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RESEARCH PAPER

Genetic control of biennial bearing in apple

Baptiste Guitton[1,2], Jean-Jacques Kelner[1], Riccardo Velasco[3], Susan E. Gardiner[2], David Chagné[2] and Evelyne Costes[1,*]

[1] INRA, UMR AGAP, Equipe Architecture et Fonctionnement des Espèces Fruitières, Avenue Agropolis-TA-A-108/03, 34398 Montpellier Cedex 01, France
[2] The New Zealand Institute for Plant & Food Research Limited, Private Bag 11600, Palmerston North, 4442, New Zealand
[3] IASMA Research and Innovation Centre, Foundation Edmund Mach, Via E. Mach 1, 38010 San Michele all'Adige, Trento, Italy

* To whom correspondence should be addressed. E-mail: costes@supagro.inra.fr

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Abstract

Although flowering in mature fruit trees is recurrent, floral induction can be strongly inhibited by concurrent fruiting, leading to a pattern of irregular fruiting across consecutive years referred to as biennial bearing. The genetic determinants of biennial bearing in apple were investigated using the 114 flowering individuals from an F_1 population of 122 genotypes, from a 'Starkrimson' (strong biennial bearer)×'Granny Smith' (regular bearer) cross. The number of inflorescences, and the number and the mass of harvested fruit were recorded over 6 years and used to calculate 26 variables and indices quantifying yield, precocity of production, and biennial bearing. Inflorescence traits exhibited the highest genotypic effect, and three quantitative trait loci (QTLs) on linkage group (LG) 4, LG8, and LG10 explained 50% of the phenotypic variability for biennial bearing. Apple orthologues of flowering and hormone-related genes were retrieved from the whole-genome assembly of 'Golden Delicious' and their position was compared with QTLs. Four main genomic regions that contain floral integrator genes, meristem identity genes, and gibberellin oxidase genes co-located with QTLs. The results indicated that flowering genes are less likely to be responsible for biennial bearing than hormone-related genes. New hypotheses for the control of biennial bearing emerged from QTL and candidate gene co-locations and suggest the involvement of different physiological processes such as the regulation of flowering genes by hormones. The correlation between tree architecture and biennial bearing is also discussed.

Key words: Auxin, floral induction, gibberellin, irregular production, *Malus×domestica*, precocity.

Introduction

Once a woody perennial plant has passed the juvenile period when it cannot be induced to flower and has reached its adult phase of reproductive competence, a proportion of its meristems will initiate floral organs annually. Flowering in temperate tree species can be divided into several stages that include flower induction, flower initiation, flower differentiation, and blooming. Flower initiation is the key developmental stage for fruit trees, particularly for horticultural crops such as the apple (*Malus×domestica* Borkh.), because it determines the success of commercial orchards (Buban and Faust, 1982) by its influence on fruit quantity and quality (Link, 2000), as well as stability of production from year to year (Schmidt *et al.*, 1989). Flower initiation can be strongly limited by an excessive crop, leading to the phenomenon known as biennial bearing (Jonkers, 1979; Monselise and Goldschmidt, 1982). Commonly used terms related to alternate bearing include biennial bearing and irregular bearing. Biennial bearing is characterized by large

Abbreviations: AIC, Akaike Information Criterion; BBI, Biennial Bearing Index; BLAST, Basic Local Alignment Search Tool; BLASTP, protein–protein BLAST; BLUP, Best Linear Unbiased Predictor; CK, cytokinin; CY, cumulative yield; EST, expressed sequence tag; FI, floral induction; GA, gibberellic acid; GS, 'Granny Smith'; HRM, high resolution melting; MRM, multiple QTL mapping; NFI, number of fruit per inflorescence; NSF, number of seed per fruit; NSI, number of seed per inflorescence; PI, Precocity Index; QTL, quantitative trait loci; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; STK, 'Starkrimson'; WGD, whole-genome duplication.

yields of small sized fruit in 'on' years, and low yields, sometimes even no fruit, in 'off' years. This alternation is a widely spread phenomenon, occurring in both deciduous and evergreen trees, and in different tree families and species such as nuts (hazelnuts, pecans, pistachios, and walnuts), temperate fruits (apple, apricot, pears, and prunes), subtropical fruits (avocados, citrus, and olives), tropical fruits (litchis and mangos), and forest trees (beeches, oaks, pines, and spruces) (Monselise and Goldschmidt, 1982).

Several reliable parameters have been proposed to phenotype biennial bearing, its intensity, and the synchrony in different parts of the tree (Monselise and Goldschmidt, 1982). Hoblyn *et al.* (1936) originally proposed an index to estimate the intensity of deviation in yield during successive years that has been renamed by Wilcox (1944) as the Biennial Bearing Index (BBI). The BBI has been widely used to study fruit yield (i.e. mass of fruit) over orchards, individual trees, or branches (Wilcox, 1944; Singh, 1948; Pearce and Dobersek-Urbane, 1967; Jonkers, 1979). Recent examples used BBI in apple (Barrit *et al.*, 1997), mango (Reddy *et al.*, 2003), coffee (Cilas *et al.*, 2011), citrus (Smith *et al.*, 2004), pecan (Wood *et al.*, 2004), and pistachio (Rosenstock *et al.*, 2010).

Although generations of scientists have tried to understand this phenomenon, the cause of alternate bearing is still largely unknown (Singh, 1948; Hoad, 1978; Jonkers, 1979; Monselise and Goldschmidt, 1982; Bangerth, 2006, 2009). External factors (photoperiod, temperature, and water stress), internal factors such as the carbon-to-nitrogen ratio and hormones [auxins, cytokinins (CKs), abscisic acid, ethylene, and gibberellins (GAs)], as well as interaction with other organs (leaves, terminal shoot growth, and fruit) affect flower formation in apple (for reviews, see Hanke *et al.*, 2007; Bangerth, 2009). The negative relationship between fruit development and flower bud differentiation is one of the most investigated causes of flower set variability in apple, as the differentiation of flower buds in apple overlaps with embryo development in the previous season's fruit (Harley, 1942; Foster *et al.*, 2003), leading to competition between flower initiation and fruit formation.

Experiments using 'Spencer Seedless', which can bear both parthenocarpic and seeded fruit, suggested that seed development rather than nutritional competition may be a factor in alternate bearing (Chan and Cain, 1967; Neilsen and Dennis, 2000). The number of seed per fruit or per bourse (flowering growth unit) has an effect on biennial bearing, which can be overcome by a high vegetative growth rate of the bourse shoot itself (Chan and Cain, 1967; Grochowska and Karaszewska, 1976; Hoad, 1978; Neilsen and Dennis, 2000). Seed are known to contain relatively large amounts of hormones (Luckwill, 1974), and auxin [indole acetic acid (IAA)], GA, and CK have been implicated separately, and in combination, as being responsible for hormonal control of floral induction (FI). IAA and GA may act together or independently to inhibit FI in perennial fruit trees, whereas CK is likely to be the hormone enhancing FI (Bangerth, 2006). Although the spur (short fruiting shoot) tissues of biennial bearing cultivars receive more GA through the pedicel than annual bearing cultivars do (Hoad, 1978), and the peak activity of GA in apple seed coincides with FI (Luckwill, 1970), it has been difficult to obtain convincing evidence for the transport of GA from seed in sufficient quantities to inhibit FI. Bangerth (2006) proposed that auxin could be the mobile signal and might stimulate GA synthesis in the meristem. In this model, GA and auxin could potentially act as FI-inhibiting signals working in concert, GA as the primary messenger that stimulates the synthesis/transport of the second messenger auxin. However, characterization and quantification of both GA and auxin in the meristem still need to be performed and, moreover, an inhibitory effect of GA/auxin and stimulation by CK on the expression of genes related to FI remain to be demonstrated (Bangerth, 2006).

Since regular bearing appears to be related to FI rather than floral organ differentiation, it may be hypothesized that floral integrator and floral meristem identity genes are involved in this phenomenon. Key genes regulating floral development have been identified in model plants, such as *Antirrhinum majus* and *Arabidopsis thaliana* (Bernier and Périlleux, 2005; Tan and Swain, 2006; Corbesier *et al.*, 2007). These include the flowering promoter gene, *FLOWERING LOCUS T* (*FT*), that encodes a protein which is a major component of florigen (Kobayashi *et al.*, 1999), and the *LEAFY* (*LFY*) and *APETALA1* (*AP1*) genes, which have been identified as necessary for the determination of the floral meristem identity (Yanofsky, 1995). Other genes such as *FLOWERING LOCUS C* (*FLC*), *TERMINAL FLOWER 1* (*TFL1*), *BROTHER OF FT* (*BFT*), and *SHORT VEGETATIVE PHASE* (*SVP*) are known to be repressors of the floral pathway integrators (Boss *et al.*, 2004; Yoo *et al.*, 2010). Although there are fundamental differences in the flowering process between annual and perennial plants, the genetics of FI and floral organ formation are likely to be similar among these plants (Tan and Swain, 2006). A set of apple genes with sequence similarity to genes involved in floral meristem transition of *Arabidopsis* has been identified and subjected to expression studies (Jeong *et al.*, 1999; Sung *et al.*, 1999; Kotoda *et al.*, 2000, 2002, 2003, 2006, 2010; Van der Linden *et al.*, 2002; Wada *et al.*, 2002; Kotoda and Wada, 2005; Esumi *et al.*, 2005). Overexpression of the apple gene orthologues of *LFY*, *AFL1*, and *AFL2* (*APPLE FLORICAULA/LFY*) (Wada *et al.*, 2002), as well as *MdMADS2* and *MdMADS5*, orthologues of the *Arabidopsis FRUITFULL* (*FUL*) and *AP1*, resulted in early flowering in heterologous systems (Sung *et al.*, 1999; Kotoda *et al.*, 2002). Conversely, overexpression of the *TFL1* orthologue gene of apple, *MdTFL1*, in *Arabidopsis* delayed flowering (Kotoda and Wada, 2005). Kotoda *et al.* (2006) further showed that transgenic 'Orion' apple trees with a reduced *MdTFL1* transcript level flowered 8 months after grafting, whereas non-transformed 'Orion' plants still had not flowered nearly 5 years after grafting.

Both progeny segregation patterns and differences in bearing behaviour among cultivars strongly suggest the

involvement of alleles transmissible by both regular and non-regular types, together with a possible modification of expression by a genotype by environment interaction effect (Monselise and Goldschmidt, 1982). However, there has been no attempt to identify the genetic and molecular determinants of biennial bearing, and apple flowering genes and their allelic variants have never been evaluated for phenotypic variations in segregating populations or within the wider *Malus* germplasm.

The goal of the present study was to investigate the genetic determinants of biennial bearing in a segregating population using a combination of quantitative genetics analysis, quantitative trait locus (QTL) detection, and candidate gene mapping. A segregating population from a cross between contrasted genotypes for bearing behaviour, 'Starkrimson' and 'Granny Smith' (STK×GS) (Segura *et al.*, 2006, 2008, 2009), was phenotyped over six consecutive years and quantification of biennial bearing was based on yield at the whole-tree scale. QTLs and candidate genes for the control of flowering and its regularity in apple were identified and mapped. It was demonstrated that candidate genes involved in flowering do not co-locate with these QTLs, whereas several genes related to control of amounts of the hormones auxin and GA co-located with the QTL intervals for biennial bearing. Although flowering genes may not directly determine biennial bearing, their control by plant hormones might be one of the processes leading to biennial bearing.

Materials and methods

Plant material

The F_1 progeny used in this study were previously used for studying tree architecture during the juvenile phase (Segura *et al.*, 2006, 2007, 2008, 2009). The population was derived from a cross between two cultivars with contrasted tree and fruiting habits: 'Starkrimson' and 'Granny Smith' (STK×GS). The female parent is characterized by an erect growth habit with many short shoots and a tendency to biennial bearing, whereas the male parent, GS, has a weeping growth habit with long shoots and exhibits fruit-bearing regularity (Lespinasse, 1992). This population consists of 122 genotypes and each is replicated twice. However, three genotypes had only one tree replicate since the second tree replicate died at the beginning of the experimentation. Two-year-old seedlings were grafted on the semi-dwarfing rootstock 'Pajam 1' and the grafted trees planted in March 2004 at the Melgueil INRA Montpellier Experimental station using a random experimental design. These trees were not pruned and neither were the fruit thinned.

Phenotyping

The 122 genotypes were observed during six consecutive years, from their second to their seventh years. A total of 241 trees were phenotyped in 2005 and 239 trees in 2010, since two trees died during the 5 years of the study. From 2005 to 2010, the number of inflorescences and the number of fruit were recorded, and the harvested mass of fruit determined at the whole-tree scale. These variables were recorded for each year of production during the 6 years of the experiment, except for the harvested mass of fruit, which was not available for year 2.

A range of descriptors was calculated from the variables measured (number of inflorescences, number of harvested fruit, and harvested mass) (Table 1). The cumulative yield (CY) is a sum of the production for each year during the whole length of the experiment (Smith *et al.*, 2004). The Precocity Index (PI) was calculated by applying Bartlett's index for earliness of germination (Sivasubramanian, 1962) also called the Earliness Index (EI) by Cilas *et al.* (2011) (Table 1). This index weights yields according to the year considered, giving a higher weight to the early years of production and less to the latter ones. The alternate bearing behaviour of each genotype was quantified by the BBI, since trees exhibit a biennial pattern. The BBI was calculated using the formula developed by Hoblyn *et al.* (1936). BBI values vary from 0 to 1, where 0 denotes equal yields in successive years and 1 alternate yield (Hoblyn *et al.*, 1936). CY indexes were calculated from 6 years of data, from 2005 to 2010, whereas PIs were calculated from 5 years of data (from 2005 to 2009), since no trees flowered for the first time during the seventh year of the study (Fig. 1). Because only 36 trees flowered in 2005, compared with 212 in 2006, the BBI was calculated on 5 years of data (from year 3 to year 7) excluding year 2 in order to treat genotypes equally. Genotypes that began to flower during year 4, 5, or 6 were excluded from the BBI calculation. Bearing behaviour has been graphically represented for the whole population based on the average of phenotypic values for the total number of inflorescences and fruit, and the harvested mass of fruit per tree, per year (Fig. 2).

In 2010, the numbers of fruit per inflorescence (NFI) were counted for 20 inflorescences per tree. Inflorescences were sampled in the terminal position of spurs located laterally on 2- to 6-year-old wood of long axillary shoots. Then 10 fruit per tree were harvested and the number of aborted and fully developed seed per fruit (NSF) counted. To obtain the number of seed per inflorescence (NSI), phenotypic averages were calculated per tree for NFI and NSF and were multiplied together.

Clustering of bearing behaviour

Within the population, contrasted bearing behaviours were graphically identified based on the average of phenotypic values for the total number of inflorescences per genotype and per year (Fig. 3). To establish this classification, trees that began to flower during year 4, 5, or 6 were not considered and only 114 genotypes were included (93.4% of the population) that began to flower in year 2 or 3. The classification was performed manually and relied on the study of the evolution of yield through time by the construction of sequences composed of '+' and '–' symbols reflecting the direction of variation of the yield for each pair of years. Yields that were higher in year n+1 than in year n were symbolized by '+', whereas yields that were lower in year n+1 than in year n were symbolized by '–'. These sequences were used to sort genotypes into clusters with the same pattern. Finally the average yield per year of all genotypes was calculated for each cluster.

Differences between tree replicates within genotypes were investigated using the same sequential method as described above. Three groups were formed: the first group included genotypes having no differences in sequence between trees, the second group was composed of genotypes for which tree replicates showed one difference in the sequence, and the third group was composed of genotypes showing at least two differences in the sequence.

Genotypes for which production during year n+1 was equal or superior to year n were considered as regular. Genotypes exhibiting production in year n+1 inferior to year n were considered as irregular, or biennial when the pattern of alternation was biennial.

Statistical analyses

Statistical analyses were performed using R software v.2.9.2. (R Development Core Team, 2009). Data sets were analysed using a two-step method: first, the statistical effects were estimated by an

Table 1. Descriptors used to study inflorescence and fruit production in the 'Starkrimson'×'Granny Smith' segregating population over 6 years.

The formula used to calculate each descriptor is shown in relation to the type of data measured, such as the number of inflorescences, the number of fruit harvested, and the mass of fruit harvested. Y_i represents yield for year i, and n represents the number of years studied. With n=7 for Cumulative Yield and Biennial Bearing Index and n=6 for Precocity index.

Trait	Formula	Variable abbreviation			References
		Number of inflorescences	Number of fruit harvested	Mass of fruit harvested	
Yield		*Y_inf_n*	*Y_fruit_n*	*Y_mass_n*	
Cumulative yield	$\sum_{i=2}^{n} Y_i$	*CY_inf*	*CY_fruit*	*CY_mass*	Smith *et al.* (2004)
Precocity index	$\frac{\sum_{i=2}^{n} Y_i \times (n+1-i)}{\sum_{i=2}^{n} Y_i \times (n-1)}$	*PI_inf*	*PI_fruit*	*PI_mass*	Sivasubramanian (1962)
Biennial bearing index	$\frac{\sum_{i=3}^{n} (\lvert y_i - y_{i-1} \rvert) / (y_i + y_{i-1})}{n-1}$	*BBI_inf*	*BBI_fruit*	*BBI_mass*	Hoblyn *et al.* (1936)

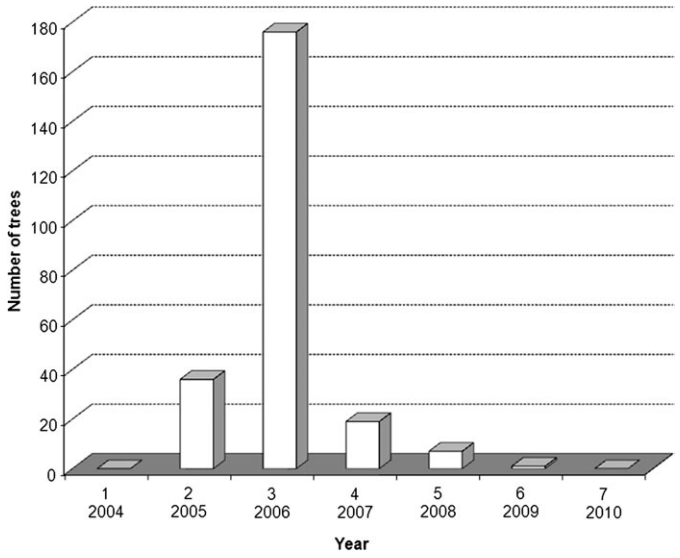

Fig. 1. Number of trees flowering for the first time according to the year after grafting. Years 1–7 on the *x*-axis correspond to 2004 to 2010, respectively.

analysis of variance (ANOVA) and then the significant effects were used to construct a linear model that estimated the genotypic value of the trait for each genotype. Three models were considered: one for yield data, which have been observed over 6 years, a second model for the CY index, PI, and BBI, which have one value for the whole study, and a third model for NFI, NSF, and NSI, which have 1 year of data, with repetitions within the tree.

Data sets for annual yield indexes were analysed by mixed linear models that included the year (Y), the genotype (G), the interaction between genotype and year (G×Y), and the nested effect of the tree within the genotype (G[T]). For the CY index, PI, and BBI, a linear model was built considering only the genotype (G). For NFI, NSF, and NSI, the model considered the genotype (G), the nested effect of the tree within the genotype (G[T]), and the nested effect of the fruit within the tree (T[F]). Significance of the effects was estimated by a type III ANOVA (function *lm*) because of unbalanced data. Then, the linear models were constructed for each variable, considering the significant effects detected by the ANOVA as fixed effects (Y) and as random effects (G, G×Y, G×T, and F×T). A model selection was performed based on the Akaike Information Criterion (AIC) minimization. For each trait, when the G effect was significant in the model selected (Table 2), BLUPs (Best Linear Unbiased Predictors) were extracted using the *ranef* function. Normal distributions of the residual errors were analysed to control the correct estimation by the model of the genotypic value. Because of the non-significant effect of the genotype, BLUPs were not extracted for *BBI_fruit* and *BBI_mass*, and QTL detection was based on phenotypic mean values for these variables. Genetic correlations were performed based on the BLUP using the Pearson coefficient, procedure 'cor' (Supplementary Table S1 available at *JXB* online).

Variable nomenclature

All BLUP variable names, except for *NFI*, *NSF*, and *NSI*, are composed of a short trait name followed by a suffix indicating if the variable was based on the number of inflorescences (*inf*), the number of harvested fruit (*fruit*), or the mass of harvested fruit (*mass*) (Table 2). BLUP variable names for annual yield indexes are followed by a suffix representing the year of the measurement. For example, the inflorescence yield measured during the second year is *Y_inf_2*. No numbers were attributed to BLUP variables that are independent of the year effect (e.g. *Y_inf*).

QTL mapping

The QTL analysis was performed using BLUP values extracted per genotype for each variable. The consensus and the parental genetic maps of STK and GS were used for QTL mapping. QTL analyses were carried out using MapQTL® 5.0. (Van Ooijen, 2004). First, a permutation test was performed to determine the logarithm of the odds (LOD) threshold at which a QTL was declared significant, using a genome-wide error rate of 0.01, 0.05, and 0.1 with 1000 permutations of the data (Van Ooijen, 2004). In the second step, an interval mapping analysis was carried out, with a step size of 1 cM, to detect potential genomic regions associated with the trait, with a LOD score higher than the threshold. The nearest marker to each QTL peak was then selected as a cofactor to perform multiple QTL mapping (MQM), with a step size of 1 cM (Van Ooijen, 2004). Each significant QTL was characterized by its LOD score, its percentage of explained phenotypic variation, and its confidence interval in cM corresponding to a LOD score drop of 1 or 2 on either side of the likelihood peak.

Allelic effects were estimated as $A_f=[(\mu_{ac}+\mu_{ad})-(\mu_{bc}+\mu_{bd})]/4$ for female additivity, $A_m=[(\mu_{ac}+\mu_{bc})-(\mu_{ad}+\mu_{bd})]/4$ for male additivity, and $D=[(\mu_{ac}+\mu_{bd})-(\mu_{ad}+\mu_{bc})]/4$ for dominance, where μ_{ac}, μ_{ad}, μ_{bc}, and μ_{bd} are estimated phenotypic means associated with each of the four possible genotypic classes ac, bc, ad, and bd, deriving from a <ab×cd> cross.

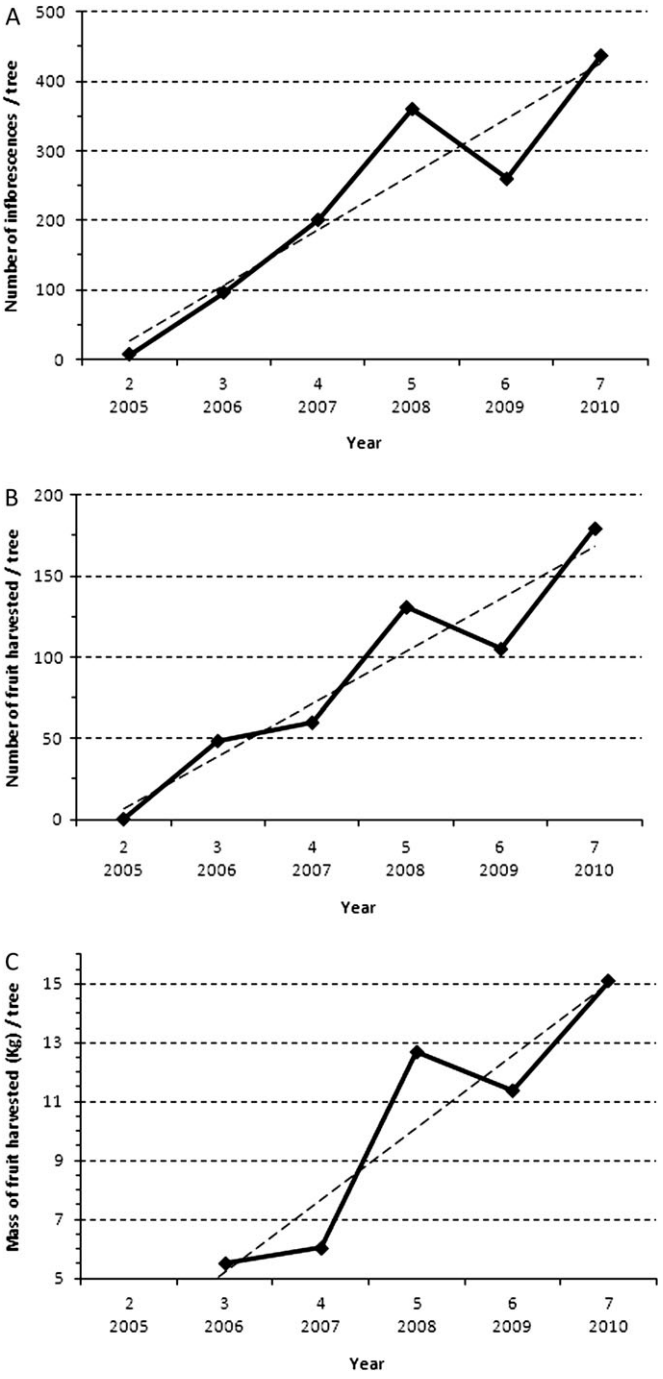

Fig. 2. Average production per tree calculated for the population (239 trees) over the 6 years of experiments. (A) Number of inflorescences, (B) number of fruit harvested, and (C) the mass of fruit harvested. Dashed lines correspond to the increasing trend estimated from a linear regression over years.

When a multilocus QTL was detected with at least two cofactors, models considering markers and their interactions as cofactors were constructed using a backward procedure under R software v2.8.1. Models were selected based on the AIC values. The location of QTLs was illustrated on the genetic maps based on the peak LOD–1 and LOD–2 intervals using MapChart® (Voorrips, 2002).

Two rounds of QTL detection were performed. The first round was performed on a genetic map comprising simple sequence repeats (SSRs) only, in order to detect the genomic regions of interest for the candidate gene mapping. The second round of QTL detection was performed on the genetic map including the candidate genes, and the results of this QTL detection are presented in Fig. 4.

Candidate gene mapping

An exhaustive *in silico* inventory of floral and hormone-related genes in apple was performed in order to establish a list of candidate genes that are possibly involved in biennial bearing. Protein sequences of *Arabidopsis* corresponding to genes involved in floral integration and meristem identity were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). A number of genes involved in plant response, synthesis, and transport of GA and CK were also selected. In total, sequences from 196 accessions from *Arabidopsis* were searched *in silico* within the 'Golden Delicious' whole-genome sequence (Velasco *et al.*, 2010) using BLASTP (protein–protein BLAST) versus apple gene predictions (amino acid). Ten gene predictions having the best BLAST (Basic Local Alignment Search Tool) expected values were selected for each *Arabidopsis* gene searched. Their positions and their protein sequences were retrieved on the *Malus*×*domestica* genome browser (http://genomics.research.iasma.it/gb2/gbrowse/apple/), and then their protein sequences were blasted against the Swiss-Prot protein reference sequences (http://expasy.org/tools/blast/) in order to identify the best *Arabidopsis* protein and *Malus* cDNA related to each apple gene prediction (E-value <1E-30). Alignments and phylogenetic trees analyses were carried out using the deduced amino acid sequences in order to remove redundant gene predictions and to determine the number of gene copies present in the apple genome per *Arabidopsis* gene (Supplementary Figs S1, S2 at *JXB* online).

A physical map was generated that included the positions in Megabases (Mb) of the predicted genes and of the SSR markers present on the STK×GS genetic map published by Segura *et al.* 2007 (Supplementary Fig. S3 at *JXB* online). The positions of the QTLs detected on the STK×GS consensus map without candidate genes were then compared with the physical map. Candidate genes co-locating with QTLs were studied in detail: phylogenetic trees were built in order to clarify the relationships among the members of each family and predicted genes were named based on their similarity with *Arabidopsis* proteins.

Amino acid sequences were analysed using the Phylogeny.fr platform (http://www.phylogeny.fr) including the pipeline chaining programs: MUSCLE 3.7 for multiple alignment, Gblocks 0.91b for automatic alignment curation, PhyML 3.0 for tree building, and TreeDyn 198.3 for tree drawing (Dereeper *et al.*, 2008). The tree building was based on an approximation of the standard likelihood ratio test.

An exhaustive inventory of genes related to auxin (ARF, AUX/IAA, and TIR) in the 'Golden Delicious' whole-genome sequence was performed by R Schaffer and K David (2011, unpublished). The position of the genes on the genome provided by this study was compared with the position of the QTLs.

The genes co-locating *in silico* with QTLs were considered as potential candidates, and specific markers were developed in order to position them on the genetic map to test their relationship with the QTLs. PCR primer pairs were designed for the candidate genes using Primer 3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The conditions were set up to amplify short fragments (100–200 bp), if possible spanning putative single nucleotide polymorphisms (SNPs). These potential SNPs were detected within the 'Golden Delicious' contig sequences from the apple genome primary assembly (Velasco *et al.*, 2010) or by aligning expressed sequence tag (EST) sequences from different cultivars. The primer pairs for *NZmsMdMYB12* and *MdCENa* were as in Chagné *et al.* (2007) and Mimida *et al.* (2009), respectively. PCRs were carried out with a real-time PCR instrument (LightCycler 480®, Roche), combined with high

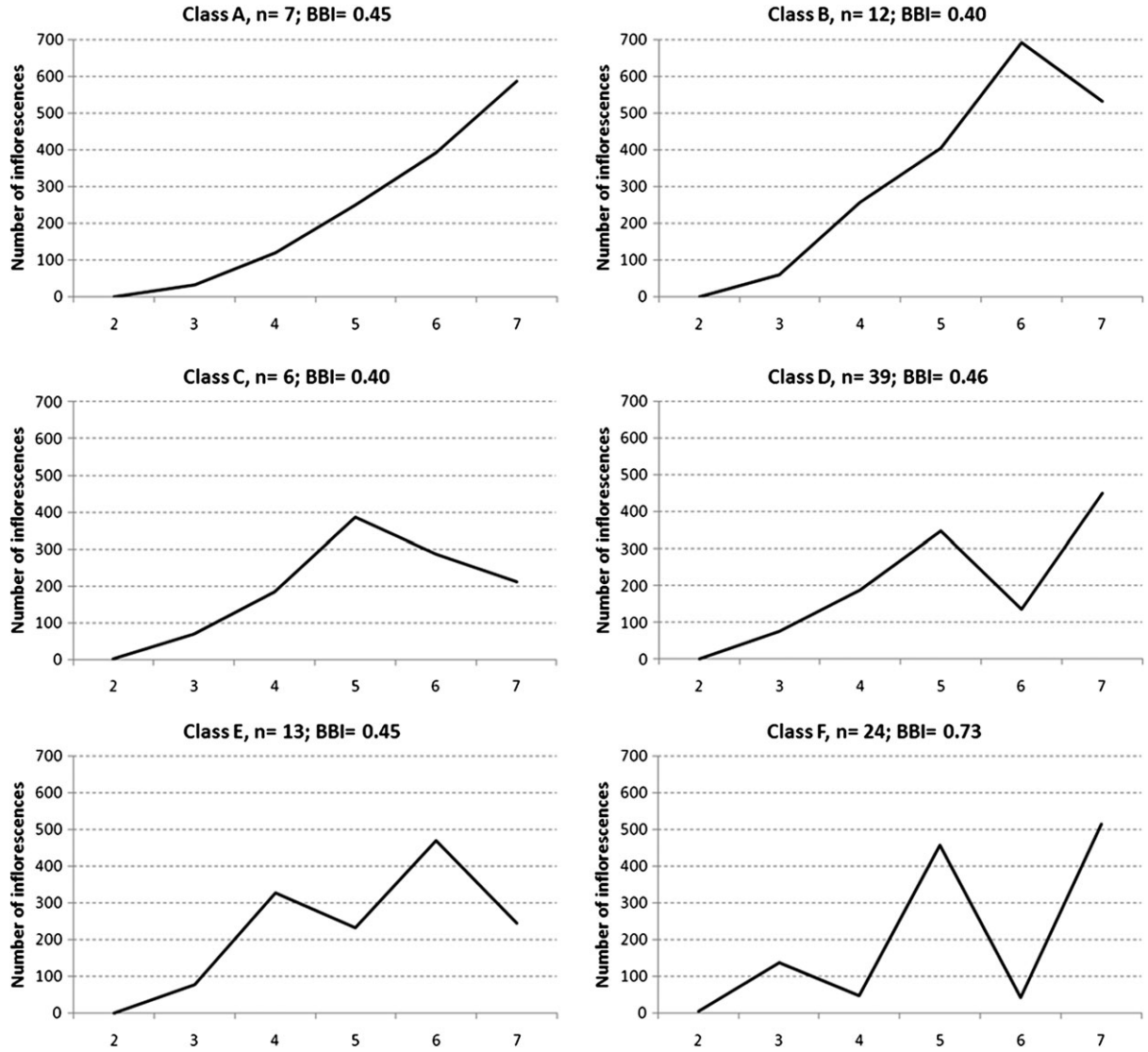

Fig. 3. Six bearing behaviours identified among genotypes within the population based on the average phenotypic values for the number of inflorescences. Class effective and average BBI values are indicated in the legend for each graph.

resolution melting (HRM) analysis for the detection of DNA polymorphisms (Liew *et al.*, 2004).

The PCRs were performed in the presence of a generic double-stranded DNA dye (LCGreen), which binds to double-stranded DNA only (Wittwer *et al.*, 2003), using a total volume of 7 µl for each well (1× LightCycler® 480 HRM Mastermix, with 2.5 mM $MgCl_2$, 0.2 mM for each primer, and 2 ng of genomic DNA). After activation at 95 °C for 5 min, the reactions underwent 40 PCR cycles of: 95 °C for 10 s; 55 °C for 30 s; 72 °C for 15 s. The HRM analysis was performed immediately after the PCR amplification, with single steps at 95 °C for 1 min; 40 °C for 1 min; 65 °C for 1 s; and then a slow increase of the temperature to reach 95 °C over 15 min, with continuous measurement of the fluorescence intensity (25 data points per degree Celsius). The Roche software identified sequence variants as groups that exhibit similar melting profiles (Hoffmann *et al.*, 2008).

Genetic map construction

One hundred and twenty-three individuals of the population and the parents were genotyped using 168 genetic markers, of which 107 were microsatellite markers (SSRs) and 61 were SNPs. STK and GS parental maps comprised 119 and 124 markers, respectively. Marker names were followed by the suffix 'SG' when polymorphic for both parents and followed by 'S' or 'G' when polymorphic for STK or GS, respectively. JoinMap 3.0 (Van Ooijen and Voorips, 2001) was used for constructing linkage maps using five segregation types: ab×cd, ef×eg, hk×hk, lm×ll, and nn×np for the consensus map. Two segregation types were used to build parental maps, hk×hk for both maps, and lm×ll and nn×np for the STK and GS map, respectively. Linkage groups (LGs) were constructed using a LOD score of 6 for grouping both the STK and GS maps. The data were analysed as population CP, and map distances were calculated using the Kosambi function.

Results

Phenotypic expression of biennial bearing in a segregating population

Of the 242 trees observed in the STK×GS segregating population, 36, 176, 19, and seven set flowers for the first time during the second to fifth year after grafting, respectively (Fig. 1). Only one tree set flowers during the

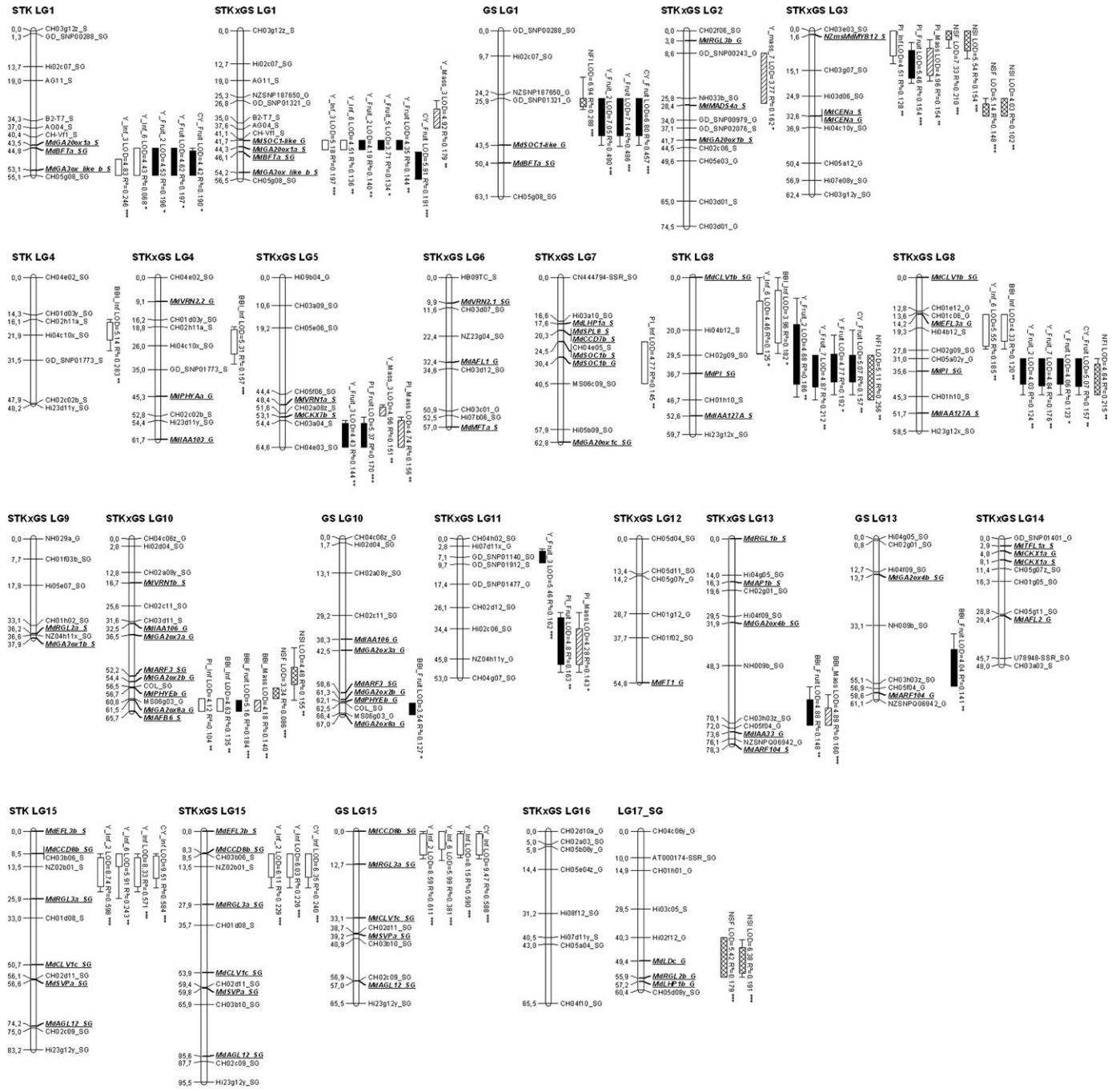

Fig. 4. Genomic positions of the QTLs detected on the consensus 'Starkrimson'×'Granny Smith' (STK×GS) and parental-maps: 'Starkrimson' maternal map (STK) and 'Granny Smith' pollen parent map (GS). QTLs are represented by boxes, in which length represents the LOD–1 confidence interval and extended lines represent the LOD–2 confidence interval. Boxes representing QTLs for the number of inflorescences are white, number of harvested fruit traits are black, mass of harvested fruit traits are hatched, and number of fruit and seed per inflorescence are double hatched. For trait abbreviations, see Table 2. Mapped candidate genes are in bold underlined. For candidate gene abbreviations, see Supplementary Table S3 at *JXB* online.

sixth year and three trees still did not flower during their seventh year after grafting. The average production of the population for both numbers of inflorescences and harvested fruit and mass of harvested fruit increased continuously from the second to the seventh year after grafting. However, compared with the upward trend of production in the segregating population, a biennial pattern was observed, as the fifth and seventh years were above the average trend, whereas the fourth and sixth years were below (Fig. 2).

Within the subset of 114 genotypes that had set flowers during the second to seventh year after grafting, six bearing behaviours were graphically identified between genotypes, based on the average phenotypic values per year for the number of inflorescences for each genotype (Fig. 3). Bearing

patterns A–F include genotypes that began to flower during year 2 or 3. A fraction of the population (5.7%) was regular bearing (class A), with production increasing consistently during the experiment. The remaining five classes were alternate bearing, and different 'on' and 'off' years were identified. Class B (9.8% of the population) were characterized by only one 'off' year during the seventh year. Class C (4.9% of the population) increased their production until the fifth year and then decreased it during the sixth and seventh year. Class D (32% of the population) began their biennial pattern during the sixth year and were characterized by one 'off' year and two 'on' years. Classes E (10.6% of the population) and F (19.7% of the population) had a clear biennial pattern but were in opposite phase. The remaining 10.6% of the population had irregular production, where 'on' and 'off' years were identified, but could not be grouped to form a homogenous class.

Differences between tree replicates within genotypes were investigated based on inflorescence yield per tree. The results showed that tree replicates of 48 genotypes had identical bearing patterns, 47 genotypes had tree replicates discriminated by 1 year of production, and 15 genotypes by 2 years of production (Supplementary Fig. S4 at *JXB* online).

Significance of genotypic and year effects on production traits

The genotypic effect on biennial bearing was evaluated using a set of measured variables including inflorescence, fruit, and mass annual yields, and indexes calculated from the measured variables. Annual yield consisted of 17 measured variables: six annual yields from year 2 to year 7 for both the number of inflorescences (*Y_inf*) and the number of fruit harvested (*Y_fruit*), and five annual yields from year 3 to year 7, for the mass of fruit harvested (*Y_mass*). Different effects were considered in ANOVAs depending on the measured variables and indexes. On annual variables, ANOVA using years and trees as repetitions showed that G, Y, G×Y, and G[T] effects were highly significant (P <0.001; Table 2). G[T] was less significant for fruit mass yield (*Y_mass*) and not significant for fruit yield (*Y_fruit*). As a result of the significant G×Y effect, BLUPs including this interaction were extracted for each measured year (*n*) (*Y_inf_n*, *Y_fruit_n*, and *Y_mass_n*), as well as BLUPs specific to the genotype effect (*Y_inf*, *Y_fruit*, and *Y_mass*).

Calculated variables such as the CY, PI, and BBI showed highly significant G effects in the ANOVA (Table 2). When calculated on the number and the mass of fruit harvested (*BBI_fruit* and *BBI_mass*), BBIs showed G effects slightly above the chosen *P*-value threshold. The PIs for number and mass of fruit harvested (*PI_fruit* and *PI_mass*) and the CY for mass of fruit harvested (*CY_mass*) exhibited a moderate genotypic effect compared with inflorescence variables. BLUPs were calculated for each index with significant genotype effect (*BBI_inf*, *PI_inf*, *PI_fruit*, *PI_mass*, *CY_inf*, *CY_fruit*, and *CY_mass*).

Table 2. Significance of the genotype effect (G), the year (Y), the tree (T), the fruit (F), and their interactions: G×Y, G[T] (i.e. T nested in G), and T[F] (i.e. F nested in T) in type III ANOVAs performed on traits phenotyped.

Trait	Name of variable	G	Y	G×Y	G[T]	T[F]
Biennial Bearing Index	*BBI_inf*	***	–	–	–	–
	BBI_fruit	NS	–	–	–	–
	BBI_mass	NS	–	–	–	–
Yield	*Y_inf*	***	***	***	***	–
	Y_fruit	***	***	***	NS	–
	Y_mass	***	***	***	*	–
Cumulative yield	*CY_inf*	***	–	–	–	–
	CY_fruit	***	–	–	–	–
	CY_mass	**	–	–	–	–
Precocity index	*PI_inf*	***	–	–	–	–
	PI_fruit	**	–	–	–	–
	PI_mass	**	–	–	–	–
Number of fruit per inflorescence	NFI	***	–	–	***	***
Number of seed per fruit	NSF	***	–	–	***	NS
Number of seed per inflorescence	NSI	***	–	–	NS	–

NS, non-significant; *P <0.05; **P <0.01; ***P <0.001).

The NFI and NSF measured in the seventh year showed highly significant effects of G, G[T], and T[F], except for NSF, for which T[F] was not significant. For the NSI, only the genotypic effect was significant (Table 2). BLUPs specific to the genotype effect were extracted for NFI, NSF, and NSI.

Correlations between variables

Negative moderate correlations were found for the number of inflorescences of a given year to the number of fruit harvested for the previous year (–0.18 to –0.54) and to the mass of fruit harvested the previous year (–0.20 to –0.59) (Supplementary Table S1 at *JXB* online). The NFI and NSI were positively correlated to variables related to fruit yield (NFI and *Y_fruit*, 0.55; and NSI and *Y_fruit*, 0.43). High correlations were observed for indices that were calculated from a set of measured variables. The PI was positively correlated with the mass of fruit harvested in the first year of significant production (i.e. third year, 0.74) and negatively with the mass of fruit harvested in the sixth year (–0.72). The BBI was positively correlated with annual inflorescence yields of years identified as 'on' in the population, such as the third and fifth years (Fig. 2) (0.33 and 0.42, respectively), whereas it was negatively correlated with annual inflorescence yields of 'off' years, such as the fourth and sixth years (–0.62 and –0.50, respectively).

Candidate gene identification, phylogenetic analysis, and genetic mapping

Candidate genes were selected on the basis on their known function in *Arabidopsis*, and the list of selected genes included 114 genes related to flowering and 73 related to metabolism and catabolism of plant hormones. A search of

the apple whole-genome sequence (Velasco *et al.*, 2010) for candidate genes using BLASTP analysis allowed the establishment of the number of members for each family and ascertainment of the location in the genome of 120 genes putatively involved in flowering and meristem identity, 41 in metabolism and catabolism of GA and CK, and 14 in branching. Phylogenetic analyses performed for 12 gene families (six flowering, two hormones, and one branching) to determine the putative function for apple gene sets are illustrated in Supplementary Figs S1 and S2 at *JXB* online. Genes were named based on their similarity with *Arabidopsis* proteins and with cDNA from apple. The genetic map for the STK×GS population was updated from its initial version (Segura *et al.*, 2006) by mapping 64 genetic markers located in candidate genes (Fig. 4). The improved consensus STK×GS map comprised 176 genetic markers including 107 SSRs and 69 SNPs, covered all 17 apple chromosomes, and encompassed 1057 cM. It was noted that 15 (23.4%) of the 64 candidate genes did not map at the position predicted from the genome assembly.

Candidate genes related to flowering: In total, 114 sequences of genes related to flowering were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) and used to search homologous proteins predicted from the apple genome sequence (Velasco *et al.*, 2010) using BLASTP analysis (i.e. *Arabidopsis* protein queries versus the predicted protein gene set). One hundred and twenty gene predictions related to flowering were identified, including 12 *FT*/*TFL1*, 49 *MADS-box*, 23 *SQUAMOSA* protein-like, 22 flowering genes that belong to different gene families, seven *PHYTOCHROME* genes, and seven *CONSTANS* (*CO*)-*like* (*COL*) genes. These 120 gene predictions showed highly significant similarity (E-value <1E-30) to 55 *Arabidopsis* reference protein sequences (Supplementary Table S2 at *JXB* online). No gene prediction was found for *FLC*, *FLD*, *FLK*, *FRIGIDA*, *GLOBOSA*, *HASTY*, and *ZIPPY*. Two paralogues were identified in duplicated genomic regions of apple for 39 genes, such as *AP1* (LG13 and LG16), *EFL3* (LG8 and LG15), and *LFY* (LG6 and LG14), whereas three gene copies were found for *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*; LG1, LG2, and LG7) and only one copy for *PISTILLATA* (*PI*: LG8) (Supplementary Table S2).

A phylogenetic analysis of the *FT*/*TFL1* family genes indicated the presence of five distinct clades within the apple genome, with two paralogous predicted gene copies for each family member: *FT*, *TFL1*, homologues of *CENTRORADIALIS*, *MOTHER OF FT*, and *BROTHER OF FT*. Apple paralogous genes shared more homology with each other than with *Arabidopsis* genes (Supplementary Fig. S1A). Twenty-two *Arabidopsis MADS-box* genes exhibiting sequence similarities with 49 apple predicted proteins were retrieved from GenBank. A phylogenetic analysis indicated that 2–4 apple putative *MADS-box* gene had clear orthology with one *Arabidopsis MADS-box* (Supplementary Fig. S1B). Only one apple gene prediction was found for five *Arabidopsis MADS-box* genes (*AGL3*, *AGL12*, *AGL19*, *AGL21*, and *PI*), whereas there were two predictions for nine *Arabidopsis* accessions (*AGL8*, *AGL11*, *AGL24*, *AP1*, *SEP1*, *SEP2*, *SEP3*, *SVP*, and *TT16*), three copies for *SOC1* and *AGL62*, and four for *AGL15*, *AGL80*, *AP2*, and *AP3* (Supplementary Table S2). A phylogenetic analysis of genes from distinct gene families related to flowering revealed separated clades for each *Arabidopsis* gene. Two paralogous genes were present in duplicated genomic regions for *LFY*, *EFL3*, *FCA*, *GI*, and *VRN2* because of the *Maloideae* whole-genome duplication (WGD) (Velasco *et al.*, 2010) (Supplementary Fig. S1C). A total of 23 apple predicted proteins were similar to 12 *Arabidopsis SQUAMOSA protein-like* (*SPL*) genes (Supplementary Table S2) and a phylogenetic analysis indicated the presence of nine clades (Supplementary Fig. S1D). The *PHYTOCHROME* family clustered in four clades with seven apple predicted genes (Supplementary Fig. S1E). The *COL* family comprised seven apple predicted genes that matched with three *Arabidopsis* genes (Supplementary Fig. S1F).

Genetic markers developed from 30 flowering genes were mapped using the STK×GS mapping population (Supplementary Table S3 at *JXB* online). Paralogous genes positioned in homoeologous genomic regions based on the WGD hypothesis of Velasco *et al.* (2010) included: *MdSOC1-like*, *MdSOC1a*, and *MdSOC1b* located on LG1, LG2, and LG7, *MdAFL1* and *MdAFL2* on LG6 and LG14, *MdVRN2.1* and *MdVRN2.2* on LG4 and LG6, *MdVRN1a* and *MdVRN1b* on LG5 and LG10, *MdCLV1a* and *MdCLV1b* on LG8 and LG15, and *MdEFL3a* and *MdEFL3b* on LG8 and LG15, respectively (Fig. 4). Only one copy of *MdPI* was mapped, on LG8.

Candidate genes related to hormones: Seventy-three sequences of genes related to metabolism and catabolism of GAs and CKs were used to search homologous proteins predicted from the apple genome: 33 and 40 genes related to GAs and CKs, respectively.

Malus×domestica possessed several copies of gibberellin oxidases, including *GA2ox*, *GA3ox*, and *GA20ox*. Fourteen *MdGA2ox*, 10 *MdGA3ox*, and seven *MdGA20ox* were identified in the predicted apple gene set (Supplementary Table S4 at *JXB* online), whereas seven, four, and five copies, respectively of these genes have been reported in *Arabidopsis*. A phylogenetic analysis indicated four separate clades, one each for *MdGA3ox* and *MdGA20ox*, and two for *MdGA2ox* (Supplementary Fig. S2A). *Malus×domestica* paralogous gene copies shared more sequence similarity with each other than with *Arabidopsis* genes. Ten apple putative cytokinin oxidases showed high orthology with five *Arabidopsis* cytokinin oxidases, and the phylogenetic analysis indicated the presence of one clade per *Arabidopsis* gene (Supplementary Fig. S2B).

Eleven auxin-related genes identified in the apple genome sequence by R Schaffer and K David (unpublished) co-located *in silico* with the QTLs, including: *MdAFB6*, *MdARF3*, *MdARF104*, *MdARF10, MdARF110*, *MdIAA4*, *MdIAA25, MdIAA33*, *MdIAA103*, *MdIAA106*, and *MdIAA127A*.

Genetic markers for 22 hormone-related candidate genes positioned on the STK×GS genetic map included four *MdGA2ox*, two *MdGA3ox*, three *MdGA20ox*, five DELLA, two cytokinin oxidases, and six auxin-related (Supplementary Table S3 at *JXB* online). Six DELLA proteins were identified as proposed by Foster *et al.* (2006), and five of them were positioned on the STK×GS genetic map, with each subgroup of paralogous gene copies located on homoeologous genomic regions: *MdRGL1a* and *MdRGL1b* on LG16 and LG13, *MdRGL2a* and *MdRGL2b* on LG9 and LG17, and *MdRGL3a* and *MdRGL3b* on LG15 and LG2, respectively (Fig. 4).

Candidate genes related to carotenoid cleavage dioxygenase (CCD): The CCD gene family involved in plant branching comprised 14 apple predicted genes that matched with six *Arabidopsis* genes (Supplementary Fig. S2C at *JXB* online). The phylogenetic analysis indicated the presence of five clades, including one common clade for *NCED3* and *NCED5*. Four gene copies were mapped *in silico* for *MdCCD4*, three for *MdCCD1*, two for *MdCCD8*, and only one copy for *MdCCD7a* on LG2. A second copy of *MdCCD7* was located on LG7 of the STK×GS map, a duplicated genomic region of LG2, whereas it was not detected *in silico*. Three CCD genes were mapped on the STK×GS genetic map: *MdCCD8a*, *MdCCD8b*, and *MdCCD7b* (Fig. 4).

QTL detection

In total, 43 QTLs spanning 12 LGs were detected on the STK×GS consensus genetic map. Twelve, 15, and eight QTLs were detected for variables related to the number of inflorescences, harvested fruit, and the mass of harvested fruit, respectively (Table 3). Seven QTLs were detected for biennial bearing, including three for the number of inflorescences (*BBI_inf*) and two for both the number and the mass of harvested fruit (*BBI_fruit* and *BBI_mass*). Seventeen and 10 QTLs were mapped on the STK and GS parental maps, respectively (Table 3, Fig. 4).

QTLs for traits related to the number of inflorescences: The 12 QTLs detected for characters related to the number of inflorescences were spread across seven different LGs (Table 3). The explained genetic variability (R^2) for each of the 12 QTLs ranged from 10.4% (precocity, *PI_inf*) to 24% (cumulative yield, *CY_inf*). Nine and four inflorescence QTLs were detected on the STK and GS parental genetic maps, respectively, with eight and three of them confirming positions identified using the consensus map. No significant QTL was detected for inflorescence yield for the years 4, 5, and 7 (*Y_inf_4*, *Y_inf_5*, and *Y_inf_7*).

Using the consensus map, three QTLs were detected for biennial bearing (*BBI_inf*): at the top of LG4 and LG8 and at the bottom of LG10. The global linear model indicated an interaction between the *BBI_inf* QTLs on LG8 and LG10 and explained 50% of the genetic variability (Table 4). The two *BBI_inf* QTLs mapped on LG4 and LG8 exhibited female effect and were confirmed on the STK parental map using the same cofactors as the consensus map, Hi04c10x_SG and Hi04b12_S, respectively. The third *BBI_inf* QTL detected on LG10 mainly resulted in male effect and co-located with a QTL for precocity (*PI_inf*) (Fig. 4).

Strong effect QTLs for inflorescence yield and cumulative yield (*Y_inf_2*, *Y_inf*, and *CY_inf*) clustered at the top of LG15 of the STK×GS map and explained 22.9, 22.6, and 24%, respectively, of the genetic variability. These QTLs were confirmed on both parental maps in the same genomic regions (Fig. 4). Although QTLs were detected on LG15 for *Y_inf_6* on both parental maps, when the consensus map was used, QTLs for this trait were identified on other genomic regions (LG1 and LG8). NZ02B01_S was used as the cofactor for the QTLs detected on LG15 on both consensus and female maps, and the *MdCCD8b_SG* marker was the cofactor for the QTLs detected on the male parental map.

The QTLs detected on LG1 for annual inflorescence yield for years 3 and 6 (*Y_inf_3* and *Y_inf_6*) mapped in the same genomic region on the consensus map and used the same cofactor (*MdGA20ox1a_S*) (Table 3). They explained 19.7% and 13.6% of the variability, respectively, and both were confirmed on the STK genetic map using CH05g08_SG as the cofactor. A second QTL identified on LG8 for *Y_inf_6* was confirmed on the STK map; both maps used Hi04b12_S as the cofactor and co-located with a QTL for *BBI_inf*. The two QTLs on LG1 and LG8 were not involved in any epistasic effect and explained 24% of the genetic variability (Table 4).

Three QTLs were detected on the consensus genetic map for precocity (*PI_inf*) on LG3, LG7, and LG10 (Table 3). None of these QTLs was confirmed on parental maps. The selected global linear model for this character showed no interactions among the three QTLs and they together explained 31% of the variability (Table 4). The LG3 and LG10 QTLs resulted in a male additivity effect, whereas the QTL mapped on LG7 mainly resulted in female effect.

QTLs for traits related to the number and the mass of harvested fruit: Fifteen and eight QTLs were mapped on the consensus map for variables related to the number and to the mass of harvested fruit, respectively (Table 3). For the number of harvested fruit, QTLs were spread across seven different LGs, with LG1 and LG8 exhibiting the highest number of QTLs. Their explained genetic variability ranged from 12.3% (fruit yield independent of year effect, *Y_fruit*) to 19.1% (cumulated yield, *CY_fruit*). Seven and four QTLs were confirmed using the parental genetic maps STK and GS, respectively. No significant QTLs were detected for fruit yield of years 4 and 6 (*Y_fruit_4* and *Y_fruit_6*). For the mass of harvested fruit, no QTLs were mapped using the parental maps. QTLs were spread over six different LGs and were related to four variables: annual mass yield for years 3 and 7 (*Y_mass_3*, *Y_mass_7*), precocity (*PI_mass*) and biennial bearing (*BBI_mass*).

Two genomic regions were identified for the BBI (*BBI_fruit* and *BBI_mass*) on LG10 and LG13 (Fig. 4). QTLs for

Table 3. QTLs detected on the consensus STK×GS map by MQM mapping for the number of inflorescences, the number of fruit harvested, and the mass of fruit harvested phenotyped over 6 years-in the STK×GS apple progeny. For trait abbreviations, see Table 2.

QTLs	LG	LOD	R^2	Cofactor	Allelic effect	A_f	A_m	D	Parental map detection
BBI_inf	4	5.31***	0.157	Hi04c10x_SG	A_f	0.039	–0.006	–0.008	STK
	8	4.33**	0.120	Hi04b12_S	A_f	–0.027	–0.013	–0.018	STK
	10	4.63**	0.135	*MdGA2ox8a_G*	A_m, D	0.013	0.032	0.023	
Y_inf_2	15	6.11***	0.229	NZ02b01_S	A_f, A_m, D	9.46	–9.30	6.31	STK/GS
Y_inf_3	1	5.18***	0.197	*MdGA20ox1a_S*	A_m	1.958	16.13	–2.149	STK
Y_inf_6	1	4.51**	0.136	*MdGA20ox1a_S*	A_m	–7.57	–38.2	6.46	STK
	8	5.55***	0.185	Hi04b12_S	A_f, A_m, D	35.0	26.7	11.96	STK
Y_inf	15	6.03***	0.226	NZ02b01_S	A_f, A_m, D	–19.3	19.0	–13.2	STK/GS
CY_inf	15	6.35***	0.240	NZ02b01_S	A_f, A_m, D	–117	103	–71	STK/GS
PI_inf	3	4.51**	0.128	*NZmsMdMYB12_S*	A_m, D	0.005	0.013	–0.010	
	7	4.77**	0.145	*MdSOC1b_S*	A_f, A_m, D	0.014	–0.011	0.002	
	10	4.12**	0.104	MS06g03_G	A_m	0.001	0.017	0.002	
BBI_fruit	10	5.16***	0.184	*MdAFB6_S*	A_m	0.027	0.052	0.023	GS
	13	4.87***	0.148	CH03h03z_SG	A_f, A_m, D	0.021	–0.004	–0.045	GS
Y_fruit_2	1	4.19**	0.140	*MdGA20ox1a_S*	A_f, A_m, D	1.471	–3.662	1.914	STK/GS
	8	4.03**	0.124	*MdPI_SG*	A_f, A_m, D	2.417	3.373	–1.674	STK
Y_fruit_3	5	4.43**	0.144	CH04e03_SG	A_f, D	–4.477	0.112	2.280	
	11	5.46***	0.162	GD_SNP01140_SG	A_f, A_m, D	–3.330	4.330	–2.869	
Y_fruit_5	1	3.71*	0.134	*MdSOC1-like_G*	A_f, A_m, D	–3.674	5.513	–7.144	
Y_fruit_7	8	4.84**	0.176	*MdPI_SG*	A_f, A_m, D	–12.52	–13.51	6.064	STK
Y_fruit	1	4.35**	0.144	*MdGA20ox1a_S*	A_f, A_m, D	–4.682	11.80	–6.186	STK/GS
	8	4.06*	0.123	*MdPI_SG*	A_f, A_m, D	–8.017	–10.40	5.481	STK
CY_fruit	1	5.91***	0.191	*MdGA3ox_like_b_S*	A_f, A_m, D	–49.00	99.7	–51.43	STK/GS
	8	5.07**	0.157	*MdPI_SG*	A_f, A_m, D	–62.98	–83.53	50.44	STK
PI_fruit	3	5.46***	0.154	CH03g07_SG	D	0.001	0.003	–0.012	
	5	5.37***	0.170	CH03a04_S	A_f, D	–0.010	0.003	0.007	
	11	4.80**	0.163	NZ04h11y_G	A_m	0.002	0.011	–0.005	
BBI_mass	10	4.18**	0.144	*MdAFB6_S*	A_f, A_m, D	0.030	0.045	0.016	
	13	4.89***	0.160	CH03h03z_SG	A_f, D	0.021	–0.003	–0.049	
Y_mass_3	1	4.92**	0.179	B2-T7_S	A_m	–0.088	0.363	–0.063	
	5	4.96**	0.151	CH02a08z_S	A_f	–0.314	0.076	0.103	
Y_mass_7	2	3.78*	0.161	NH033b_SG	A_m	–0.013	0.818	0.181	
PI_mass	3	4.96**	0.154	CH03g07_SG	D	0.001	0.004	–0.013	
	5	4.74**	0.156	CH03a04_S	A_f	–0.011	0.005	0.007	
	11	4.28*	0.143	NZ04h11y_G	A_m	0.000	0.013	–0.004	
NFI	8	4.64**	0.215	*MdPI_SG*	A_f, A_m, D	–0.057	–0.155	0.038	STK
NSF	3	7.33***	0.210	CH03e03_SG	A_f, A_m, D	–0.406	0.640	–0.395	
	3	5.14***	0.148	*MdCENa_S*	A_f, A_m, D	0.603	–0.245	–0.228	
	17	5.42***	0.179	MS06g03_G	A_f, A_m	0.575	–0.446	–0.115	
NSI	3	5.54***	0.154	*NZmsMdMYB12_S*	A_f, A_m, D	0.071	1.120	–0.664	
	3	4.03**	0.102	*MdCENa_S*	A_f, D	0.788	–0.360	0.782	
	10	4.48**	0.155	MS06g03_G	A_m, D	0.354	–1.311	–1.807	
	17	6.38***	0.191	*MdLD_G*	A_f, A_m	1.047	–1.016	–0.237	

BBI_fruit and *BBI_mass* were similarly located and the same cofactors were used for both traits. The interactions between the cofactors were significant for *BBI_fruit* in the global linear model and explained 37% of the genotypic variability (Table 4). The QTLs detected on LG10 displayed mainly male and female effects, whereas on LG13 the QTLs were mainly due to dominance and female effects (Table 3). The LG10 and LG13 *BBI_fruit* QTLs were confirmed using the GS genetic map.

Five QTLs related to fruit yield were detected on LG1 using the consensus map: four for the yield (*Y_fruit_2*, *Y_fruit_5*, *Y_fruit*, and *Y_mass_3*) and one for the CY (*CY_fruit*) (Fig. 4). The QTLs detected on the consensus map for *Y_fruit_2, Y_fruit*, and *CY_fruit* were confirmed on both parental maps using *MdGA3ox-like-b_S* and *MdSOC1-like_G* markers on STK and GS maps, respectively. Both QTLs displayed female, male, and dominant effects. The *Y_fruit_5* QTL resulted in female, male, and dominant effects and was not confirmed on the parental maps, probably because of its low LOD score (Table 3).

A second QTL cluster was identified on LG8 for variables related to annual and cumulated yields of harvested fruit: *Y_fruit_2*, *Y_fruit_7*, *Y_fruit*, and *CY_fruit*. Their explained genotypic variability ranged from 12.2% (*Y_fruit*) to 17.6%

Table 4. Global model estimations for traits with several QTLs detected by MQM with P, the effect probability, and global R^2, the proportion of variation explained by the model.
Models were selected according to AIC values. Some of the markers used in MapQTL as cofactors were replaced by their nearest marker with four genetic classes (ab, bc, ad, and bd, or ef, eg, fg, and ee) for the model construction. For trait abbreviations, see Table 2.

Trait	LG	Effects	Cofactor	*P*-value	Global R^2
BBI_inf	4	Hi04c10×_SG	Hi04c10x_SG	0.0017	0.49
	8	Hi04b12_S	CH02g09_SG	0.0068	
	10	*MdGA2ox8a_G*	COL_SG	3.81E-05	
	8*10		CH02g09_SG*COL_SG	0.0134	
Y_inf_6	1	*MdGA20ox1a_S*	CH05g08_SG	6.54E-05	0.24
	8	Hi04b12_S	CH02g09_SG	0.0539	
PI_inf	3	*NZmsMdMYB12_S*	CH03e03_SG	9.72E-05	0.31
	7	MdSOC1b_S	Hi03a10_SG	0.0024	
	10	MS06g03_G	COL_SG	0.0094	
BBI_fruit	10	*MdAFB6_S*	COL_SG	0.0002	0.37
	13	CH03h03z_SG	CH03h03z_SG	0.0003	
	10*13		CH03h03z_SG*COL_SG	0.0267	
Y_fruit	1	*MdGA20ox1a_S*	CH05g08_SG	4.11E-05	0.29
	8	*MdPI_SG*	CH02g09_SG	0.0022	
Y_fruit_2	1	*MdGA20ox1a_S*	CH05g08_SG	4.83E-05	0.29
	8	*MdPI_SG*	CH02g09_SG	0.0026	
Y_fruit_3	5	CH04e03_SG	CH04e03_SG	5.12E-05	0.49
	11	GD_SNP01140_SG	GD_SNP01140_SG	1.95E-06	
	5*11		CH04e03_SG*GD_SNP01140_SG	3.37E-05	
CY_fruit	1	*MdGA3ox_like_b_S*	CH05g08_SG	2.90E-05	0.40
	8	*MdPI_SG*	CH02g09_SG	0.0010	
	1*8		CH05g08_SG:CH02g09_SG	0.0626	
PI_fruit	3	CH03g07_SG	CH03g07_SG	0.0001	0.71
	5	CH03a04_S	CH04e03_SG	0.0033	
	11	NZ04h11y_G	CH04g07_SG	0.1705	
	3*5*11		CH03g07_SG:CH04e03_SG:CH04g07_SG		
BBI_mass	10	*MdAFB6_S*	COL_SG	0.0042	0.23
	13	CH03h03z_SG	CH03h03z_SG	0.0006	
Y_mass_3	1	B2-T7_S	CH05g08_SG	0.0338	0.15
	5	CH02a08z_S	CH05f06_SG	0.0056	
PI_mass	3	CH03g07_SG	CH03g07_SG	0.0002	0.70
	5	CH04e03_SG	CH04e03_SG	0.0098	
	11	CH04g07_SG	CH04g07_SG	0.1999	
	3*5*11		CH03g07_SG:CH04e03_SG:CH04g07_SG		
NSF	3	CH03e03_SG	CH03e03_SG	0.0002	0.65
	3	*MdCENa_S*	Hi04c10y_SG	0.0000	
	17	*MdLD_G*	CH05d08y_SG	0.0002	
	3*3*17		CH03e03_SG:Hi04c10y_SG:CH05d08y_SG		
NSI	3	*NZmsMdMYB12_S*	CH03e03_SG	0.0037	0.85
	3	*MdCENa_S*	Hi04c10y_SG	0.0010	
	10	MS06g03_G	COL_SG	0.2828	
	17	*MdLD_G*	CH05d08y_SG	0.0031	
	3*3*10		CH03e03_SG:Hi04c10y_SG:COL_SG		
	3*3*17		CH03e03_SG:Hi04c10y_SG:CH05d08y_SG		

(*Y_fruit_7*) (Table 3). These four QTLs were confirmed on the STK genetic map and *MdPI_SG* was used as the cofactor on both maps.

For the *Y_fruit_2*, *Y_fruit*, and *CY_fruit* QTLs mapped on both LG1 and LG8, the global linear model included interaction between LG1 and LG8 only for CY (*CY_fruit*), explaining 40% of the genetic variability (Table 4).

The annual yield of year 3 (*Y_mass_3*) QTLs on LG1 mainly resulted in male additivity and explained 17.9% of the variability (Table 3). A second *Y_mass_3* was detected on LG5 and displayed a female effect. For this variable, the interaction between the cofactors was not significant in the global linear model and explained 15% of the genetic variability (Table 4).

Two QTLs were detected on the consensus map for fruit yield of year 3 (*Y_fruit_3*) on LG5 and LG11, with epistatic effect explaining together 49% of the genetic variability. No QTLs were mapped on the parental maps for this variable. However, the QTLs for both fruit and mass yields of year 3 (*Y_fruit_3* and *Y_mass_3*) on LG5

co-located with QTLs for precocity (*PI_fruit* and *PI_mass*) (Fig. 4).

Three QTLs were detected for the PI of fruit and mass (*PI_fruit* and *PI_mass*) on LG3, LG5, and LG11 of the consensus map. The global linear model showed that the LG11 QTL was only involved in epistasic effect and showed significant epistatic effect, with the three QTLs explaining together 71% and 70% of the genetic variability for *PI_fruit* and *PI_mass*, respectively (Table 4). The QTLs displayed female and male additivity, and also important dominant effects, and were not detected on the parental maps.

Number of fruit per inflorescences and number of seed per fruit: The QTL detected for the NFI on LG8 resulted mainly in male additivity effect and explained 21.5% of the genetic variability (Table 3). *MdPI_SG* was used as the cofactor and the QTL co-located with QTLs mapped for fruit yield.

Three QTLs were mapped for the NSF: two on LG3 and one on LG17 (Fig. 4). The QTLs exhibited female, male, and dominance effects, but none of these QTLs was confirmed on parental maps. The global linear model included an interaction among the three QTLs and explained 65% of the genetic variability (Table 4). The QTL mapped on the top of LG3 co-located with the QTL for the PI of inflorescences (*PI_inf*).

The QTLs detected for the NSI were very similar to those described for NSF; however, a fourth QTL was detected at the bottom of LG10. The global linear model included an interaction among the four QTLs and explained 85% of the genetic variability (Table 4).

Co-location between candidate genes and QTLs

The QTL cluster detected for inflorescence and fruit yields at the bottom of LG1 overlaid four candidate genes genetically mapped in a small genomic region of 13 cM: *MdSOC1-like*, *MdGA20ox1a*, *MdBFTa*, and *MdGA3ox-like-b*. According to the 'Golden Delicious' genome sequence, a *COL* gene, *MdCOL1*, is also located in the same genomic region; however, this could not be genetically mapped in the present population. On the consensus map, *MdSOC1-like* and *MdGA20ox1a* were located within the QTL interval of annual yields (*Y_inf_3*, *Y_inf_6*, *Y_fruit_2*, *Y_fruit_5*, and *Y_fruit*), while *MdBFTa* and *MdGA3ox-like-b* co-located with the cumulative fruit yield (*CY_fruit*) QTL. When the parental maps were used, the candidate gene co-location with the QTL differed slightly. On the STK genetic map, only *MdBFTa* and *MdGA3ox-like-b* co-located with the QTL cluster, and on the GS parental map, *MdSOC1-like* was at the limit of the LOD significance for the QTL cluster.

The candidate gene *MdMADS4a* mapped on LG2 was located at the external border of the LOD significance for the yield mass QTL of year 7 (*Y_mass_7*).

On LG3, the peak LOD score for the QTLs for precocity (*PI_inf*), NSF, and NSI was located right above the transcriptional factor *MdMYB12*. Although QTLs for precocity (*PI_fruit* and *PI_mass*) were located in the same genomic region, *MdMYB12* did not map within the QTL confidence interval. The LOD peaks of the NSF and NSI QTLs located in the middle of LG3 were positioned directly above *MdCENa*. For the second *PI_inf* QTL on LG7, the *MdSOC1b* candidate gene was located within the LOD score interval.

MdEFL3a mapped within the limit of the QTL intervals for *Y_inf_6* and *BBI_inf* QTLs on LG8. *In silico* mapping revealed that five other candidate genes, *MdARF10*, *MdARF110*, *MdIAA4*, *MdIAA25*, and *MdGA3ox1a*, were present within these QTL intervals.

The candidate gene *MdPI* was located right in the middle of the LOD significance interval for the QTL cluster mapped on LG8 relating to fruit production and to the number of fruit per inflorescence. On LG10, the QTL cluster related to BBI and to the precocity of flowering spanned *MdGA2ox8a* and *MdAFB6*. For these four QTLs, the LOD score was higher at the *MdAFB6* locus than it was at *MdGA2ox8a*. The NSI QTL mapped above this QTL cluster, and several candidate genes were located within the QTL interval: *MdARF3*, *MdGA2ox2b*, *MdPHYEb*, and *MdGA2ox8a*. On LG15, the LOD score of the inflorescence QTL cluster fell 2 cM before the position of *MdCCD8b* on both male and consensus maps. The LG17 QTLs for NSF and NSI co-located with the *MdLD* candidate gene.

Flowering genes such as *MdFT*, *MdMFT*, *MdTFL1*, *MdCEN*, *MdLHP1*, *MdAFL*, *MdAP1*, and *MdMADS4*, and hormone-related genes such as *MdRGL* did not co-locate with any QTL mapped.

Discussion

The challenge of quantifying alternate bearing

Quantifying biennial bearing, a physiological phenomenon that occurs over a range of years, is a complex task. The approach here utilized data collected over 7 years from an apple segregating population, including the juvenile phase and the entrance into mature phase. The calculation of indices to quantify biennial bearing was essential both to describe the genetic variability and to identify the genomic regions linked to this trait.

Most of the trees first flowered during their third year and their production increased during the experiment. BBI was calculated using 5 years of yield (i.e. from the third to the seventh year), although Huff (2001) recommended using BBI over a minimum of 6 years during the trees' mature phase. Indeed, the BBI values can change, depending on the number of annual yields included in the calculation, and between the juvenile and mature phases (Smith *et al.*, 2004). However, these recommendations do not allow the early evaluation of biennial bearing tendency among genotypes, which would be useful for breeders. The results suggest that an early evaluation is possible, since genotypes having a clear alternate behaviour; that is, characterized by two 'on' and two 'off' years, had higher BBI values than genotypes having a clear regular behaviour (Fig. 3). However, intermediate behaviours might be difficult to

characterize based on BBI. One major difficulty derived from the fact that this index includes positive yield differences due to the ontogenic increasing trend of tree production. Estimation of biennial bearing during this period would certainly be improved by removing the increasing trend and accounting for yearly fluctuations only.

In addition to indexes, yearly variables were also studied. Most of them were significantly affected by the year factor effect and its interaction with the genotype factor (Table 2). However, the experimental design did not allow a distinction to be made between the ontogenic and climatic year effects within the year effect, as previously proposed by Segura *et al.* (2008). The interaction G×Y is illustrated by the graphic representations of bearing behaviours over years (Fig. 3), which show that 'on' and 'off' years can occur during the same climatic year, depending on the genotype. In addition, genotype by environment interactions can also be expected, since previous studies demonstrated different bearing behaviours for the same cultivar, depending on the cultivation site (for a review, see Monselise and Goldschmidt, 1982).

BLUPs were used as a tool to predict the genetic merit of trees based on their field performance for the traits studied. QTL detection was performed based on BLUP values, in order to improve the statistical power to detect significant QTLs (see Segura *et al.*, 2009). Among the traits studied, the number of inflorescences per tree was the most accurate for quantification of production and its regularity, because this trait is less subject to environmental effect. The significance of the genetic effects was higher for the variables related to the number of inflorescences than for the variables related to the number and the mass of harvested fruit (Table 2). This might be due to environmental effects that would induce variability during fruit set, self-thinning, and fruit development.

QTL detection, clustering, and trait correlation

The higher number of QTLs for STK suggests a greater effect of this parent on these traits and is consistent with the strong tendency towards the biennial bearing characteristic of STK compared with the regular bearing GS (Lespinasse, 1992). This also suggests that biennial bearing in the studied population may be due to alleles that have a negative effect rather than to positive regular bearing alleles.

QTL clusters were identified on eight genomic regions, and several of these clusters were due to indices calculated from a set of measured variables, resulting in QTL co-location between the index and the variables. For instance, production precocity was calculated from measured yearly fruit and mass yields. Strong correlations (0.66 and 0.74 for fruit and mass, respectively) were found between the yield in the third year (i.e. the first year of significant production) and the calculated production precocity, which in turn resulted in a QTL cluster mapping on LG5 (Fig. 4; Supplementary Table S1 at *JXB* online). This emphasized the third year of production as being highly determinant for precocity.

However, several statistical correlations and QTL co-locations occurred for independent variables that result from common physiological processes. This can be exemplified by QTLs for inflorescence yield of a given year that co-located with QTLs for fruit yield of the next year on LG1 and were consistent with negative correlations between the variables, for example *Y_fruit_5* and *Y_Inf_6*, –0.54. These results corroborate the main hypothesis for biennial bearing in apple that the presence of fruit influences the formation of inflorescences the following year, and point to the base of LG1 as being highly determinant for biennial bearing.

Another example is the fruit yield QTLs that clustered with a QTL for the NFI on LG8. These co-locations are supported by significant correlations between variables (ranging from 0.55 to 0.99). However, lower correlations were found between the number of inflorescences and fruit yield (from 0.18 to 0.51). Therefore, the fruiting yield appears to be influenced more by the NFI than by the number of inflorescences per tree.

Most interestingly, a number of QTL clusters resulted from non-correlated variables. For instance, the NSI mapped adjacent to QTLs for biennial bearing for inflorescence, fruit number, and mass on LG10, despite a low correlation between these variables (correlations ranging from 0.10 to 0.16). Similarly, the co-location between a QTL for flowering precocity with QTLs for biennial bearing on LG10 opens up interesting breeding perspectives. Indeed, these variables showed moderate correlations (0.32), meaning that some genotypes are precocious and regular, whereas others are precocious and biennial. Genotypes that are both precocious and irregular represent the largest proportion within the population, suggesting that in general trees producing flowers in early stages might enter in a biennial bearing cycle. Despite this, breeders wish to select genotypes that combine precocity and regularity, and these were present in the population studied.

Emerging hypotheses for the control of biennial bearing

This study used QTL detection in a segregating population combined with a candidate gene mapping strategy to identify potential genetic determinants of biennial bearing. It was demonstrated that biennial bearing involves interactions between independent genomic regions spanning genes of various functions. While stable genetic transformation to overexpress or knock out genes often provides solid proof of function for plant genes, it is believed that using genetic transformation would be extremely challenging for dissecting biennial bearing because of the control system's complexity. It is impossible to generate phenotypes equivalent to biennial bearing in annual model plants using genetic transformation. Knocking out *TFL1* in apple resulted in an extreme reduction of the juvenile phase with collateral effects on inflorescence architecture and chilling requirements (Kotoda *et al.*, 2003, 2006). However, in the present study, QTLs for precocity did not co-locate with *TFL1*, suggesting that mechanisms upstream of *TFL1*, and

possibly controlling *TFL1*, are determinants in the decision to set flowers.

The availability of the apple genome sequence enabled a comprehensive search for a set of candidate genes involved in flowering, hormones, and branching to be performed and their position could be compared with those of the QTLs detected for biennial bearing in the STK×GS genetic map. This enabled the fact to be highlighted that co-location between QTL clusters and candidate genes, which is not definitive evidence, provides pertinent new information on putative genetic control of biennial bearing in apple. While the approach used here was based on a systematic search of candidate genes using *in silico* analysis, it was shown that 24% of the genetic markers mapped to a different location from that in the genome assembly, which points at discrepancies in the apple genome assembly. This indicates that some relevant candidate genes might have been left out from the QTLs because of wrong genome location.

Flowering integrator genes and biennial bearing: It has been shown that genes described as key flowering genes in *Malus*, such as *MdFT* and *MdTFL1* (Mimida *et al.*, 2009; Kotoda *et al.*, 2010), were not present within QTL intervals for annual yields, precocity, and biennial bearing. Although it is suggested that these genes are likely not to be directly responsible for biennial bearing in apple tree, their control and regulation could be determinant. In contrast, other flowering genes such as *MdBFTa*, *MdSOC1-like*, and *MdCOL1* were located within QTL intervals for inflorescence and fruit production mapping on LG1. In *Arabidopsis*, *BFT* possesses a *TFL1-like* activity and functions redundantly with *TFL1* in inhibition of inflorescence meristem development (Yoo *et al.*, 2010). *SOC1* co-located with QTLs on LG1 and LG7 for inflorescence and fruit production and precocity, respectively. In annual plants, *SOC1* enhances FI in response to GAs (GA_4) (Eriksson *et al.*, 2006). In apple, GA_4 has been shown to promote flowering during 'off' years when applied the year before (Looney *et al.*, 1985). Similarly, *CO* positively regulates the expression of two floral integrators, *LFY* and *SOC1*, via *FT* in *Arabidopsis* (Samach *et al.*, 2000; Parcy, 2005). However, based on QTL mapping, it cannot be determined which gene among *MdSOC1*, *MdBFTa*, and *MdCOL1* is causative for the LG1 QTL. Further study, including mRNA expression during FI with different applications of hormones (e.g. different forms of GA), is needed.

Homeotic genes and fruit yield: Homeotic genes that are involved downstream of FI would not be *a priori* candidate genes for FI. However, *MdPI* co-located with a QTL cluster for fruit production and for the number of fruit per inflorescence on LG8. Previous studies have suggested that *MdPI* could be responsible for seed development, after the observation that a seedless apple mutant has a mutated *PI* gene (Yao *et al.*, 2001), whereas Tanaka *et al.* (2007) proposed that the *MdPI* gene was related to the development of petals and stamens and had function equal to *Arabidopsis PI* (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Consistently with Tanaka *et al.* (2007), the QTL cluster on LG8 does not control the NSF but the NFI.

Plant hormones and biennial bearing: Several genes involved in the GA biosynthesis pathway were located in QTL cluster intervals for production and its alternation: *MdGA20ox1a* and *MdGA3ox-like-b* on LG1 and *MdGA2ox8a* on LG10. These genes are known to determine the final amount of bioactive GA through their influence on key steps of GA synthesis (reviewed by Hedden and Phillips, 2000). Furthermore, auxin-related genes (*MdAFB6*) were mapped in the interval of QTLs for biennial bearing on LG10, and *in silico* mapping suggested that some AUX/IAA and ARF genes might be also located on LG8 and LG13. In pea, auxin has an important role in regulating GA biosynthesis by inducing the accumulation of *PsGA3ox1* mRNA and reducing the *PsGA2ox1* transcript, increasing the amount of bioactive GA_1 (Ross *et al.*, 2000). Bioactive GA might also directly target key flowering genes in the shoot apical meristem, including *SOC1* and *LFY*, which have been shown to be regulated by GA_4 in *Arabidopsis* (Eriksson *et al.*, 2006). *LFY* has been proposed to have a role in apple tree architecture and be responsible for columnar phenotype (Flachowsky *et al.*, 2010). In the monocot *Lolium temulentum*, it has been shown that GA_5 and GA_6 are the active GAs in the induction of flowering (King *et al.*, 2003). However, in apple, there is no evidence about which GAs are active in FI, although GAs are known to have an opposite effect on FI in perennial and annual plants (Jackson and Sweet, 1972). Indeed, applications of GA to apple trees showed that GA_7 is the most inhibitory GA on FI (Tromp, 1982), and horticultural practices commonly involve the application of GA during 'off' years to prevent an excessive FI and so attenuate the biennial bearing cycle (Schmidt *et al.*, 2009). Bioactive GAs might thus be expected to have an inhibitory effect on key flowering genes/steps in apple.

Is there a common genetic determinism for tree architecture and biennial bearing? Branching intensity and spur extinction have been demonstrated to be correlated with biennial bearing in a set of apple cultivars (Lauri *et al.*, 1995, 1997). More precisely, spur-type cultivars have often been described as having an irregular fruiting behaviour (Looney and Lane, 1984). Since the STK×GS population was previously used for dissecting the genetic control of scion architecture during the first 3 years of growth (Segura *et al.*, 2009), the comparison of QTL positions between the two studies enabled a number of QTLs mapping to common locations to be highlighted. QTLs for branching intensity were found to co-locate with QTL clusters for biennial bearing on LG4 and LG13, as well as with QTLs for flower and fruit production on LG1. This may result from statistical correlation between traits, since an increase in branching intensity increases the number of flowering sites. This is an important component of flowering yield that can be managed by horticultural practices such as 'spur artificial

extinction' (short fruiting shoot thinning) (Lauri, 2002), which mitigates biennial bearing. Moreover, the *CCD8* gene, which is involved in branching in petunia (Snowden *et al.*, 2005), was located at the border of the QTL cluster for inflorescence yield on LG15. Although no QTL for vegetative branching traits was located on this LG in the previous study during the first years of tree development, it is suggested that variations in the *MdCCD8b* gene might possibly have an effect on axillary bud activity and on the number of flowering sites.

A QTL for mean internode length of proleptic axillary shoots was co-located with a biennial bearing QTL on LG4. This corroborates the previous assumption of a positive correlation between bourse shoot length (shoots growing from inflorescence bases) and return bloom (consecutive occurrence of flowering in years n and n+1 on two successive shoots). That resulted from the observation that bourse shoot length >10 mm appeared to override the negative effect of seed on FI (Neilsen and Dennis, 2000). Finally, the biennial bearing QTLs on LG10 were located close to the genomic region that includes the columnar locus responsible for compact growth habit (Hemmat *et al.*, 1997) and a pleiotropic effect for architectural traits in apple (Conner *et al.*, 1998; Kenis and Keulemans, 2007). As previously discussed, this region also included candidate genes for GA biosynthesis and degradation. Numerous studies have demonstrated the implication of GAs in the cell elongation process. In particular, GA_4 has been shown to be the active GA in the regulation of cell elongation and shoot growth in *Arabidopsis* (Xu *et al.,* 1997), as well as in the regulation of stem elongation in *L. temulentum* (King *et al.,* 2001).

Taken together, these factors, including QTL co-locations, and mapping of candidate genes associated with vegetative growth, branching, and FI to QTLs associated with biennial bearing, strongly support the hypothesis of common molecular controls for tree architecture and biennial bearing in apple.

Conclusion

This study of biennial bearing in segregating apple progeny has provided new knowledge concerning the genetic architecture of this complex character. Biennial bearing is clearly a multigenic trait that is influenced by plant age and year effect as well as genetic effects. The comparison of locations of QTLs with candidate genes has given a clear indication that biennial bearing is unlikely to be directly controlled by floral integrator or meristem identity genes. However, their control by hormones might be the determinant factor in the decision to flower, consequently leading to biennial bearing. Even if not a definite indication of the exact physiological process, several genes related to metabolism, degradation, and transport of GA and auxin co-located with QTLs for biennial bearing, and these genes could regulate the amounts of substances inhibiting floral induction in the shoot apical meristem.

It is proposed that the candidate genes may act on physiological processes believed to be involved in biennial bearing and might be the genetic determinants of biennial bearing. However, further analyses are needed to narrow down the list of candidate genes and to confirm their implication in biennial bearing.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Phylogenetic analysis of flowering genes from *Arabidopsis thaliana* and *Malus*×*domestica.*

Figure S2. Phylogenetic analysis of hormone- and branching-related genes from *Arabidopsis thaliana* and *Malus*×*domestica*.

Figure S3. Physical position (Mb) of genetic markers (black) and candidate genes on the 17 chromosomes (Chr) of the apple genome ('Golden Delicious').

Figure S4. Variability of the number of inflorescences per tree between replicates of the same genotype.

Table S1. Genetic correlations between variables observed for the number of inflorescences, the number of fruit harvested, and the mass of fruit harvested.

Table S2. List of 120 candidate genes related to flowering identified *in silico* in the 'Golden Delicious' apple genome.

Table S3. Accession numbers of gene predictions in the apple genome and sequences of primers developed for candidate gene mapping in the population STK×GS.

Table S4. List of 55 candidate genes related to gibberellin and cytokinin oxidases and to carotenoid cleavage dioxygenase identified *in silico* in the 'Golden Delicious' apple genome.

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