs grew off the second-order sylleptic and proleptic branches. This branching habit was repeated in the following growing cycle, resulting in a very complex tree structure which could be ranked into three vigour classes based on overall tree size, with 55 small, 200 moderate and 148 vigorous phenotypes. Flowering first occurred at the beginning of the third year of growth after grafting for 257 individuals (63%) of the OHxLBJ population. The following spring (year 4), 398 trees flowered. In year 5, 56 of the trees (14%) did not flower, of which only five (1%) had never flowered before. Flowering occurred mainly on spurs and terminal buds, with an average of 10.5 flower clusters per tree in year 3 and 113.6 in year 4 for the OHxLBJ population.

High numbers of axillary (one-year-old lateral bud) flower clusters were found on the scions grafted onto the QC controls in year 3, with an average of 22.5 axillary buds and 116 spurs and terminal buds per tree. The trees grafted onto OHxLBJ showed only minimal axillary flowering in year 3 and year 4, with averages of 2.2 and 4.3 respectively. In total, 247 rootstocks exhibited root suckering, while 161 did not. Root suckering was detected for 38 (69%) out of 55 of the trees classified as small, 128 (64%) out of 199 moderate trees, and 77 (52%) out of 148 vigorous trees. Trees with root suckering had a significantly smaller average TCA than those without (3.65cm^2 and 4.28cm^2, respectively; $p = 0.002$).

Table 5: Phenotypic variability for scion architecture and flowering in the pear OHxLBJ segregating population and Quince C (QC) controls grafted with 'Comice'. Trunk cross-sectional area (TCA); Number of non missing values (N); Standard error of mean (SE Mean); Standard deviation (StDev); First quartile (Q1); Third quartile (Q3).

Variable	Year		N	Mean	SE Mean	StDev	Min	Q1	Median	Q3	Max
Number of branches per tree	1	OHxLBJ	385	6.8	0.2	4.6	0	3	7	10	20
		QC	49	8.4	0.7	4.9	0	5	8	11	21
	2	OHxLBJ	389	37.5	0.8	15.9	0	26	38	47	107
		QC	50	36.2	2.1	14.6	10	25	37	47	74
	3	OHxLBJ	276	60.4	2.0	32.6	0	38	59	81	169
		QC	49	42.7	3.3	23.3	7	27	39	58	108
Total tree height	1	OHxLBJ	382	127.5	1.3	25.0	14	121	134	142	183
		QC	47	110.5	3.7	25.3	52	101	118	126	157
	2	OHxLBJ	382	205.5	1.7	33.5	65	188	203	226	303
		QC	47	191.5	4.3	29.5	129	168	190	206	251
	3	OHxLBJ	379	298.4	2.6	50.4	74	270	302	332	443
		QC	46	284.8	5.7	38.4	181	260	285	318	354
	4	OHxLBJ	403	376.8	4.6	91.7	29	350	400	433	546
		QC	50	347.4	13.0	91.9	58	330	374	400	449
Length of the new main axis growth	2	OHxLBJ	388	78.0	1.2	24.5	10	60	69	97	154
		QC	50	82.1	2.5	17.8	47	70	81	91	121
	3	OHxLBJ	402	93.3	1.4	27.9	6	75	99	114	144
		QC	49	93.2	3.1	21.5	52	77	96	112	127
	4	OHxLBJ	400	96.9	0.9	18.5	11	92	101	108	127
		QC	47	90.9	2.7	18.6	18	82	94	105	116
Inflorescence	3	OHxLBJ	405	12.5	1.1	23.0	0	0	2	15	136
		QC	50	45.9	6.1	43.1	0	10	35	67	176
	4	OHxLBJ	403	117.9	4.3	85.6	0	44	110	181	458
		QC	50	119.6	8.9	62.7	0	71	114	161	293

Axillary inflorescence	2	OHxLBJ	405	2.2	0.4	7.1	0	0	0	0	63
		QC	50	22.5	4.1	28.8	0	2	11	36	123
	4	OHxLBJ	402	4.3	0.4	8.5	0	0	1	5	69
		QC	49	5.9	0.9	6.0	0	1	4	11	22
Number of nodes per tree	1	OHxLBJ	383	42.6	0.4	7.1	8	40	44	47	58
		QC	47	38.0	1.0	7.1	18	34	38	43	50
	2	OHxLBJ	386	32.6	0.4	7.4	9	27	30	40	50
		QC	50	32.8	0.8	5.8	22	28	32	38	42
Number of spurs per tree	1	OHxLBJ	385	10.1	0.3	6.8	0	5	9	14	42
		QC	49	5.6	0.6	3.9	0	3	5	9	16
	2	OHxLBJ	389	43.3	1.7	33.0	0	15	36	66	179
		QC	50	42.7	4.4	31.2	0	16	39	57	124
	3	OHxLBJ	276	197.5	5.0	83.5	15	134	195	253	557
		QC	49	242.8	14.2	99.7	56	159	238	315	454
TCA of the trunk	1	OHxLBJ	383	0.6	0.0	0.2	0	0	1	1	1
		QC	47	0.7	0.0	0.3	0	1	1	1	1
	2	OHxLBJ	387	1.7	0.0	0.7	0	1	2	2	5
		QC	50	1.9	0.1	0.6	1	1	2	2	3
	3	OHxLBJ	404	3.8	0.1	1.6	0	3	4	5	10
		QC	50	4.0	0.2	1.2	2	3	4	5	7
	4	OHxLBJ	402	5.9	0.1	2.6	0	4	6	8	14
		QC	49	5.4	0.2	1.6	2	4	5	7	10
TCA of the rootstock	3	OHxLBJ	404	7.5	0.1	2.8	1	6	7	9	18
		QC	50	3.3	0.1	1.0	2	3	3	4	6
	4	OHxLBJ	402	10.6	0.2	4.1	1	8	10	13	27
		QC	49	5.1	0.2	1.7	2	4	5	6	11
TCA secondary main axis growth	3	OHxLBJ	399	1.1	0.0	0.5	0	1	1	1	4
		QC	49	1.3	0.1	0.4	1	1	1	2	2
TCA tertiary main axis growth	3	OHxLBJ	399	0.6	0.0	0.3	0	0	1	1	3
		QC	49	0.7	0.0	0.2	0	1	1	1	1

3.4.2 Correlation between traits

The raw phenotypic data were used to look at relationships between traits. Figure 12 shows some selected correlation graphs, while the correlation matrix for all traits, with Pearson correlations (r) and their significance values, can be found in Supplementary material 3.

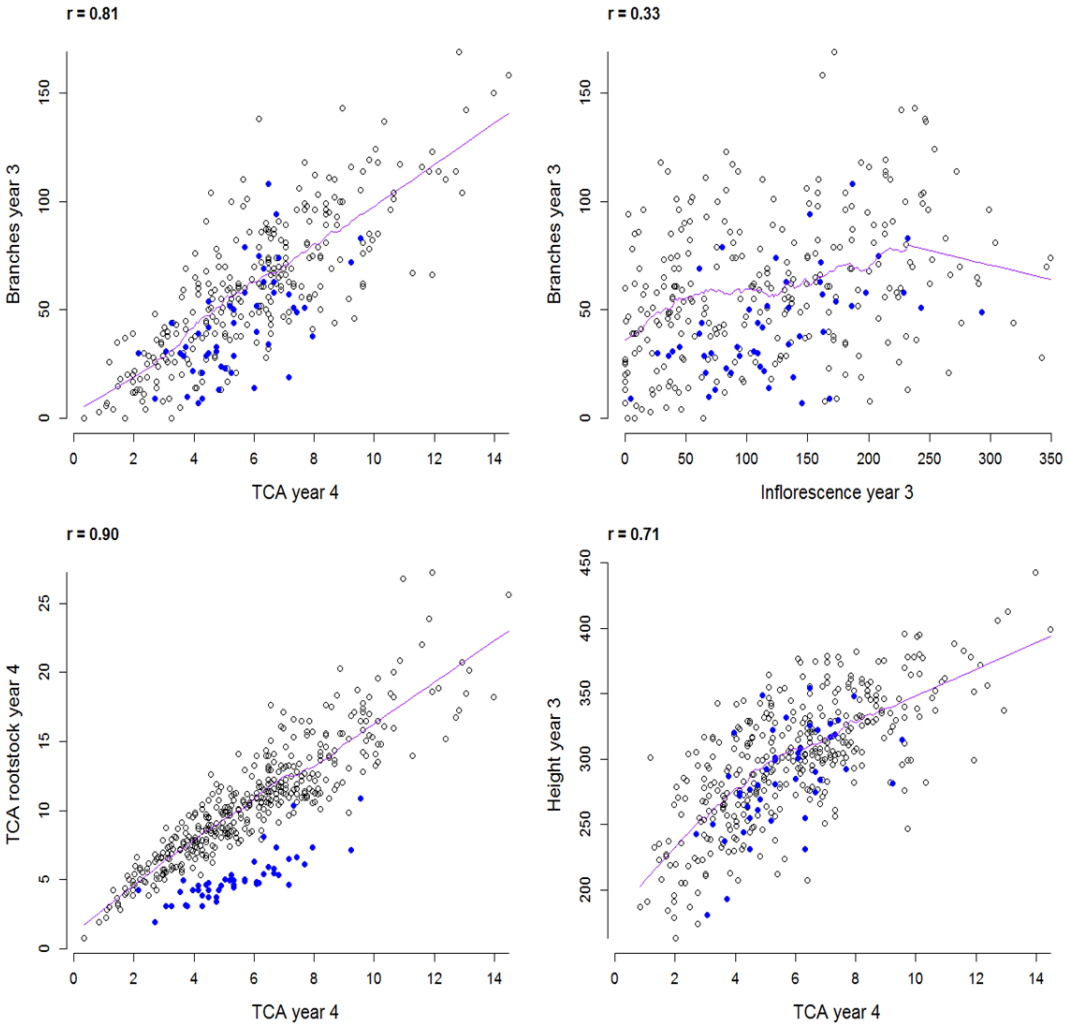

Figure 12: Scatterplots between different pear architectural and flower traits designed with RStudio. TCA: trunk cross-sectional area. Black circles represent 'Old Home' x 'Louise Bonne de Jersey' (OHxLBJ) seedlings and blue dots 'Quince C' controls. The purple line shows a "Friedman's super smoother" (span = 0.2). The correlation coefficients (shown at the top left of each plot) were calculated for the OHxLBJ values only.

A significant positive correlation (r = 0.81) was observed between *Branches_year3* and the *TCAtrunk_year4* and between the *Height_year3* and the *TCAtrunk_year4* (r = 0.71). As expected, the highest correlation (r = 0.9) was found between the *TCAtrunk_year4* and the *TCAroot_year4*, showing the consistency in the measurements.

No strong positive correlation between flowering and architectural traits was found. However, trees that flowered early (year 3) had significantly more sylleptic branches than those that did not (Chi-square = 31.49, p-value = <0.0005) (Figure 13).

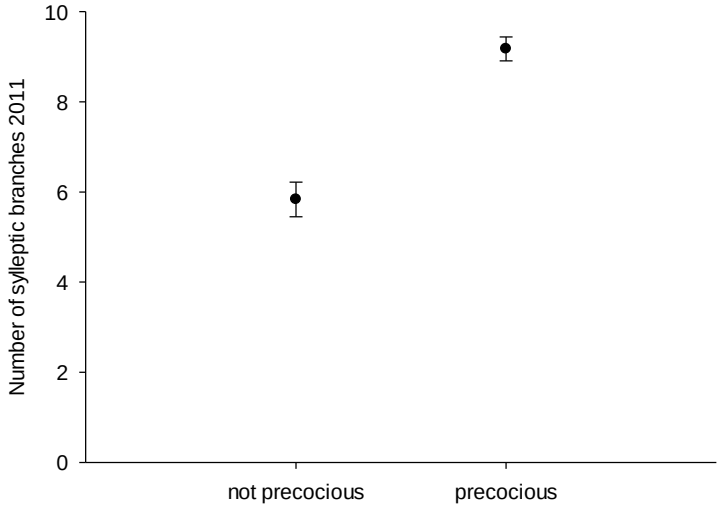

Figure 13: Interval plot of first-year (2011) sylleptic branching of the 'Old Home' x 'Louise Bonne de Jersey' (OHxLBJ) pear population comparing precocious and non precocious trees (p-value=0.000) Symbols show the mean (precocious=9.2; not precocious=5.8) and the error bars of the mean (precocious=0.38; not precocious=0.27).

The TCA showed the strongest correlation with other traits and was therefore a representative measurement for tree vigour, becoming a stronger indicator for overall tree size with each annual growth cycle (Figure 14). The variation in vigour of the scions budded onto the QC rootstocks indicates the environmental influence within the orchard.

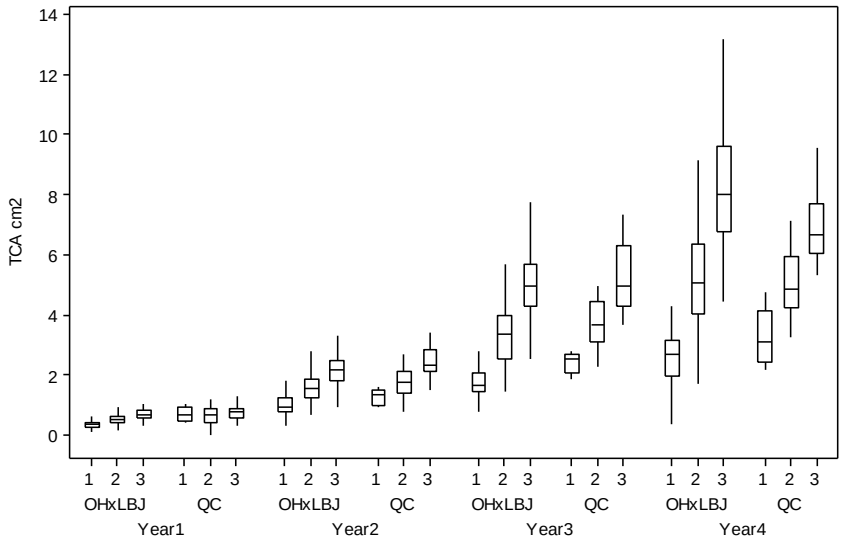

Figure 14: Box-plots of each year's trunk cross-sectional area (TCA) comparing three vigour classes 1) small, 2) moderate, 3) vigorous within the 'Old Home' x 'Louise Bonne de Jersey' (OHxLBJ) pear population and the 'Quince C' (QC) controls. Box-plot symbols show the median, Q1 and Q3, and the highest and lowest values.

3.4.3 Analysis of the phenotypic variability within the orchard and among genotypes

Positional effects within the orchard were accounted for by using three different linear mixed models: Model 1: first-order autocorrelation for both row and plant position within the row; Model 2: first-order autocorrelation for only the plant position; Model 3: no autocorrelation for both row and plant position. For *Branches_year1-3*, *Height_year1+3*, *Inflorescence_year2+3*, *Spurs_year2+3*, *TCA_year3+4*, *TCAroot_year3+4* and *TCAsec_year3*, the row and plant position auto-correlation did not improve the fit. For the *Height_year4*, *LNG_year2-4*, *Nodes_year2*, *Spurs_year1*, *TCA_year2* and the *TCAtert_year3*, the plant position improved the fit and Model 2 was used for bivariate models for QTL detection. The clonal QC controls should arguably be fitted as fixed effects. This was tested with a few key variates and the breeding values obtained were very similar to those obtained from the model described. Square root transformation was necessary to normalise the data for all traits recorded, except for *Branches_year2*.

However, some variables (*LNG_year2+3*, *Height_year1+2+3*, *Nodes_year2*, *Spurs_year1+3*, *TCA_year2* and the *Inflorescence_year2+4*) showed marked deviations from normality, even after transformation.

3.4.4 Genetic Map construction

High density genetic maps were constructed for both parents using 597 and 113 polymorphic pear and apple SNP markers (Montanari *et al.*, 2013) respectively (Table 6). The OH map consists of 17 linkage groups (LG) representing the 17 chromosomes of the pear genome. Only 16 linkage groups were obtained for LBJ, with LG17 being absent. The genetic maps of OH and LBJ were aligned with parental maps of 'Moonglow' (Moon) and PEAR1 (Montanari *et al.*, 2013) which contain SSR markers derived from apple (Supplemental material 2).

Table 6: Number of pear and apple markers in 'Old Home' (OH) and 'Louise Bonne de Jersey' (LBJ) genetic maps. LG: number of linkage groups; cM: total length of the genetic map in centiMorgans.

	Apple	**Pear**	**total**	**LGs**	**cM**
OH	58	341	399	17	913
LBJ	64	382	446	16	1044
Common	9	126	135		

3.4.5 QTL detection

QTLs were detected using the OH and LBJ parental genetic maps for the tree architecture and flowering traits across four years (Tables 7 and 8, Supplementary material 4). Significant QTLs for the control of the number of branches were detected in three successive years on LG5 and LG6 of OH. A small-effect QTL controlling *Branches_year1* was located on LG6 of LBJ and was also detected in year 3.

In the first year of growth after grafting, significant QTLs were detected for the *TCAtrunk_year1* on LG16 and 6 of OH. The LG6 QTL was confirmed in years 2, 3 and 4, whereas the LG16 QTL was not reproducible.

A QTL influencing *TCAtrunk* was detected on LG5 of OH in both years 3 and 4. Additional smaller-effect QTLs controlling *TCAtrunk*, inherited from LBJ, were detected on LG13 and LG6. QTLs influencing *LNG* were detected on OH LG5 in years 2-4 and these co-located with the *TCAtrunk* QTL. Smaller-effect QTLs controlling the *LNG* from LBJ were located on LG6 and LG7; however, only the LG6 QTL could be replicated across two years. QTLs controlling the *TCAsec_year3* and *TCAtert_year3* (only measured in year 3), *Height* and the *Spurs* per tree were detected on LG5 and LG6 of OH and LG6 and LG1 of LBJ. A QTL controlling *Size_year3* was detected on LG5 and LG6 of OH, co-locating with the *TCAtrunk* and *Height* QTLs. The architectural OH LG5 QTLs explained between 5.44% and 16.6% of the variability for *Spurs_year2* and *TCAsec_year3*, respectively. The variance explained for the OH LG6 QTLs ranked from 3.98% for *TCAtrunk_year3* to 16.42% for *Height_year3*. The highest variance explained by any LBJ LG6 QTL was 7.72% for the QTL controlling the *TCAtert_year3*, and the lowest was 4.25% for the QTL influencing *Branches_year1*. A QTL controlling *Inflorescence* phenotyped at the beginning of the third year after grafting was detected on LG5 of OH, co-locating with the tree architecture QTLs. No flowering-related QTLs were detected segregating from LBJ. A QTL controlling *Suckers_year3* was detected on LG5 of OH.

Table 7: QTLs detected for pear architectural and precocity traits for predicted and normalised (bivariate analysis, square root transformation) data coming from 'Old Home' (OH) and 'Louise Bonne de Jersey' (LBJ). LOD score indicates the genome-wide significance of the QTL *: 90%, **: 95% and ***: 99%. Percentage of the phenotypic variance explained by each QTL (% Expl.) was calculated using ANOVA. See Table 4 for an explanation of the variables.

Parent	LG	Traits	Marker with highest LOD	Marker position (cM)	LOD		% Expl.
OH	5	*Branches_year1*	ss475878191	2.2	6.70	***	10.01
OH	5	*Branches_year2*	ss475878191	2.2	3.53	***	5.48
OH	5	*Inflorescence_year4*	ss475878191	2.2	13.06	***	18.31
OH	5	*LNG_year4*	ss475878191	2.2	11.13	***	16.16
OH	5	*Spurs_ year2*	ss475878191	2.2	3.51	***	5.44
OH	5	*TCAtrunk_year3*	ss475878191	2.2	12.37	***	15.99
OH	5	*TCAtrunk_ year4*	ss475878191	2.2	11.31	***	16.42
OH	5	*Branches_ year3*	ss527788221	1.6	6.74	***	11.16
OH	5	*Height_ year3*	ss527789077	0.0	8.57	***	12.69
OH	5	*Nodes_ year1*	ss527789704	1.1	5.67	***	9.54
OH	5	*TCAroot_ year4*	ss527789704	1.1	7.89	***	12.45
OH	5	*TCAsec_ year3*	ss527789704	1.1	10.97	***	16.6
OH	5	*TCAtert_ year3*	ss527789704	1.1	10.61	***	15.23
OH	5	*TCAroot_ year3*	ss527789704	1.1	5.55	***	8.97
OH	6	*Height_ year3*	ss475883025	5.2	4.37	***	16.42
OH	6	*Branches_ year1*	ss527789305	6.5	5.29	***	7.79
OH	6	*Branches_ year2*	ss527789305	6.5	5.04	***	7.93
OH	6	*Branches_ year3*	ss527789305	6.5	4.93	***	7.24
OH	6	*Nodes_ year1*	ss527789305	6.5	2.95	**	4.72
OH	6	*Spurs_ year2*	ss527789305	6.5	5.00	***	7.48
OH	6	*TCAroot_ year4*	ss527789305	6.5	3.21	***	4.81
OH	6	*TCAsec_ year3*	ss527789305	6.5	4.37	***	6.25
OH	6	*TCAtert_ year3*	ss527789305	6.5	3.46	***	6.46
OH	6	*TCAtrunk_ year1*	ss527789305	6.5	5.08	***	7.3
OH	6	*TCAtrunk_ year3*	ss527789305	6.5	3.46	***	3.98
OH	6	*TCAtrunk_ year4*	ss527789305	6.5	4.66	***	6.71
OH	6	*TCAroots_ year3*	ss527789305	6.5	3.51	***	5.28
LBJ	6	*Branches_ year1*	ss475878560	47.8	2.58	*	4.25
LBJ	6	*Spurs_ year2*	ss475878560	47.8	2.78	*	4.68
LBJ	6	*TCAtrunk_ year4*	ss475878560	47.8	3.72	**	6
LBJ	6	*Branches_ year3*	ss527787860	60.6	2.75	*	5.27

LBJ	6	*Height_ year3*	ss527787915	59.4	2.77	*	5.65
LBJ	6	*TCAsec_ year3*	ss527789084	21	4.14	***	6.33
LBJ	6	*TCAtert_ year3*	ss527789084	21	5.05	***	7.72
LBJ	7	*TCAtert_ year3*	ss527789229	31.0	3.25	**	4.79
LBJ	16	*TCAroot_ year4*	ss527788231	56.2	3.02	**	4.2

Table 8: QTLs detected for pear architectural and precocity traits for predicted (bivariate analysis), non-normally distributed data derived from 'Old Home' (OH) and 'Louise Bonne de Jersey' (LBJ), showing the closest marker and its position on the linkage group (LG). Significance was calculated using the Kruskal-Wallis (K value) analysis. See Table 4 for an explanation of the variables.

Parent	LG	Trait	Marker with highest LOD	Marker position (cM)	K value	p-value
OH	5	*Size_year3*	ss475878225	4.0	24.6	0.0001
OH	5	*Height_year1*	ss527788221	1.6	25.1	0.0001
OH	5	*Height_year2*	ss527788221	1.6	32.2	0.0001
OH	5	*Suckers_year3*	ss527788221	1.6	20.9	0.0001
OH	5	*Inflorescence_year3*	ss527789278	0.7	46.6	0.0001
OH	5	*Height_year4*	ss527789704	1.1	37.4	0.0001
OH	5	*Inflorescence_year5*	ss527789704	1.1	60.8	0.0001
OH	5	*LNG_year2*	ss527789704	1.1	22.2	0.0001
OH	5	*LNG_year3*	ss527789704	1.1	47.7	0.0001
OH	5	*Nodes_year2*	ss527789704	1.1	22.0	0.0001
OH	5	*Spurs_year3*	ss527789704	1.1	38.4	0.0001
OH	6	*Height_year1*	ss527789305	49.0	19.2	0.0001
OH	6	*Height_year2*	ss527789305	49.0	17.6	0.0001
OH	6	*Height_year4*	ss527789305	49.0	16.4	0.0001
OH	6	*Size_year3*	ss527789305	49.0	16.1	0.0001
LBJ	1	*Height_year2*	ss475876925	54.0	11.5	0.001
LBJ	1	*Height_year4*	ss527789822	32.1	9.4	0.005
LBJ	6	*TCA_year2*	ss475876015	37.3	12.5	0.0005
LBJ	6	*Height_year4*	ss475878560	12.8	13.0	0.0005
LBJ	6	*LNG_year2*	ss527787800	41.7	16.2	0.0001
LBJ	6	*Spurs_year3*	ss527787800	41.7	17.6	0.0001
LBJ	6	*Height_year2*	ss527787860	0.0	12.0	0.001
LBJ	6	*LNG_year3*	ss527788212	27.8	8.4	0.005
LBJ	6	*Nodes_year2*	ss527788579	41.7	14.0	0.0005
LBJ	6	*Height_year1*	ss527789592	9.3	12.1	0.001
LBJ	7	*LNG_year3*	ss527789852	35.5	13.2	0.0005
LBJ	7	*Suckers_year3*	ss527789852	35.5	11.0	0.001
LBJ	10	*Size_year3*	ss527788181	48.4	14.7	0.0005

3.4.6 Synteny between apple and pear dwarfing QTLs

Alignment of the top of LG5 of the apple and pear genomes (Figure 15) showed that the OH LG5 QTL for rootstock control of architecture and flowering traits is syntenic to the dwarfing and precocity *Dw1* QTL detected in apple 'M9' rootstocks (Foster et al. 2015). The pear LG5 QTL markers with the highest LOD scores are located on scaffolds 3 and 4 on LG5 of the 'Golden Delicious' v1.0 genome (Velasco *et al.*, 2010). After filtering, 20 *Pyrus* scaffolds mapped to three *Malus* scaffolds (Scaffold3, 4 and 5) on LG5. Only alignments longer than 2kbp and with >90% identity are drawn on Figure 15. Three of the markers with the highest LOD scores for the total number of flowers (year 3) and the TCA of the trunk (year 3) were located on 'Bartlett' v1.0 Scaffold00014, and could be aligned with loci on Scaffold3 and Scaffold4 of the 'Golden Delicious' v1.0 LG5. Two other markers mapped to 'Bartlett' v1.0 Scaffold00214 and Scaffold00116, and were both aligned to Scaffold4 in 'Golden Delicious' v1.0 LG5. 'Golden Delicious' Scaffold3, 4 and 5 span approximately 1.33Mbp of the apple genome and the 20 'Bartlett' v1.0 scaffolds cover 3.45Mbp of the European pear genome in total.

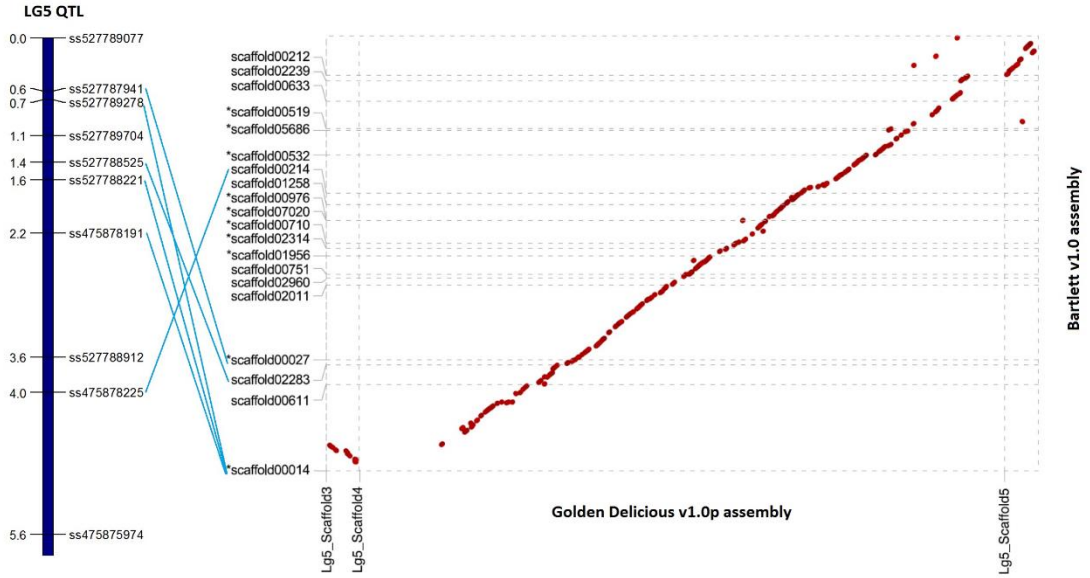

Figure 15: Alignment of the pear ('Bartlett' v1.0) and apple ('Golden Delicious' v1.0p) genomes in the orthologous QTL region. The Dot-plot shows the alignments (longer than 2kbp and with >90% identity) of the apple (X-axis) and European pear (Y-axis) genomes in the LG5 QTL region controlling vigour and precocity in apple and pear. "*" in front of the scaffold name represents the scaffold is aligned in the opposite direction.

3.4.7 Dwarfing and precocity

Architectural QTLs were mainly detected on LG5 and LG6 of OH. QTLs for the control of the total number of inflorescences co-located with the architectural QTLs on LG5 of OH. The effects of the QTLs indicate that smaller trees tended to have delayed flowering. Analysis of the genotype of the marker with the highest LOD score (ss475878191) of the LG5 QTL (Table 9) demonstrated that individuals carrying the high vigour genotype (AA) had a higher tendency for precocity, with 74% being precocious, while 50% of the individuals with the low vigour genotype (AB) were precocious. However, only 14% of the total population had the desired low vigour and precocious phenotype, with more individuals carrying the AB allelotype.

Table 9: Segregation of dwarfing and precocity among the seedlings in the OHxLBJ pear population using the LG5 SNP marker ss475878191. Low vigour individuals are represented by a trunk cross-sectional area (TCA) smaller than 3cm^2 and high vigour have a TCA greater than 4.5cm^2. The ss475878191 SNP segregates as ABxAA in the OHxLBJ population.

Year 3	TCA (cm^2)	no inflorescence	+ inflorescence
AA	<3	11 (10%)	12 (11%)
	3-4.5	10 (9%)	28 (26%)
	>4.5	8 (7%)	41 (37%)
AB	<3	40 (25%)	25 (16%)
	3-4.5	27 (17%)	34 (22%)
	>4.5	11 (7%)	21 (13%)
total population	<3	51 (19%)	37 (14%)
	3-4.5	37 (14%)	62 (23%)
	>4.5	19 (7%)	62 (23%)

3.4.8 Detection of the LG5 precocious allele in a pear germplasm set

The microsatellite marker Hi01c04 that was located within the QTL region on LG5 (Supplementary material 4) was heterozygous in both OH (116bp and 121bp alleles) and LBJ (113bp and 117bp alleles). The 121bp fragment was more frequent in precocious OHxLBJ segregating individuals (Table 10). This allele was also detected in 17 European pear cultivars (*P. communis* and *P. syriaca*), including *Pyrus* rootstocks such as 'Pyriam', 'Fox' and some rootstocks of the 'Old Home' x 'Farmingdale' (OHxF) series. The 116bp fragment was more frequent in OHxLBJ individuals, conferring low vigour to the scion. This allele was also detected in the dwarfing rootstock 'Pyrodwarf', also derived from an OHxLBJ population.

Table 10: Genotyping of the SSR marker Hi01c04 in pear germplasm accessions. Pear rootstock cultivars are underlined. Hi01c04 is linked to *Dw1* in apple. The alleles are represented by the fragment size amplified and analysed using the ABI377 instrument. The 116 bp allele linked to low vigour in OHxLBJ is indicated in bold. Accessions with a OHxLBJ prefix are individuals from the OHxLBJ segregating population and are presented with their vigour and precocity phenotype.

Accession	vigour/ precocity	Germplasm	Family	Allele size (bp)
'Old Home' (OH)		PFR, NZ	*P. communis*	**116**;121
'Louise Bonne de Jersey' (LBJ)		PFR, NZ	*P. communis*	113;117
OHxLBJ105	high/ early	*P. communis*	*P. communis*	117;121
OHxLBJ109	high/ early	*P. communis*	*P. communis*	113;121
OHxLBJ185	high/ early	*P. communis*	*P. communis*	113;121
OHxLBJ129	high/ early	*P. communis*	*P. communis*	117;121
OHxLBJ118	low/ early	*P. communis*	*P. communis*	**116**;-
OHxLBJ122	low/ late	*P. communis*	*P. communis*	113;**116**
OHxLBJ176	low/ late	*P. communis*	*P. communis*	**116**;-
OHxLBJ172	low/ late	*P. communis*	*P. communis*	113;**116**
'Bartlett'		Corvallis, USA	*P. communis*	113;117
'Beurre Hardy'		PFR, NZ	*P. communis*	113;-
'Beurre Hardy - Royal Red'		Corvallis, USA	*P. communis*	**116**;-
'Bishops Thumb'		PFR, NZ	*P. communis*	113;-
'Bon Chrétien d'Hiver'		Corvallis, USA	*P. communis*	115;-
'Bosc'		PFR, NZ	*P. communis*	113;-
BP 1		INRA, France	*P. communis*	112;**116**

QTLs controlling dwarfing and precocity

BP 3	INRA, France	*P. communis*	113;-
‘Brokmal’	Bundessortenamt, Germany	*P. communis*	113;121
BU 2-33	Bundessortenamt, Germany	*P. communis*	113;121
BU 3	Bundessortenamt, Germany	*P. communis*	115;127
‘Comice’	PFR, NZ	*P. communis*	113;124
‘Conference’	PFR, NZ	*P. communis*	113;**116**
‘Crimson Gem’	PFR, NZ	*P. communis*	113;124
‘Farmingdale’	Corvallis, USA	*P. communis*	113;-
‘Fox’	Corvallis, USA	*P. communis*	**116**;121
‘Fox 11’	INRA, France	*P. communis*	113;117
‘Fox 16’	INRA, France	*P. communis*	115;-
‘Joey's Red Flesh Pear’	Corvallis, USA	*P. communis*	115;-
‘Le Nain Vert’	Corvallis, USA	*P. communis*	117;133
OHxF* 40	Corvallis, USA	*P. communis*	115;122
OHxF* 112	Corvallis, USA	*P. communis*	113;121
OHxF* 198	Corvallis, USA	*P. communis*	113;121
OHXF* 230	Corvallis, USA	*P. communis*	113;121
OHxF* 266	Corvallis, USA	*P. communis*	115;118
OHxF* 267	Corvallis, USA	*P. communis*	113;121
OHxF* 288	Corvallis, USA	*P. communis*	113;121
OHXF* 333	Corvallis, USA	*P. communis*	113;121
OHxF* 340	Corvallis, USA	*P. communis*	115;123
OHxF* 361	Corvallis, USA	*P. communis*	113;121
OHxF* 40	Corvallis, USA	*P. communis*	113;121

OHXF* 51	Corvallis, USA	*P. communis*	113;121
OHXF* 87	Corvallis, USA	*P. communis*	113;121
OHxF* 9	Corvallis, USA	*P. communis*	113;121
OHxF* 97	Corvallis, USA	*P. communis*	113;121
‘Packham's Triumph’	PFR, NZ	*P. communis*	113;-
‘Patrick Barry’	Corvallis, USA	*P. communis*	**116**;-
‘Pyriam’	INRA, France	*P. communis*	113;121
‘Pyrodwarf’	Corvallis, USA	*P. communis*	114;**116**
QR 708-12	East Malling	*P. communis*	**116**;-
QR 708-2	East Malling	*P. communis*	115;-
QR 708-36	East Malling	*P. communis*	113;**116**
‘Red Bartlett’	PFR, NZ	*P. communis*	113;-
‘Red Pear’	Corvallis, USA	*P. communis*	117;-
‘Rousselet de Reims’	Corvallis, USA	*P. communis*	119;125
‘Sanguinole’	Corvallis, USA	*P. communis*	113;128
‘Verbelu’	Corvallis, USA	*P. communis*	112;117
‘Williams bon Chrétien’	PFR, NZ	*P. communis*	111;113
OSU 3-6	INRA, France	*P. betulifolia*	119;129
OPR 249	Corvallis, USA	*P. calleryana*	109;124
OPR 255	INRA, France	*P. calleryana*	125;-
OPR 264	Corvallis, USA	*P. calleryana*	117;119
Pyrus calleryana	PFR, NZ	*P. calleryana*	118;120
G28-120	INRA, France	*P. nivalis*	113;124
G54-11	INRA, France	*P. nivalis*	113;**116**

‘Poire Branche’	INRA, France	*P. nivalis*	113;124
RV134	INRA, France	*P. nivalis*	113;-
‘Naspati’	Corvallis, USA	*P. pashia*	124;-
‘Kosui’	PFR, NZ	*P. pyrifolia*	115;125
‘Sotoorihime’	Corvallis, USA	*P. pyrifolia*	125;133
‘Eilon’	INRA, France	*P. syriaca*	113;121
B II-3-25-27'	Corvallis, USA	*P. ussuriensis*	**116**;129
‘Ping Ding Li’	Corvallis, USA	*P. ussuriensis*	131;-
‘Tse Li’	Corvallis, USA	*P. ussuriensis*	126;-
‘Lantai Jujuli’	Corvallis, USA	*P. x sinkiangensis*	114;-

* Postman *et al.* (2013) found that the ‘Old Home’ x ‘Farmingdale’ (OHxF) was actually a cross between ‘Old Home’ and ‘Bartlett’.

3.5 Discussion

Tree architecture and productivity are complex traits that are expressed only after several years of growth following grafting and are influenced by both genetics and environmental factors (Barthélémy and Caraglio, 2007; Segura *et al.*, 2009a). Knowledge about genetic factors that confer rootstock-induced dwarfing of the scion in fruit trees is limited because of the difficulty in generating large segregating rootstock populations, as well as the requirement for robust and time-consuming phenotyping across multiple years, and the construction of dense genetic maps. Our systematic attention to these factors has enabled us to identify QTLs that control vigour and precocity in the grafted scion in pear rootstocks for the first time.

3.5.1 TCA is a strong indicator of tree vigour

TCA was found to be a strong indicator for overall tree size in 'Comice' scions grafted to our seedling rootstocks, first becoming evident in year 2 and becoming stronger with each successive annual cycle, as previously reported for apple (Westwood and Roberts, 1970). High correlations between the TCA and other vegetative growth traits, such as the height of the tree and the number of branches, substantiate our finding.

3.5.2 QTLs controlling precocity and vigour

QTLs controlling both precocity and architectural traits were located on LG5 and LG6 of OH and LG6 for architectural traits segregating from LBJ. These QTLs are considered robust, as they were detected for successive years in the same position and often with the same marker having the highest LOD score. The co-location of all QTLs on LG5 and LG6, respectively, of OH indicates that the overall dwarfing effect is controlled by at least two loci.

The variance explained by each QTL is low (4-18%) indicating that the traits under investigation may be controlled by more loci than identified in this study. Furthermore, a strong environmental influence and a lack of replicates hampered the detection of larger effect loci controlling precocity and architectural traits.

A small effect QTL for root suckering was detected on LG5 in the same genomic region as the architecture QTLs. Rootstocks of 69% of the trees classified as dwarf produced suckers, indicating a strong correlation between dwarfing and suckering, and suggesting that the same physiological mechanisms might control both traits. We hypothesise that a reduced auxin transport from the scion to the roots, induced by *Pyrus* rootstocks, may promote root suckering and reduce scion vigour. This hypothesis is consistent with the findings that polar auxin transport in the xylem parenchyma inhibits suckering (Farmer, 1962; Wan *et al.*, 2006), and that a reduced auxin transport in the rootstock stem occurs in dwarfed apple trees (Soumelidou *et al.*, 1994; van Hooijdonk *et al.*, 2010).

3.5.3 Synteny between apple and pear QTLs controlling scion vigour

The QTL controlling tree architecture and flowering on pear LG5 is in a genomic region orthologous to that of *Dw1*, which is the major locus for dwarfing conferred on apple scions by the 'M9' rootstock (Rusholme Pilcher *et al.*, 2008; Foster *et al.*, 2015). These results are consistent with our hypothesis that orthologous loci in apple and pear control scion growth and precocity conferred by the rootstock. We found that the proximal marker flanking the *Dw1* locus in apple also segregates for dwarfing and precocity in pear, and that the 116bp pear allele linked to low vigour in the rootstock mapping population is carried by the dwarfing pear rootstock 'Pyrodwarf'.

Our findings raise the possibility that the apple dwarfing locus *Dw1* and the OH LG5 QTL are derived from the same source, and therefore probably existed before the divergence of apple and pear. The conservation of synteny for QTLs involved in tree architecture has enabled us to align the genomic regions of interest.

This will facilitate the identification of candidate genes for dwarfing in both species and enable the testing of our hypothesis of a common origin for this locus, either before the *Pyrus-Malus* speciation or due to hybridization. Indeed, *Pyrus* and *Malus* are known to infrequently cross-hybridize (Crane and Marks, 1952; Fischer *et al.*, 2014) and *P. communis* is sympatric with *M. sylvestris*, a related species that contributed genetically to the ancestry of modern *M.* x *domestica* (Yamamoto and Chevreau, 2009; Velasco *et al.*, 2010; Cornille *et al.*, 2012; Cornille *et al.*, 2013), including the dwarfing rootstock 'M9'. It is interesting to note that a second pear QTL corresponding to apple *Dw2* was not detected on LG11 in the present study. Phenotypic analysis of scions grafted to segregating rootstock populations has demonstrated that the combination of *Dw1* and *Dw2* confers the greatest degree of rootstock-induced dwarfing in apple rootstocks (Fazio *et al.*, 2014; Foster *et al.*, 2015).

3.5.4 Tree size and precocity

We found that precocious and non-precocious trees differed significantly in the number of sylleptic branches grown in the first year, with a lower sylleptic branch number correlating with a delay in flowering. Although studies in pear indicate that the scion cultivar has a greater influence on sylleptic shoot formation than the rootstock (Tadeusz, 2004; Milošević and Milošević, 2010), Watson *et al.* (2012) found that both the rootstock and the scion influence the number of first-year sylleptic branches. In this study we have confirmed the influence of the rootstock on early branch development in pear; however, we could not evaluate the effect of the scion. Watson *et al.* (2012) also found that increased flowering did not result in early growth reduction in pear, and they suggested that the difference between the apple and pear rootstock dwarfing effects might lie in the length of the juvenile period. The QTL conferring reduced sylleptic branching, tree size and TCA in pear co-locates with the QTL on LG5 conferring precocity. However, the effects of the detected QTLs are in *trans*, unlike the situation in apple, meaning that a smaller tree takes longer to flower.

In terms of breeding, this means that it is difficult to breed for rootstocks conferring both reduced tree size and precocity to the scion cultivar. However, 14% of the trees in our segregating rootstock population did exhibit low vigour and precocity in their grafted scions. Assuming the application of marker assisted selection (MAS) on the basis of the ss475878191 AA allelotype only 11% of the individuals would have the desired low vigour and precocious phenotype. However, selection for the AB allelotype would result in a slightly higher percentage (16%) of individuals with both desired traits in the progeny. Hence, selecting for the ss475878191 AB allele would increase the proportion of rootstocks in a breeding population that confer both precocity and a reduced vigour to the scion. However, the percentage of rootstocks conferring low vigour and precocity was generally low (14%).

It is noteworthy that in this respect, our findings for the pear rootstock QTLs differ from the effects of the apple QTL on LG5 which confers both precocity and a reduced tree size. The dwarfing effect of 'M9' apple rootstocks is correlated with an increase in the proportion of floral buds relative to vegetative buds and sylleptic shoots that develop within the first year of growth (Seleznyova *et al.*, 2008; Foster *et al.*, 2014). The up-regulation of key flowering genes in 'M9' rootstocks may be responsible for this shift from vegetative to floral development. As a high proportion of axillary floral shoots leads to reduced vegetative growth in the next growth cycle, the tree becomes more dwarfed over time. A substantial difference between flowering in apple scions grafted onto 'M9' and the pear scions in this study was the position of the floral buds on the developing tree. In apple, flowering occurs in axillary and terminal buds, and, as the tree ages, is more common on two-year-old spurs. In pear, flowering mostly occurs on two-year-old spurs and terminal buds. This biological difference has profound implications to the subsequent development of tree architecture. Apple scions grafted onto 'M9' flower at the beginning of the second year of growth after grafting, while pear scions on pear rootstocks do not flower until the third year of growth and thus have a longer period of vegetative growth before flowering.

In pear, an increase in branching in years 1 and 2 would generate the potential for more spurs for flowering in year 3, and hence the relationship between early vegetative vigour and flowering would be opposite to that observed in apple. No QTL was detected for the percentage of axillary inflorescence in this study. It might be predicted that crossing a rootstock that increases the number of axillary buds on the scion cultivar with OH might result in a dwarfing pear rootstock, similar to 'M9', conferring both low vigour and precocity to the scion.

3.6 Conclusion

In this study we detected the first pear rootstock QTLs associated with control of architectural and flowering traits in scions. Furthermore, we found that these orthologous loci control scion growth and precocity in apple and pear rootstocks. These findings will facilitate the identification of candidate genes for control of scion traits by rootstocks in both species. Future research may focus on finding the common origin for the dwarfing locus in apple and pear. The application of our results in pear rootstock breeding may assist developing a marker for MAS for breeding a pear rootstock that confers both vigour control and precocity to the grafted scion cultivar.

3.6.1 Acknowledgements

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4 QUANTITATIVE TRAIT LOCI MAPPING FOR VEGETATIVE PROPAGATION TRAITS IN EUROPEAN PEAR ROOTSTOCKS (*PYRUS COMMUNIS* L.)

4.1 Introduction

Vegetative propagation is widely used in horticultural crops to multiply established cultivars. It avoids sexual outbreeding and overcomes sterility, and unlike seedling propagation it ensures uniformity in growth and cropping (Hartmann *et al.*, 1990). Vegetative propagation is possible as plants are able to produce adventitious roots (AR), which are roots growing from plant parts other than the root apical meristem. Some plants produce ARs naturally from preformed roots, however in other plants they must be induced by stress such as wounding (Hartmann *et al.*, 1990). This results in the activation of previously differentiated cells to meristematic cells, followed by the induction phase for root initiation, which leads to cell division, the formation and elongation of root primordia, and finally root elongation and the growth of vascular connections (Hartmann *et al.*, 1990; De Klerk *et al.*, 1995; De Klerk *et al.*, 1999). Endogenous auxin plays a major role in AR growth, activating the differentiated cells and stimulating the primordium growth (Legue *et al.*, 2014). Application of exogenous auxin in the form of Indole-3-butyric acid (IBA) is a widely used practice to improve the propagation success in many difficult-to-root cultivars (Diaz-Sala *et al.*, 1996; De Klerk *et al.*, 1999; Fett-Neto *et al.*, 2001). Callus (the mass of parenchyma cells) often appears concurrently with adventitious roots. Even though rooting and callus have been thought to be independent processes (Hartmann *et al.*, 1990), it has been shown that ARs can develop out of callus tissue in species that are difficult to propagate vegetatively (Girouard, 1967; Cameron and Thomson, 1969).

There are many factors influencing the ability of a cutting to develop roots via vegetative propagation, starting with the physiological state and treatment of the mother plant (age, health, management), the state of the propagation material (length, thickness, number of nodes, etiolation, position along a shoot), the treatment of the cutting (storage, auxin treatment) and the propagation conditions (humidity, temperature, soil, light) (Hartmann *et al.*, 1990; Webster, 1995b). Optimising the conditions for vegetative propagation can enhance the strike rate of some species. However, many fruit tree rootstocks exhibit difficulty in development of adventitious roots, even after wounding and when treated under optimal conditions (Webster, 1998).

While a number of genes related to rooting have been identified in *Arabidopsis thaliana* (Willemsen *et al.*, 1998; Sorin *et al.*, 2005; Sorin *et al.*, 2006; Okushima *et al.*, 2007), little is known about genes involved in AR formation in woody plants. An apple gene up-regulated during the induction phase of AR formation, the *Adventitious Rooting Related Oxygenase* (*ARRO-1*) was identified by gene expression analysis (Butler and Gallagher, 1999). Further studies by Smolka *et al.* (2009) and Li *et al.* (2012) suggested an involvement of *ARRO-1* in auxin regulation during AR induction. However, further investigation is needed to confirm these results and identify other genes involved in rooting in woody plants.

In commercial pipfruit production, clonal scions are grafted onto rootstocks. A rootstock influences scion traits such as vigour and precocity, as well as fruit yield and quality (Jayawickrama *et al.*, 1991; Webster, 2003, 2004; Hancock *et al.*, 2008). Vigour controlling rootstocks are used to restrict the size of the scion and shorten the juvenile period in many perennial cultivars (Lockard and Schneider, 1981; Bell and Zimmermann, 1990; Itai, 2007). As a rootstock that confers the desired traits to the scion must be easily propagated, identification of the genetic factors involved in rooting ability in pear will assist greatly in improving the efficiency of pear rootstock breeding.

The genetic loci controlling complex traits can be identified using quantitative trait locus (QTL) analysis. To develop an understanding of the genetic background of vegetative propagation in perennial plants, genetic studies have been conducted for oak (Scotti-Saintagne *et al.*, 2005), poplar (Han *et al.*, 1994) eucalyptus (Borralho and Wilson, 1994; Marques *et al.*, 1999; Marques *et al.*, 2005), pine (Shepherd *et al.*, 2006) citrus (Siviero *et al.*, 2003) and apple (Moriya *et al.*, 2015) trees. No genetic study of vegetative propagation traits has been reported for *Pyrus* yet.

In the present study we investigate the genetic basis of rooting ability using a F1 segregating population of *Pyrus communis* obtained from a cross between 'Old Home' and 'Louise Bonne de Jersey' (OHxLBJ), using dormant hardwood cuttings, a frequently used, low-cost and fast method of vegetative propagation of woody perennials (Webster, 1995b, 1998).

4.2 Materials and Methods

4.2.1 Plant material and rooting assessment

A rootstock population of 421 F1 individuals from an OHxLBJ cross, previously established for genetic analysis of vigour and precocity control of pear rootstocks (Chapter 3), was used to evaluate the ease of propagation. For this purpose, shoots removed from the segregating rootstock population, grown as described in (Chapter 3), were grafted onto *Pyrus calleryana* seedling rootstocks in 2009 in order to establish mother plants for the provision of cutting material and leaf material for DNA extraction. Cutting material was collected when trees were dormant, during June/July. The trees had completed their third year of growth after grafting, when cuttings were first collected. Ten cuttings from one year old sylleptic shoots of each individual tree were collected over three successive years and stored in plastic bags at 4°C until used for the propagation assessment (0-4 days). The sylleptic shoots were cut from the primary axis of the tree to preserve as many of the proximal-most non-extended internodes as possible.

The shoots were then cut to approximately 20cm in length, from the base of each shoot, cutting above a node at the top and just below a node at the bottom. The base of each cutting was wounded by making three vertical cuts with a scalpel down the sides of the cutting, and thereby removing the bark to expose a greater surface area for absorption of rooting hormone. The basal 2cm of each cutting was dipped into a 1:1 solution of water: 10g/l indolebutyric acid (LIBA^{TM} 10000, Zelam Ltd, New Plymouth, New Zealand) for 30s to stimulate AR formation; while Greenseal^{TM} ULTRA, a pruning paint, was applied to the cut apical surface of each cutting to prevent desiccation. The prepared cuttings were placed approximately 6cm deep in a bed containing 15cm of sand in a polythene tunnel house. The bed was constantly heated to 25^{o}C with an electric heating cable system and regularly irrigated by hand. Air temperature inside the tunnel house was kept cool via fogging system. After six weeks the cuttings were evaluated for rooting ability. The absence or presence of callus and roots was recorded for all cuttings of each individuals (Figure 16). The percentage of cuttings that developed roots, callus, or both was calculated for each F1 individual and used for QTL analysis.

Figure 16: Rooting assessment in a pear segregating population. Successful rooting (left), callus development (middle) and no callus or roots (right) of three individuals from the 'Old Home' x 'Louise Bonne de Jersey' segregating population after six weeks of cultivation.

4.2.2 Data analysis

For data analysis, R 3.0.1 (R Core Development Team, 2013) and lme4 package version 1.1-7 (Bates *et al.*, 2012) were used to account for the effect of the genotype and the year on vegetative propagation traits in a linear mixed effects analysis. Best linear unbiased prediction (BLUP) and best linear unbiased estimator (BLUE) analysis were used to account for the year effect, taking year as a random and fixed effect, respectively. The genotype was added as another random effect into both models. Residual plots were visually examined to reveal any obvious deviations from homoscedasticity or normality. Likelihood ratio tests of the full model with the year/genotype effect against the model without the year/genotype effect were used to obtain p-values.

4.2.3 Linkage map construction and QTL analysis

A genetic map was constructed for both parents separately using a 9K Illumina SNP array as previously reported in Montanari *et al.* (2013) and chapter 2. The female (OH) map consists of 399 markers located on 17 linkage groups (LGs) with a total distance of 913cM. The male (LBJ) map comprises of 446 markers spanning 1044cM on 16 LGs.

QTL detection was carried out using the Kruskal-Wallis test, Interval Mapping (IM), Multiple QTL Mapping (MQM) and permutation test (1000 permutations) with MapQTL5 (van Ooijen, 2004). Minitab® version 16.1.1 was used for plotting phenotypic data and QTL results. For QTLs derived from the same loci in both parents, allelic effects were calculated using phenotypic means (μ) corresponding to the phenotypic classes ac, ad, bc and bd , form a abxcd cross. Female additivity was calculated with $A_f = [(\mu_{ac} + \mu_{ad}) - (\mu_{bc} + \mu_{bd})]/4$, male additivity with $A_m = [(\mu_{ac} + \mu_{bc}) - (\mu_{ad} + \mu_{bd})]/4$ and dominance with $D = [(\mu_{ac} + \mu_{bd}) - (\mu_{ad} + \mu_{bc})]/4$.

4.3 Results

4.3.1 Phenotypic variation

Of the 421 F1 individuals of the OHxLBJ population grafted onto *Pyrus calleryana*, 18 individuals died and 55 individuals did not have suitable shoots for propagation in year 1 of the experiment, so only 348 individuals were used of which 148 rooted. In year 2, 365 individuals were employed for the propagation assessment of which 107 rooted. In year 3, a proportion of the trees were removed from the orchard due to the emergence of European canker (*Neonectria ditissima*) and 299 individuals were used for the experiment, of which 210 exhibited the ability to root. Consistent rooting results (rooting or not rooting) were observed for 84 trees between year 1 and year 2, 127 trees between year 1 and year 3, and 106 trees between year 2 and year 3. Sixty-eight individuals (19%) of the progeny had a consistent rooting result (rooting or no rooting) in all three years.

Rooting was mostly associated with callus development. Only 27%, 13% and 6% of the rooted cuttings failed to show callus development in year 1, year 2 and year 3, respectively. The data for the percentage of rooting in the population was not normally distributed (Figure 17), and square root transformation did not improve normality.

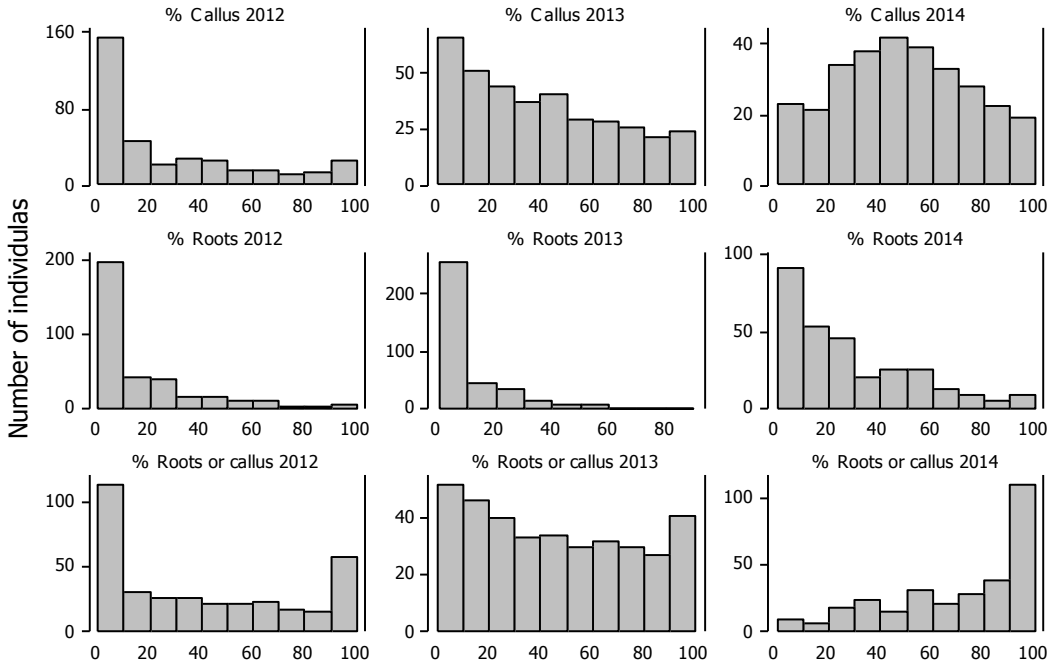

Figure 17: Frequency distribution of the percentage of 'callused', 'rooted' and 'rooted and/or callused' cuttings per individual of the 'Old Home' x 'Louise Bonne de Jersey' population. Graphs are made with non-transformed data.

There was a strong variability of the rooting successes among the three years of assessment (Figure 18, A). Especially year 3 differs from the two other years substantially showing a higher success of rooting. For this reason we accounted for the year effect by calculating BLUE and BLUP values taking the year effect as random and fixed effect, respectively. The values obtained by BLUE and BLUP analysis were almost identical (p-value = 1.0; correlation coefficient = 1.0) and therefore only the predicted BLUP data was used for further analysis.

The likelihood ratio test showed that both the year ($x^2(1) = 131.2$, p-value = 0.000) and the genotype affected rooting ($x^2(1) = 40.2$, p-value = 0.000). While the genotype and the year have a very similar variability (Standard Deviation (Std.Dev.: genotype = 9.5; year = 8.7) there was a strong variability (residual = 17.3) that was not due to genotype or year. The genotype affects the growth of callus ($x^2(1) = 6.5$, p-value = 0.01) less than the year affects callus growth ($x^2(1) = 72.6$, p-value = 0.000) and the variability of the year (Std.Dev. = 10.2) is stronger than the variability of the genotype (Std.Dev. = 8.526). However, the residual variability (Std.Dev. = 27.3) was again twice as high as both genotype and year variability. The growth of roots and/or callus was found to be equally strongly affected by both genotype ($x^2(1) = 33.6$, p-value = 0.000) and year ($x^2(1) = 165.8$, p-value = 0.000), and the variability was again strongest for residuals (Std.Dev. = 28.9) followed by year (Std.Dev. = 16.8) and genotype (Std.Dev. = 14.9). Taking the year and genotype into account as random effects increased normality in the data and the predicted data was used for QTL analysis (Figure 18, B).

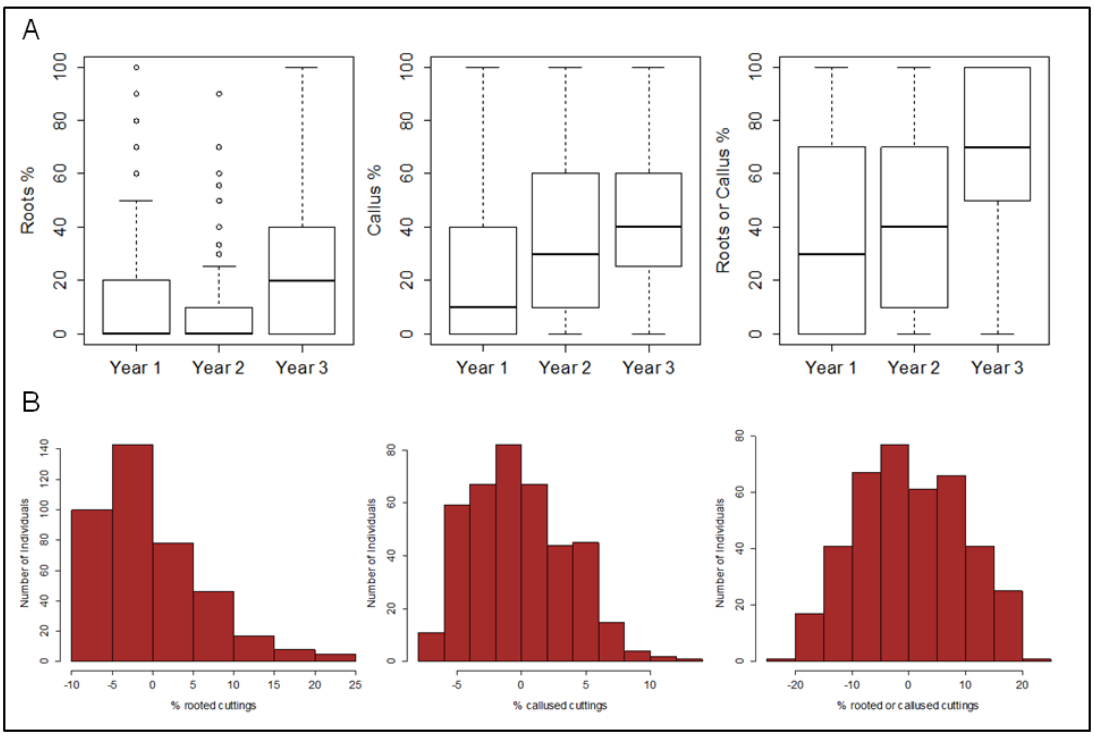

Figure 18: A) variability between years and B) BLUP distribution graph of 'rooted', 'callused' and 'rooted and/or callused' individuals.

4.3.2 QTL detection

QTLs associated with the percentage of rooted cuttings were detected on LG7, LG8 and LG10 of OH, and LG7 and LG15 of LBJ (Table 11). A QTL controlling root or callus development was detected on LG7 of both LBJ and OH. Callus development was found to be controlled by a single QTL on LG15 of OH, while no such QTL was detected for LBJ (Figure 19).

Table 11: QTLs detected for vegetative propagation traits derived from 'Old Home' (OH) and 'Louise Bonne de Jersey' (LBJ), showing the marker with the highest LOD score and its position on the linkage group (LG).

Parent	Trait %	LG	Position	Marker with highest LOD score	K-value	p-value	LOD		% Expl.
LBJ	Roots	7	0	ss527789100	11.3	0.001	3.6	***	5.8
LBJ	Roots	15	0	ss527789764	15.6	0.0001	3.43	***	5.5
LBJ	Roots or callus	7	0	ss527789100	11.3	0.001	2.81	*	4.5
OH	Roots	10	24	ss527787893	14	0.0005	3.4	***	5.5
OH	Roots	7	15.8	ss527788659	14.4	0.0005	3.19	**	5.1
OH	Roots	8	2	ss527787780	11.4	0.001	3.12	**	5
OH	Callus	15	67.6	ss527789869	12.5	0.0005	2.76	*	4.5
OH	Roots or callus	7	15.8	ss527788659	11.4	0.001	2.84	**	4.6

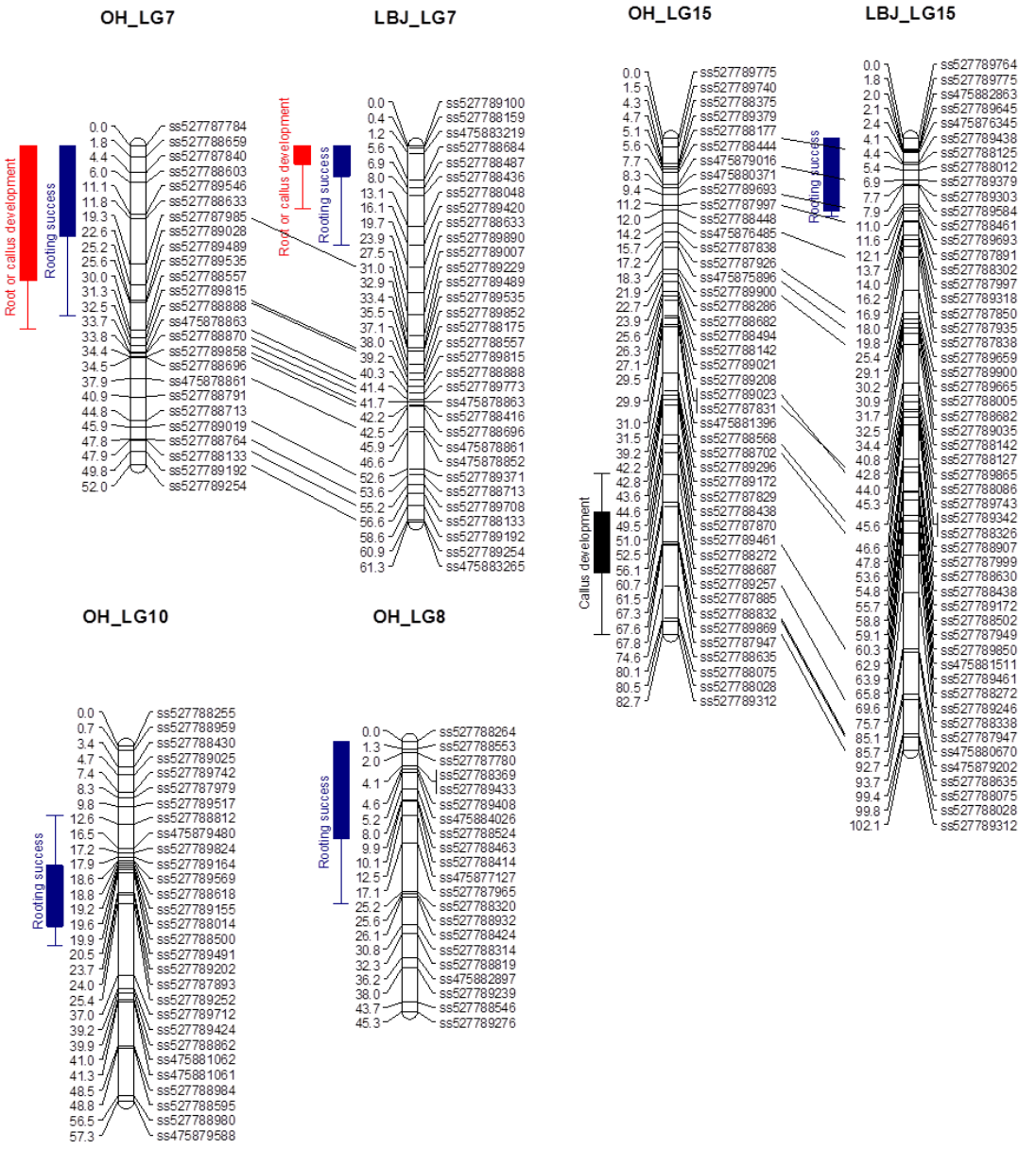

Figure 19: Identified QTLs controlling adventitious rooting and alignment of linkage groups from 'Louise Bonne de Jersey' (LBJ) and 'Old Home' (OH). The markers are named using the NCBI dbSNP accessions and their positions are indicated in centiMorgan. The linkage group (LG) numbering system is consistent with the apple LG numbering.

The LG7 QTLs associated with rooting are located in homologous regions at the top of LG7 of both OH and LBJ. For the marker with the highest LOD score for the OH LG7 QTL (ss527788659), the AB genotype has a higher tendency to root in all years when compared with the AA genotype (Figure 20A). For the LBJ LG7 QTL, the AA genotype of the marker with the highest LOD score (ss527789100) is associated with a higher tendency to root in all years of assessment (Figure 20A). The allelic effect for the LG7 marker from both parents was calculated and showed male and female additive and dominance effects for all years and the BLUP data (Table 12). These additive and dominance effects are confirmed in Figure 20B where the *bc* genotype has the highest propensity to root across years.

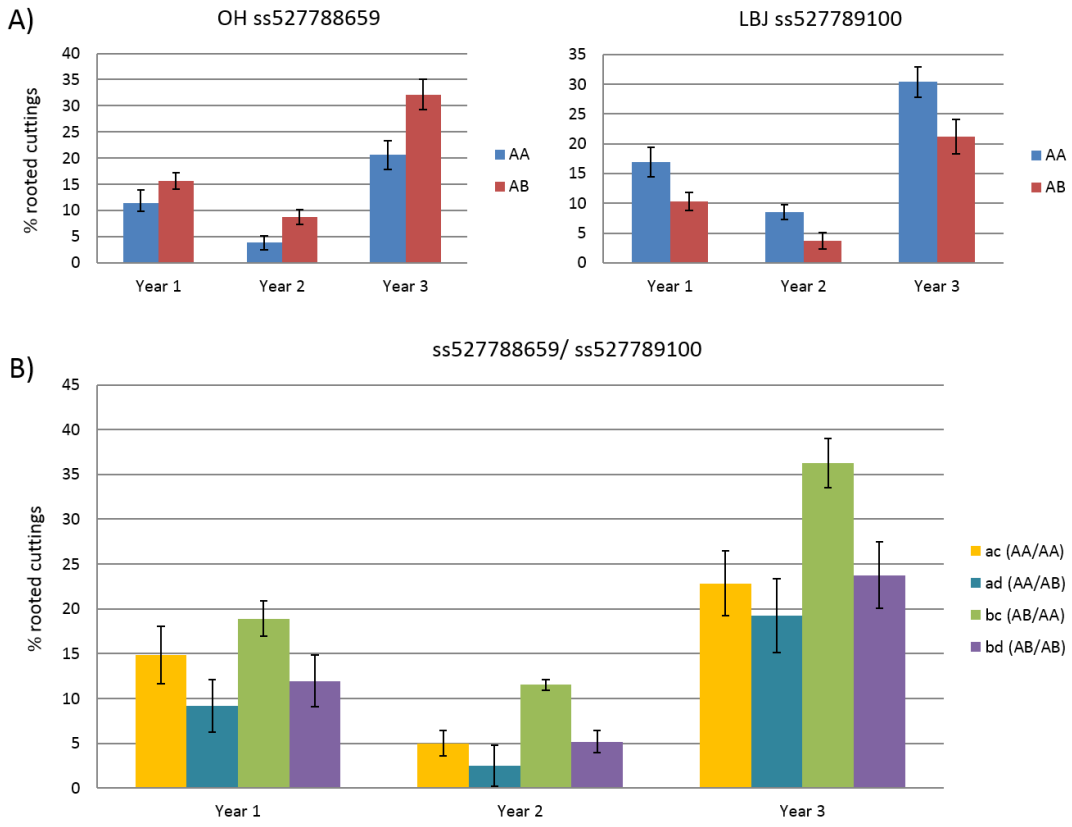

Figure 20: Bar charts of the allelotypes of the LG7 QTL markers controlling the percentage of rooted cuttings per individual from 'Old Home' (OH) and 'Louise Bonne de Jersey' (LBJ). Bars show the mean and the error bars show the standard error of the mean.

Table 12: Allelic effects for the LG7 QTL for estimated means of the percentage of rooting from both 'Old Home' and Louise Bonne de Jersey'. Female additivity (A_f), male additivity (A_m) and dominance (D).

	A_f	A_m	D
Year 1	-1.71	3.15	-0.32
Year 2	-2.3025	2.2025	-0.9575
Year 3	-4.4775	4.0275	-2.2175
BLUP	-1.225	1.36	-0.535

Results for allelotype vs phenotype comparisons for all remaining QTLs, for each year of assessment are shown in Figure 21. In OH the marker ss527787893 (LG10) exhibited a homozygous genotype (AB) linked to a slightly higher rooting rate for year 1 and year 3. A higher percentage of callused cuttings can be associated with the homozygous AA genotype of the marker ss527789869 (LG9, OH) in year 2 and year 3. The heterozygous genotype (AB) of marker ss527789859 (LG8, OH) is linked to a higher percentage of cuttings that produce roots or callus in all years. For LBJ the marker ss527789100 (LG7) shows homozygous (AA) genotypes linked to more root and callus development in all years, while the homozygous (AA) genotype of marker ss52779764 (LG15, LBJ) is linked to a higher percentage of rooting in year 3.

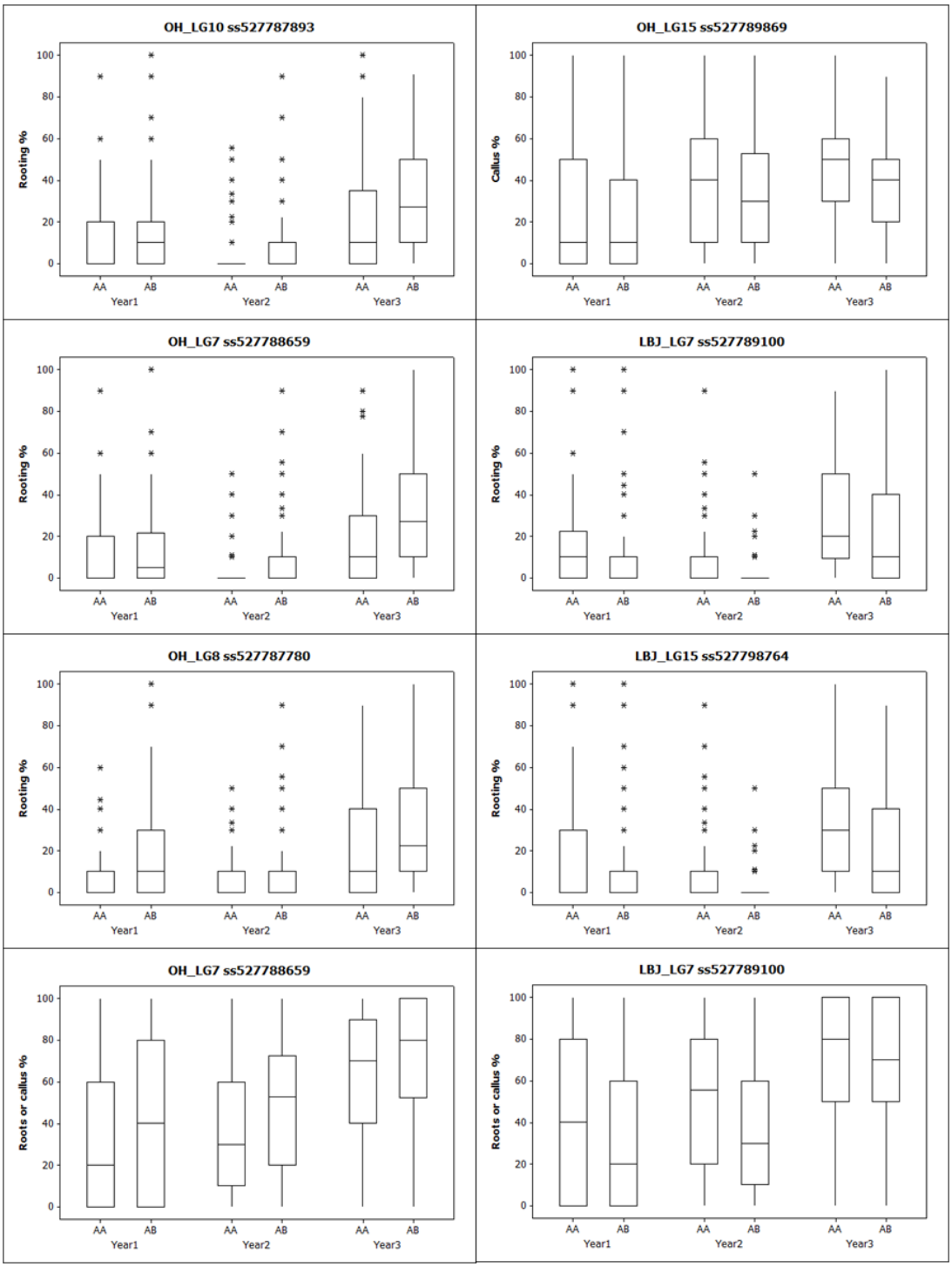

Figure 21: Highest LOD score marker for QTLs controlling vegetative propagation traits applied to three years' data. Symbols show the median, Q1, Q3 and the highest and lowest value.

4.4 Discussion

Although the ability to produce ARs is a complex trait, genetically controlled and influenced by many endogenous and exogenous factors, small effect QTLs associated with the ability of pear hardwood cuttings to develop roots have been detected in this study. The results underline the complexity of control of this trait, showing variation of the ability to root not only between individuals, but for the same individual in different years. The low number of individuals showing the same results in all three years demonstrates the sensitivity of rooting in pear cuttings to environmental conditions. Even though the assessment was repeated under similar conditions across three years and using ten replicates per genotype, small changes in the time of harvest, condition of the mother plants or handling of the samples may have influenced the results. When accounting for the genotypic and year effect in a linear mixed analysis we observed a strong variability in the data that was not due to either genotype or year. This indicates that there must be some factors affecting the growth of ARs that we did not account for in our experiment. The variation in adventitious rooting ability within species and individuals, depending on quality of the cuttings and environmental conditions, is well known. For example, a study in oak found a significant year effect on AR when repeating the experiment in four different years under the same conditions (Scotti-Saintagne *et al.*, 2005). Hence, segregating populations for QTL analysis for vegetative propagation should be large and the experiment should be repeated in different years to avoid the detection of unstable QTLs linked to this trait. For this reason, we repeated the experiment in three successive years using a large population of more than 300 segregating individuals across the three years. However, there are improvements that might be made in future propagation assessments of pear. The cuttings should be randomised to account for variability in the planting bed, such as differences in temperature. Replicates within years could be used to calculate repeatability of the experiment within the same growing season and eliminate the year effect.

Furthermore, the choice of parents might not be ideal for this experiment. OH and LBJ have both been reported as easy to propagate by hardwood cuttings (Hartmann and Hansen, 1958; Webster, 1998; Jacob, 2002). To develop a segregating population optimal for genetic studies, the parents should differ for the trait of interest. However, in this case the cross was designed to detect QTLs for dwarfing pear rootstocks (Chapter 3), and the rooting ability experiment was conducted to evaluate potential rootstocks for their vegetative propagation ability. While not ideal for the identification of large effect QTLs controlling adventitious rooting, it is important to reveal the genetic background of vegetative propagation traits in breeding populations useful for the development of new rootstock cultivars for commercial production.

Here we have identified small effect QTLs for rooting ability derived from both OH and LBJ. The analysis of the most significant QTL markers with respect to phenotypic data from all three years demonstrates that the same genotype is linked to the rooting phenotype in at least two of the three years of assessment. A QTL controlling the percentage of rooting in hardwood cuttings was detected on LG7 of OH and LBJ. We found that the additive and dominance effects of both QTLs lead to a higher percentage of rooted cuttings. When calculating the allelic effect, we confirmed both a male and a female additive effect and a dominance effect. Our results raise the possibility that a double recessive gene with recessive alleles inherited from both parents controls the ability to root.

In this study adventitious rooting was demonstrated to be controlled by small effect QTLs and explained a low percentage of variance. Our results confirm the oligogenic architecture controlling adventitious rooting in some woody perennial plants as reported previously for oak (10 QTLs 4.4- 13.8% variance explained) (Scotti-Saintagne *et al.*, 2005) and eucalyptus (7 QTLs 3.11- 6.36 % explained) (Thumma *et al.*, 2010). However, other studies have shown vegetative propagation to be controlled by a few major QTLs explaining a high percentage of the phenotypic variance. For instance, only one large effect QTL controlling adventitious rooting was detected on LG17 of the closely related genus apple (Moriya *et al.*, 2015).

Han *et al.* (1994) detected one major QTL controlling adventitious rooting in poplar with 51.1% genetic variance explained and in citrus, two QTLs (15.8 and 20.9% explained) controlling rooting traits were identified by Siviero *et al.* (2003). Similar results were obtained for eucalyptus (one QTL 66%) (Shepherd *et al.*, 2006), pine (one QTL 40%) and apple (one QTL 57.1%) (Moriya *et al.*, 2015). The difference between these studies and the first two quoted is that interspecific crosses were used in the studies detecting major QTLs and the parents were selected for low and high rooting. Shepherd *et al.* (2008) and Moriya *et al.* (2015) suggested that a species effect was responsible for strong effect QTLs.

Only one QTL associated with callus development was detected, on LG15 of OH, while QTLs associated with rooting were detected on LG7 and LG10 of OH, indicating that the formation of callus and roots are controlled by different genes. However, as the LG15 QTL is minor, we cannot yet conclude that callus growth is genetically controlled in pear even though it has been shown to be under genetic control in other plant species such as wheat (Jia *et al.*, 2007) and barley (Mano *et al.*, 1996; Mano and Komatsuda, 2002). Combining rooting and callus growth as one trait, as presented in some parts of the present study, might therefore lead to wrong assumptions. Indeed, although the QTLs detected for the combined traits are in the same region as the rooting QTLs, they have a lower significance (lower LOD score and percentage of variance explained) indicating that the QTL detected may be actually the QTL controlling adventitious rooting.

4.5 Conclusion

This study reports the first QTLs associated with vegetative propagation ability in pear. The presented results are the first step towards understanding the genetic control of this complex trait, and may assist in the finding of markers suitable for MAS for adventitious rooting. MAS for vegetative propagation, together with other important rootstock traits such as disease resistance and vigour control, may substantially shorten the time required for breeding new pear rootstocks. Future research on vegetative propagation may focus on identifying the candidate genes controlling adventitious rooting in pear, and on developing an understanding the molecular and physiological mechanisms involved.

5 ARABIDOPSIS FLOWERING GENES IN *PYRUS COMMUNIS* AND COMPARISON TO APPLE

5.1 Introduction

5.1.1 The role of *FT*-like genes in the transition to flower

Precocity is an important trait in commercial fruit production because it determines the length of time before an orchard becomes profitable for the producer. Precocity is defined as the time required before a plant transitions from the vegetative to the reproductive growth stage. Many endogenous and external factors influence this change in plant development. The most important external factors regulating precocity are day length (photoperiodism) and temperature (vernalization). *FLOWERING LOCUS* (*FT*) and *FT*- homologues have been shown to stimulate the transition to flowering in plants in response to environmental inputs (Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999). *FT* expression is activated by the zinc finger protein *CONSTANS* (*CO*) as a response to long days (Valverde *et al.,* 2004). *FT* proteins then move long distances through the phloem to the shoot meristem were they interact with the bZIP transcription factor (FD) (Abe *et al.,* 2003; Turnbull, 2011). FD and SQUAMOSA BINDING PROTEIN LIKE (SPL) transcription factors (also activated by FT and FD) activate the expression of *FRUITFUL* (*FUL*), *SUPRESSOR OF OVER-EXPRESSION OF CONSTANS 1* (*SOC1*), *APETALA 1* (*AP1*) and *LEAFY* (*LFY*) which leads to the initiation of flower development. The interaction of *SOC1* with the MADS box transcription factor AGK24 promotes the transcription of *LFY* (Schultz and Haughn, 1991; Yoo *et al.*, 2005; Andrés and Coupland, 2012) (Figure 22 and 23).

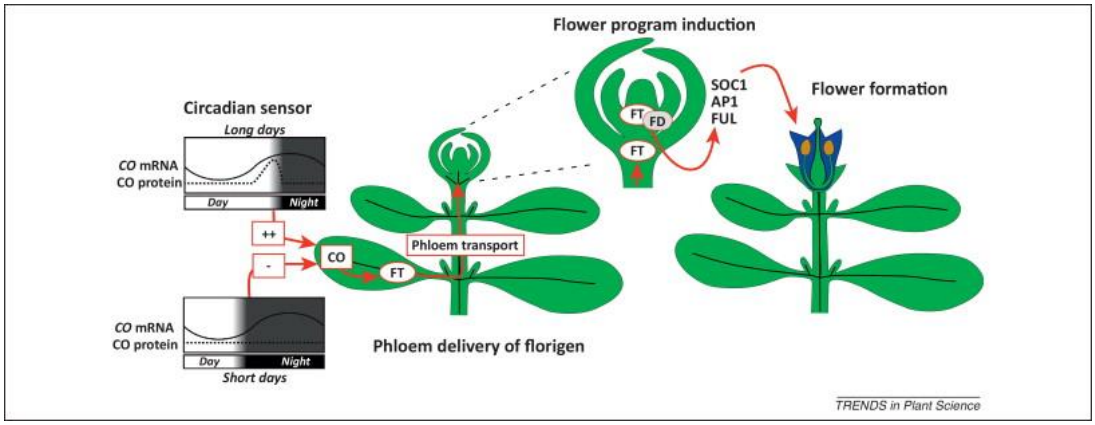

Figure 22: Flowering initiation as a response to day length (McGarry and Kragler, 2013)

TERMINAL FLOWER1 (TFL1) is a homologue of *FT* and both genes have very similar sequences. Nevertheless, *TFL1* acts as a repressor of flower transition and therefore promotes vegetative growth and juvenility (Bradley *et al.*, 1993; Ratcliffe *et al.*, 1998). *TFL1* represses the expression of *LFY* and *AP1* in the centre of the shoot meristem. Once flowering is initiated, *LFY* and *AP1* repress the *TFL1* transcription (Andrés and Coupland, 2012) (Figure 23).

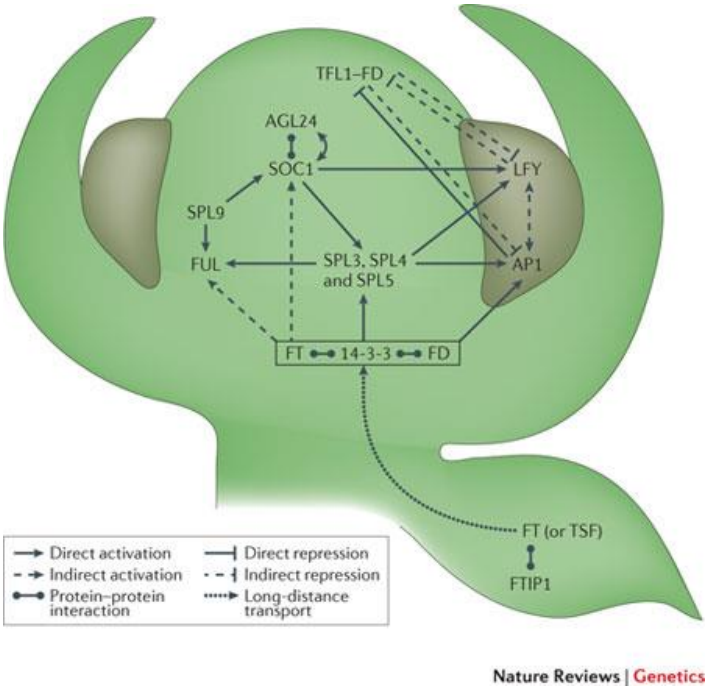

Figure 23: *FT* triggered pathway in shoot apex leading to flower initiation in *Arabidopsis thaliana* (Andrés and Coupland, 2012)

The *Arabidopsis thaliana FT/TFL1* gene family consists of six genes: *FLOWERING LOCUS* (*FT*), *TERMINAL FLOWER1* (*TFL1*), *CENTRORADIALIS* (*AtCEN*), *TWIN SISTER OF FT* (*TSF*), *BROTHER OF FT* (*BFT*) and *MOTHER OF FT* (*MFT*). While the role of *FT* and *TFL1* is well studied, less is known about the other members of the family. *BFT* may have similar functions as *TFL1* as a study in *Arabidopsis* suggested (Yoo *et al.*, 2010). The over expression of *BFT* in transgenic *Arabidopsis* plants was found to delay flowering, and reduced the expression of *LFY* and *AP1* expression (Yoo *et al.*, 2010). Another repressor of the transition to flower in *Arabidopsis* is *ATC*, which inhibits flowering on short days. Like *FT*, *ATC* interacts with *FD* and thereby affects *AP1*, but in the opposite way (Huang *et al.*, 2012).

TSF has shown to function very similarly to *FT* as a flowering promoter responding to long days. However, *TSF* and *FT* seem to promote flowering independently of each other (Yamaguchi *et al.*, 2005). The role of *MFT* is not well studied but Yoo *et al.* (2004) suggests that it promotes flowering, as they found that transgenic *Arabidopsis* plants over-expressing *MFT* flower slightly earlier than wild-types.

5.1.2 Flowering genes in woody perennials

While the role of flowering genes in *Arabidopsis* is well studied, the homologous flowering genes in perennial woody plants are still mostly unknown. Hsu *et al.* (2006) studied the expression of the *Arabidopsis FT* homologue, *FT2*, in juvenile and mature poplar trees and found that *FT2* is significantly more highly expressed in mature poplar trees, and that an over-expression of *FT2* shortens the juvenile period. The findings suggest that *FT2* plays a major role in the transition of a tree from the juvenile to the reproductive phase as found in *Arabidopsis*. In *Pyreae* several studies have found two homologues of each of the genes from the *Arabidopsis FT*/*TFL1* family (Esumi *et al.*, 2005; Kotoda and Wada, 2005; Mimida *et al.*, 2009; Kotoda *et al.*, 2010). This may be explained by the proposed whole genome duplication event in *Pyreae* and the rearrangement of the chromosomes, resulting in the 17 chromosomes in the *Pyreae* species (Velasco *et al.*, 2010) (Figure 24).

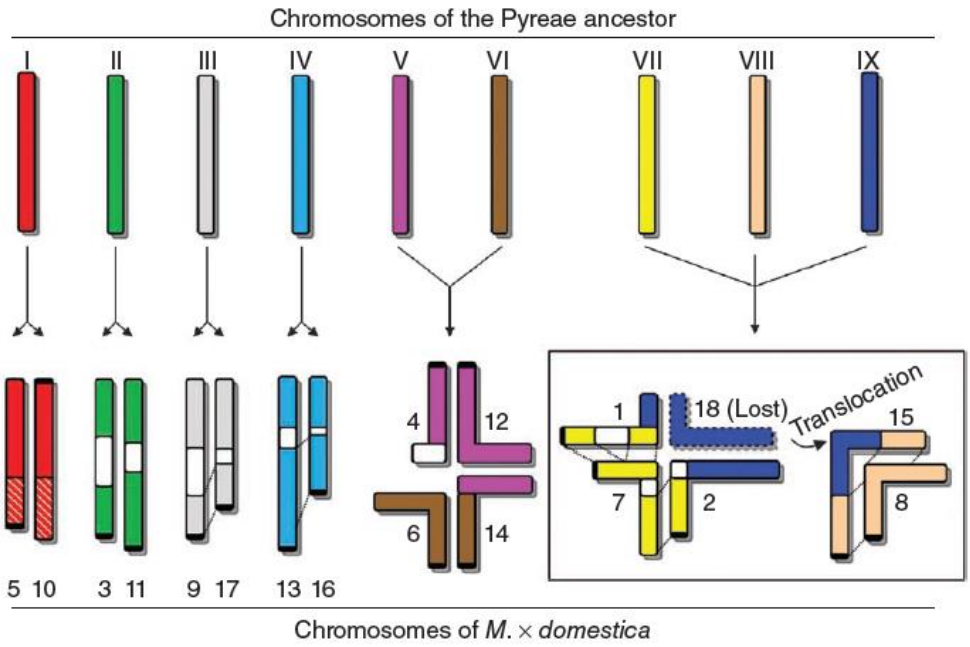

Figure 24: Model of the genome wide duplication and chromosome recombination event in *Pyreae*. Colours indicate homology between extant chromosomes. The white-hatched fragments indicate partial homology. White portions indicate lack of a duplicated counterpart. (Velasco *et al.*, 2010).

Apple homologues of the six genes from the *Arabidopsis FT/TFL1*- family have been identified in different studies. Kotoda *et al.* (2010) published the *AtFT* homologues *MdFT1* and *MdFT2*. Homologues of *AtTFL1* and *AtCEN* were identified and called *MdTFL1a*, *MdTFL1b*, *MdCENa* and *MdCENb* (Kotoda and Wada, 2005; Mimida *et al.*, 2009). The over-expression of *MdTFL1* in transgenic *Arabidopsis* delayed flowering (Kotoda and Wada, 2005), and a reduction of the *MdTFL1* level in apple trees has shown to shorten juvenility drastically (Kotoda *et al.*, 2006).

Foster *et al.* (2014) published the apple homologues of *BFT* and *MFT*: *MdBFTa*, *MdBFTb*, *MdMFTa* and *MdMFTb,* and constructed a phylogenetic tree showing the relationship of the *Arabidopsis* and apple *FT*-like genes (Figure 25). They found that *MdFT1* and *MdFT2* are more upregulated in vascular tissue of the dwarfing and precocity inducing apple rootstocks than in the vigorous rootstocks. This confirms the role of *FT* in shortening the juvenile period in trees (Foster *et al.*, 2014). Interestingly, *MdBFT* was found to be upregulated in dwarfing rootstocks too, showing *FT*-like activities of *MdBFT* rather than *TFL1* like activities, as found for *BFT* in *Arabidopsis* (Yoo *et al.*, 2010).

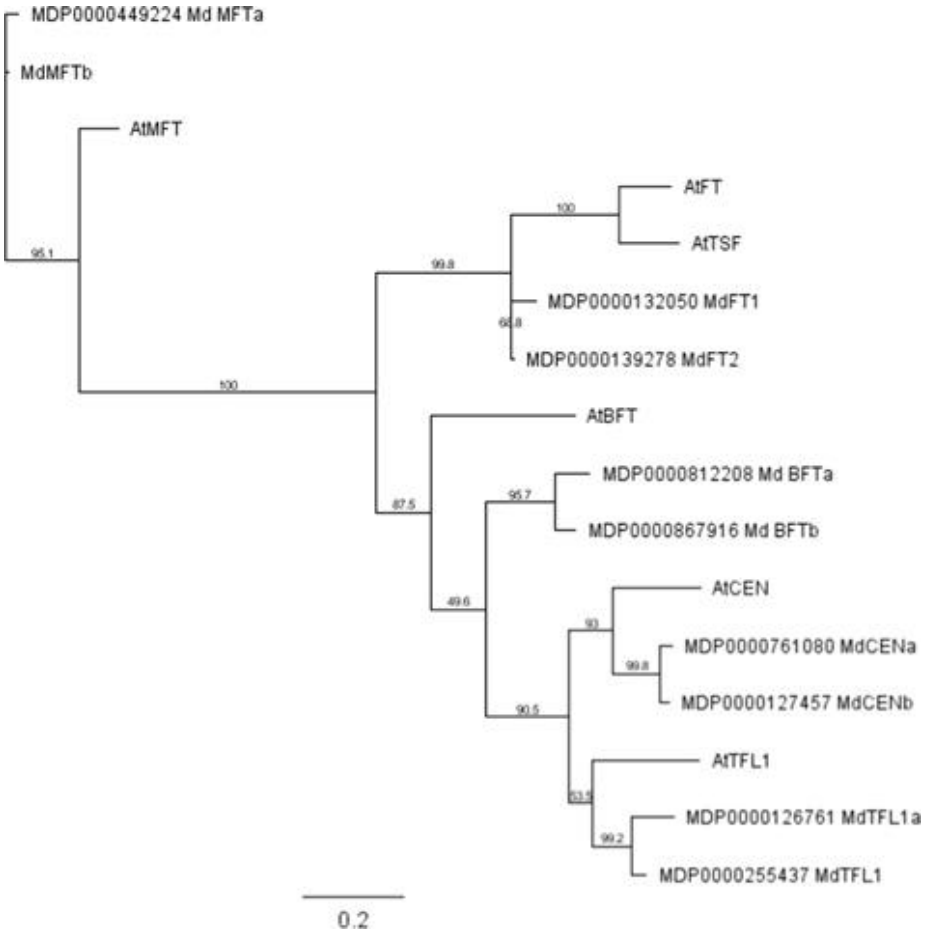

Figure 25: Phylogenetic tree of *FT*/*TFL1* protein family constructed with PHYML, using JTT substitution model and 1,000 bootstrap iterations. The scale indicates 0.2 substitutions per site; bootstrap proportion is shown for each branch (Foster *et al.*, 2014).

In pear Esumi *et al.* (2005) isolated *LFY* and *TFL1* from European and Japanese pear and found two homologues of both genes in both species. The over-expression of a citrus *FT* (*CiFT*) gene in transgenic *P .communis* trees lead to precocious flowering (Matsuda *et al.*, 2009), while the silencing of *PcTFL1-1* and *PcTFL1-2* had the same effect (Freiman *et al.*, 2012). These studies show that members of the *FT*- gene family in pear have a similar role as in *Arabidopsis* and apple.

The purpose of this study was to identify the homologue *FT*- like genes in *P. communis* and their relation to the *Arabidopsis* and *Malus FT/TFL1*- family in phylogenetic analysis. Quantitative RT-PCR was performed to study gene expression levels in different plant tissues and different vigour controlling rootstocks.

5.2 Materials and methods

5.2.1 Phylogenetic analysis

The protein sequence of the *Arabidopsis* flowering genes of the *FT*/*TFL1*- family was obtained from the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The protein sequence was searched using BLASTP within the apple 'Golden Delicious' genome and the pear 'Bartlett' genome. All sequences with a BLASTP score greater than 100 were considered as putative orthologues and blasted back against the *Arabidopsis* genome (www.arabidopsis.org). Only apple and pear putative proteins that retrieved the original Arabidopsis protein sequence with a BLASTP score greater than 100 were used for phylogenetic analysis. A phylogenetic tree was constructed in Geneious 6.1.8 © (2005-2015 Biomatters Limited) with the PHYML tree building tool using the JTT substitution model and 1000 bootstraps. PHYML estimates the maximum likelihood phylogenies from alignments of sequences.

5.2.2 Gene expression analysis

Samples for gene expression analysis using quantitative PCR (qPCR) were taken at two time points from vegetative and floral buds on spurs and floral buds at terminal positions of two five years old clonal 'Bartlett' trees. Two biological replicates were taken on the 19th of August 2014 just before bud break (Figure 26 A,C) and on the 9th of September 2014 when buds opened up (Figure 26 B,D). The trees are the same age and were grown under the same conditions in 50l plastic bags.

Figure 26: Pictures of vegetative and floral buds from 'Bartlett' trees before and after bud break. A) floral bud before bud brake, B) floral bud after bud brake, C) vegetative bud before bud break, D) Vegetative bud after bud break.

Samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. RNA extraction was performed following a modified version of the hot borate RNA extraction protocol (Wan and Wilkins, 1994). RNA quantity and quality was measured using the NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific Inc.). Reverse transcription of the RNA into cDNA was conducted using the PrimeScript^{TM} RT reagent Kit (Takara Bio inc.).

Quantitative real-time PCR (qRT-PCR) was performed on a Roche LightCycler 480 using a KAPA sybrfast qRT-PCR mastermix. The cDNA was diluted 20 times and three technical replicates were used. The following PCR cycle settings were used: pre-incubation for 5 min at 95°C followed by 45 amplification cycles (95°C for 10s, 55°C for 15s, 72°C for 10s) and a melting curve analysis (95°C for 5s, 65°C for 1min, 97°C with a continuous acquisition every 6°C). PCR primers were designed using Genious 6.1.8 targeting *PcFT1,* and *PcFT2* by blasting *Malus* genes to the 'Bartlett' genome (Table 13). *PP2a* was used as a reference gene. ANOVA was performed to calculate the significance of the gene expression levels using Minitab 16 Statistical Software (2010 Minitab Inc.)

Table 13: Primer used for qRT-PCR designed with Genious 6.1.8 targeting *PcFT1* and *PcFT2*

Type	PCP gene	Sequence	Blast score	Tm	%GC	Product Size
PcFT2 F	PCP023373	TCTGGGTCATGGTGGATCCT	32	60	55	189
PcFT2 R	PCP023373	TTGGCCGTGGACTTTCGTAA	40	60	50	189
PcFT1 F	PCP004421	GGCGACGATCTCAGGACTTT	40	59.8	55	171
PcFT1 R	PCP004421	TGTTGGCCGTGGACTTTCAT	40	60.2	50	171

5.3 Results

5.3.1 Identification of the *Pyrus FT*/*TFL1* gene family

Pear homologues of the *FT/TFL1* gene family were found using BLASTP with the *Arabidopsis FT* family genes [GenBank accession numbers]: *FLOWERING LOCUS* (*AtFT*) [NM_105222], *TERMINAL FLOWER1* (*AtTFL1*) [NM_120465], *CENTRORADIALIUS* (*ATCEN*) [NM_128315], *TWIN SISTER OF FT* (*AtTSF*) [NM-_118156], *BROTHER OF FT* (*AtBFT*) [NM_125597] and *MOTHER OF FT* (*AtMFT*) [NM_101672] against the pear genome and compared to apple in a phylogenetic analysis (Figure 27).

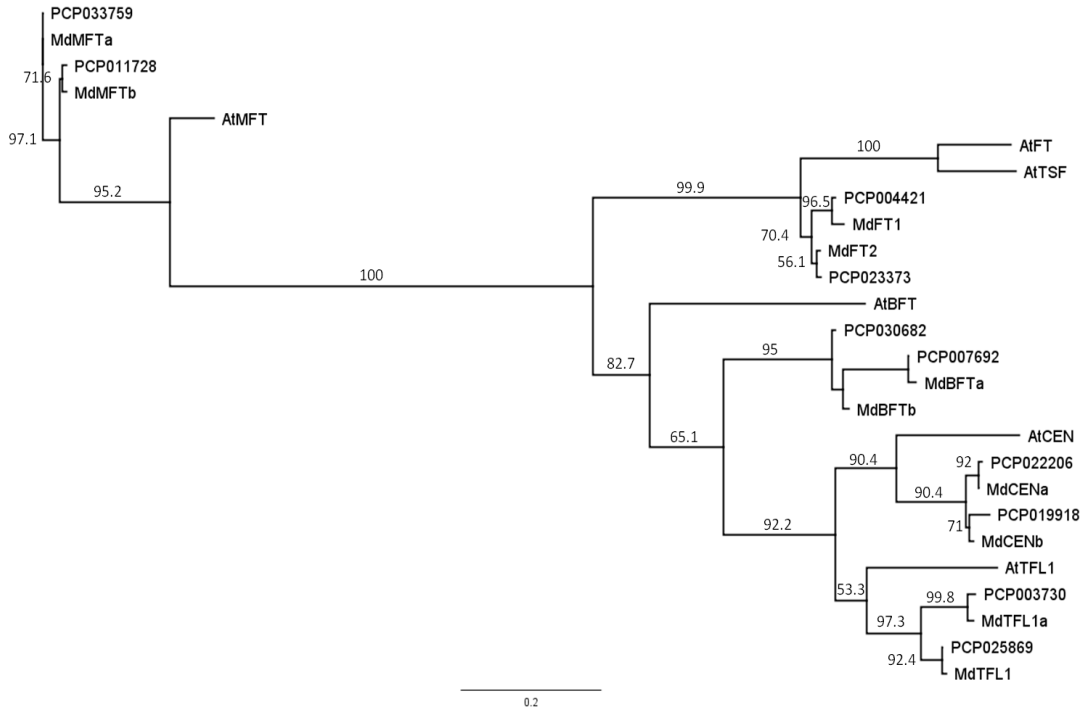

Figure 27: Phylogenetic tree of the *FT*/*TFL1* protein family. Tree was constructed in Geneious 6.1.8 with PHYML using 1000 bootstraps in the JTT substitution model width. The tree shows 0.2 substitutions per site (scale) and the bootstrap proportions for each branch.

Two pear homologues were identified for each *FT*-like Arabidopsis gene adding ten pear homologous genes to the ten apple genes identified earlier by Foster *et al.* (2014). The homology of each gene with one of the other genes from the phylogenetic analysis is shown in Table 14.

The predicted protein from the 'Bartlett' genome PCP003730 and PCP025869 are most likely homologues of *AtTFL1* with 73.6% and 74.7% sequence similarity, and of *MdTFL1a* (98.3%) and *MdTFL1b* (99.4%), respectively. The pear homologues of *AtCEN* are PCP022206 (78.3%) and PCP019918 (77.1%) which are highly similar to *MdCENa* (99.4%) and *MdCENb* (96.6%), respectively. PCP007692 is the most similar predicted pear protein to *MdBFTa* with 98.8% homology and PCP030682 shares 97.1% homology with *MdBFTb*. Both genes are homologous to *AtBFT* with 67.2% sequence similarity.

Table 14: Percentage of homology of *Pyrus*, *Malus* and *Arabidopsis* genes form the *FT*/*TFL1* gene family calculated with phylogenetic analysis.

	PCP004421	PCP023373	PCP030682	PCP025869	PCP022206	PCP019918	PCP003730	PCP007692	PCP033759	PCP011728	*AtFT*	*AtTSF*	*MdFT1*	*MdFT2*	*AtCEN*	*MdCENa*	*MdCENb*	*AtTFL1*	*MdTFL1*	*MdTFL1a*	*AtBFT*	*MdBFTa*	*MdBFTb*	*AtMFT*	*MdMFTa*	*MdMFTb*
PCP004421		95.4	61.3	58.7	57.8	56.6	58.7	57.8	50.3	50.3	74.1	75.3	97.7	95.4	54.6	57.8	57.8	57.5	58.7	58.7	56.8	58	60.7	52.9	50.3	49.7
PCP023373	95.4		62.4	59.3	59	57.8	59.3	59	49.1	49.1	75.3	75.3	94.3	98.9	55.7	59	59	58.6	59.3	59.9	58	59.2	61.8	52.3	49.1	48.6
PCP030682	61.3	62.4		69.4	69.4	67.1	66.5	89	47.7	48.9	54.9	54.3	60.7	63	66.1	69.4	68.8	65.1	69.4	66.5	67.2	89	97.1	50.3	47.7	47.7
PCP025869	58.7	59.3	69.4		74	72.3	90.7	67.1	50.9	50.9	55.8	56.4	58.1	59.9	73.6	74	74	74.7	99.4	90.1	62.6	67.1	68.2	53.4	50.9	50.3
PCP022206	57.8	59	69.4	74		94.8	72.3	64.7	51.1	52	56.9	56.3	57.2	59.5	78.3	99.4	97.1	72.7	74	71.7	60.3	64.7	68.8	49.1	51.1	51.7
PCP019918	56.6	57.8	67.1	72.3	94.8		71.7	63	50.6	51.4	55.7	55.2	56.1	58.4	77.1	94.8	96.6	69.9	72.3	71.1	58.6	63	66.5	48.6	50.6	51.1
PCP003730	58.7	59.3	66.5	90.7	72.3	71.7		65.3	53.2	53.2	54.7	55.8	58.7	59.9	71.3	72.3	72.8	73.6	90.1	98.3	62.1	65.3	65.3	55.2	53.2	52.6
PCP007692	57.8	59	89	67.1	64.7	63	65.3		48.9	50	56.6	55.5	57.2	59.5	64.4	64.7	64.7	61.7	67.1	65.3	67.2	98.8	90.2	52	48.9	48.9
PCP033759	50.3	49.1	47.7	50.9	51.1	50.6	53.2	48.9		96.5	44.5	48	50.3	49.7	51.1	51.1	52.3	47.1	50.9	53.2	46.6	48.9	47.1	79.8	100	96.5
PCP011728	50.3	49.1	48.9	50.9	52	51.4	53.2	50	96.5		43.1	47.1	50.3	49.7	51.4	52	53.1	46.1	50.9	53.2	47.1	50	48.3	79.2	96.5	98.8
AtFT	74.1	75.3	54.9	55.8	56.9	55.7	54.7	56.6	44.5	43.1		81.7	72.4	75.9	54.9	56.9	56.9	56.6	55.8	55.2	56.8	56.3	54.9	45.4	44.5	43.4
AtTSF	75.3	75.3	54.3	56.4	56.3	55.2	55.8	55.5	48	47.1	81.7		74.1	75.9	54.9	56.3	56.3	54.9	56.4	55.8	53.4	55.2	54.3	47.7	48	47.4
MdFT1	97.7	94.3	60.7	58.1	57.2	56.1	58.7	57.2	50.3	50.3	72.4	74.1		94.3	54	57.2	57.2	56.9	58.1	57.6	55.7	57.5	60.1	52.3	50.3	49.7
MdFT2	95.4	98.9	63	59.9	59.5	58.4	59.9	59.5	49.7	49.7	75.9	75.9	94.3		56.3	59.5	59.5	59.2	59.9	60.5	58	59.8	62.4	52.9	49.7	49.1
AtCEN	54.6	55.7	66.1	73.6	78.3	77.1	71.3	64.4	51.1	51.4	54.9	54.9	54	56.3		78.3	78.9	67	73	71.3	58	64.4	65.5	49.7	51.1	51.1
MdCENa	57.8	59	69.4	74	99.4	94.8	72.3	64.7	51.1	52	56.9	56.3	57.2	59.5	78.3		97.1	72.7	74	71.7	60.3	64.7	68.8	49.1	51.1	51.7
MdCENb	57.8	59	68.8	74	97.1	96.6	72.8	64.7	52.3	53.1	56.9	56.3	57.2	59.5	78.9	97.1		72.2	74	72.3	60.3	64.7	68.2	49.7	52.3	52.9
AtTFL1	57.5	58.6	65.1	74.7	72.7	69.9	73.6	61.7	47.1	46.1	56.6	54.9	56.9	59.2	67	72.7	72.2		75.3	74.1	59.4	61.7	64	48.9	47.1	46.6
MdTFL1	58.7	59.3	69.4	99.4	74	72.3	90.1	67.1	50.9	50.9	55.8	56.4	58.1	59.9	73	74	74	75.3		89.5	62.6	67.1	68.2	53.4	50.9	50.3
MdTFL1a	58.7	59.9	66.5	90.1	71.7	71.1	98.3	65.3	53.2	53.2	55.2	55.8	57.6	60.5	71.3	71.7	72.3	74.1	89.5		62.1	65.3	65.3	55.2	53.2	52.6

AtBFT	56.8	58	67.2	62.6	60.3	58.6	62.1	67.2	46.6	47.1	56.8	53.4	55.7	58	58	60.3	60.3	59.4	62.6	62.1		66.9	67.8	49.1	46.6	46.6
MdBFTa	58	59.2	89	67.1	64.7	63	65.3	98.8	48.9	50	56.3	55.2	57.5	59.8	64.4	64.7	64.7	61.7	67.1	65.3	66.9		89	52	48.9	48.9
MdBFTb	60.7	61.8	97.1	68.2	68.8	66.5	65.3	90.2	47.1	48.3	54.9	54.3	60.1	62.4	65.5	68.8	68.2	64	68.2	65.3	67.8	89		49.7	47.1	47.1
AtMFT	52.9	52.3	50.3	53.4	49.1	48.6	55.2	52	79.8	79.2	45.4	47.7	52.3	52.9	49.7	49.1	49.7	48.9	53.4	55.2	49.1	52	49.7		79.8	79.2
MdMFTa	50.3	49.1	47.7	50.9	51.1	50.6	53.2	48.9	100	96.5	44.5	48	50.3	49.7	51.1	51.1	52.3	47.1	50.9	53.2	46.6	48.9	47.1	79.8		96.5
MdMFTb	49.7	48.6	47.7	50.3	51.7	51.1	52.6	48.9	96.5	98.8	43.4	47.4	49.7	49.1	51.1	51.7	52.9	46.6	50.3	52.6	46.6	48.9	47.1	79.2	96.5	

The most likely pear homologue of *MdMFTa* is PCP033759 (100%) and PCP011728 of *MdMFTb* (98.8%). PCP033759 and PCP011728 share 79.8% and 79.2% sequence homology with *AtMFT*, respectively. PCP004421 is homologous to apple *MdFT1* (97.7 %), while PCP023373 has a higher homology with *MdFT2* (98.9 %). Both pear genes cluster with both *Arabidopsis AtFT* (74.1% and 75.3%) and *AtTSF* (75.3% and 75.3%).

Two key protein motifs are conserved between the homologues of Arabidopsis, apple and pear as well as between both paralogous pear genes (Figure 28). The single amino acid differences in a ligand binding pocket and in an external P-loop domain are responsible for the opposite function of *FT* and *TFL1*. Tyr-85/Gln-140 promotes flowering in *FT* and His-88/Asn-144 inhibits flowering in *TFL1* in Arabidopsis and apple (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). Both amino acid differences were found in the same location in the pear sequences.

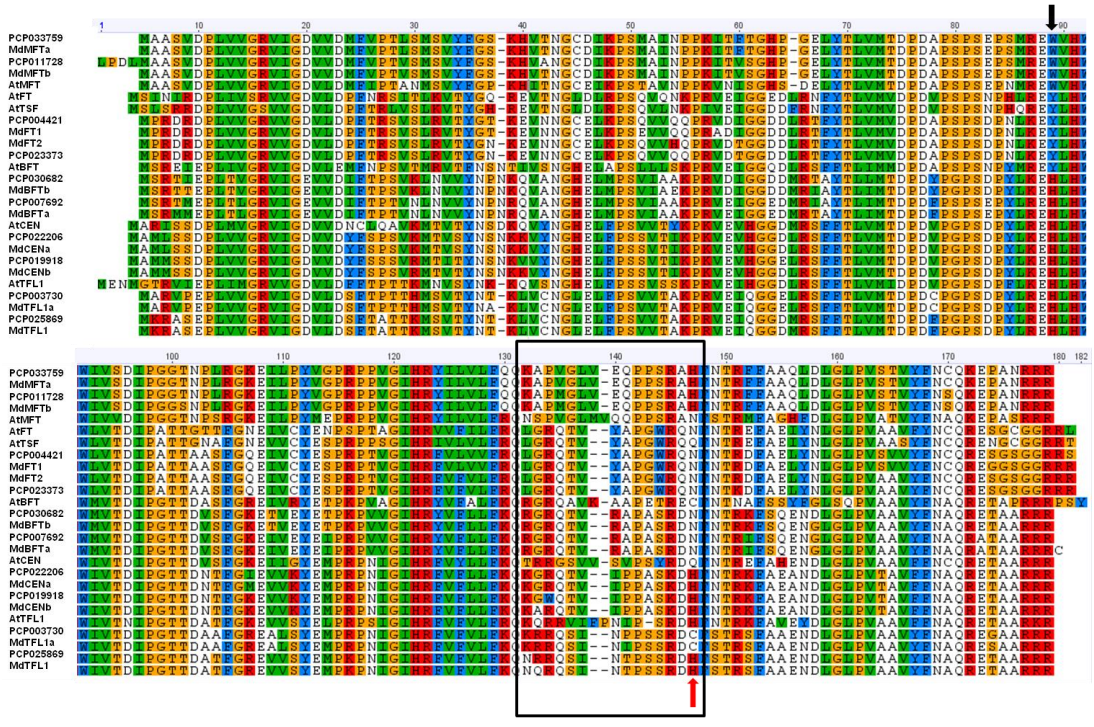

Figure 28: Alignment of the amino acid sequences of the *FT*/*TFL1* gene family from Arabidopsis, apple and pear. Black arrow shows Tyr-85/His-88 and red arrow shows Gln-140/Asn-144. Both amino acids are responsible for the opposite functions of *FT* and *TFL1*. The black square shows the ligand binding pocket in *FT*/*TFL1* proteins.

Comparing the *TFL1* homologues PCP003730 and PCP025869 found in this study with *PpTFL1.1/2 and PcTFL1.1/2* identified earlier by Esumi *et al.* (2005) results in a 100% alignment of PCP003730 with *PpTFL1.2* and *PcTFL1.2* while PCP025869 aligns 100% with *PcTFL1.1* and 99% with *PpTFL1.1* (Figure 29).

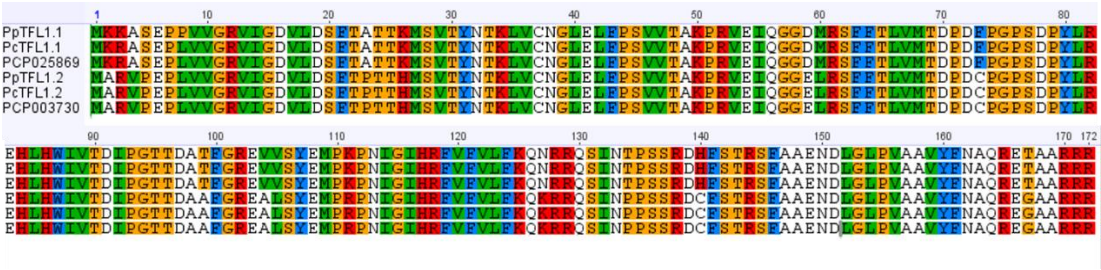

Figure 29: Alignment of the amino acid sequences of *PpTFL1.1/2 and PcTFL1.1/2* published by (Esumi *et al.*, 2005) with PCP025869 and PCP003730

5.3.2 Gene expression

5.3.2.1 *FT1* and *FT2* expression in vegetative and floral buds

The qRT-PCR analysis indicates that both pear homologues of *MdFT1* (PCP004421) and *MdFT2* (PCP023373) are expressed in floral and vegetative buds. *FT2* is expressed at higher levels than *FT1* for all time points and tissues (Figure 30). There was no significant difference in the expression of *FT1* or *FT2* amongst different time points (FT1: *p=0.833;* FT2: *p=0.99*). Furthermore, *FT1* and *FT2* expression does not differ between spur and terminal floral buds (FT1: *p=0.327;* FT2: *p=0.43*). However, *FT1* is expressed at higher levels in floral buds compared to vegetative buds (*p=0.038*), while *FT2* is more highly expressed in vegetative buds compared to floral buds (*p=0.001*).

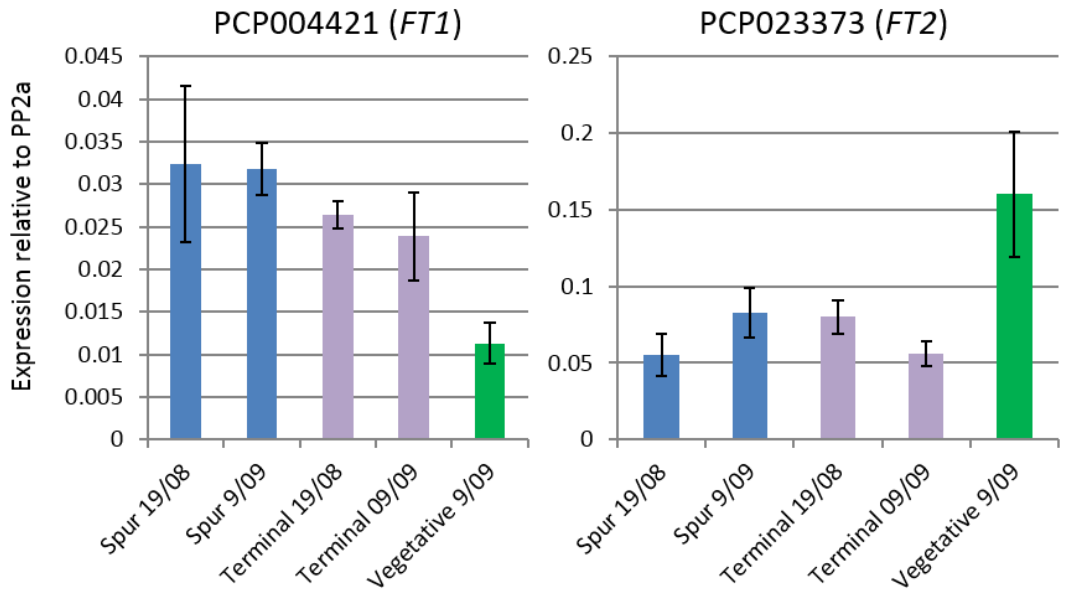

Figure 30: Gene expression results of *FT1* and *FT2* by quantitative qRT-PCR. Error bars indicate the standard error of biological replicates.

5.4 Discussion

We identified key flowering genes from the *Arabidopsis FT/TFL1*- gene family in *Pyrus communis* by phylogenetic analysis and found a high synteny of these genes with their apple homologues. Two homologues of each of the *AtFT*, *AtTFL1*, *AtCEN*, *AtBFT* and *AtMFT* genes were identified in the European pear genome due to the whole genome duplication event in *Pyreae* (Velasco *et al.*, 2010). Two homologous copies of these genes were also reported in apple (Foster *et al.*, 2014) and other *Pyreae* species. Esumi *et al.* (2005) isolated *LFY* and *TFL1* from apple, quince, Chinese quince, loquat, Japanese pear and European pear using PCR amplification of genomic DNA, (RT)-PCR and rapid amplification of cDNA ends (RACE). They found two homologues of *LFY* (*LFY-1* and *LFY-2*) and *TFL1* (*TFL1-1* and *TFL1-2*) in all *Pyreae* species studied. The *PcTFL1 and PcTFL1a* homologues presented in the present study showed a 100% alignment with *PcTFL1.1* and *PcTFL1.2* published by Esumi *et al.* (2005).

Only two pear genes (PCP004421 and PCP023373) clustered with *AtFT* and *AtTSF*. The same was found earlier for apple while those genes were called *MdFT1* and *MdFT2* (Foster *et al.*, 2014). These results may show that one gene copy of *TSF* and *FT* has disappeared during the whole genome duplication of *Pyreae*. PCP004421 shares a slightly higher homology with *AtTSF* than with *AtFT*, while PCP023373 shares the same percentage of homology with both *AtTSF* and *AtFT*. In a phylogenetic analysis, Esumi *et al.* (2005) found that the homologues of the different species cluster together (*PpLFY-1, PcLFY-1, MdLFY-1, CoTFL1-1*) and have a higher amino acid sequence identity than the homologues of the same species (*PcLFY-1* and *PcLFY-2*). In this study I confirm this phenomenon. For example I found a 98.8% amino acid sequence identity between PCP007692 and *MdBFTa*. PCP030682 shares 97.1% homology with *MdBFTb*, while the identity between PCP007692 and PCP030682 is 89%. These circumstances raise the possibility that there are different functions of the homologues of these genes. Indeed, I found that *PcFT1* is more highly expressed in floral buds than in vegetative buds, while *PcFT2* expression was upregulated in vegetative buds relative to floral buds. This might indicate that the *PcFT1* is involved in floral development while *PcFT2* is involved in vegetative growth. Similar results have been reported before in apple by Kotoda *et al.* (2010). They found that *MdFT1* was higher expressed in apical buds of fruit bearing shoots than in floral organs or juvenile trees, while the expression of *MdFT2* was highest in floral organs and young fruits. A study in poplar also revealed different functions of the *FT1* and *FT2* poplar homologues due to seasonal temperature and day length changes (Hsu *et al.*, 2011). The transcription of poplar *FT1* was found to be activated at cold temperatures, but not affected by day length, while *FT2* was upregulated in warm temperatures and by long days. *FT1* was found to promote the formation of floral buds, while *FT2* signalling promotes vegetative growth. The *FT* homologues therefore control seasonal reproductive and vegetative growth in poplar trees (Hsu *et al.*, 2011). However, the different functions of the *FT* pear homologues need to be further investigated by using more biological replicates and different time points of development and tissue.

Different functions of pear homologues of other genes have also been under investigation in another study.

PpLFY-1 and *PcLFY-1* have been found to be only expressed in buds, while their homologues *PpLFY-2* and *PcLFY-2* were also transcribed in leaves (Esumi *et al.*, 2005). Therefore *LFY-2* might be involved in different regulation pathways than its homologue. However, both homologues were found to be involved in the floral bud development.

While *FT*-like genes were proven to be involved in the transition from the vegetative to the reproductive phase in woody perennials it is still unknown what leads to first flowering after a long juvenile phase. The level of *FT* in juvenile trees was found to be much lower than in trees in their reproductive phase, while an increase of the *FT* level in transgenic plants shortens the juvenile phase drastically. *FT* initiates flowering as a response to long days, but only when the tree has reached a certain life stage or size. It is not known what triggers the change in the response to long days and the transition from juvenile to reproductive phase (Hsu *et al.*, 2006). Further gene expression studies in pear might help to reveal the role of *FT/TFL*- like genes in the transition from the vegetative to the reproductive phase.

5.5 Conclusion

I identified the pear homologues of *FLOWERING LOCUS* (*FT*), *TERMINAL FLOWER1* (*TFL1*), *CENTRORADIALIUS* (*ATC*), *TWIN SISTER OF FT* (*TSF*), *BROTHER OF FT* (*BFT*) and *MOTHER OF FT* (*MFT*) and their relatedness to the *Arabidopsis* and *Malus FT/TFL1*-family in phylogenetic analysis. I reported a high homology of the apple and pear *FT*- like genes. These results confirm the high synteny of the apple and pear genome. A gene expression study indicated differing roles for *PcFT1* and *PcFT2* in flowering and vegetative growth, building a foundation for further investigations about the role of these key flowering genes in the transition of a tree from the juvenile to the reproductive phase.

6 GENERAL CONCLUSION AND PERSPECTIVES

During my PhD project I developed an understanding of the genetic determinants of vigour control and precocity by pear rootstocks. To achieve this, I used a segregating population of 405 F1 *P. communis* seedlings from a cross between 'Old Home' and 'Louise Bonne de Jersey' (OHxLBJ) grafted as rootstocks with 'Doyenne du Comice' scions. The population was comprehensively phenotyped over four growing seasons for traits related to tree architecture and flowering. Genetic and QTL mapping in this population enabled me to identify genomic regions responsible for the control by rootstocks of dwarfing and precocity in pear. This is a significant finding for the future of commercial pear production.

In apple, dwarfing rootstocks such as 'M9' have made commercial production more cost effective by reducing scion vigour and inducing precocity. Minimising the length of the juvenile period makes the orchard become profitable sooner, and smaller trees allow a high-density plantings resulting in a higher yield per hectare.

Currently, there are no *Pyrus* rootstocks available that are able to dwarf pear scions to the same level as super-dwarfing ('M27') or dwarfing ('M9') apple rootstocks, or induce precocity, making pear cultivation less profitable for growers than apple. The traditional method of breeding rootstocks is time consuming and costly because of the time end expenses involved in phenotyping large numbers of seedling rootstocks for their effect on grafted scions. In order to develop new systems for breeding pear rootstocks more cost effectively and faster, it has been necessary to first understand the mechanisms involved in vigour control and precocity in pear, and then to identify the genetic control of such desired traits. My thesis describes the development of this understanding.

6.1 Tree architecture

Description of the cumulative growth of a tree and assessment of its vigour is time consuming and involves expensive measurements over successive years. Hence the development of standardized measurements representing the overall tree size and which predict the future growth of the tree during early growing stages is essential to reduce the time and labour required both for further rootstock research and for rootstock breeding. Recently developed coding languages, mathematical methods, analytical software and image analysis have aided the evaluation of phenotypic variability and the identification of correlations between variables (Godin *et al.*, 1999; Seleznyova *et al.*, 2003; Costes *et al.*, 2004b; Costes *et al.*, 2006; Guo *et al.*, 2011). In the last decade, such architectural analysis has enabled the study of complex trees including species such as olive (Hammami *et al.*, 2012), walnut (Rezaee *et al.*, 2009) peach (Carrillo-Mendoza *et al.*, 2010) and apple (De Wit *et al.*, 2002; Segura *et al.*, 2006; Segura *et al.*, 2007; Segura *et al.*, 2009b).

In my study, I found that not only is the TCA an easy trait to phenotype, but it is a good indicator of overall tree size for pear scions grafted onto *Pyrus* rootstocks. I suggest that TCA should be used in further studies as a standard measurement. The phenotyping of other traits that are more time consuming to quantify did not bring many further insights into the genetics of vigour control and precocity in this investigation.

6.2 Genetic map

New high throughput genotyping and sequencing technologies have made genotyping fast and cost effective in the last few years, resulting in a rapid knowledge gain in plant genomics. My PhD project benefited from these rapidly developing technologies. The Infinium® II 9K apple & pear SNP array was available for the genotyping of 297 individuals of the OHxLBJ population and enabled the construction of high density genetic maps within a short period of time. The maps constructed for OH and LBJ were amongst the first five high- density SNP based genetic maps constructed for pear.

The SNP array was successfully used in interspecific pear crosses and has been shown to be transferable between the genera *Malus* and *Pyrus* (Montanari *et al.*, 2013). The high density of the genetic maps constructed made them highly suitable for the detection of QTLs for pest and disease resistance (Montanari *et al.*, 2015) and storage related disorders (Saeed *et al.*, 2014), as well as for the identification of QTLs controlling scion vigour and precocity in this study. Comparable SNP arrays have been successfully used in other Rosaceae species such as the 6K sweet cherry Illumina Infinium® II SNP array (Peace *et al.*, 2012) and the 9K Illumina Infinium® II SNP array for peach (Verde *et al.*, 2012) and more recently higher density SNP arrays, such as the 20K Illumina Infinium® II SNP array for apple (Bianco *et al.*, 2014) and the 90K Axiom® SNP array for strawberry (Bassil *et al.*, 2015). These arrays also promise to be useful for Genome Wide Association Studies (GWAS), Pedigree-Based Analysis and Genomic Selection (GS) (Kumar *et al.*, 2013). However, the construction of a SNP array is time consuming, because it requires SNP detection and validation in a small subset of samples prior to the array development. In comparison, genotyping-by-sequencing identifies and screens novel markers simultaneously, requires less work, and is more cost effective than the array based method (Ward *et al.*, 2013). I constructed the first GBS based genetic map for pear, improving the anchoring of the 'Bartlett' v1.0 genome (Chagné *et al.*, 2014) to the 17 *Pyreae* LGs. Combining the SNP array method and the GBS approach resulted in a high density SNP based consensus map for pear with markers transferable between *P. communis*, *P. bretschneideri* and *Malus*, which assigned linkage groups anchored to the 'Bartlett' genome. The newly discovered SNPs should be useful in the improvement of the 'Bartlett' genome assembly, which is part of an ongoing collaboration between Plant & Food Research (PFR), Institut National de Recherche Agronomique (INRA), Fondazione Edmund Mach (FEM) and the University of California, Davis (UC Davis). Improving the 'Bartlett' genome assembly will aid in revealing the genetic control of further horticulturally important traits and enhance the detection of functional and positional candidate genes.

6.3 Pear *DW1*-QTL

I utilized my high density SNP based genetic maps to identify the first QTLs controlling architectural and precocity traits in pear rootstocks, finding that vigour control and precocity are controlled by two loci on LG5 and LG6 of OH, and one on LG6 of LBJ. Phenotyping of the segregating population performed in four successive years has ensured the robustness of the detected QTLs. The co-location of the OH LG5 QTL controlling scion dwarfing by pear rootstocks in the same genomic region as the apple 'M9' *Dw1* locus (Rusholme Pilcher *et al.*, 2008; Foster *et al.*, 2015) leads to the hypothesis that orthologous loci in apple and pear control scion growth and precocity conferred by the rootstock. The alignment of the LG5 QTL controlling scion dwarfing by rootstocks in *Malus* and that for *Pyrus* in this study revealed a high degree of synteny between the genomes of these closely related genera in this region (Fig. 31). This is a first step towards the aligning of the entire apple and pear genomes, which will on one hand reveal the extent of their synteny and on the other, identify regions that are unique and therefore specific to one or other genus. The high synteny of the QTLs will facilitate the identification of candidate genes in both genera. Further work might concentrate on the detection of candidate genes underlying these QTLs using expression analysis techniques, such as qPCR and RNA sequencing, bioinformatics analysis of their putative function, as well as orthology of position between apple and pear, utilizing the published Chinese (Wu *et al.*, 2013) and the European (Chagné *et al.*, 2014) pear genomes, as well as the apple (Velasco *et al.*, 2010) genome to enhance the identification process.

The follow up project could be structured to include RNA sequencing of pear rootstocks that conferred extreme phenotypes to the scions. For this purpose ten small/precocious, ten small/not precocious, ten vigorous/precocious and ten vigorous/not precocious trees may be chosen to identify differentially expressed genes, followed by qPCR to narrow down the candidates. Targeted exon sequencing of genomic DNA of OH and 'M9' might be conducted to detect similarities and differences in the sequence of the QTL LG5 region in apple and pear.

At present there are a number of possible candidate gene for apple dwarfing under investigation. The detected LG5 QTL will help with the understanding of dwarfing in both species.

Due to a lack of biological replicates in the 'OHxLBJ' population, gene expression analysis comparing extreme phenotypes was not possible in this project. In order to be able to conduct gene expression analysis in this population in the future, critical plants in the population should be replicated vegetatively. Even though members of this population were difficult to propagate with hardwood cuttings, other methods such as stool bed propagation or even *in vitro* propagation might help to overcome this problem. Furthermore, having replicates of the whole population available would make it possible to account for environmental variability when repeating the phenotyping and might result in the detection of larger effect QTLs. The GxE effect could be studied by planting the replicates at PFR sites with differing enviroments (Motueka, Hawkes Bay and Palmerston North).

6.4 Pear flowering genes

I identified the pear homologues of the Arabidopsis *FT*/*TFL*- flowering genes in the 'Bartlett' genome assembly. Because *FT*/*TFL-1* like genes are known to be involved in the transition from juvenility to the reproductive phase in model plants (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999) and woody perennials (Hsu *et al.*, 2006) these results may help in developing an understanding the mechanism controlling precocity in pear. However, none of the *FT*/*TFL1*-like genes were located on LG5 in the same region as the precocity QTL detected in the OHxLBJ population (Table 15). However this does not necessarily mean that *FT*-like genes are not involved in the rootstock control of precocity in pear. In fact *FT* has been shown to be upregulated in vascular tissue of the dwarfing and precocity inducing apple rootstocks compared to vigorous rootstocks (Foster 2014). The LG5 locus controlling precocity in pear may interact with the *FT* pathway.

However, this is speculative and future work on the genetic control of precocity should focus on the detection of genes involved in the flowering pathway underlying the LG5 precocity QTL detected in this study. This might be done by fine mapping the confidence interval of the QTLs in a larger population with a greater number of markers, combined with gene expression analysis of the detected candidate genes to reveal their function.

Table 15: Map position of the apple and pear *FT*/*TFL1*- gene family

Apple FT-family	IASMA predicted genes	LG Apple	pear hybrid genes	'Bartlett' scaffolds	LG OHxLBJ	SNP pear
MdBFTa	MDP0000812208	7	PCP007692	scaffold01885	7	
MdBFTb	MDP0000867916	1	PCP030682	scaffold00240	1	
MdMFTa	MDP0000449224	6	PCP033759	scaffold00047	6	ss527788597
MdMFTb	MDP0000208806	14	PCP011728	scaffold00228	14	ss527788362
MdFT1	MDP0000132050	12	PCP004421	scaffold01463	12	
MdFT2	MDP0000139278	4	PCP023373	scaffold00915	4	
MdCENa	MDP0000761080	10	PCP022206	scaffold03074	--	ss475876242
MdCENb	MDP0000127457	13	PCP019918	scaffold00473	11	ss475876412
MdTFL1a	MDP0000255437	12	PCP003730	scaffold00223	14	
MdTFL1	MDP0000126761	4	PCP025869	scaffold00057	12	

6.5 Marker assisted selection for breeding pear rootstocks

Marker assisted selection can shorten the time of breeding new cultivars of fruit trees significantly by enabling the culling unwanted plants based on their genotype, instead of waiting until their phenotype is exhibited, hence reducing costs for labour and space. The marker flanking the *Dw1* locus in apple, Hi01c04 (Foster *et al.*, 2015), was found to segregate with vigour control and precocity in the OHxLBJ pear population and may enable MAS in pear rootstock breeding to breed better rootstocks faster. However, this marker needs to be validated in different rootstock breeding populations to ensure that it is robust across different genetic backgrounds.

Future identification of the gene/s controlling precocity and dwarfing in pear rootstocks will enable the development of 'ideal' gene-based markers for pear, with the tightest possible linkage between marker and trait.
Once validated closely linked markers are available, rootstock breeding for precocity and vigour control may be possible in pear in combination with other horticulturally important traits conferred to the scion by a rootstock, such as pest and disease resistance and high yield. MAS for dwarfing is already applied successfully in apple rootstock breeding at PFR, amongst other traits that are difficult and time consuming to phenotype, such as resistance to fire blight (*Erwinia amylovora*) and woolly apple aphid (*Eriosoma lanigerum*).

Although the LG5 QTL controlling vigour in pear co-locates with the QTL conferring precocity, the effects of the detected QTLs are in *trans,* meaning that a smaller tree takes longer to flower. In terms of breeding, this makes it difficult to work out how to use markers to select for rootstocks conferring both reduced tree size and precocity to the scion cultivar. However, the more important trait for commercial production is precocity, as this reduces the time before the orchard becomes profitable for the producer. In addition, flowering and fruiting will most likely lead to a growth reduction in the following growing cycle. The number of preformed nodes in vegetative buds in apple (9-12) was found to be higher than in floral buds (5-6) (Rivals, 1965) resulting in the growth of shorter floral (sympodial) and longer vegetative (monopodial) annual shoots during the following growing season (Seleznyova *et al.*, 2008). Precocious trees with a high number of inflorescences will therefore result in less vigorous trees in the years following first flowering, when compared with non-precocious trees. I predict that the ideal pear rootstock/ scion combination will develop strong sylleptic branches in the first year of growth after grafting, leading to precocity at the beginning of the third year of growth, and eventually a reduced growth in the following season. I suggest that breeders should select for precocity using MAS, to reduce costs and substantially shorten the time to breed new rootstocks.

The aim of this PhD project was to develop an understanding of the genetic determinants of vigour control and precocity by pear rootstocks. To accomplish the aim I comprehensively phenotyped the OHxLBJ segregating population for scion growth and flowering in four successive years. I constructed high density genetic maps using high throughput genotyping tools. QTL-analysis was conducted to identify genetic regions controlling vigour control, precocity and vegetative propagation ability. QTL regions detected were compared to the apple genome sequence and genomic regions of interest were aligned. Future work may focus on the identification of candidate genes for precocity in pear and apple.

The ease of vegetative propagation, a crucial trait for rootstock breeding, was assessed in rooting experiments and small effect QTLs were identified. More work is needed to understand the genetic control of this complex trait in order to develop markers suitable for MAS.

In conclusion, my project has played a major role in improving the previously poor knowledge in the genetic control of precocity and vigour control of pear rootstocks. Breeding for precocious *Pyrus* rootstocks, easy to propagate vegetatively and resistant to major pest and diseases with a robust marker assisted selection system is now feasible. The availability of a precocity inducing and vigour controlling *Pyrus* rootstocks that are compatible with newly developed scion cultivars may transform commercial pear production in the near future.

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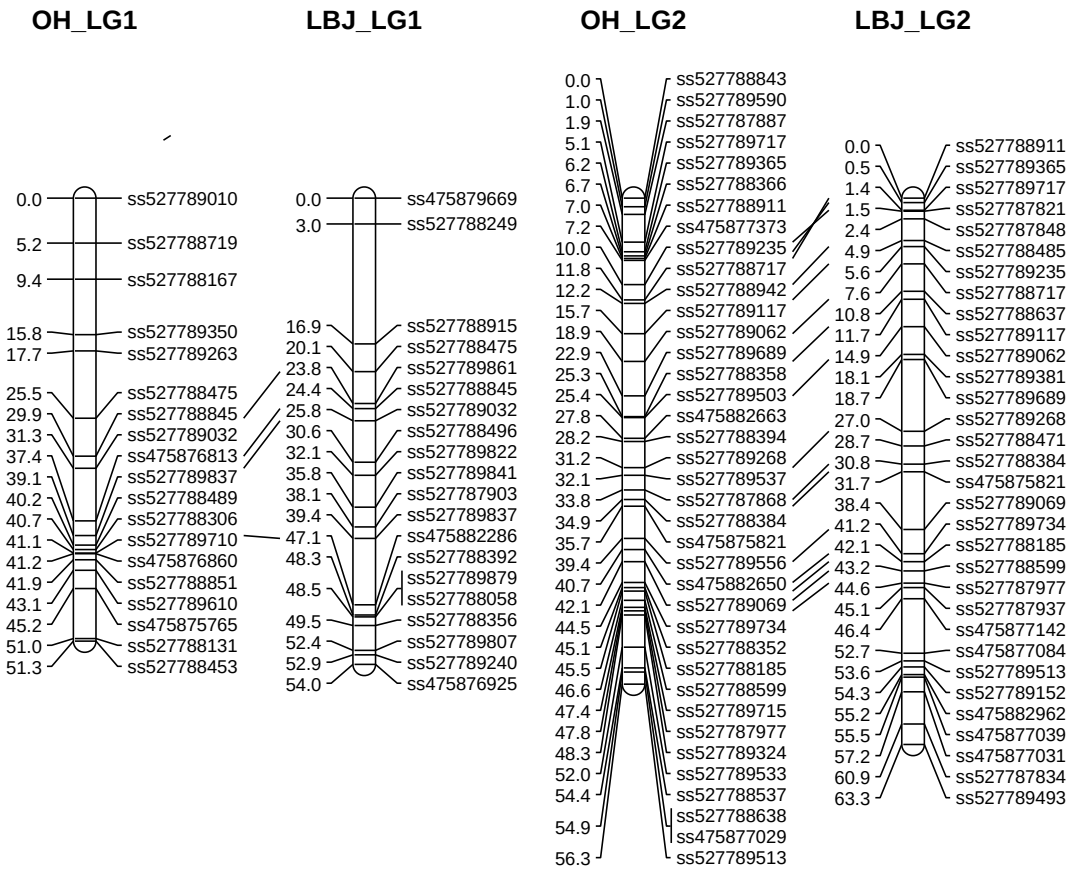
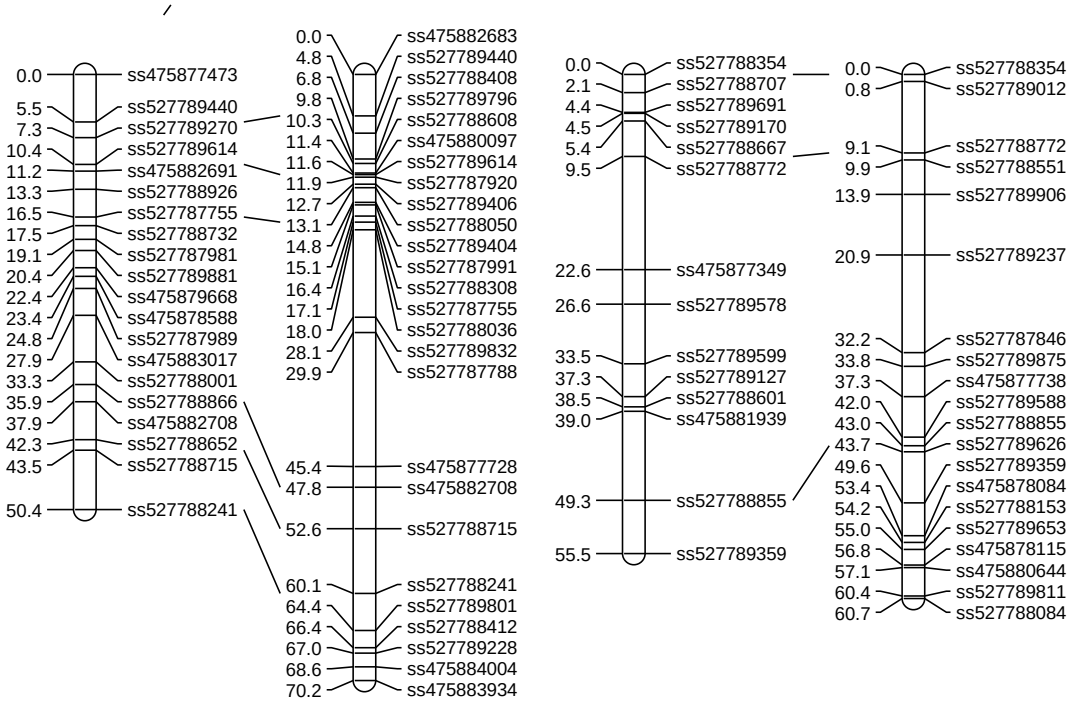
APPENDICES

Supplemental material 1: Aligned parental maps from ‘Old Home’ and ‘Louise Bonne de Jersey’ constructed with the apple and pear Infinium® II 9K SNP. SS names are the NCBI dbSNP accessions. The marker positions are indicated in centiMorgan (cM).

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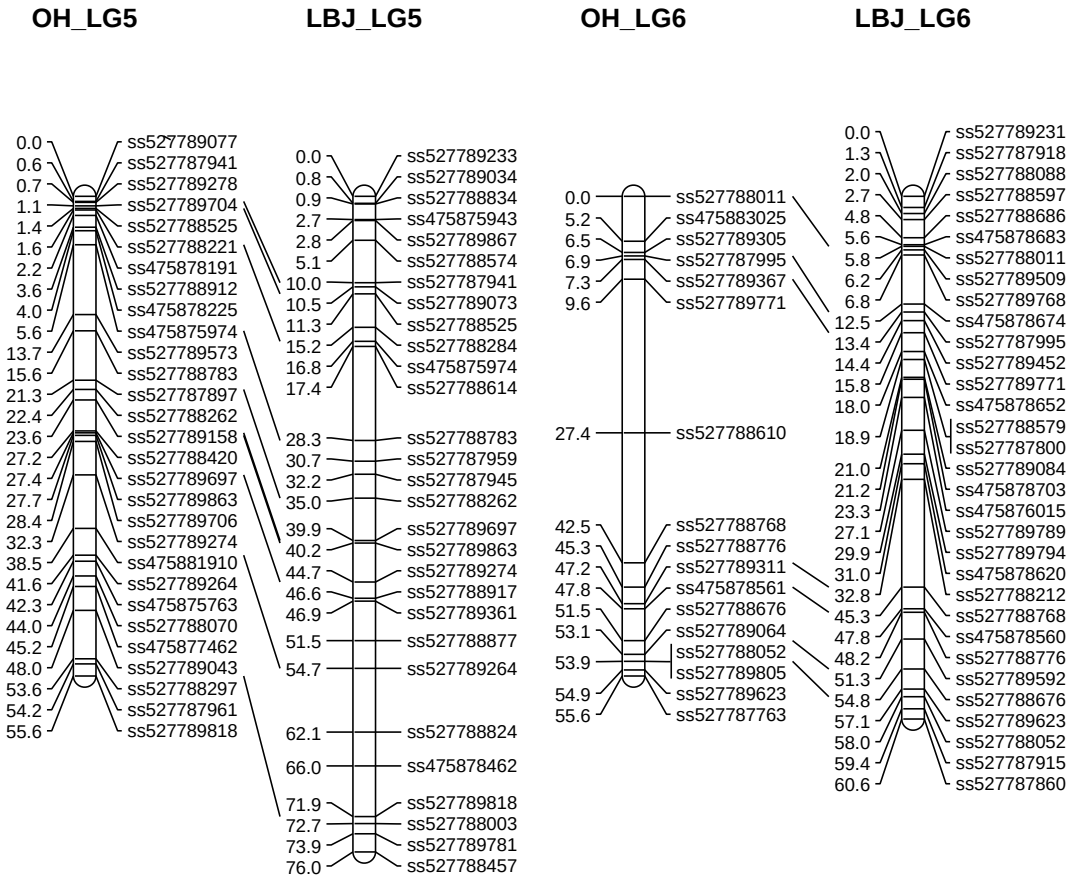
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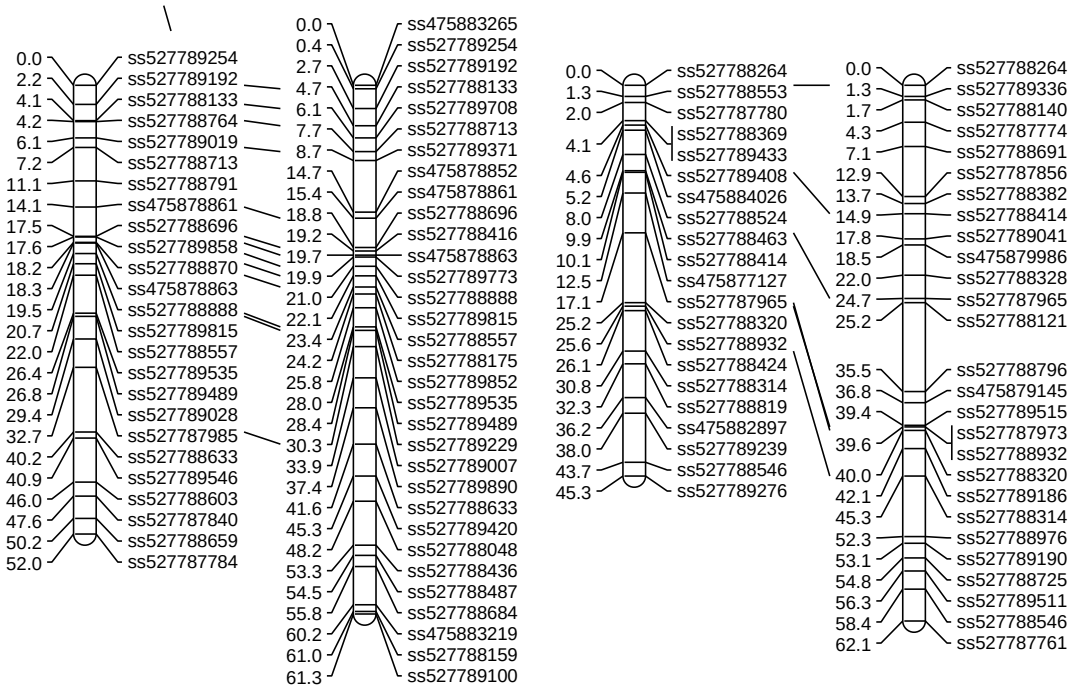
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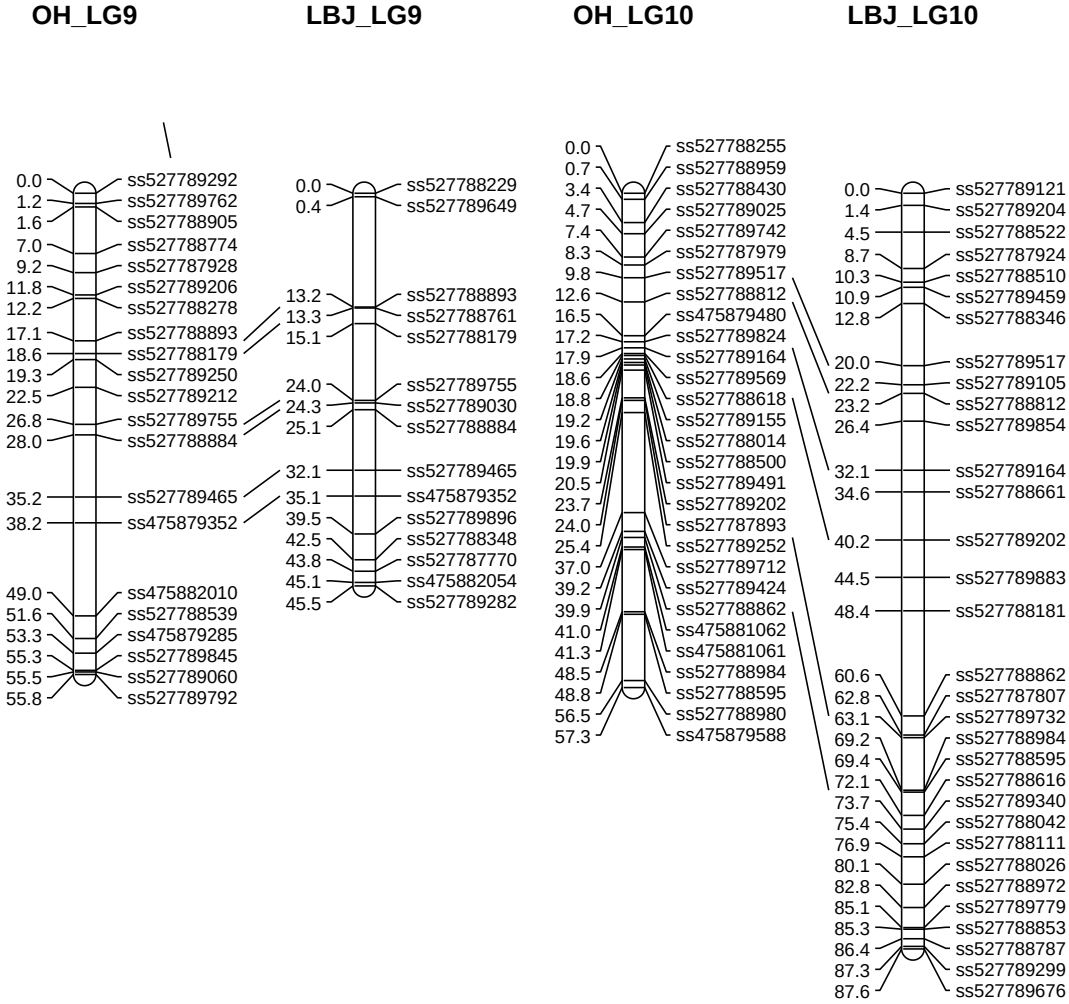
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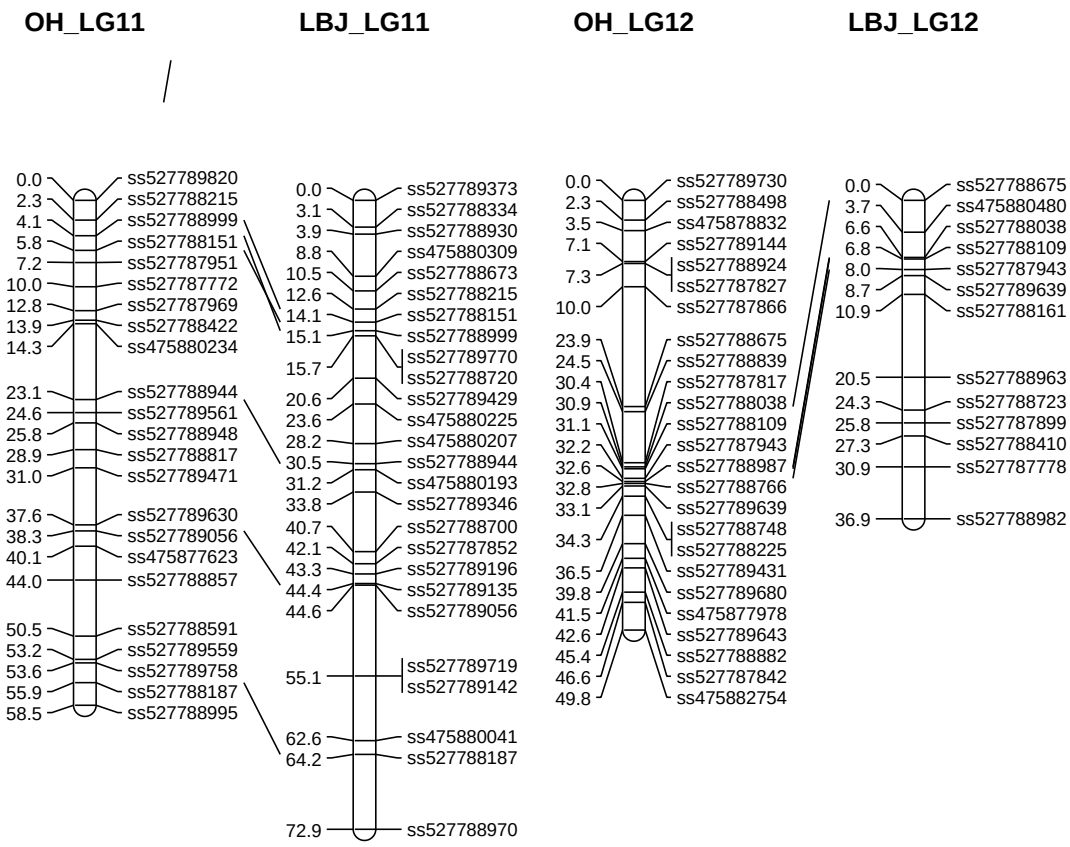
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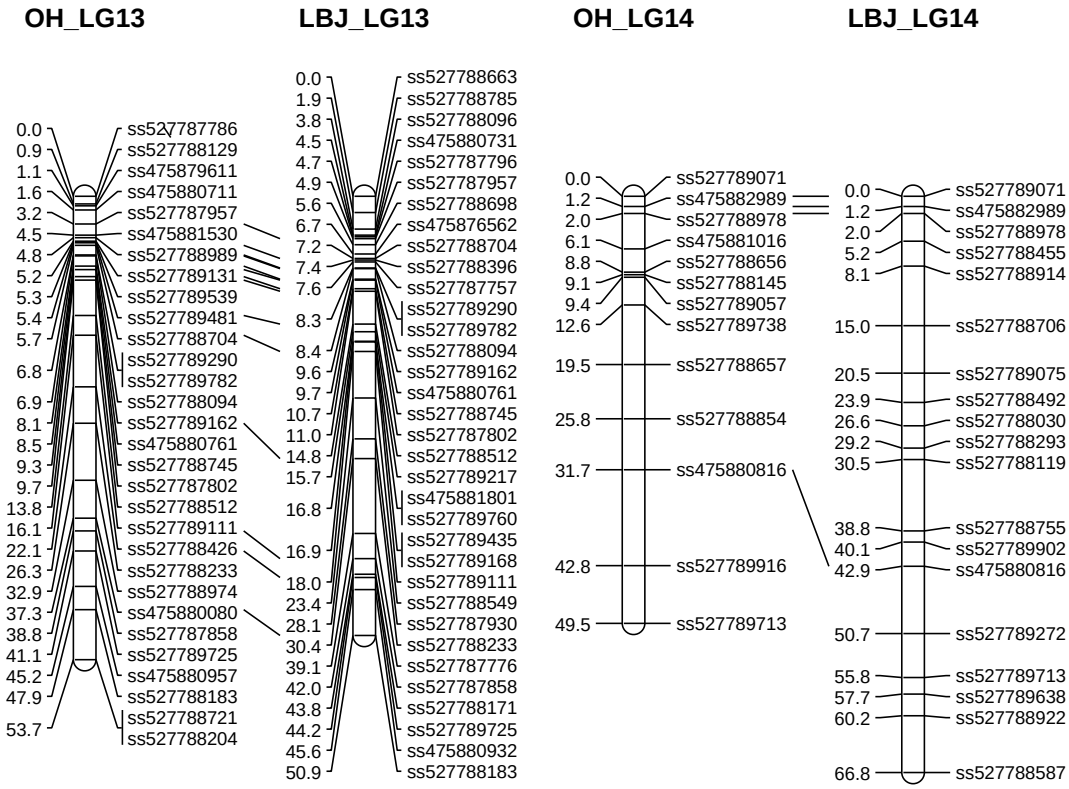
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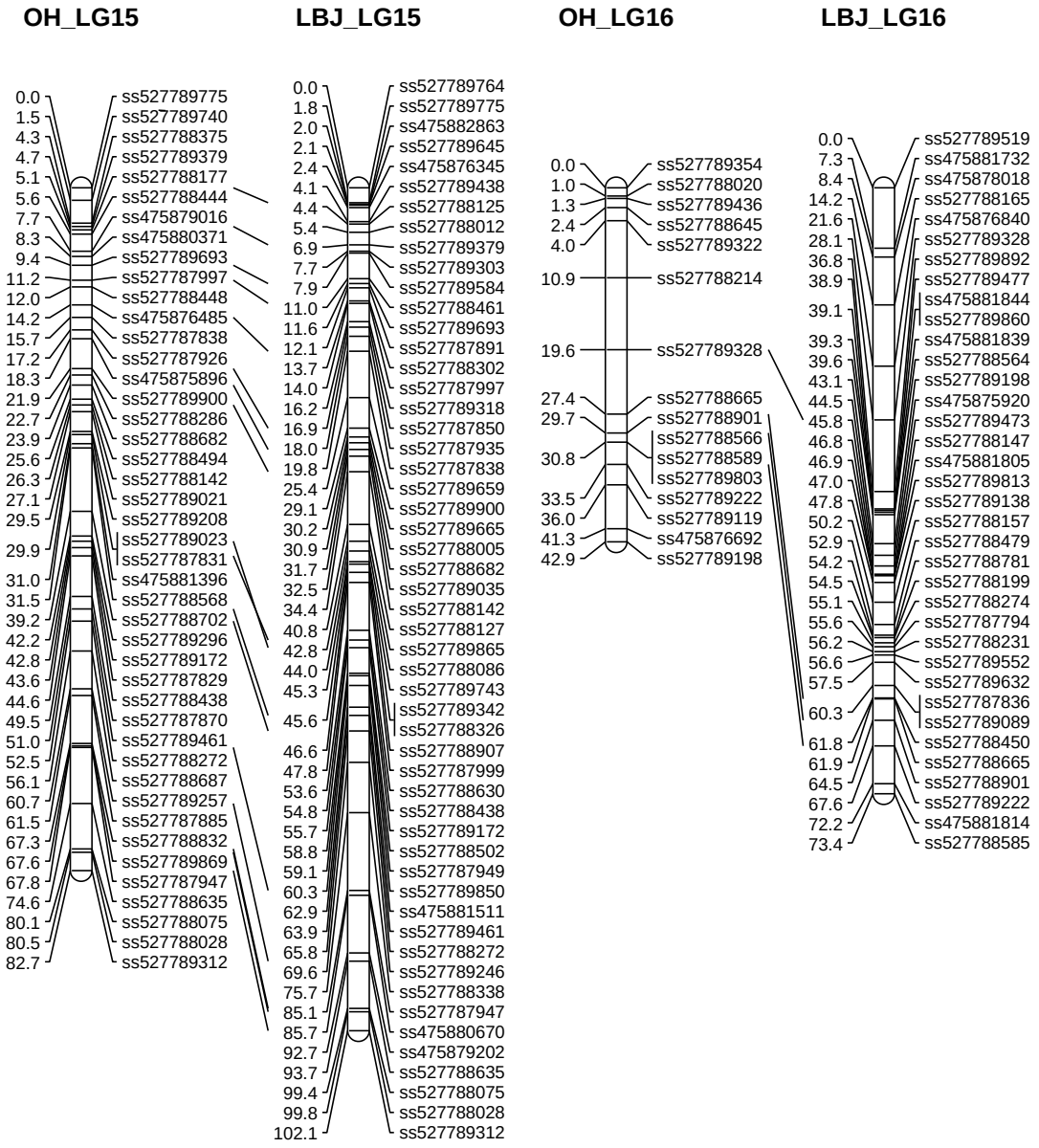
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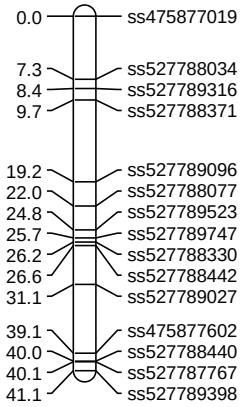
OH_LG17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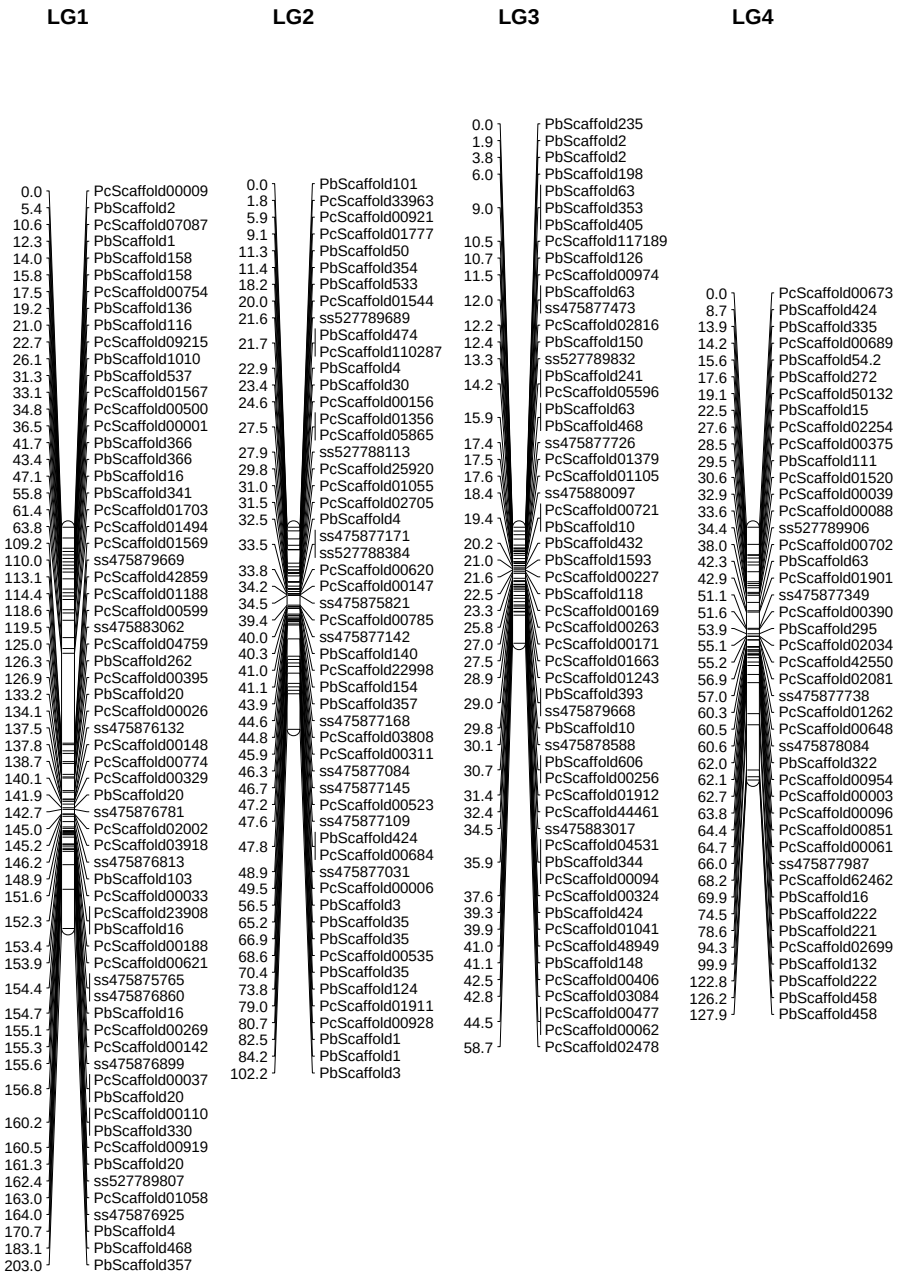
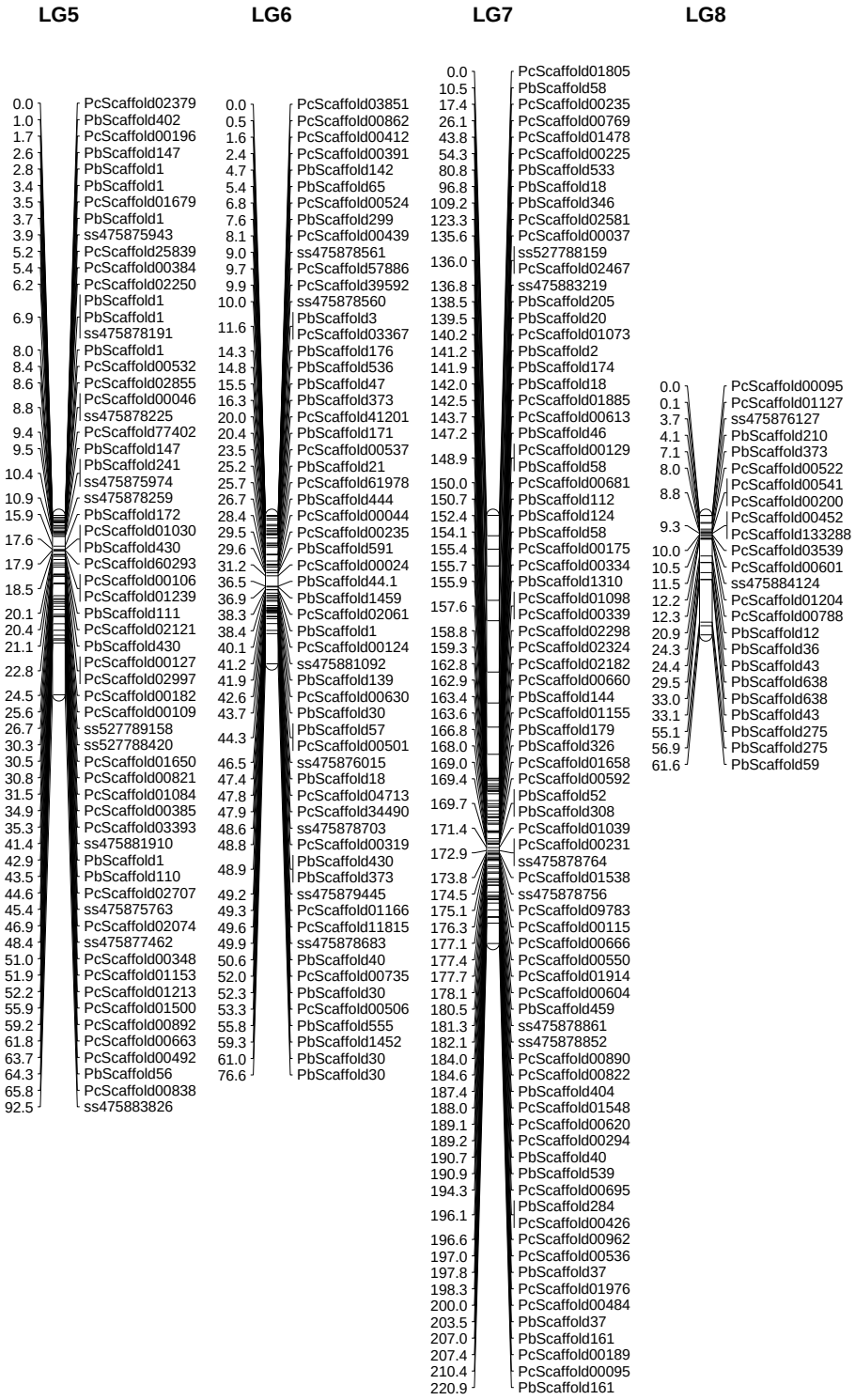
Supplemental material 2: Consensus map constructed for ‘Old Home’ and ‘Louise Bonne de Jersey’ using the genotyping-by-sequencing approach. Displayed are the scaffolds of *Pyrus bretschneideri* (Pb) and *Pyrus communis* (Pc). SS markers are NCBI dbSNP accessions from the SNP array map. The marker positions are indicated in centiMorgan (cM). The map shows one scaffold per cM position only.

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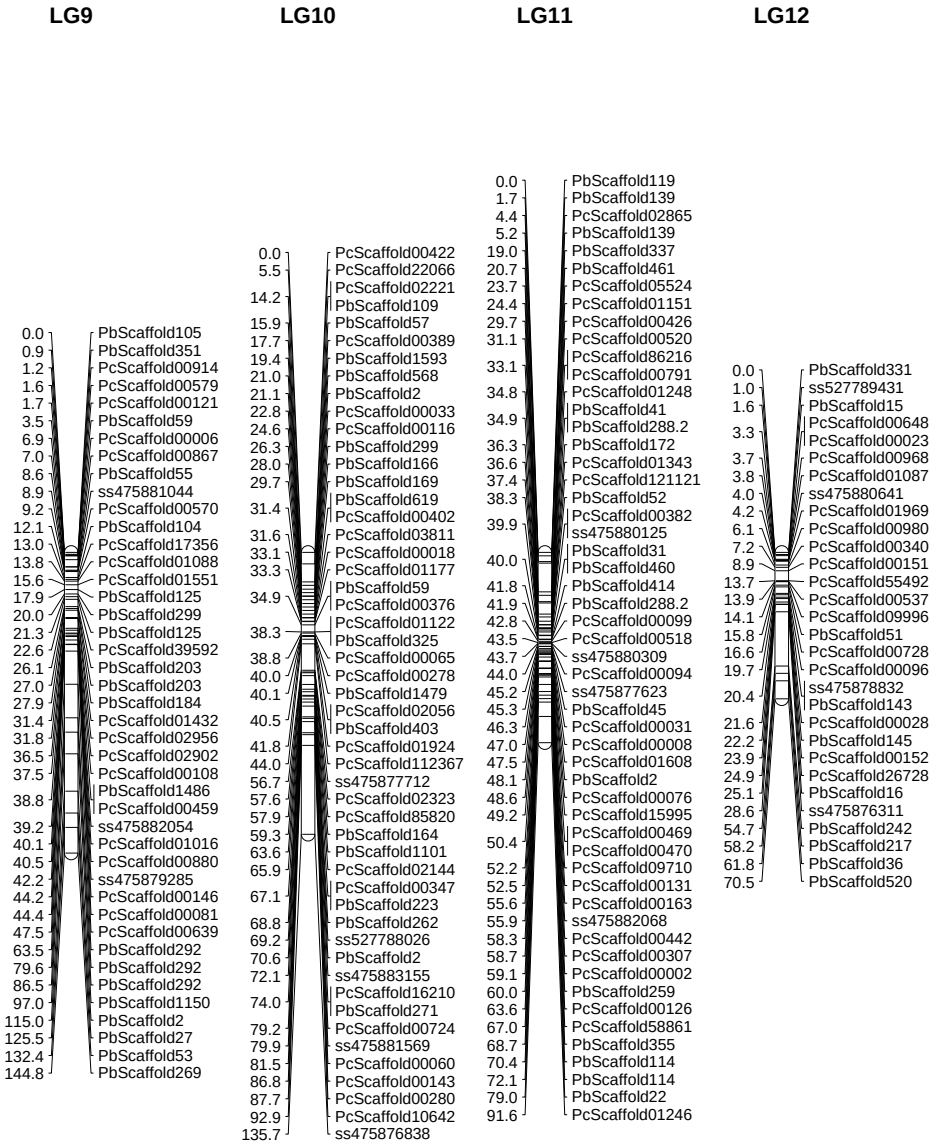
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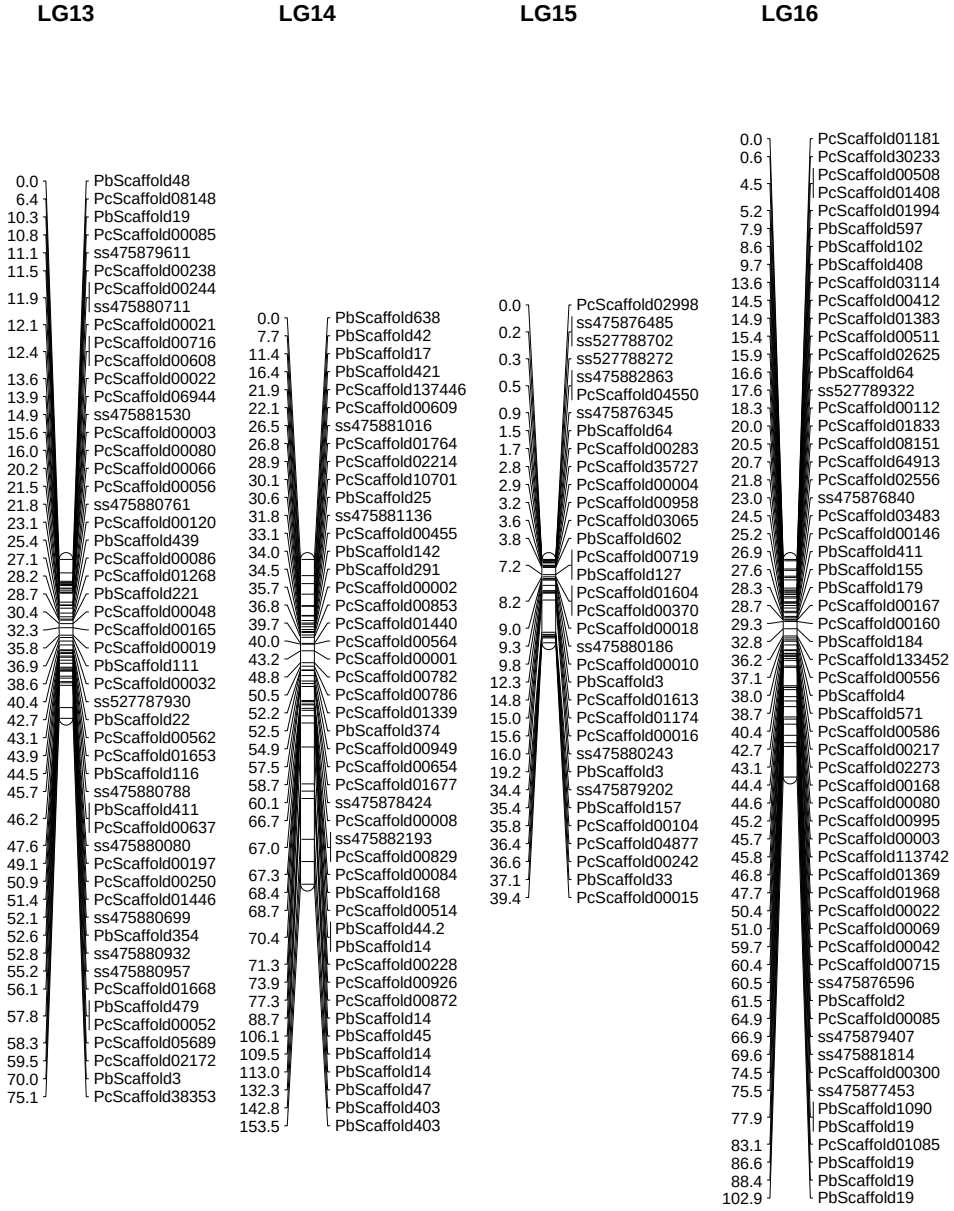
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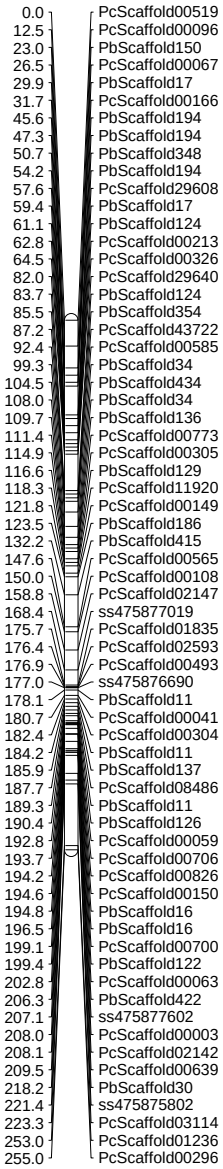
88.4 PbScaffold19
102.9 PbScaffold19

LG17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45.6 PbScaffold194
47.3 PbScaffold194
50.7 PbScaffold348
54.2 PbScaffold194
57.6 PcScaffold29608
59.4 PbScaffold17
61.1 PbScaffold124
62.8 PcScaffold00213
64.5 PcScaffold00326
82.0 PcScaffold29640
83.7 PbScaffold124
85.5 PbScaffold354
87.2 PcScaffold43722
92.4 PcScaffold00585
99.3 PbScaffold34
104.5 PbScaffold434
108.0 PbScaffold34
109.7 PbScaffold136
111.4 PcScaffold00773
114.9 PcScaffold00305
116.6 PbScaffold129
118.3 PcScaffold11920
121.8 PcScaffold00149
123.5 PbScaffold186
132.2 PbScaffold415
147.6 PcScaffold00565
150.0 PcScaffold00108
158.8 PcScaffold02147
168.4 ss475877019
175.7 PcScaffold01835
176.4 PcScaffold02593
176.9 PcScaffold00493
177.0 ss475876690
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180.7 PcScaffold00041
182.4 PcScaffold00304
184.2 PbScaffold11
185.9 PbScaffold137
187.7 PcScaffold08486
189.3 PbScaffold11
190.4 PbScaffold126
192.8 PcScaffold00059
193.7 PcScaffold00706
194.2 PcScaffold00826
194.6 PcScaffold00150
194.8 PbScaffold16
196.5 PbScaffold16
199.1 PcScaffold00700
199.4 PbScaffold122
202.8 PcScaffold00063
206.3 PbScaffold422
207.1 ss475877602
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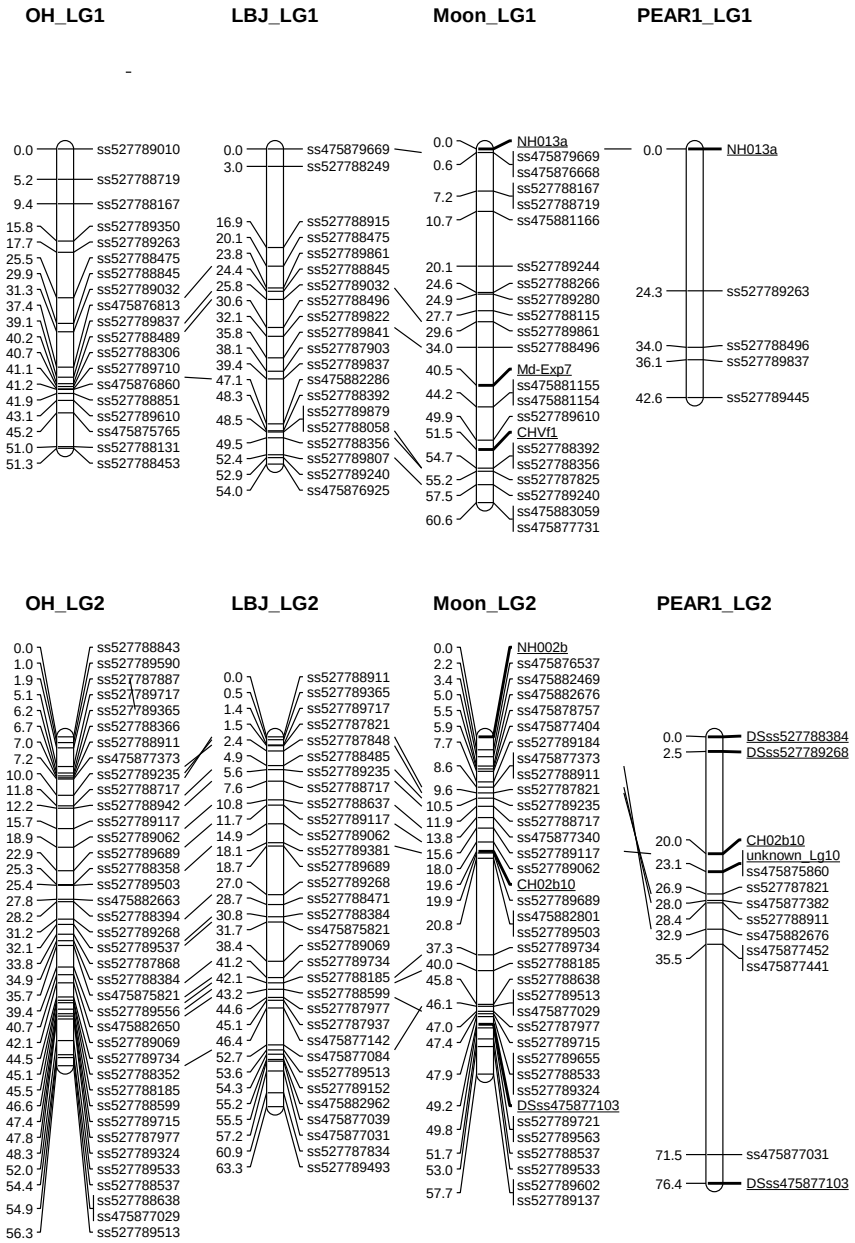
209.5 PcScaffold00639
218.2 PbScaffold30
221.4 ss475875802
223.3 PcScaffold03114
253.0 PcScaffold01236
255.0 PcScaffold00296

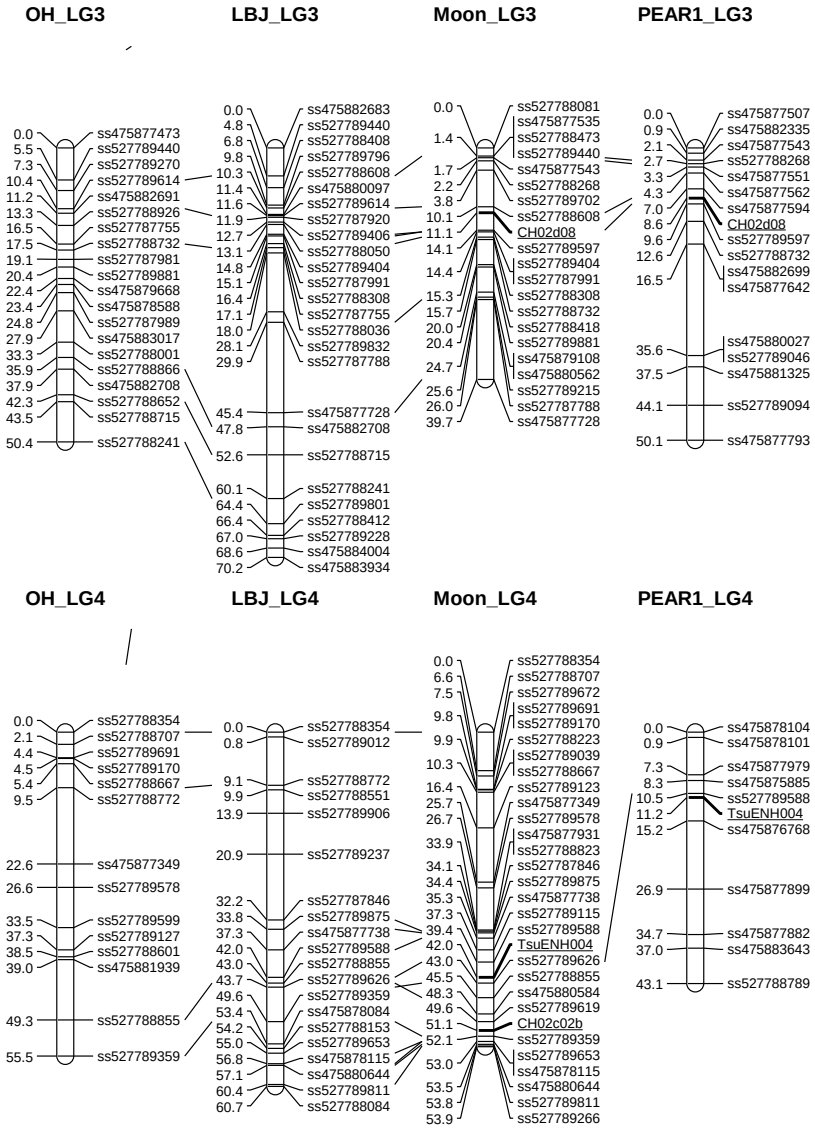
Supplementary material 3: Pearson correlation (first cell) and P-value (second cell) of all the traits measured over four years in the 'Old Home' x 'Louise Bonne de Jersey' OHxLBJ segregating pear population. Branches: branches per tree; height: total tree height; inflorescence: inflorescences per tree; nodes: nodes per tree; Spurs: spurs per tree; TCAtrunk: trunk cross-sectional area 20cm above graft unit; TCAroot: TCA of rootstock; TCAsec: TCA secondary growth of the main axis; TCAtert: TCA tertiary growth of the main axis.

	Branches year 1	Branches year 2	Branches year 3	Height year 1	Height year 2	Height year 3	Inflor-escence year 3	Inflor-escence year 4	LNG year 2	LNG year 3	LNG year 4	Nodes year 1	Nodes year 2	Spurs year 1	Spurs year 2	Spurs year 3	TCA year 1	TCA year 2	TCA year 3	TCA year 4	TCA root year 3	TCA root year 4	TCA sec year 3
Branches year 2	0.757 0.000																						
Branches year 3	0.599 0.000	0.635 0.000																					
Height year 1	0.371 0.000	0.534 0.000	0.364 0.000																				
Height year2	0.552 0.000	0.795 0.000	0.524 0.000	0.898 0.000																			
Height year 3	0.485 0.000	0.467 0.000	0.614 0.000	0.529 0.000	0.569 0.000																		
Inflorescence year 3	0.366 0.000	0.456 0.000	0.144 0.017	0.296 0.000	0.407 0.000	0.192 0.002																	
Inflorescence year 4	0.318 0.000	0.398 0.000	0.360 0.000	0.342 0.000	0.389 0.000	0.302 0.000	0.546 0.000																
LNG year 2	0.263 0.000	0.085 0.174	0.276 0.000	-0.106 0.089	-0.021 0.740	0.559 0.000	-0.064 0.295	0.061 0.326															
LNG year 3	0.336 0.000	0.301 0.000	0.513 0.000	0.121 0.050	0.224 0.000	0.761 0.000	0.134 0.025	0.162 0.007	0.251 0.000														
LNG year 4	0.274 0.000	0.244 0.000	0.406 0.000	0.074 0.233	0.117 0.060	0.443 0.000	0.074 0.218	0.113 0.061	0.162 0.008	0.554 0.000													
Nodes year 1	0.327 0.000	0.424 0.000	0.285 0.000	0.800 0.000	0.687 0.000	0.348 0.000	0.211 0.001	0.307 0.000	-0.137 0.027	0.069 0.267	0.048 0.441												
Nodes year2	0.165 0.007	-0.033 0.600	0.134 0.031	-0.210 0.001	-0.175 0.005	0.381 0.000	-0.157 0.010	-0.010 0.876	0.818 0.000	0.179 0.003	0.217 0.000	-0.235 0.000											

Spurs	0.189	0.150	0.158	0.295	0.238	0.146	0.107	0.194	-0.121	0.101	0.054	0.418	-0.213										
year 1	0.002	0.016	0.012	0.000	0.000	0.020	0.083	0.002	0.051	0.103	0.390	0.000	0.001										
Spurs	0.797	0.739	0.563	0.322	0.529	0.463	0.379	0.310	0.269	0.325	0.289	0.267	0.179	0.031									
year 2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.615									
Spurs	0.721	0.815	0.646	0.486	0.659	0.562	0.430	0.553	0.181	0.387	0.337	0.448	0.061	0.191	0.732								
year 3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.327	0.002	0.000								
TCAtrunk	0.740	0.720	0.576	0.433	0.580	0.470	0.358	0.310	0.183	0.305	0.195	0.363	0.076	0.134	0.638	0.719							
year 1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.002	0.000	0.225	0.031	0.000	0.000							
TCAtrunk	0.606	0.629	0.611	0.295	0.422	0.427	0.302	0.370	0.158	0.353	0.323	0.263	0.258	0.031	0.608	0.663	0.665						
year 2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.617	0.000	0.000	0.000						
TCAtrunk	0.698	0.733	0.834	0.409	0.558	0.714	0.320	0.414	0.314	0.620	0.462	0.331	0.186	0.129	0.701	0.764	0.733	0.752					
year 3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.037	0.000	0.000	0.000	0.000					
TCAtrunk	0.666	0.675	0.812	0.352	0.496	0.724	0.281	0.344	0.341	0.653	0.526	0.261	0.226	0.101	0.633	0.701	0.662	0.698	0.964				
year 4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.104	0.000	0.000	0.000	0.000	0.000				
TCAroot	0.697	0.735	0.795	0.430	0.574	0.680	0.316	0.436	0.276	0.569	0.446	0.348	0.168	0.163	0.701	0.775	0.674	0.714	0.921	0.889			
year 3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.008	0.000	0.000	0.000	0.000	0.000	0.000			
TCAroot	0.653	0.694	0.788	0.377	0.515	0.685	0.269	0.364	0.292	0.601	0.503	0.284	0.191	0.135	0.638	0.718	0.625	0.681	0.919	0.931	0.958		
year 4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
TCAsec	0.443	0.396	0.618	0.139	0.254	0.704	0.143	0.267	0.600	0.609	0.436	0.114	0.486	0.014	0.426	0.492	0.395	0.535	0.693	0.725	0.638	0.684	
year 3	0.000	0.000	0.000	0.025	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.065	0.000	0.823	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
TCAtert	0.422	0.380	0.567	0.140	0.261	0.758	0.198	0.254	0.464	0.795	0.458	0.105	0.395	0.018	0.413	0.458	0.395	0.437	0.652	0.679	0.604	0.633	0.791
year 3	0.000	0.000	0.000	0.024	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.091	0.000	0.770	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Supplementary material 4: Alignment of linkage groups from 'Louise Bonne de Jersey' (LBJ) and 'Old Home' (OH) pears with the maps of 'Moonglow' (Moon) and PEAR1 (Montanari *et al.*, 2013). The markers are named using the NCBI dbSNP accessions and their positions are indicated in centiMorgan. Microsatellite markers mapped in the 'Moonglow' x PEAR1 population are underlined. The linkage group (LG) numbering system is consistent with the apple LG numbering. Identified QTLs are shown with blue symbols coming from OH and brown symbols from LBJ. The *Dw1* flanking marker Hi01c04 (underlined and red) mapped to LG5 of OH.

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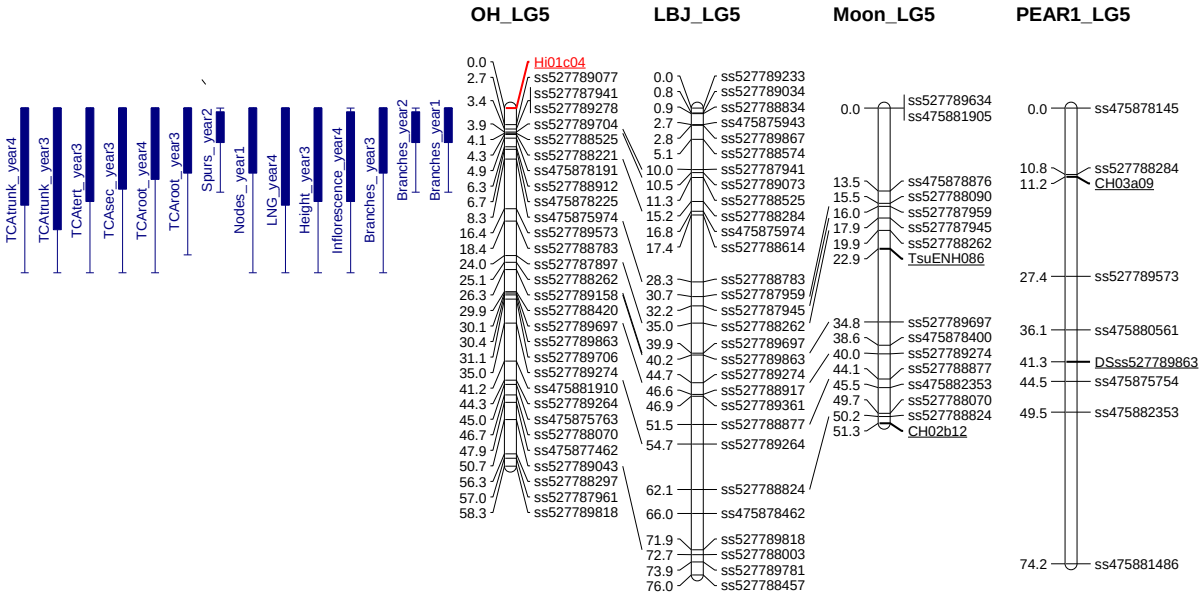
LBJ_LG3
Moon_LG3
PEAR1_LG3
OH_LG4
LBJ_LG4
Moon_LG4
PEAR1_LG4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TCAtert_year3
TCAsec_year3
TCAroot_year4
TCAroot_year3
Spurs_year2
Nodes_year1
LNG_year4
Height_year3
Inflorescence_year4
Branches_year3
Branches_year2
Branches_year1
OH_LG5
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3.4 ss527787941
ss527789278
3.9 ss527789704
4.1 ss527788525
4.3 ss527788221
4.9 ss475878191
6.3 ss527788912
6.7 ss475878225
8.3 ss475875974
16.4 ss527789573
18.4 ss527788783
24.0 ss527787897
25.1 ss527788262
26.3 ss527789158
29.9 ss527788420
30.1 ss527789697
30.4 ss527789863
31.1 ss527789706
35.0 ss527789274
41.2 ss475881910
44.3 ss527789264
45.0 ss475875763
46.7 ss527788070
47.9 ss475877462
50.7 ss527789043
56.3 ss527788297
57.0 ss527787961
58.3 ss527789818
LBJ_LG5
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0.8 ss527789034
0.9 ss527788834
2.7 ss475875943
2.8 ss527789867
5.1 ss527788574
10.0 ss527787941
10.5 ss527789073
11.3 ss527788525
15.2 ss527788284
16.8 ss475875974
17.4 ss527788614
28.3 ss527788783
30.7 ss527787959
32.2 ss527787945
35.0 ss527788262
39.9 ss527789697
40.2 ss527789863
44.7 ss527789274
46.6 ss527788917
46.9 ss527789361
51.5 ss527788877
54.7 ss527789264
62.1 ss527788824
66.0 ss475878462
71.9 ss527789818
72.7 ss527788003
73.9 ss527789781
76.0 ss527788457
Moon_LG5
0.0 ss527789634
ss475881905
13.5 ss475878876
15.5 ss527788090
16.0 ss527787959
17.9 ss527787945
19.9 ss527788262
22.9 TsuENH086
34.8 ss527789697
38.6 ss475878400
40.0 ss527789274
44.1 ss527788877
45.5 ss475882353
49.7 ss527788070
50.2 ss527788824
51.3 CH02b12
PEAR1_LG5
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10.8 ss527788284
11.2 CH03a09
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49.5 ss475882353
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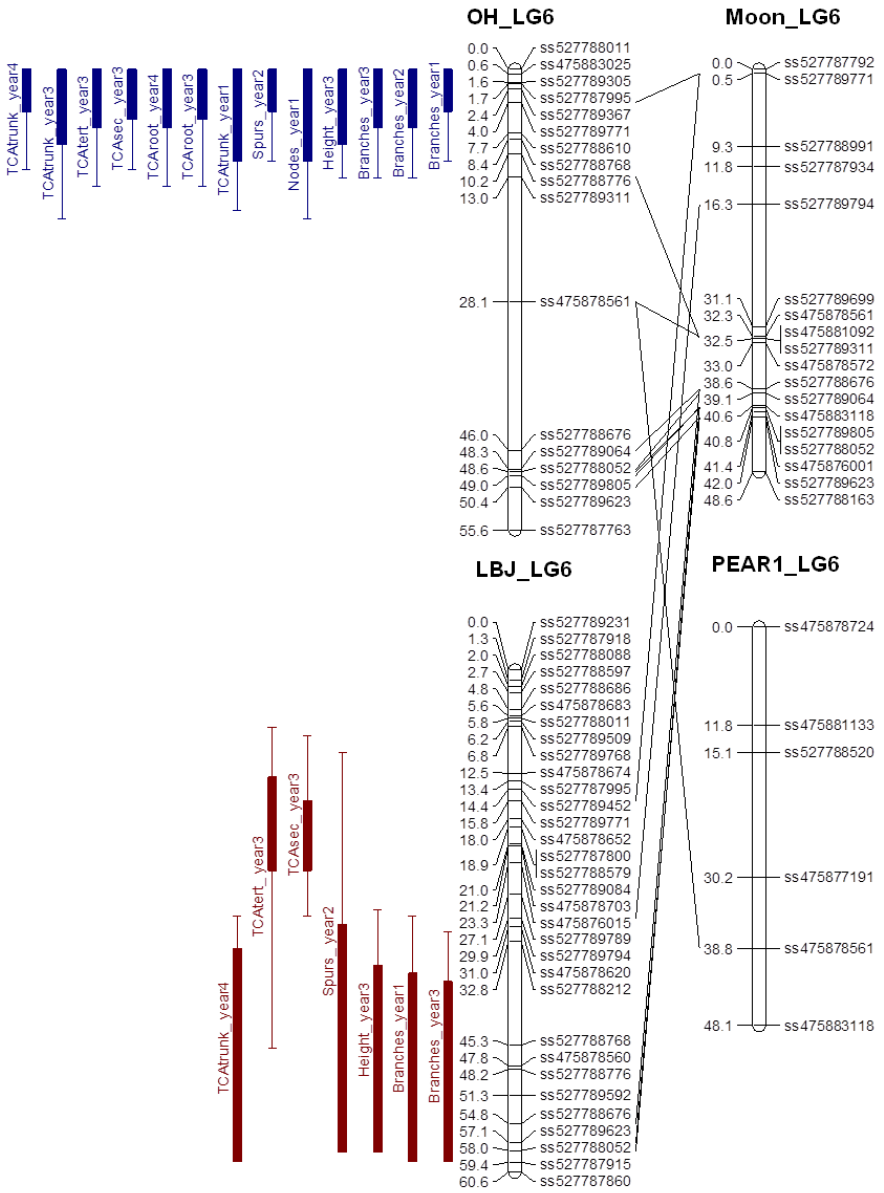
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Moon_LG6
LBJ_LG6
PEAR1_LG6
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TCAtert_year3
TCAsec_year3
TCAroot_year4
TCAroot_year3
TCAtrunk_year1
Spurs_year2
Nodes_year1
Height_year3
Branches_year3
Branches_year2
Branches_year1
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1.6 ss527789305
1.7 ss527787995
2.4 ss527789367
4.0 ss527789771
7.7 ss527788610
8.4 ss527788768
10.2 ss527788776
13.0 ss527789311
28.1 ss475878561
46.0 ss527788676
48.3 ss527789064
48.6 ss527788052
49.0 ss527789805
50.4 ss527789623
55.6 ss527787763
0.0 ss527787792
0.5 ss527789771
9.3 ss527788991
11.8 ss527787934
16.3 ss527789794
31.1 ss527789699
32.3 ss475878561
32.5 ss475881092
ss527789311
33.0 ss475878572
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39.1 ss527789064
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40.8 ss527789805
ss527788052
41.4 ss475876001
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48.6 ss527788163
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1.3 ss527787918
2.0 ss527788088
2.7 ss527788597
4.8 ss527788686
5.6 ss475878683
5.8 ss527788011
6.2 ss527789509
6.8 ss527789768
12.5 ss475878674
13.4 ss527787995
14.4 ss527789452
15.8 ss527789771
18.0 ss475878652
18.9 ss527787800
ss527788579
21.0 ss527789084
21.2 ss475878703
23.3 ss475876015
27.1 ss527789789
29.9 ss527789794
31.0 ss475878620
32.8 ss527788212
45.3 ss527788768
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57.1 ss527789623
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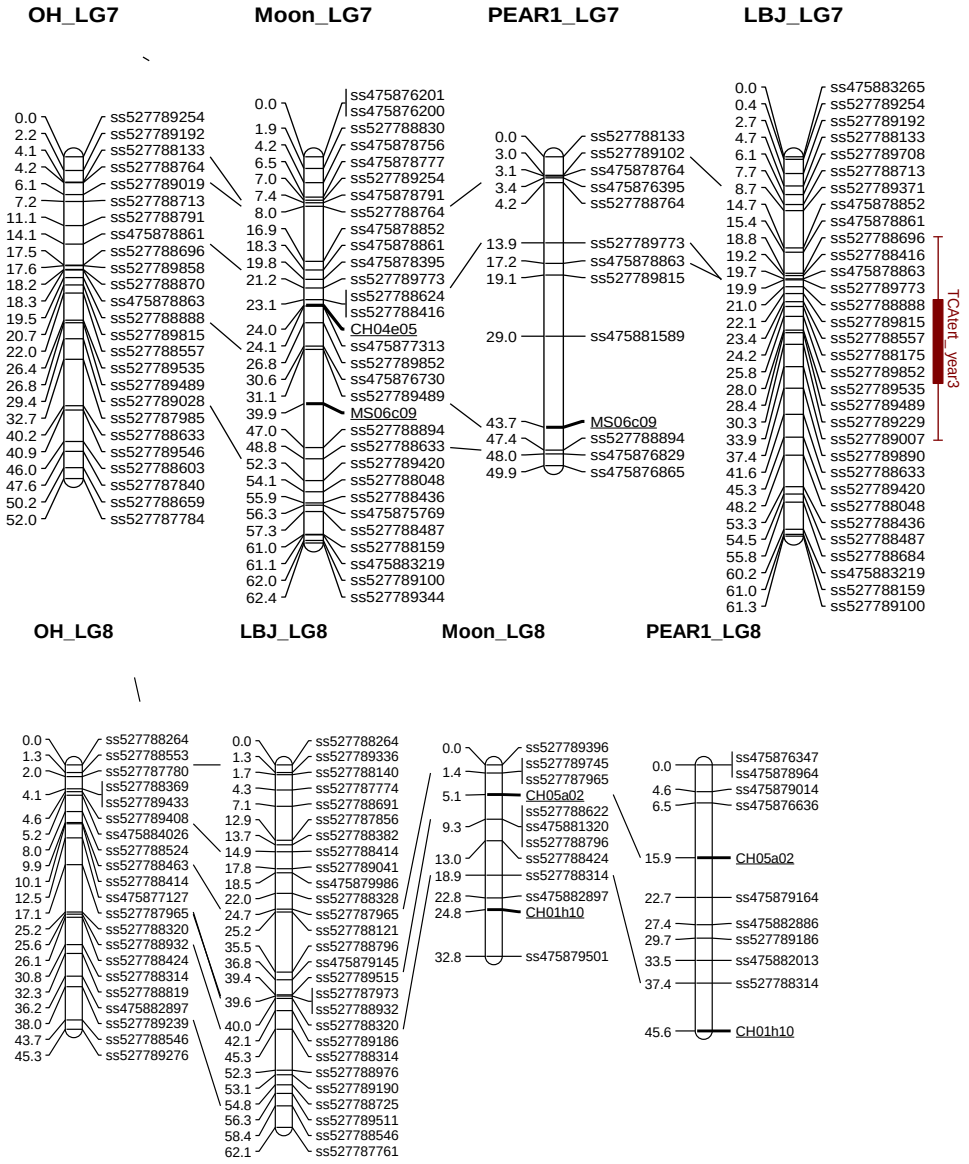
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Moon_LG7
PEAR1_LG7
LBJ_LG7
OH_LG8
LBJ_LG8
Moon_LG8
PEAR1_LG8
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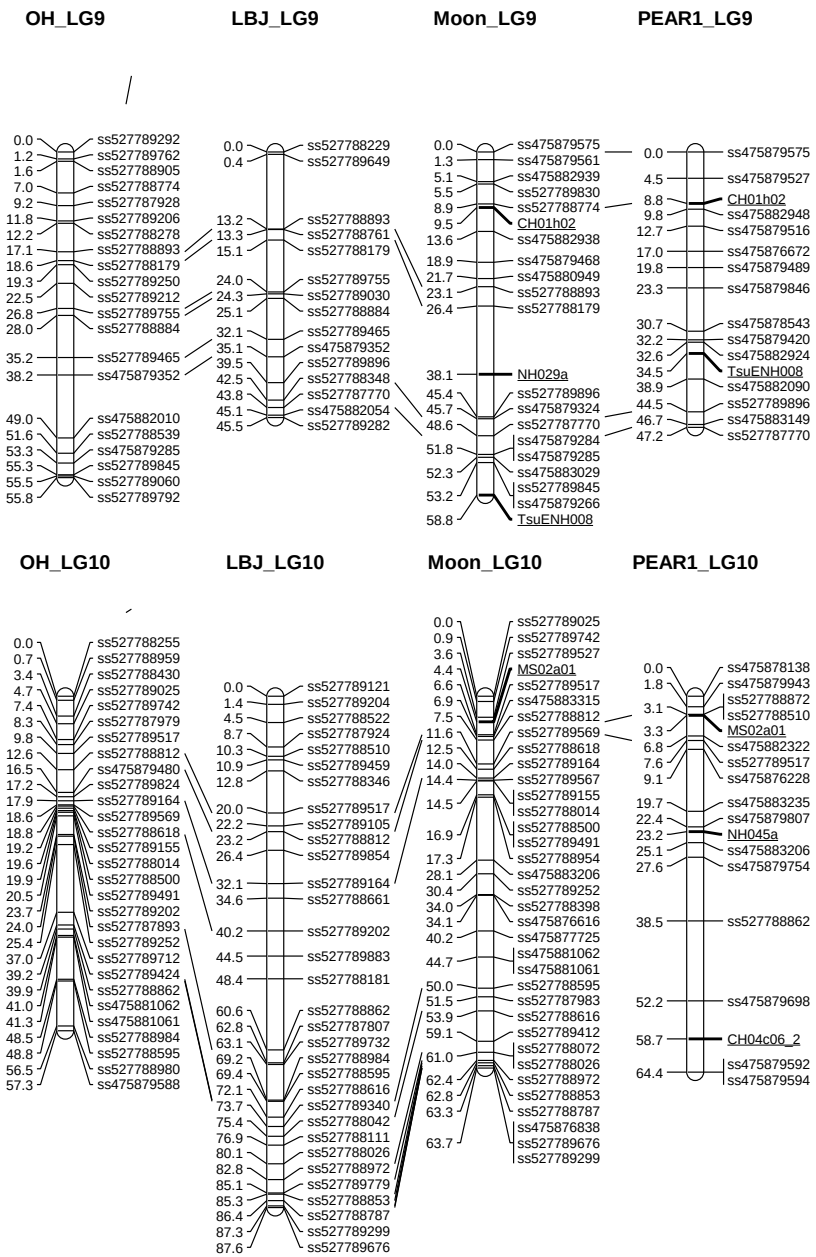
CH04e05
MS06c09
CH05a02
CH01h10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LBJ_LG9
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PEAR1_LG9
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LBJ_LG10
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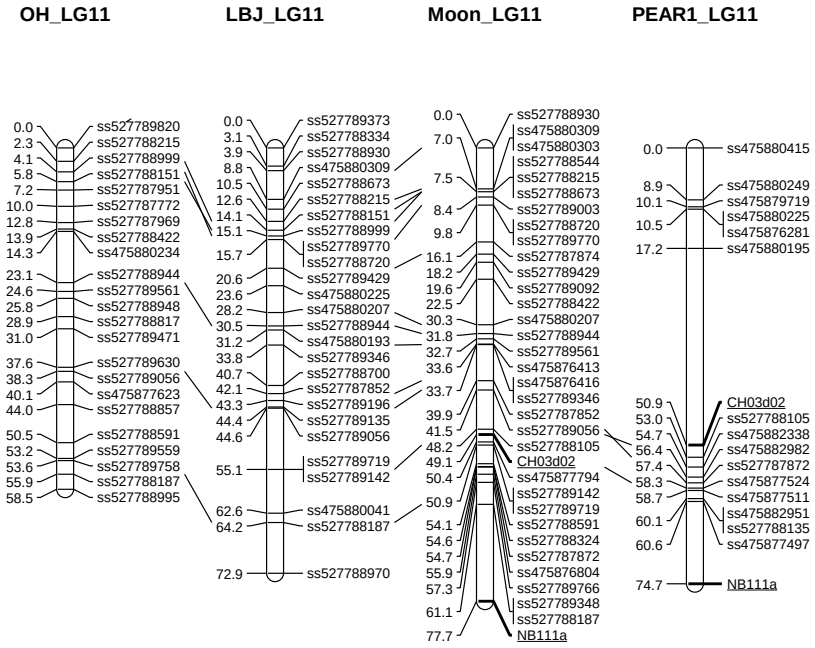
PEAR1_LG10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LBJ_LG11
Moon_LG11
PEAR1_LG11
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4.1 ss527788999
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7.2 ss527787951
10.0 ss527787772
12.8 ss527787969
13.9 ss527788422
14.3 ss475880234
23.1 ss527788944
24.6 ss527789561
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28.9 ss527788817
31.0 ss527789471
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38.3 ss527789056
40.1 ss475877623
44.0 ss527788857
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55.9 ss527788187
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10.5 ss527788673
12.6 ss527788215
14.1 ss527788151
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ss527788720
20.6 ss527789429
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44.4 ss527789135
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ss527789142
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ss475880303
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18.2 ss527789429
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48.2 ss527788105
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ss475880225
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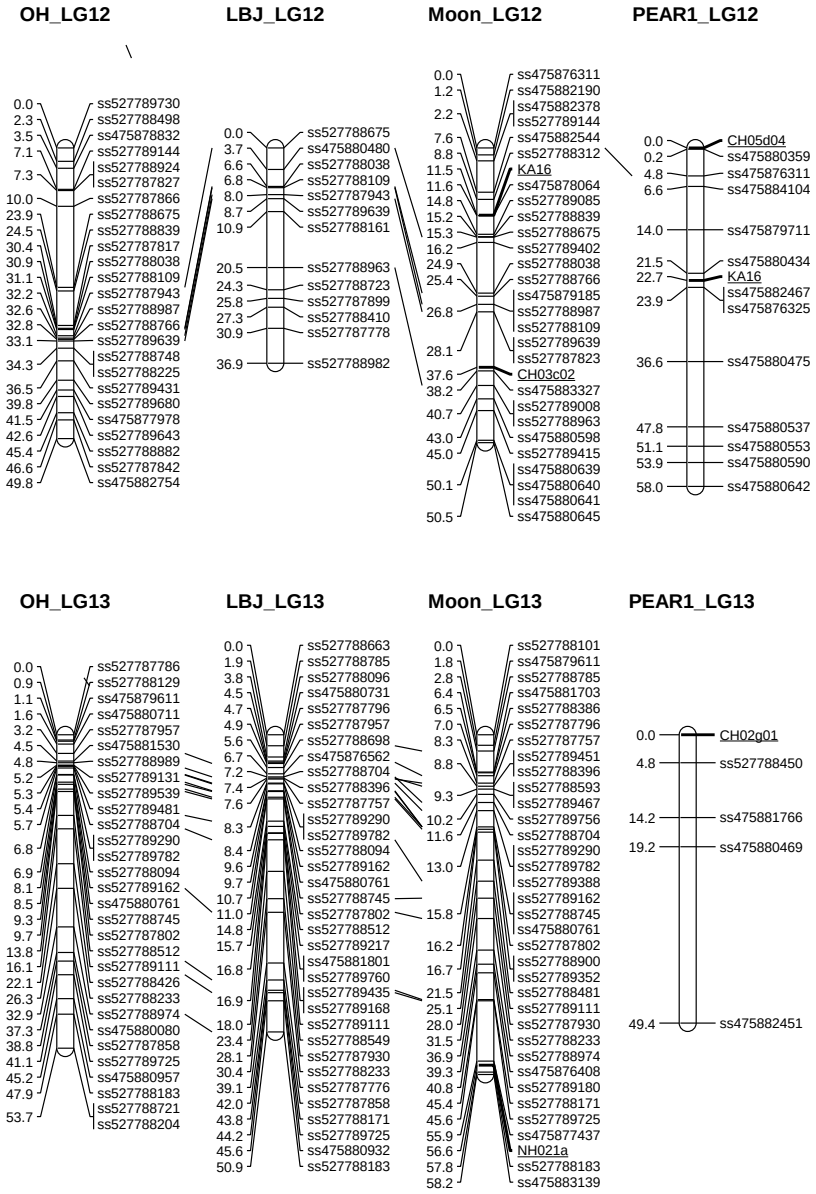
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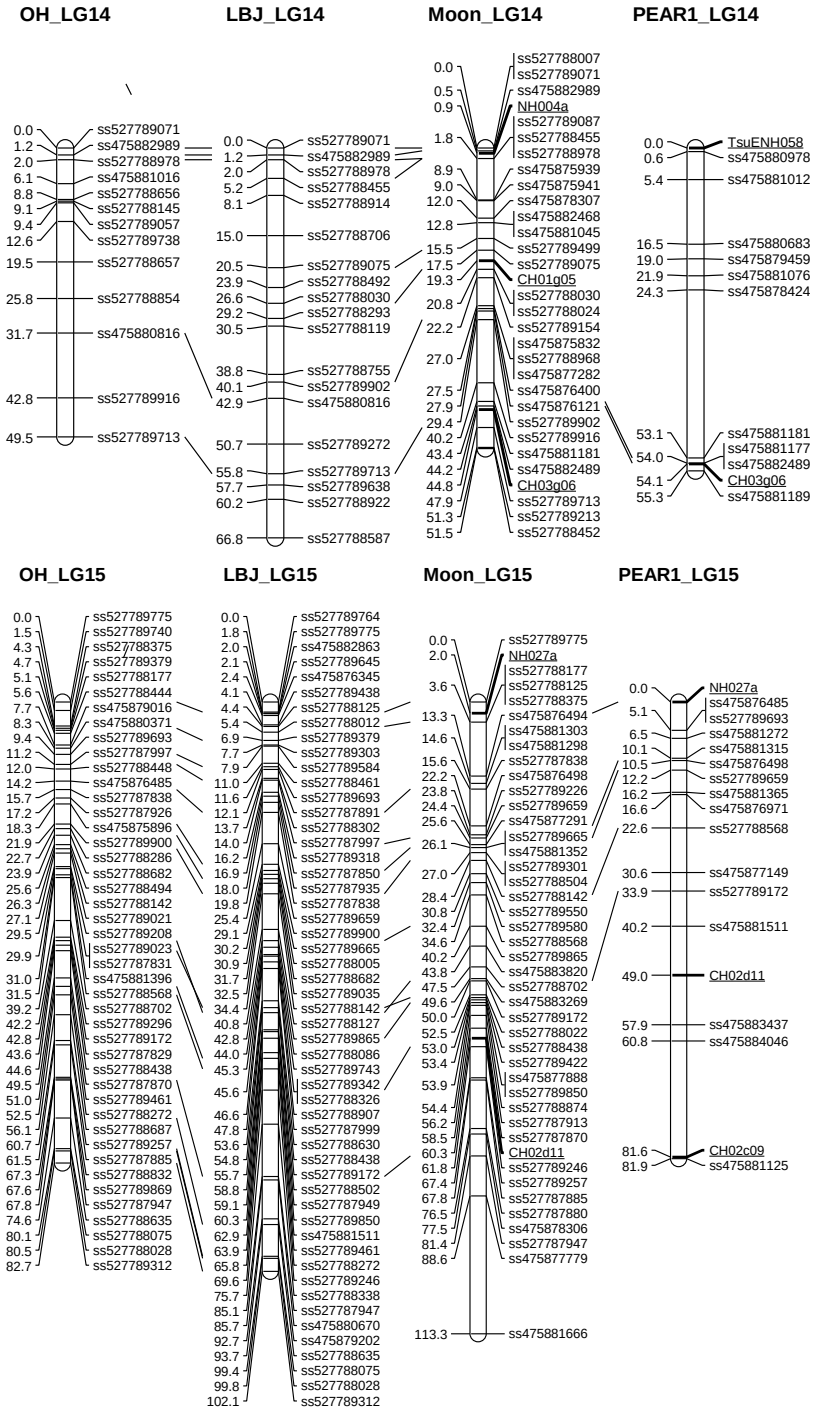
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PEAR1_LG14
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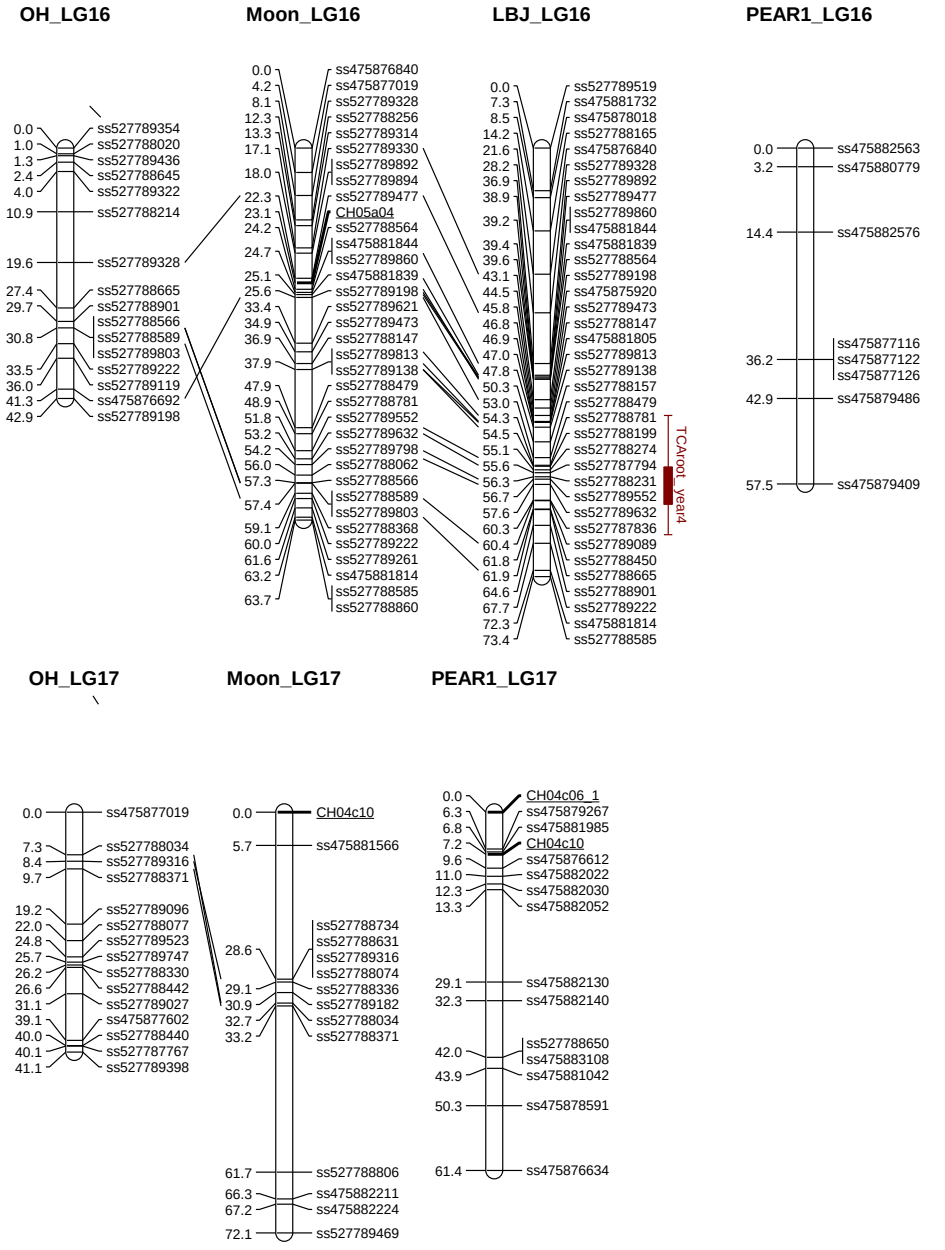
LBJ_LG15
Moon_LG15
PEAR1_LG15


OH_LG16
Moon_LG16
LBJ_LG16
PEAR1_LG16
0.0 ss527789354
1.0 ss527788020
1.3 ss527789436
2.4 ss527788645
4.0 ss527789322
10.9 ss527788214
19.6 ss527789328
27.4 ss527788665
29.7 ss527788901
ss527788566
30.8 ss527788589
ss527789803
33.5 ss527789222
36.0 ss527789119
41.3 ss475876692
42.9 ss527789198
0.0 ss475876840
4.2 ss475877019
8.1 ss527789328
12.3 ss527788256
13.3 ss527789314
17.1 ss527789330
ss527789892
18.0 ss527789894
ss527789477
22.3 CH05a04
23.1 ss527788564
24.2 ss475881844
24.7 ss527789860
25.1 ss475881839
25.6 ss527789198
33.4 ss527789621
34.9 ss527789473
36.9 ss527788147
37.9 ss527789813
ss527789138
47.9 ss527788479
48.9 ss527788781
51.8 ss527789552
53.2 ss527789632
54.2 ss527789798
56.0 ss527788062
57.3 ss527788566
57.4 ss527788589
ss527789803
59.1 ss527788368
60.0 ss527789222
61.6 ss527789261
63.2 ss475881814
63.7 ss527788585
ss527788860
0.0 ss527789519
7.3 ss475881732
8.5 ss475878018
14.2 ss527788165
21.6 ss475876840
28.2 ss527789328
36.9 ss527789892
38.9 ss527789477
39.2 ss527789860
ss475881844
39.4 ss475881839
39.6 ss527788564
43.1 ss527789198
44.5 ss475875920
45.8 ss527789473
46.8 ss527788147
46.9 ss475881805
47.0 ss527789813
47.8 ss527789138
50.3 ss527788157
53.0 ss527788479
54.3 ss527788781
54.5 ss527788199
55.1 ss527788274
55.6 ss527787794
56.3 ss527788231
56.7 ss527789552
57.6 ss527789632
60.3 ss527787836
60.4 ss527789089
61.8 ss527788450
61.9 ss527788665
64.6 ss527788901
67.7 ss527789222
72.3 ss475881814
73.4 ss527788585
TCAroot_year4
0.0 ss475882563
3.2 ss475880779
14.4 ss475882576
ss475877116
36.2 ss475877122
ss475877126
42.9 ss475879486
57.5 ss475879409
OH_LG17
Moon_LG17
PEAR1_LG17
0.0 ss475877019
7.3 ss527788034
8.4 ss527789316
9.7 ss527788371
19.2 ss527789096
22.0 ss527788077
24.8 ss527789523
25.7 ss527789747
26.2 ss527788330
26.6 ss527788442
31.1 ss527789027
39.1 ss475877602
40.0 ss527788440
40.1 ss527787767
41.1 ss527789398
0.0 CH04c10
5.7 ss475881566
28.6 ss527788734
ss527788631
ss527789316
ss527788074
29.1 ss527788336
30.9 ss527789182
32.7 ss527788034
33.2 ss527788371
61.7 ss527788806
66.3 ss475882211
67.2 ss475882224
72.1 ss527789469
0.0 CH04c06_1
6.3 ss475879267
6.8 ss475881985
7.2 CH04c10
9.6 ss475876612
11.0 ss475882022
12.3 ss475882030
13.3 ss475882052
29.1 ss475882130
32.3 ss475882140
42.0 ss527788650
ss475883108
43.9 ss475881042
50.3 ss475878591
61.4 ss475876634

R Scripts:

OneMap

```
library(onemap)
setwd("K:/Mapping/Pears/Old Home x
LBJ/2014/GBS/PearGBS_EW_Ref_Pcomm")
mapeuro<-read.outcross("K:/Mapping/Pears/Old Home x
LBJ/2014/GBS/PearGBS_EW_Ref_Pcomm","Group5-8.txt")

twoptseuro<-rf.2pts(mapeuro)
# Assigning markers to LGs
mark.alleuro<-make.seq(twoptseuro,"all") #all markers will be used for analysis
LGseuro<-group(mark.alleuro) # Estimating two point recombination fractions
LOD3 and recombination fraction (RF) 0.50
LGseuro<-group(mark.alleuro,LOD=7,max.rf=0.4) # changing LOD score and RF
LGseuro
#genetic mapping of LGs
set.map.fun(type="kosambi") #setting mapping function
LG1euro<-make.seq(LGseuro,1)
LG1euro
LG2euro<-make.seq(LGseuro,2)
LG2euro
#for large LGs
LG1euro.ord<-order.seq(LG1euro,n.init=6,touchdown=TRUE)
LG1euro.ord
(LG1euro.final <- make.seq(LG1euro.ord,"force"))
ripple.seq(LG1euro.final) #check the final map
LG1euro.final
```

LPmerge

```
setwd("K:/Mapping/Pears/Old Home x LBJ/2014/GBS/GBS map com and bret")
library(LPmerge)
library(PAAUtils)
# Read in Linkage Groups
SNPOHLG1<- readX('OHSNP.xlsx', 'a') # readX is part of PAAUtils all data is read as a character no factor
SNPLBJLG1<- readX('LBJSNP.xlsx', 'a')
GBSLG1h1<- readX('GBSmap.xlsx', 'LG1h1')
GBSLG1h2<- readX('GBSmap.xlsx', 'LG1h2')
# Convert data into data frames
SNPOHmapI <- data.frame(marker=SNPOHLG1)
SNPLBJmapI <- data.frame(marker=SNPLBJLG1)
GBSh1mapI <- data.frame(marker=GBSLG1h1)
GBSh1mapIX <- data.frame(marker=GBSLG9h1)
GBSh2mapI <- data.frame(marker=GBSLG1h2)
# make list of all matching Linkage Groups
map1 <- list(I=GBSh1mapI,II=SNPOHmapI,III=GBSh2mapI,IV=SNPLBJmapI)
# make consensus map with LPmerge
CON1 <- LPmerge(map1)
```

Accounting for environmental infulence in block

```
## @knitr getData
setwd("K:/Mapping/Pears/Old Home x LBJ/2013/Data analysis with r")
library(PAAUtils)
oh13 <- readX('OHLBJ.xlsx', 'Sheet2') # readX is part of PAAUtils all data is read as
a character no factor

# First phenotypic data
phenData <- readX('OHLBJ.xlsx', namedRange='AllData') # readX is part of
PAAUtils
str(phenData) # structure of the data.frame
# Good to simplify names
names(phenData) <- caseChange(tolower(names(phenData)), 'c') # caseChange is
part of PAAUtils
names(phenData) <- paste0(tolower(substring(names(phenData), 1, 1)),
substring(names(phenData), 2)) #new words have capital letter at beginning
names(phenData) <- gsub("'", '', names(phenData)) #names have no ' or space
names(phenData) <- gsub('\\(cm\\)', '', names(phenData)) #removes (cm)
names(phenData) <- paste0(tolower(substring(names(phenData), 1, 1)),
substring(names(phenData), 2))
ends(phenData)

# Next the planting plan
pPlan <- readX('Planting plan.xlsx', 'Sheet1') # readX is part of PAAUtils
str(pPlan)
ends(pPlan)
```

```
pPlan <- data.frame(id=unlist(pPlan[,2:4]), row=rep(1:3, each=nrow(pPlan)),
tree=rep(pPlan[,1], ncol(pPlan)-1), stringsAsFactors=FALSE) #takes colum 2,3,4
unlist ant puts everything ito one collumn. Tells R not to take data as factors
pPlan[pPlan$id=='QC','id'] <- 'QC/BH' #replaces QC wit QC/BH
if (all.equal(phenData$id, pPlan$id)) phenData <- data.frame(pPlan, phenData[,-1])
row.names(phenData) <- 1:nrow(phenData)
for (i in c('id','row','tree')) phenData[,i] <- factor(phenData[,i])

## @knitr edaPlots
with(phenData, myPlot(tcaTrunk, lengthNewGrowth, xlab='TCA at trunk',
ylab='Length of new growth', prCor=TRUE))
with(phenData[phenData$id=='QC/BH',], points(tcaTrunk, lengthNewGrowth,
pch=16))
lines(with(phenData, supsmu(phenData$tcaTrunk, phenData$lengthNewGrowth,
span=0.2)), col='purple')
# Could do this sort of thing to other pairs of variates as well

pairs(phenData[,c('size','sucker','lengthNewGrowth','tcaTrunk')]) # for a scatterplot
matrix

# Find 0 tca in 2011 which have positive tca in 2013
phenData[phenData$trunkTca11 %in% 0 & !(phenData$tcaTrunk %in% 0),
c('id','row','tree','trunkTca11','tcaTrunk')]

# Identify 'special' points in totalLength vs lengthNewGrowth graph
with(phenData, plot(lengthNewGrowth, totalLength))
spPts <- with(phenData, identify(lengthNewGrowth, totalLength))
phenData[spPts, c('id','row','tree','totalLength','lengthNewGrowth')]
```

```
## @knitr allRandom
library(asreml)

ttASR0 <- asreml(lengthNewGrowth~1, random=~id, rcov=~ar1(row):ar1(tree),
data=phenData, maxiter=50)
plot(ttASR0, aspect=1)
plot(variogram(ttASR0))
summary(ttASR0)$varcomp

ttASR1 <- asreml(lengthNewGrowth~row+lin(tree), random=~id,
rcov=~id(row):ar1(tree), data=phenData, maxiter=50)
ttASR2 <- asreml(lengthNewGrowth~row+lin(tree), random=~id,
rcov=~ar1(row):ar1(tree), data=phenData, maxiter=50)
ttASR3 <- asreml(lengthNewGrowth~row+lin(tree), random=~id,
rcov=~id(row):id(tree), data=phenData, maxiter=50)
lrt(ttASR1, ttASR2)
lrt(ttASR1, ttASR3)
plot(ttASR1, aspect=1)
plot(variogram(ttASR1))
plot(fitted(ttASR1), resid(ttASR1))
outL <- identify(fitted(ttASR1), resid(ttASR1))
phenData[outL, c('id','row','tree','lengthNewGrowth')]
summary(ttASR1)$varcomp

ttPred1 <- predict(ttASR1, classify='id', maxiter=1)$pred$pval
myPlot(predict(ttASR0, classify='id', maxiter=1)$pred$pval$pred, ttPred1$pred,
      xlab='No linear row', ylab='With linear row', panel.first=abline(0, 1,
col='grey70'))

ttMerge <- merge(phenData[,c('id','lengthNewGrowth')], ttPred1)
```

```
with(ttMerge, myPlot(lengthNewGrowth, predicted.value, xlab='Raw data',
ylab='BLUPs', panel.first=abline(0, 1, col='grey70')))
with(ttMerge[ttMerge$id=='QC/BH',], points(lengthNewGrowth, predicted.value,
pch=16, col='blue2'))

# Interested in size, sucker, lengthNewGrowth and tcaTrunk

# Need to consider have QC/BH as fixed effects; also bi-variate analysis and analysis
of score data

# Treat QC/BH checks as fixed effects
phenData$progeny <- phenData$check <- phenData$id
phenData[phenData$id %in% 'QC/BH','progeny'] <- NA
phenData[!(phenData$id %in% 'QC/BH'),'check'] <- NA

ttASR1a <- asreml(lengthNewGrowth~check+row+lin(tree), random=~progeny,
rcov=~id(row):ar1(tree), data=phenData, maxiter=50, na.method.X='include')
plot(fitted(ttASR1), fitted(ttASR1a), panel.first=abline(0, 1, col='gold')) # i.e., makes
little difference how QC/BH checks are treated

# Bi-variate analysis
ttASR2 <- asreml(tcaTrunk~row+lin(tree), random=~id, rcov=~id(row):ar1(tree),
data=phenData, maxiter=50)
ttPred2 <- predict(ttASR2, classify='id', maxiter=1)$pred$pval

biData <- phenData[rep(1:nrow(phenData), 2), c('id','row','tree')]
biData$prop <- rep(c('lengthNewGrowth','tcaTrunk'), each=nrow(phenData))
biData$value <- c(phenData$lengthNewGrowth, phenData$tcaTrunk)
ends(biData)
```

```
ttASRb <- asreml(value~prop*(row+lin(tree)), random=~corgh(prop):id,
rcov=~at(prop):id(row):ar1(tree), data=biData)
ttPredb <- predict(ttASRb, classify='prop:id', maxiter=1)$pred$pval

summary(ttASR1)$varcomp
summary(ttASR2)$varcomp
summary(ttASRb)$varcomp

par(mfrow=c(2,2))
plot(ttPred1$pred, ttPredb[ttPredb$prop=='lengthNewGrowth','predicted.value'],
panel.last=abline(0, 1, col='gold'),
    xlab='Uni-variate', ylab='Bi-variate', main='Length new growth')
identify(ttPred1$pred, ttPredb[ttPredb$prop=='lengthNewGrowth','predicted.value'],
ttPred1$id)
plot(ttPred2$pred, ttPredb[ttPredb$prop=='tcaTrunk','predicted.value'],
panel.last=abline(0, 1, col='orange'),
    xlab='Uni-variate', ylab='Bi-variate', main='TCA of trunk')
ttMerge <- merge(biData, ttPredb)
with(ttMerge[ttMerge$prop=='lengthNewGrowth',], plot(value, predicted.value,
panel.last=abline(0, 1, col='gold'),
                                    xlab='Raw data', ylab='Bi-variate', main='Length
new growth'))
with(ttMerge[ttMerge$prop=='tcaTrunk',], plot(value, predicted.value,
panel.last=abline(0, 1, col='gold'),
                                    xlab='Raw data', ylab='Bi-variate', main='TCA of
trunk'))
ends(ttMerge)
```

BLUP analysis

```
setwd("K:/Mapping/Pears/Old Home x LBJ/propagation/BLUP")
qualdat = read.csv("rooting.csv", header=T)
## Check to ensure data imported correctly
str(qualdat)
head(qualdat)
tail(qualdat)
## Attach dataset
attach(qualdat)
## Examine distribution of data
hist(roots, col="gold")
boxplot(roots~Year, xlab="Location", ylab="Degrees Brix", main="Degrees Brix by
Location", col="pink")
# Rename variables for ease of use
roots = as.numeric(roots)
callus = as.numeric(callus)
nothing = as.numeric(nothing)
roots.or.callus = as.numeric(root.or.callus)
Year = as.factor(Year)
genotype = as.factor(genotype)
## Calculate variance components
# requires lme4 package
library(lme4)
# Linear Model with random effects for variance components
rootsvarcomp = lmer(roots~ (1|genotype) + (1|Year))
# Extract variance components
summary(rootsvarcomp)
```

```
#likelihood ratio test by comparing two models
rootsmodel = lmer(roots ~ (1|genotype) + (1|Year))
rootsnullyear = lmer(roots ~ (1|genotype))
anova(rootsmodel,rootsnullyear)
rootsnullgenotype = lmer(roots ~ (1|Year))
anova(rootsmodel,rootsnullgenotype)

## BLUPS
# fit the model
rootsmodel = lmer(roots ~ (1|genotype) + (1|Year))
# estimate BLUPS
rootsblup = ranef(rootsmodel)
# look at output structure
str(rootsblup)
# extract blup for roots and genotype
rootsgenotypeblup = rootsblup$genotype
# see the structure of the blup for each genotype
str(rootsgenotypeblup)
# save the rootsgenotypeblup output to a separate .csv file
write.csv(rootsgenotypeblup, file="rootsgenotypeblup.csv")
## Creating plots with the BLUPs
# Create a numeric vector with the BLUP for each genotype
rootsgenotypeBLUP = rootsgenotypeblup [,1]
# Create a histogram with the BLUP for each genotype
hist(rootsgenotypeBLUP, col="brown")
## Compare BLUP to line averages on a scatterplot
lmean = tapply(roots, genotype, na.rm=T, mean)
plot(rootsgenotypeBLUP, lmean, col="blue")
```