Identification of a major QTL for time of initial vegetative budbreak in apple (Malus x domestica Borkh.).

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ABSTRACT

In the Western Cape region of South Africa dormancy release and the onset of growth does not occur normally in apple (Malus x domestica Borkh.) trees during spring due to the mild winter conditions experienced and fluctuations in temperatures experienced during and between winters. In this region the application of chemicals to induce the release of dormancy forms part of standard orchard management. Increasing awareness of the environmental impact of chemical sprays and global warming has led to the demand for new apple cultivars better adapted to local climatic conditions. We report the construction of framework genetic maps in two F1 crosses using the low chilling cultivar 'Anna' as common male parent and the higher chill requiring cultivars 'Golden Delicious' and 'Sharpe's Early' as female parents. The maps were constructed using 320 simple sequence repeats (SSR), including 116 new markers developed from expressed sequence tags (ESTs). These maps were used to identify quantitative trait loci (QTLs) for time of initial vegetative budbreak (IVB), a dormancy related characteristic. Time of IVB was assessed 4

times over a 6-year period in ‘Golden Delicious’ x ‘Anna’ seedlings kept in seedling bags under shade in the nursery. The trait was assessed for 3 years on adult full-sib trees derived from a cross between ‘Sharpe’s Early’ and ‘Anna’ as well as for 3 years on replicates of these seedlings obtained by clonal propagation onto rootstocks. A single major QTL for time of IVB was identified on linkage group (LG) 9. This QTL remained consistent in different genetic backgrounds and at different developmental stages. The QTL may co-localize with a QTL for leaf break identified on LG 3 by Conner et al. (1998), a LG that was, after the implementation of transferable microsatellite markers, shown to be homologous to the LG now known to be LG 9 (Kenis and Keulemans, 2004). These results contribute towards a better understanding regarding the genetic control of IVB in aplle and will also be used to elucidate the genetic basis of other dormancy related traits such as time of initial reproductive budbreak and number of vegetative and reproductive budbreak.

INTRODUCTION

The domesticated apple (Malus x domestica Borkh.) has been distributed into diverse climatic conditions worldwide for commercial production of fruit. Apple trees need exposure to cold temperatures, referred to as chill unit (CU) accumulation during winter, in order for budbreak to occur promptly and uniformly after winter (Cook and Jacobs, 2000). In warmer production areas, such as the Western Cape region of South Africa, the application of dormancy breaking chemicals, forming part of standard orchard management, enable successful production of high chilling requiring apple cultivars in suboptimal environmental conditions. Failure to apply dormancy breaking chemicals can result in prolonged dormancy symptoms (PDS), which include extended rest, less synchronised breaking of buds and reduced branching (Labuschagné et al., 2002b). An increasing awareness of both global temperature increase and the negative effects associated with the use of chemical sprays (for both pest and disease resistance and growth regulation) has resulted in the need to breed cultivars better adapted to current and future environmental conditions.

The breeding of new cultivars using conventional breeding methods is a time consuming process, especially in perennial tree species with a long juvenile phase such as apple.

Markers linked to genes involved in apple disease resistance for a variety of pests and pathogens have been identified (Gardiner et al., 2007) and are already in use in breeding programs (Kellerhals et al., 2008, Tartarini and Sansavini, 2003, Tartarini et al., 2000), through the implementation of marker-assisted-breeding (MAB) and selection (MAS) that enables the selection of favourable genotypes at a very early seedling stage. The genetic determinants of dormancy related characteristics, such as time of initial vegetative budbreak (IVB), are still poorly understood, and this hampers the genetic improvement of such characters using MAB. Dormancy characteristics can be controlled by factors residing within the bud itself, referred to as endodormancy, by factors in the plant but outside of the bud (paradormancy) and control by environmental factors (ectodormancy) (Khan, 1997, Lang et al., 1985). Although our study focused on time of IVB, a character related to endodormancy (Bradshaw and Stettler, 1995), various other characteristics can be associated with dormancy, such as position and number of budbreak and budbreak duration.

Unravelling of the genetic basis of complex traits such as dormancy, can be undertaken through the construction of a genetic linkage map followed by QTL identification (Falconer and Mackay, 1996, Young, 1996). A first attempt towards understanding the genetic control of 'leaf break' in apples through the identification of QTLs, was performed by Conner et al. (1998) using a population of 172 trees derived from a cross between 'Wijcik McIntosh' and NY 75441-58. Eight genomic regions on 7 linkage groups (LGs) could be associated with time of budbreak. The genetic linkage map constructed during their investigation, however did not include transferable simple sequence repeat (SSR) markers, resulting in their inability to align this map with the now more commonly used LG numbering for apple genetic linkage maps (Maliepaard et al., 1998). Further investigation resulted in alignment of three LGs from these two maps, including one (LG 3) that was homologous to LG 9 of Maliepaard et al. (1998) and carried a QTL for leaf break (Kenis and Keulemans, 2004). More recently Segura et al. (2007) used 123 seedlings derived from a cross between 'Starkrimson' and 'Granny Smith' to identify 2 QTLs for time of budbreak. The first on LG 8, corresponded to that identified on the corresponding LG 7 by Conner et al. (1998) (see Kenis and Keulemans, 2004). The second QTL for time of budbreak identified by Segura et al. (2007) was on LG 6. In the present study, genetic

linkage maps were constructed for two mapping pedigrees with the low chilling requiring cultivar 'Anna' as common male parent. 'Anna' is one of only a few cultivars worldwide characterized by a low chilling requirement (CR) and with 'Dorsett Golden' was reported as varieties needing less than 300 hours of chilling in Southern California (http://ucce.ucdavis.edu/files/filelibrary/5764/33384.pdf) and North and North Central Florida (Andersen and Crocker, 2000). Both published SSR markers (Celton et al., 2009, Guilford et al., 1997, Hemmat et al., 2003, Hemmat et al., 1997, Liebhard et al., 2002, Silfverberg-Dilworth et al., 2006, Yamamoto et al., 2002a, Yamamoto et al., 2002b) and 116 new SSR markers, developed from expressed sequence tags (ESTs), were used for the construction of the genetic linkage maps used to identify a major QTL for time of IVB on LG 9.

MATERIALS AND METHODS

Plant material.

Two F1 progenies, derived from crosses between the low chilling 'Anna' (common male parent) and the higher chill requiring 'Golden Delicious' (population A) and 'Sharpe's Early' (population B), containing 87 and 92 individuals respectively, were used. Seedlings from population A were kept in seedling bags under shade netting in Groot Drakenstein (Western Cape, South Africa) (33º50'36" S 18º58'39" E). Seedlings in this population were cut back and re-grown to single shoots on a seasonal basis and no chemical treatment was used to induce budbreak. Seedlings from population B were planted in an orchard in Vyeboom (Western Cape, South Africa) (34º4'15" S 19º4'47" E) characterized by low winter chilling. Resulting trees were in their 5th growing season at the onset of this investigation. Seven clonal replicates from seedlings in population B and the two parental cultivars were grafted onto rootstocks (M793) and planted in 7 randomized blocks in an adjacent orchard (34º8'21" S 19º0'44" E). Both sites are characterized by warmer winters and fluctuating chilling accumulation between winters. At these sites chill unit (CU) accumulation varies between 500 and 1000 CU annually. Chill units were calculated according to a modified Utah model found to be more suitable for local chilling conditions where negative CU values are not carried from one day to the next (Linsley-Noakes et al., 1994). Orchard management of adult and juvenile clonal trees from population B were

typical of commercial practice, except that no pruning and tree growth manipulations, such as dormancy breaking chemicals, were applied.

Phenotypic assessment.

The time of initial vegetative budbreak (IVB) was scored as the day on which the first green leaves emerged from the vegetative buds (day 1 being the 1st of January) (Labuschagné et al., 2002a, b). Phenotypic trait assessments were performed 4 times over a period of 6 years (1999, 2000, 2002 and 2004) on the 87 seedlings from population A. Trait assessment of population B was first performed during a 3 year period, from 1996 to 1998, on 60 adult trees, initially in their 5th growing season, followed by trait assessment on the 7 clonally replicated juvenile trees of all 92 siblings from 1998 to 2000. The data obtained from population B has been used in previous studies (Labuschagné et al., 2002a, b) during which broad sense heritability of IVB was estimated between 0.62 and 0.92 in clonal trials on young seedlings and between 0.57 and 0.83 for adult seedling trees. We calculated Pearson's correlation coefficients, to determine the relationship between different years of phenotypic trait assessment.

DNA Extraction

Extraction of seedling and parental cultivar DNA were performed using the CTAB method described by Doyle and Doyle (1990) with the addition of polyvinylpyrollidone (PVP) (Kim et al., 1997) in order to bind secondary plant products such as polyphenolics.

SSR Marker development and implementation

Unigene sets obtained from the large public EST database (> 240 000) (http://www.ncbi.nlm.nih.gov) (Naik et al., 2006, Newcomb et al., 2006) for Malus, were searched for SSRs using the Tandem Repeats Finder algorithm (Benson, 1999). SSRs were selected based on length of the repeat unit, number of repeats (>10 for di-, >7 for tri-, >5 for tetra- and penta- and >3 for hexanucleotide repeats) and length of sequences flanking SSR regions. Conserved sequences flanking 196 selected SSRs (100 di-, 60 tri-, 25 tetra-,

5 penta- and 6 hexanucleotide repeats) were used to design primers resulting in amplicons ranging between 100 and 450 bp in length. Newly developed SSR markers were tested on the three parental cultivars, 'Anna', 'Sharpe's Early' and 'Golden Delicious'. Markers for map construction were selected based on map position as well as heterozygosity observed during previous studies. They included 238 previously published SSR markers (Celton et al., 2009, Guilford et al., 1997, Hemmat et al., 2003, Hemmat et al., 1997, Liebhard et al., 2002, Silfverberg-Dilworth et al., 2006, Yamamoto et al., 2002a, Yamamoto et al., 2002b), marker AG11 (unpublished data: A. Patocchi (ETH-Zürich, CH)) and marker Md-EXP7 (Costa et al., 2008) and were initially screened for polymorphism over the three parental cultivars, 'Anna', 'Sharpe's Early' and 'Golden Delicious'.

SSR Analysis

All SSR markers implemented in mapping populations were fluorescently labelled and up to 16 markers were multiplexed using both size and fluorescent dye (6-FAM™, VIC™, NE ™ and PET ™) differences. PCR reactions were performed using the Qiagen multiplexing kit (QIAGEN Ltd., West Sussex, RH10 9NQ) according to the manufacturer's instructions. Resulting PCR products were prepared for capillary electrophoresis (CE) by adding 1 µl of a 1:10 diluted PCR product to 10 µl Hi-Di formamide containing 0.15 µl GeneScan^{TM}–500 LIZ^{TM} size standard (Applied Biosystems). Genotyping was performed using the ABI Prism 310 and 3130 (16-capillary array system) Genetic Analyzers (Applied Biosystems, Foster City CA, USA). Data collection and analysis were performed using GeneMapper® 4 software (Applied Biosystems, Foster City CA, USA).

Genetic linkage map construction.

For both progenies, parental genetic linkage maps and integrated genetic linkage maps were constructed using JoinMap® 4 (Van Ooijen, 2006). A logarithm of the odds (LOD score) of 4 was used to define LGs and genetic distances between markers were calculated using the Kosambi mapping function. On the basis of previously mapped SSRs, LGs were numbered in accordance with the 17 LGs obtained by Maliepaard et al. (1998).

QTL analysis

QTL analysis was performed using MapQTL® 5 (Van Ooijen, 2004) using the average phenotypic value for the four years of phenotypic trait assessment performed on population A and the two three year periods of trait assessment performed on adult and juvenile trees from population B. Analyses were also performed separately for each year of phenotypic trait assessment and in the case of clonal replicates the mean value per genotype was used. Regions with potential QTL effects were identified using interval mapping with a step size of 1 cM. QTLs were declared significant if the maximum LOD, obtained after multiple rounds of MQM mapping, exceeded the genome wide (GW) LOD threshold (calculated with an error rate of 0.05 over 1000 permutations). QTLs were characterized by the maximum LOD score and the percentage of phenotypic variation explained. For each QTL the differences in mean time of IVB associated with the different genotypic classes, ac, ad, bc and bd, derived for an ab x cd cross, are reported. QTLs were graphically displayed as bars next to the LGs on which they were identified, with bars corresponding to a 95% confidence interval (LOD score drop of 0.5) and dotted lines corresponding to a 90% confidence interval (LOD score drop of 1).

RESULTS

Phenotypic trait assessment.

Bi-modal distribution patterns were observed during most years of phenotypic trait assessment (Figure 1). The distribution patterns indicate budbreak was occurring earlier during consecutive years as trees matured. Significant levels of correlation were found between the different years during which phenotypic trait assessment were conducted (Table 1). High broad sense heritability values ($h^2 = 0.69$) for IVB were calculated by Labuschagné et al. (2002a).

SSR Marker development and implementation

The amplification success of newly developed SSR markers was 86% (168 SSRs from a total of 196). From these a total of 116 new SSR markers were polymorphic in at least one of the three parental cultivars used and were mapped in one or both mapping populations (Table 2). Of the 240 previously published markers, including 238 SSR markers (Celton et

al., 2009, Guilford et al., 1997, Hemmat et al., 2003, Hemmat et al., 1997, Liebhard et al., 2002, Silfverberg-Dilworth et al., 2006, Yamamoto et al., 2002a, Yamamoto et al., 2002b), marker AG11 (unpublished data: A. Patocchi (ETH-Zürich, CH)) and marker Md-EXP7 (Costa et al., 2008), 232 markers yielded amplification products of which 204 markers were heterozygous in one or more of the three cultivars tested. Designing new SSR markers so that the resulting amplicons vary in size, enabled effective multiplexing of up to 16 markers in one PCR reaction, greatly reducing the cost involved in the screening of mapping populations. Markers used within each multiplex are very flexible when using the QIAGEN multiplexing kit (QIAGEN Ltd., West Sussex, RH10 9NQ) that provides optimal reaction conditions that increases specificity and minimizes the effect of primer-dimers and non-specific artifacts often associated with multiplex PCR reactions. The ease with which different multiplexes could be assembled enabled easy assembly of new multiplexes containing highly informative markers for each specific mapping pedigree.

Genetic linkage map construction.

The four parental maps constructed (Figure 2) enabled the positioning of 286 SSR markers on 17 LGs corresponding to the number of chromosomes in the apple haploid genome. The number of SSR markers per LG range from 10 SSR markers on LG 3 to 28 SSR markers on LG10, with an average of 17 SSR markers per LG. The positioning of the 116 newly developed SSR markers (Table 2) range from 2 SSR markers on LG 1 to 15 SSR markers on LG10.

Genetic linkage map construction allowed the positioning of five previously published but unmapped markers (Liebhard et al., 2002). CH01b09b was mapped to LG 4, CH01e09b was mapped to LG 10 and CH02h11b was mapped to LG 12 in both mapping populations. $CH01e12_1$ was mapped to LG 8 and CH05c02 was mapped to LG 11 in the 'Golden Delicious' x 'Anna' mapping population. Three markers were mapped to different LGs when compared to their location on previously published maps: (i) CH03e03 was mapped to LG 5 compared to LG 3 (Liebhard et al. 2002), most likely due to the amplification of a different locus as observed fragment sizes are slightly larger than published (a fragment size of 216bp was observed in 'Prima' compared to the published 186bp), (ii) Hi23g12

was mapped to LG 15 compared to LG 8 (Silfverberg-Dilworth et al., 2006) confirming results obtained by Patocchi et al. (2009); (iii) CH05d04 was mapped to LG 5 compared to LG 12 (Liebhard et al., 2002), also most likely due to the amplification of a different locus as observed fragment sizes are slightly smaller than published (fragments of 154 and 175 bp were observed in 'Prima' compared to the published 176 and 186 bp. The marker CH05g07 (Liebhard et al., 2002) was found to amplify 2 loci, both mapping to LG 12. A locus amplified by the marker Hi03a03 (Silfverberg-Dilworth et al., 2006) was confirmed to map onto LG 6 in both mapping populations used while a second locus amplified by the same marker was found to map to LG 14 in the 'Anna' x 'Sharpe's Early' mapping population, confirming structural homology between LG 6 and LG14 (Celton et al., 2009).

Population A.

Of the 285 SSR markers screened on 87 seedlings from the 'Golden Delicious' x 'Anna' mapping pedigree, 260 markers were positioned on the integrated F1 genetic linkage map (map coverage: 1376.7 cM). Genetic linkage maps constructed for the parental cultivars 'Golden Delicious' (map coverage: 1124.5 cM) and 'Anna' (map coverage: 1292.6 cM) consisted of 163 (including 72 new SSRs) and 170 (including 71 new SSRs) markers, respectively. Parental maps were aligned using 92 SSR markers in common (Fig 2).

Population B.

The 'Sharpe's Early' x 'Anna' genetic map was constructed using 230 SSRs genotyped over the 92 F1 seedlings. The integrated F1 genetic linkage map (map coverage: 1242.6 cM) consisted of 207 mapped SSR markers. Genetic linkage maps constructed for the parental cultivars 'Sharpe's Early' (map coverage: 1012.9 cM) and 'Anna' (map coverage: 1050.6 cM) consisted of 127 (including 41 new SSRs) and 126 (including 45 new SSRs) markers respectively. Parental maps were aligned using 79 SSR markers in common (Fig 2). The parental map constructed for 'Anna' has 94 SSR markers in common with the parental map for 'Anna' constructed for population A.

QTL detection and mapping

A single major QTL for time of IVB was detected on LG 9 (Fig 3). Analyses performed on

the average time of IVB for the different populations and developmental stages showed that this QTL exceeded the GW LOD threshold during phenotypic trait assessment performed on adult trees from population B. LOD scores obtained for the analyses performed on averages from population A and juvenile trees from population B were just below the GW LOD thresholds. Separate QTL analysis for the different years of phenotypic trait assessment performed on seedlings from population A resulted in GW LOD thresholds being reached during trait assessment performed on seedlings in their fourth (2002) and sixth (2004) year (Table 3). GW LOD thresholds were exceeded during all three years phenotypic trait assessment has been performed on adult trees from population B (Table 3). Separate QTL analysis for the three different years of phenotypic trait assessment performed on juvenile trees from population B resulted in GW LOD thresholds not being reached during the first three juvenile years (Table 3). One-way ANOVA indicated significant association ($P<0.0001$) between specific NZmsCN943946 alleles inherited from the parental cultivar 'Anna' and time of initial vegetative budbreak (IVB). This association was true during all years of phenotypic trait assessment on 'Golden Delicious' x 'Anna' ($30.22<F>91.73$) and 'Sharpe's Early' x 'Anna' adult ($34.39<F>49.9$) and juvenile ($30.6<F>69.27$) trees.

Differences in time of IVB associated with the four genotypic classes, ac, ad, bc and bd, derived from an ab x cd cross, indicate that the phenotypic variation can be associated with alleles inherited from the common male parent 'Anna'. This QTL explains between 4.8% and 40.1% of the phenotypic variation observed in population A and between 11.9% and 44.6% of the phenotypic variation observed in population B.

DISCUSSION

The genetic linkage maps constructed are composed entirely of SSR markers and since a very large proportion of these markers are derived from EST sequences (more than 120) these maps are the most functional maps yet available. The newly developed and mapped SSR markers will enable the expansion of the 15cM reference map, currently consisting of 86 SSR markers covering 85% of the genome, proposed by Silfverberg-Dilworth et al. (2006) with up to 11 SSR markers. Depending on polymorphic information content determined on a larger number of cultivars, some of the newly developed SSR markers

might be used to replace markers with low polymorphism now included in the reference set, due to lack of more polymorphic SSR markers in certain regions (Silfverberg-Dilworth et al., 2006).

The time of IVB showed a wide bi-modal distribution in the seedlings derived from both mapping populations. Although bi-modality could be explained by seedlings having a difference in their rapidity of response to favourable conditions after their CR was satisfied (Labuschagné et al., 2003), the distribution of time of IVB can be explained by the fact that the trait is controlled by a major QTL together with some minor QTLs. High heritability estimates, although specific to the experimental conditions in which they have been calculated, were calculated for time of IVB by both Labuschagné et al. (2002a) (h^2 = 0.69) and Segura et al. (2007) (h^2 = 0.58), indicating that the trait has a strong genetic influence and that it can be selected for using marker assisted selection. Heritability is not always related to the power of QTL detection (Segura et al., 2007), as the latter is also influenced by population size and the number of QTLs affecting the trait. The small number of individuals included in phenotypic trait assessment (87 from 'Golden Delicious' x 'Anna' and 60 and 92 for adults and juveniles from the 'Sharpe's Early' x 'Anna' mapping pedigrees) and the amount of variation observed among seedlings from the same mapping population, allowed for the detection of only one QTL with large effect. The fact that this QTL explains up to 40.1% and 44.6% of the phenotypic variation observed in populations A and B respectively, indicates that there are further QTLs affecting time of IVB. These may include several QTLs with smaller effect that are statistically not detectable due the restricted population sizes used and the phenotypic variation observed in the seedlings. During initial interval mapping (van Dyk et al., 2009) the involvement of several minor QTLs were suggested. Implementation of more markers
leading to better genome coverage and the ability to perform MQM analysis, enabled the identification of a QTL with large effect in the current study.

Genetic linkage maps constructed for both mapping populations enabled the efficient detection of a major QTL affecting the time of IVB on LG 9 (Table 3). This QTL may co-localize with one of eight QTLs involved in leaf break that was identified by Conner et al. (1998). The QTL identified on LG 3 of the genetic linkage map produced by Conner et al. (1998) was, after the implementation of transferable microsatellite markers, shown to

be homologous to the LG now known to be LG 9 (Kenis and Keulemans, 2004). In the present study the QTL on LG 9 can be associated with specific allele inheritance from the common parent 'Anna'. Performing QTL analyses on an integrated parental map when working with an outbreeder, as was done during this study, enables the determination of both the effect of alleles inherited from a single parent and the interaction between alleles inherited from both parents. Results (Table 3) indicated a clear difference in average time of IVB between seedlings that inherited allele "c" from 'Anna' (average "ac" and "bc") compared to seedlings that inherited allele "d" from 'Anna' (average "ad" and "bd"). No clear difference could be detected between seedlings that inherited different alleles from the other parental cultivar involved in each mapping pedigree or seedlings with a specific combination of parental alleles.

The power of QTL detection (LOD score) increased during consecutive years of phenotypic trait assessment being performed on seedlings from population A and juvenile trees from population B. This suggests that although the QTL can be associated with time of IVB in young seedlings, the association between the QTL and the trait becomes stronger as the tree matures. The QTL was found to be significant (LOD score exceeding GW LOD thresholds) in all three years during which phenotypic trait assessment was performed on adult trees from population B (Table 3). Although significant GW LOD thresholds are not met in juvenile trees from population B, the association between the QTL and time of IVB can be seen from obtained phenotypic means associated with each of the genotypic classes (Table 3). Budbreak occurring earlier as trees mature has not been reported before. Preliminary results suggest no correlation between the earlier time of vegetative budbreak, associated with seedling age in two apple populations studied, and the CU accumulated during different years. These results suggest that the chilling requirement (CR), which is the major determinant of time of budbreak (Bradshaw and Stettler, 1995), has been met and that the time of vegetative budbreak is also influenced by factors associated with tree age. These results need to be confirmed in future studies, including several years of phenotypic trait assessment performed during different developmental stages and on different populations.

Markers linked to the QTL identified will be used in a validation test on a larger progeny sharing common parentage. The QTL region will be saturated with markers selected for

their positioning on the genetic linkage map as a result of selective (bin) mapping on a subset of individuals (van Dyk and Rees, 2009). The ideal will be the identification of markers flanking the QTL that can be used for the implementation of MAS in breeding for cultivars that are better adapted to local climatic conditions.

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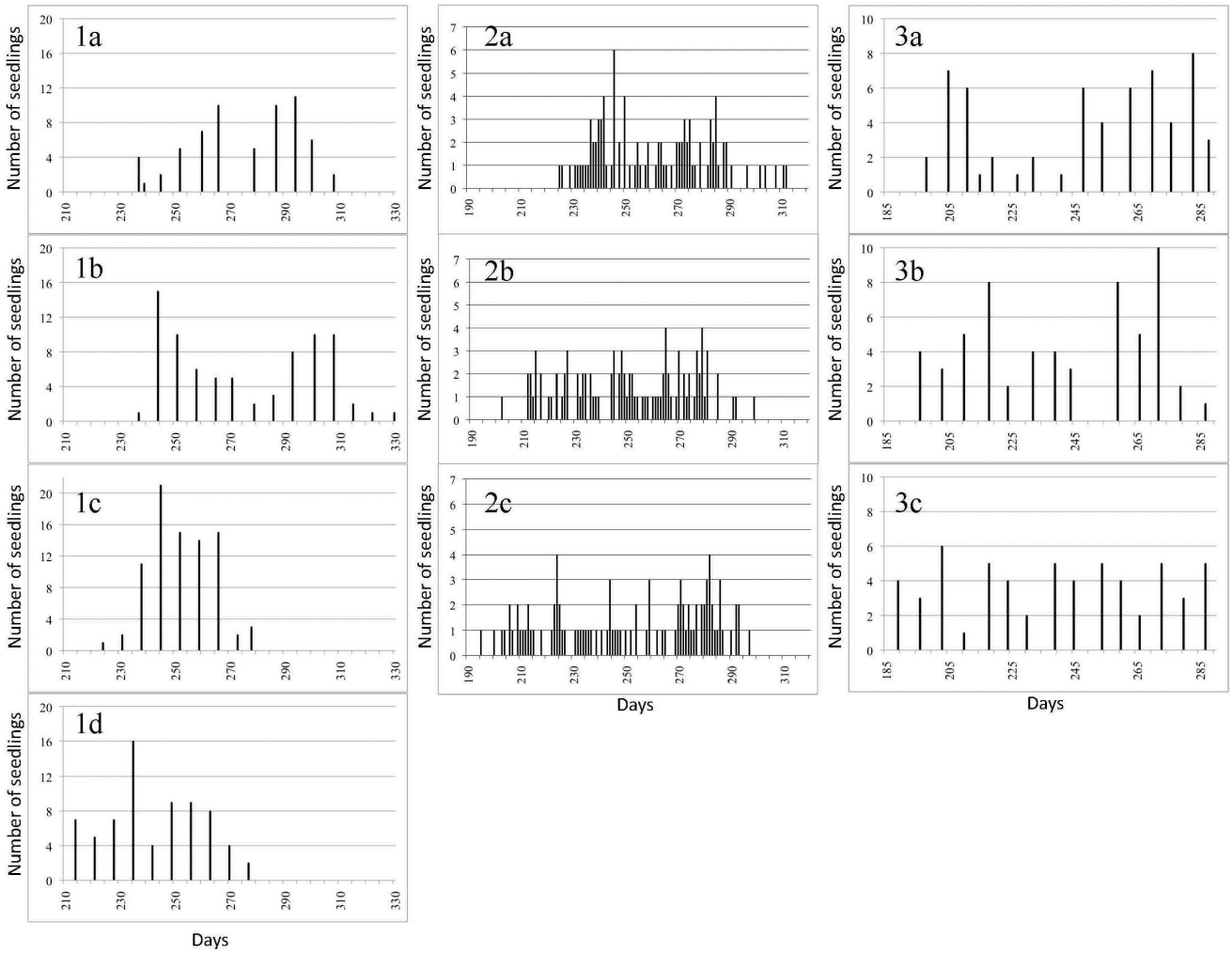
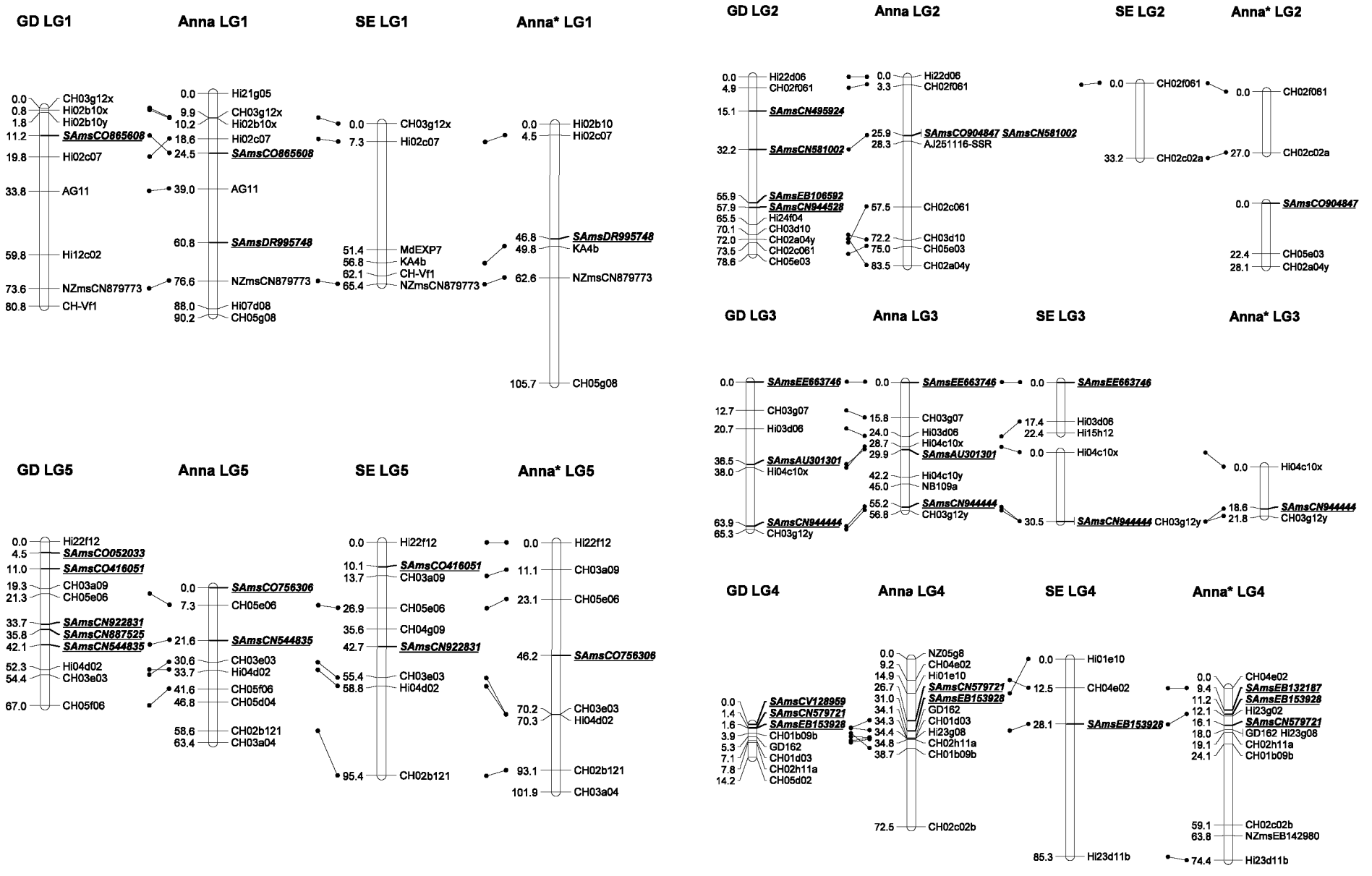


Figure 1: Histogram showing the distribution of time of initial vegetative budbreak (IVB) observed in 1: 'Golden Delicious' x 'Anna' seedlings during 4 years of phenotypic trait assessment a = 1999, b = 2000, c = 2002 and d = 2004; 2: 'Sharpe's Early' x 'Anna' juvenile trees during 3 years of phenotypic trait assessment a = 1998, b = 1999 and c = 2000; and 3: 'Sharpe's Early' x 'Anna' adult trees during 3 years of phenotypic trait assessment a = 1996, b = 1997 and c = 1998.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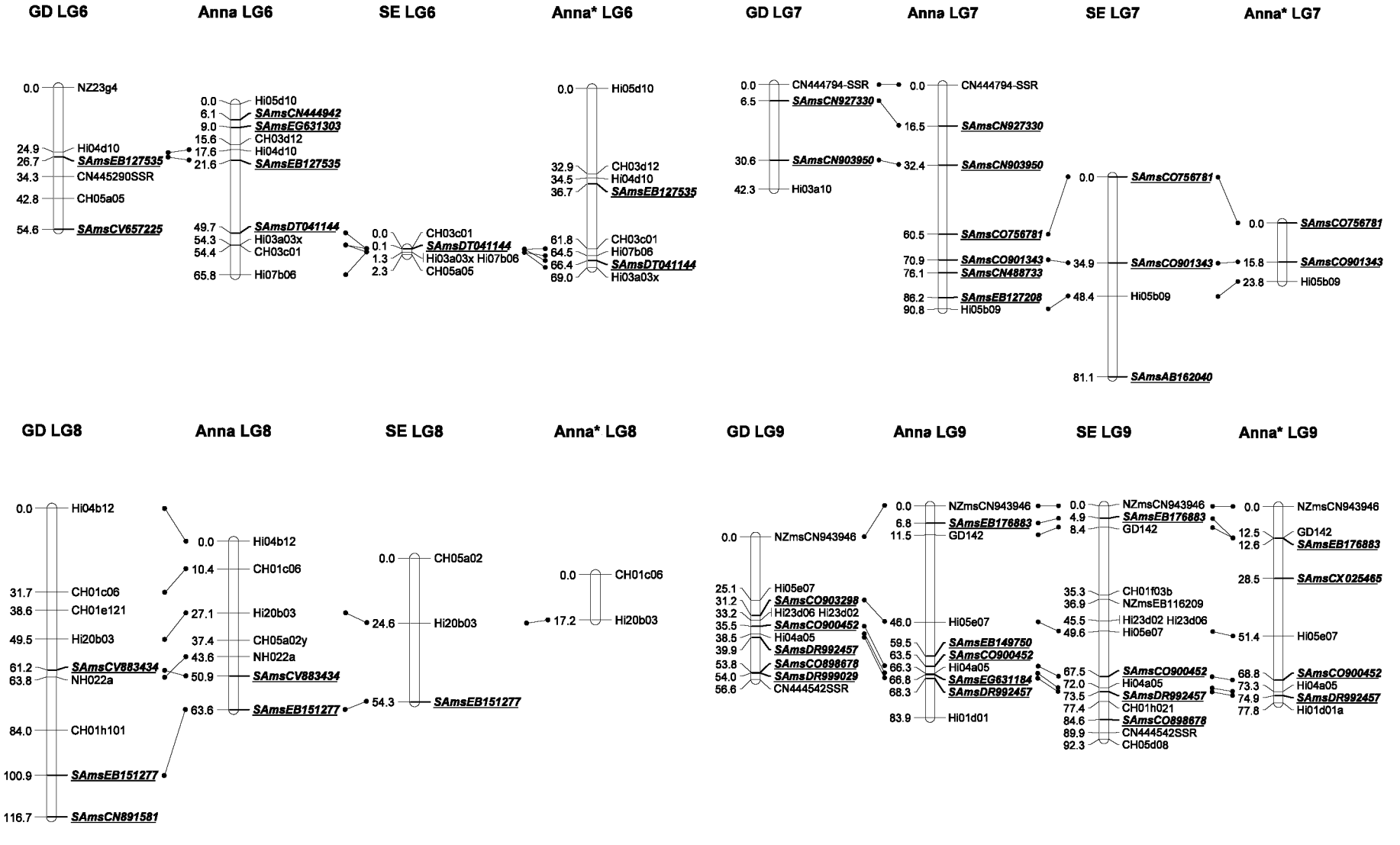
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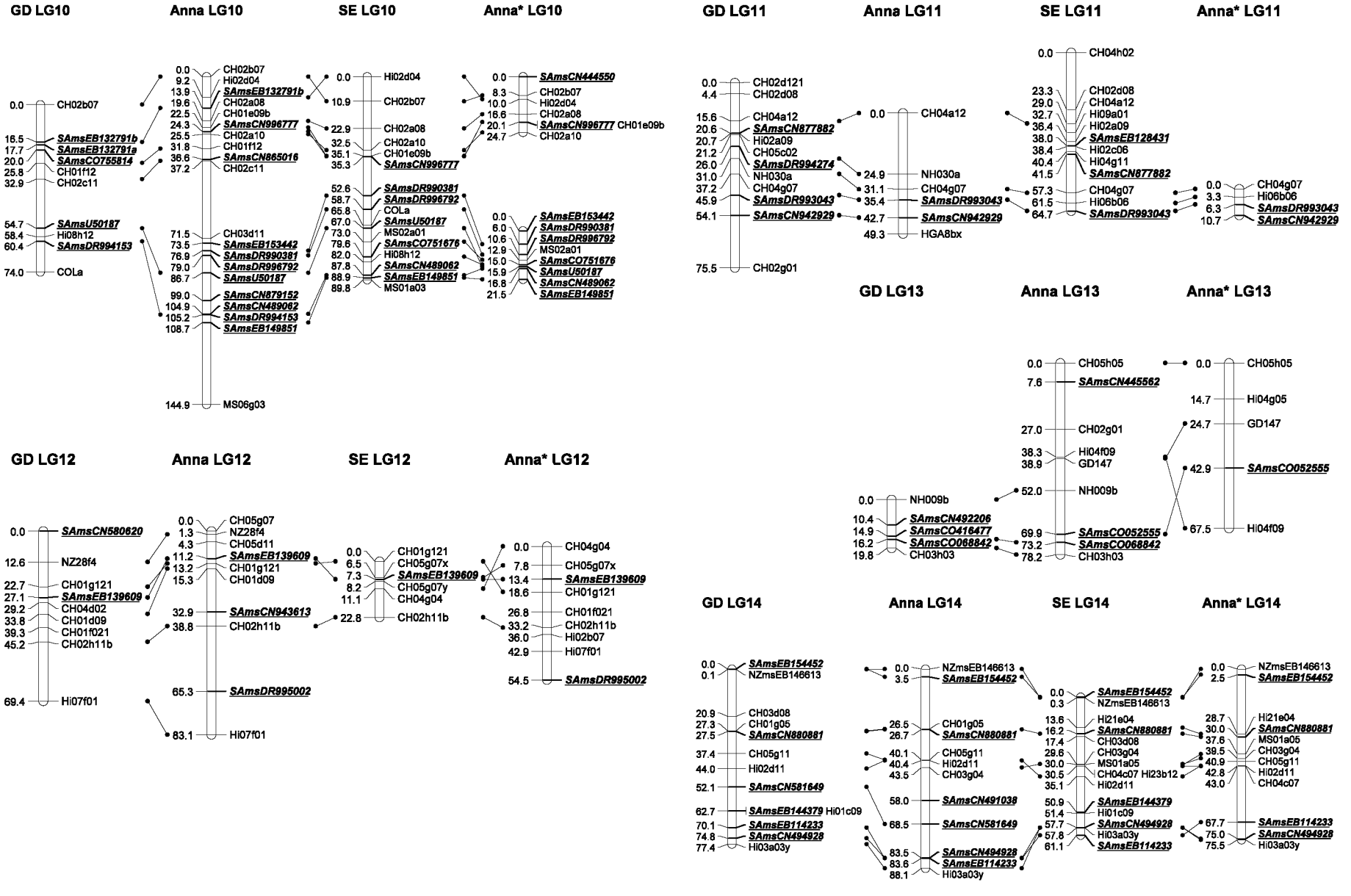
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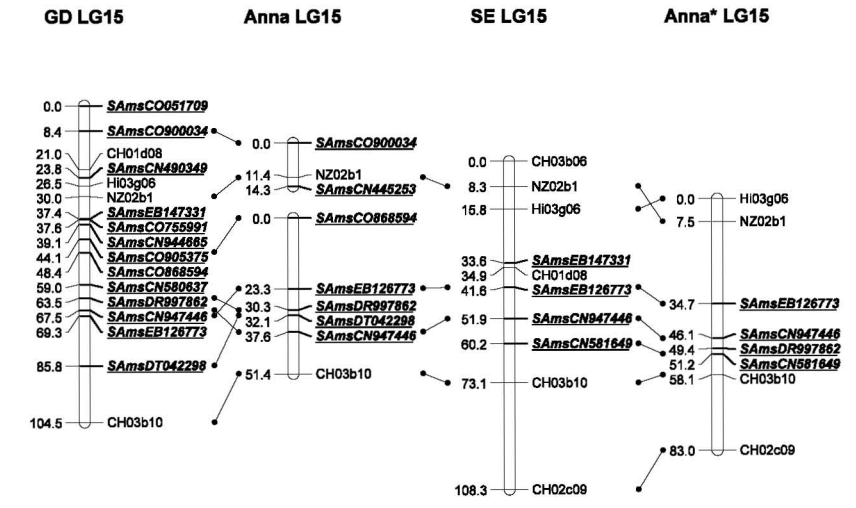

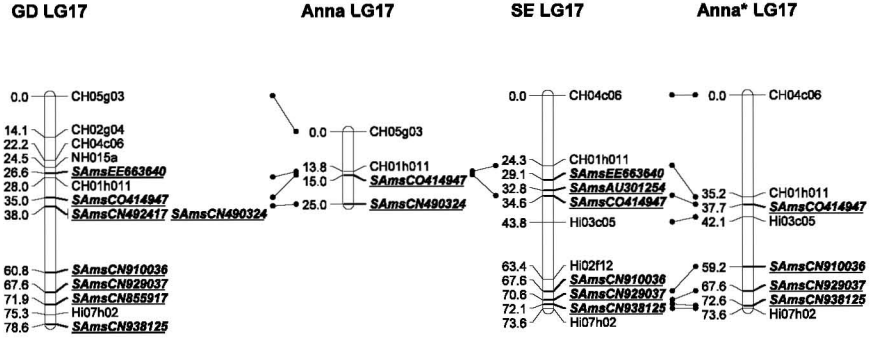

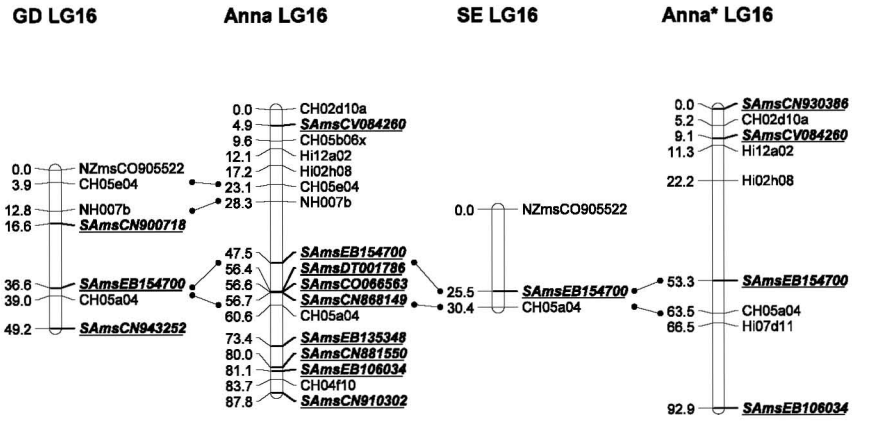

Figure 2: Parental genetic linkage maps of 'Golden Delicious' (GD) and 'Anna' from population A and 'Sharpe's Early' (SE) and 'Anna*' from population B. Numbering of LGs are according to Maliepaard *et al.* (1998). Newly developed SSRs are prefixed by SAms and are indicated in bold, italic and underlined.

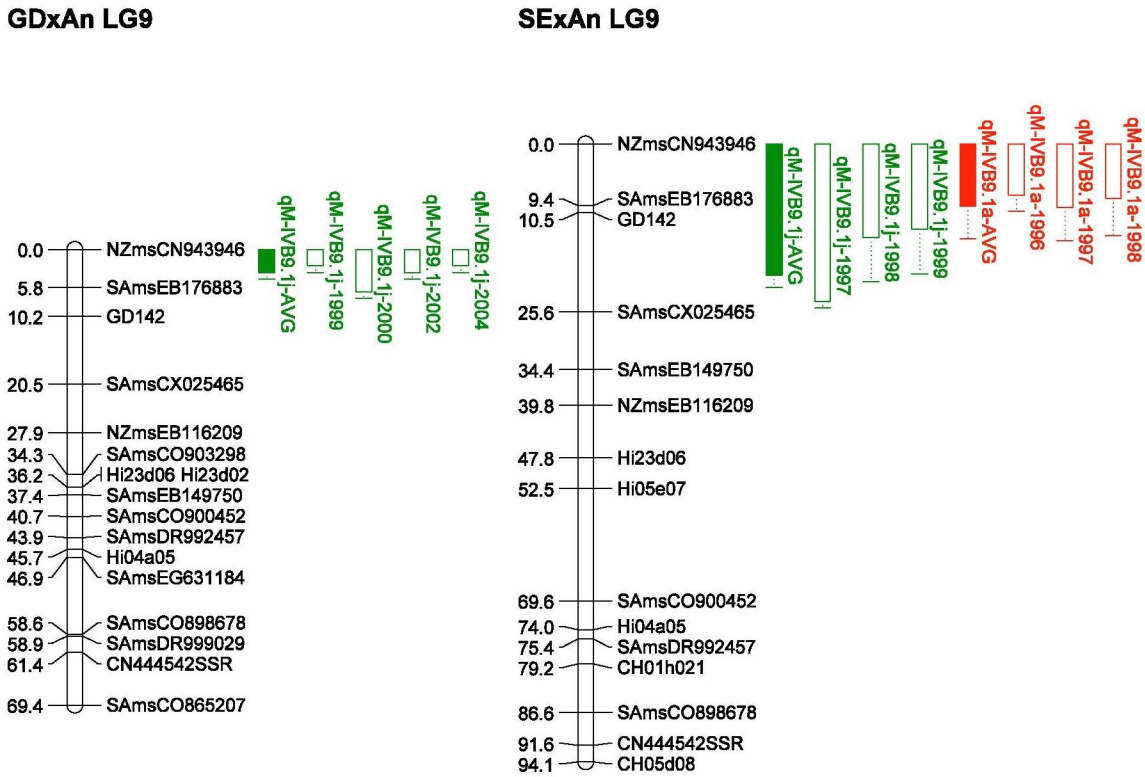

Figure 3: Position of the QTL for time of IVB detected on LG9 of the consensus 'Golden Delicious' x 'Anna' (GDxAn) map and the 'Sharpe's Early' x 'Anna' (SExAn) map. QTLs are represented by boxes where the length of the box corresponds to a 5% confidence interval and extended lines to a 10% confidence interval. Boxes representing average time of IVB are filled and boxes representing time of IVB for separate years are open. Boxes indicating QTL detected on juvenile trees are green and those indicating QTL detected on adult trees are red.

Table 1 Pearson's correlation coefficients indicating phenotypic association ($P < 0.0001$) between different years for time of initial vegetative budbreak (IVB)

Mapping population	**Association between different years of phenotypic trait assessment**					
	Years 1 + 2	**Years 1 + 3**	**Years 2 + 3**	**Years 1 + 4**	**Years 2 + 4**	**Years 3 + 4**
'Golden Delicious' x 'Anna'	0.68	0.69	0.68	0.70	0.78	0.67
'Sharpe's Early' x 'Anna' (Adult trees)	0.96	0.94	0.96			
'Sharpe's Early' x 'Anna' (Young seedlings)[a]	0.81	0.80	0.90			

[a]Clonal trial

Table 2 Summary of 116 new SSR markers, accession number, repeat motif, primers, resulting fragment sizes and genetic linkage map position

Marker	Repeat motif	Forward primer	Reverse primer	Segregating alleles scored			Genetic linkage maps						
							F1		Parental				
				'Anna'	'Golden Delicious'	'Sharpe's Early'	'Golden Delicious' x 'Anna'	'Sharpe's Early' x 'Anna'	'Golden Delicious' x 'Anna'		'Sharpe's Early' x 'Anna'		
									'Golden Delicious'	'Anna'	'Sharpe's Early'	'Anna'	
SAmsCO865608	TC (13)	CAACAAGTGTGCCTCTGTGG	AGCAAGCAACAGATCAAGCC	160-168	160-164		1		1	1			
SAmsDR995748	TC (16.5)	TACACCAGCGCCACACCG	TGGCGAGCACGATGAGCG	314-334	334	334	1	1		1		1	
SAmsCN495924	TC (14.5)	CTCTCAATGAGTCCCCTGC	AAACCCTGTGTTCATCTTCC	148-175	150-173		2		2				
SAmsCN581002	TC (10)	TGGAGGGAAAGGAGAAGCAG	CTTGGAAGCTTTCTGTCAGC	253-267	241-253		2		2	2			
SAmsCO904847	GTT (11.3)	GTGGGTGTGGTTTTTGATGG	AGCTAAAGGAGAGCTACACC	190-193	182	193	2	2		2		2	
SAmsCN944528	GAC (11.7)	GACGACGGAAAGGAAGACG	ATTACGCTGTTGCAGAGAGC	204	204-214		2		2				
SAmsEB106592	TCC (8.7)	CTTGGAAGCCCAACGAACC	AGAGGAGCTTGTTGTTGAGG	236	233-236		2		2				
SAmsEE663746	GA (15.5)	TGGCAATACCCGTTCGACC	CCATCAAATACAAGCCCACC	305-307	305-317	315-317	3	3	3	3	3		
SAmsAU301301	CT (14.5)	GGCATAGCAATGCTTGAAGG	GAATAGCACAAAGGAGGTTGC	228-234	223-241	229	3		3	3			
SAmsCN944444	AAG (8.7)	TAGTGCAAGTACTGGGGCC	CATCGATAGAATAGGACGGC	371-374	371-378	374-376	3	3	3	3	3	3	
SAmsEB13	GGA	TCTCCCTCACTCGAC	GTTGCAGGAAGGAG	243-	253	250		4				4	

2187	(8.3)	GTTG	TGTCG	250									
SAmsCV128959	TC (11.5)	AAATAGTGTGGAAGACGCGG	CAATATACTAATGAGTCCTTCG	240	232-242		4		4				
SAmsCN579721	CT (14)	GATCCAAATCTCAAACCCTCC	GTTGAAGACGTGGTTGGGC	246-259	248-259	259	4	4	4	4		4	
SAmsEB153928	CT (25)	CTCAAATCCCAGAAGATTATCC	GTCCTCGGAATCGTCCTCC	348-350	350-357	350-353	4	4	4	4	4	4	
SAmsCO052033	CT (11.5)	TTGCCAATCCGCATTCGCC	TGAGGTTCCCGCCCTTGC	118	118-196		5		5				
SAmsCO756306	AAAT (5.8)	GTAAATATCACCACCACCGC	ACACAGAACGTCGTACATCG	180-184	180	180-186	5	5		5		5	
SAmsCO416051	AG (16)	CCTCACTAAACGCATTGCAC	CGGTACGATGAGGATCATCC	120-133	120-130	130-133	5	5	5		5		
SAmsCN922831	TC (13)	TTTAGATTCGGAGAGGATACG	CTGCTTGGAATCCTCGAGC	293	290-293	293-295	5	5	5		5		
SAmsCN887525	TTTA (7.8)	TAGTAGCTACACACTCTTTCC	GCATTGCCTTGAGCTCCAG	207	207-214		5		5				
SAmsCN544835	AG (17.5)	AGGAGAGCTTTCTGCATTCC	AGCGCTATCCCCAGCTGC	301-303	303-305	301-303	5		5	5			
SAmsCN444942	CT (16.5)	GCTCTCAAAGTCTCTCCAGC	TACGGACTCTCTTTGGGGC	265-273	275		6			6			
SAmsEG631303	AT (25)	GGCATGTGAATATGGTGAGC	CCAATCAATGTCTTGCTTACC	330-351	327		6			6			
SAmsEB127535	GA (30)	AACACACACACCACCATTCG	TAGGAAGTCGACGTAGTCG	326-330	322-330	326	6	6	6	6		6	
SAmsCV657225	TAT (10.3)	TCCCTGTCATCGAATGATGC	GCAAACCCAATCAGAAGGAC	193	193-198		6		6				
SAmsDT041144	AG (15)	AAATGCTGCAGTGAGGCCC	GAATTCCATCTAAACGAGAGC	349-351	349	349-360	6	6		6	6	6	
SAmsCN927330	ACC (7.3)	TTAAACTGCCAAATTGCACGG	GTTGGGTATTTGCATGGTGG	438-443	431-438		7		7	7			
SAmsCN903950	AGA (14.3)	TTTCCCTTTTGGCCAGTGCA	GTTTGGGCCTCGATGATGG	306-319	297-319		7		7	7			

SAmsCO75 6781	CT (19.5)	ATAAGTTTAGGCTC ATCTGCC	AAACCCATCCCACTT AAGGC	355-361	333-361	346-379	7	7		7	7	7	
SAmsCO90 1343	(CT) 15.5	CACCTCTTCCCTCAT CAGTC	CGACAAAGGAGACT GAGAGG	208-222	208	210-230	7	7		7	7	7	
SAmsCN48 8733	TC (13)	CACAACCATTCCAC CAAGTC	CAGCCGGAGCAGTC TACC	127-131	131-142		7			7			
SAmsEB12 7208	AG (14.5)	ATTCCTCTCAACCCC TATCC	CACAGTGCTGTTAAA GCTGG	479-491	491	479-491	7			7			
SAmsAB16 2040	TC (39.5)	GGAGTGCTATTAGC TCCTCC	TCCTTGAATCTCAAC TCTAGG	266		266-272		7			7		
SAmsCV88 3434	TC (23)	CGAAACTGGTCGAA GAACCT	AAACTACACAGAGC AAGATGG	331-335	350-354		8		8	8			
SAmsEB15 1277	TC (29)	TCCTCAATCTCTCTC AATACC	GCGTTCTAGAGAGA GAAAGG	179-197	197-202	202-214	8	8	8	8	8		
SAmsCN89 1581	TCC (8)	CCAAAACTCCCACG ACCGC	CCAGAGCTTGTAGG ACTCG	294-297	294-null		8		8				
SAmsEB17 6883	TGCT (8.5)	AAAGCTGCTTGCTT GATTGC	ACCATCAGCTGGGTT CTCG	330-338	322	322-338	9	9		9	9	9	
SAmsCX02 5465	GAC (10.7)	TGCTAGAGCTGCGT TCTCC	TCGCAGACTGCTCGC TGC	232-238	232-238	232	9	9				9	
SAmsCO90 3298	TC (14)	TTGAGAAGCAATGC TGCCTC	TGCCACAGTTGGAA GGTGG	344	344-350	342-344	9		9				
SAmsEB14 9750	TC (19)	ATCAAGGTGTGAGT GTGTGC	AAGCTTGCATCTCTA GGTCC	258-263	255		9			9			
SAmsCO90 0452	GA (12.5)	CAAGGCATCTCCCT CATTGG	TACTACAGTTCCGAT CAAAGC	291-314	293-295	311-314	9	9	9	9	9	9	
SAmsEG63 1184	GA (10)	CTTATGGACCCTGC AAATGG	AGACTCTGTACATAC ATCTCC	447-464	447		9			9			
SAmsDR99 2457	AGC (13.7)	TCTCCAAGTGGACG AATCAG	TCCTCAGTGAAGAC AAACCC	360-370	356-368	360-365	9	9	9	9	9	9	
SAmsCO89 8678	CT (16)	CCCAAGTGCACCAC ATACAG	AGCTTCTGGCAGCA AGTGC	242	238-244	238-242	9	9	9		9		
SAmsDR99 9029	TC (14)	CGCCCTCACTCATTC AGTC	TCAACATGAACTTCA GTCGC	440	440-	440	9		9				

					443								
SAmsCO865207	GA (13.5)	TGCACCAAATAAGCCGATCC	CAAGAAGTGCAACCAGTCGA	134-138	120		9						
SAmsCN444550	TGGAT (5)	AGCATCAAGCCAATCTTTAAGC	GTATGCTCTTCTTCTTCATGG	346-351	341	341		10				10	
SAmsEB132791a	CT (17.5)	CACTACAGAACTCCTCATCC	GTGGGATGGAACCGAAACC	312	312-316		10		10				
SAmsEB132791b				344-350	340-350	344-350	10		10	10			
SAmsCO755814	CT (21)	AACATCAAGACAGAGAAGAGC	CGTCTTCTTCACAAACTCCG	263	257-263		10		10				
SAmsCN996777	CACCT (5)	TGACAACTATGATCGAAGTGG	TTTCATATCACATGACGTGGC	270-275	275	266-275	10	10		10	10	10	
SAmsCN865016	CAT (14)	TTCTTCACACCCTTCAATCC	AAAGCGCCTGCGATTGCG	340-345	334-340	340-345	10	10		10			
SAmsU50187	GA (17.5)	ACCTGAGAGAGCTCCAAACG	GTGCGCCACGTCAAATACG	160-null	149-162	149-162	10	10	10	10	10	10	
SAmsEB153442	CT (23.5)	GGTTCACAAGGCCAACTTTG	ATGGTTCGATCGGTTTAATGC	366-373	373	371-373	10	10		10		10	
SAmsDR990381	TCT (9.7)	AAACACTACTGTGCTGGTGG	AGTCCACTTACTACTCCTCC	287-300	300	294-300	10	10		10	10	10	
SAmsDR996792	CT (15)	AGGCTTCCTTCCTTTCTTCC	GGACCATTTGTGGTGGAGC	378-399	397	388-396	10	10		10	10	10	
SAmsCO751676	TC (15)	TGTGGCTCTGGATGGTTCC	TACCAGTCCATCCGTATAGC	233	218	218-228		10			10	10	
SAmsCN879152	ATC (7.3)	CGTTGGAGATGATCAGTACG	ACCTACAATAGTAGTGGAGAC	256-null	243-256		10			10			
SAmsCN489062	GA (13)	ACAACTTGGTTACGCGACAC	GAACAGATTAGGGTCGCTGG	296-300	284-314	284-296	10	10		10	10	10	
SAmsDR994153	AG (14.5)	CACGAGGCGAAACCGATC	AGGTCCTCAGAACCTGAGC	465-472	463-465	465-472	10		10	10			
SAmsEB149851	AGA (10.3)	GAA CAG AGG GAA GCA GAC G	AGA AGT GGC AAC CAT GTT GC	187-190	190-202	187-202	10	10		10	10	10	

SAmsCN87 7882	CTAG T (6.8)	AACTTGCTGAGAGA GTAATGG	CAACCAAAGGGCCT GAAGC	485	495-500	485-500	11	11	11		11		
SAmsEB12 8431	TAA (17)	ACGTAGTGATACCG GATTCG	AGAGCTAGCTAGAG ATATTCC	335	342-null	322-342		11			11		
SAmsDR99 4274	ACC (12.3)	CCACCCACAAAACA TACACC	TGCTGTTGTTGGTGA TGTGG	228	221-228		11		11				
SAmsDR99 3043	TC (13.5)	CACGAGGGTAAGCT CCCC	TTGGGGTTATTGCTC TGACG	298-314	279-304	293-307	11	11	11	11	11	11	
SAmsCN94 2929	GTTT (5)	ACGCTAGGAGAGAG GAACG	GAGCATTCCGTATTA AATCCG	519-524	524-529	524	11	11	11	11		11	
SAmsCN58 0620	CGG (7)	TGCGGTCAACGATG TCTTCG	AAGGTACAAGCCCG CAAAGG	380	377-380		12		12				
SAmsEB13 9609	AG (32)	ACCATATACATCTC TCTCTGC	TTCAGAAGCTGTTGT TGTTGG	322-334	313-358	340-358	12	12	12	12	12	12	
SAmsCN94 3613	CTT (7.3)	TAGCAGAAACCAGC AGATGG	GAAGGACCCGAATT GGAGC	165-174	174		12			12			
SAmsDR99 5002	GAT (8)	ATCTGATGGTGCAT CGGTAG	TTAGGGTCTTCTTGT CACGC	329-332	332	332	12	12		12		12	
SAmsCN49 2206	TTG (10.7)	ACATACTGGAGTCT GCGAGC	CAATACGCTAGTGA AGACGC	398	398-471		13		13				
SAmsCO05 2555	AT (12.5)	GAAGTTCTCATCAA GTCTTGC	GCTTCTGCACAATGG CTGG	232-234	236	232	13	13		13		13	
SAmsCN44 5562	TC (23.5)	CACAAACCAACCGT CTAACC	GCTCTTGATCATAGG CGTGG	139-154	150-154		13			13			
SAmsCO41 6477	CT(14)	CCACACAACACAAA CCAACC	GAGGCATTGATCCTC ATCGT	218	218-224		13		13				
SAmsCO06 8842	TC (22)	TGGTTGGAGATGTT CCATGG	ACCAGCTAGATTATC TTCTGC	455-null	401-447		13		13	13			
SAmsEB15 4452	GATC (5)	CACTCAACTCACGT TTCTCC	AGGCAGAAGGCAGA AGAGG	169-174	174-184	174-181	14	14	14	14	14	14	
SAmsCN88 0881	CCA (10)	ATAGCTCATACCGC TTCTCC	GTGACGAAAACCAA GAACCC	427-429	406-408	406-427	14	14	14	14	14	14	
SAmsCN49 1038	TC (19)	GCTCTGTCTCGTTGA TCGG	AGCTGCTTCACCCTC TTGC	498-510	510		14			14			

SAmsCN581649a	CAT (13.3)	AGCCCTGATCTTCCTCTAGC	GACAATCTTCTGAAAGTCTGG	343-351	351-354		14		14	14			
SAmsEB144379	GGCGGT (4.5)	AGCTGATGGCCAGAACTGC	GAGGGTCCAAGTTACAAAGG	418	412-418	412-418	14	14	14		14		
SAmsCN494928	ATC (14)	AATTATATCCGTCCGACTCCA	TTACTGCTACCTGATGATCC	226	209-219	209-215	14	14	14	14	14	14	
SAmsEB114233	GA (11.5)	GCATCCGCCATTGTTGTCG	TGGATTGAGACTGAGAGAGG	221-227	217-223	227-231	14	14	14	14	14	14	
SAmsEB147331	CT (26)	CCTAACTCTGACTCAGTTGC	AGTGTCGTCTGGAGCTTCC	257	261-266	264-266	15	15	15		15		
SAmsCN944665	TATG (10.8)	GTCTCTGCTTGCTTAATTCAG	AGGCCAATCCTGACTATAG	320	224-320		15		15				
SAmsCN490349	AGG (8.7)	GTACTATCAGCAGAAACTGG	GATTTGAGCACAACATACGG	200	200-206	200	15		15				
SAmsCN445253	CTG (8)	TGCAAGAATCATCCACTTCC	TTGGACCTGTGAGGACTCC	478-494	491		15			15			
SAmsCO900034	AAG (10.3)	AAAGTCCGTTTTGGGCTGAG	GCTCTCTGCTGCCATTTCC	361-367	353-367	361-367	15		15	15			
SAmsCO051709	CTCAAG (3.5)	CTGTGCCGTCATCTATATGC	AACCAAAGAGGGAAGAGACG	193	193-200		15		15				
SAmsCN580637	TC(16.5)	ACAACAGCTGACGAACAAGC	CTACTCGTCGAAGTACGCC	418	406-418		15		15				
SAmsCO415353	AG(14)	ATGAACAGTCACAGACTATGC	AACGAAGCAAAGGAAGACGG	329-333	329-333	329-333	15	15					
SAmsCN947446	CTT(8.3)	CCGTTACAGCTATCCAAACC	ATAATGGCCATTCTGTTCAGC	178-181	181-184	181-187	15	15	15	15	15	15	
SAmsEB126773	CT(23.5)	GTTTGTGTTTGAACAACGACC	GTGGTTGTTGAGGTCGTGG	447-453	441-447	455-469	15	15	15	15	15	15	
SAmsDT042298	GT(12)	AGCATGTTGTGGGAAGCCC	GCATACTCTCATACAAGTCCG	227-229	225-227	227-229	15		15	15			
SAmsDR997862	TCTG (7.8)	CACAATCATATTCCCGCACG	TTCTTCTCCGATGAGCAAGC	275-280	275-283	275	15	15	15	15		15	

SAmsCN581649b	CAT (13.3)	AGCCCTGATCTTCCTCTAGC	GACAATCTTCTGAAAGTCTGG	332-338		332-347		15			15	15	
SAmsCO868594	CT (19)	CACCTCTTCAAACAACACACC	GGGCGGAGGTAGTTTATCC	412-414	418-436	412-416	15		15	15			
SAmsCO905375	AG (23.5)	AGTCTCTGTTTTTGCTCGTTC	GAACGCCGGGTCCCTGC	407	407-427		15		15				
SAmsCO755991	TC (16.5)	AATCTCTCGTCTGCAAACCC	GGCACTGAGCGCACTTGG	154	150-154		15		15				
SAmsCN930386	AGA (13)	TTGGGTTTGTTGCTGAAAACC	TGACCGGACTGTTTACAGG	94-111	94	94		16				16	
SAmsCV084260	AG (22)	CAAAGCAAAACAGAGGATTTG	GGAGCGCATGAAATTACTGC	226-256	262	226-264	16	16		16		16	
SAmsCN900718	CAG (7.7)	AGCATCTGAACTACCAATACC	ACCGATATAGTGCTGTTGC	278	268-278		16		16				
SAmsEB154700	AG (24)	TTTGTTGGGATTGTGGGTCG	GTTGCTGAGAGTGATGATGG	229-236	229-234	234-236	16	16	16	16	16	16	
SAmsCO066563	GA (11)	ACAAAGGAACAGTGAAGACTC	TACTTGCTCTGCATAGTTTGG	422-431	425	422-431	16	16		16			
SAmsEB135348	CCA (11)	ATCCCTAACCCCAGGATGG	AGCATGTGGAAATCGTATACC	330-333	330		16			16			
SAmsCN881550	CAG (14.3)	ATCCAAACAACCCCATTGCG	AGTCGATGTTGAACGCTCCA	346-348	356		16			16			
SAmsCN868149	AT (10)	TTGCTGCTGTCTGTGTTTGC	GTCTCGTCGAAATCTTAAAGG	246-252	252		16			16			
SAmsDT001786	GA (17.5)	TTCTCTGTCTGTGAAATTGCG	GTTAACTGAGCTCCTGGTATTCC	143-147	141		16			16			
SAmsCN943252	TTC (9.7)	TCCCACTGACACTATCACC	TGCAGGAAATGAGAATGCGC	194	194-197		16		16				
SAmsEB106034	AAG (11.7)	AGAAGAAGCCCATCCCAGC	TTCACCTTCGTCGGCATGG	191-194	194	191	16	16		16		16	
SAmsCN910302	TCTG (6.8)	TTTTCAGGCATCACTGTCCC	ATCAGGATTTCCAACAGCGC	466-484	484		16			16			
SAmsEE663640	GA (12.5)	AGTGTAGCAACCAAACGCTG	TTATTTCCTCGTCGGCAAGG	486	483-488	481-483	17	17	17		17		
SAmsAU3	TC	TCCCGGAAATTTTTC	AACGCTAGGGATTG	233-	233	233-		17			17		

01254	(15)	AACGC	GTCGC	246		240							
SAmsCO41 4947	AG (12)	TTTGATTGGACCTG CAGTGG	TTAGCAGCTGCTTCA GTGTG	346-350	341-354		17	17	17	17	17	17	
SAmsCN49 2417	TC (10)	TACCATGTTTTAGC ACCATGG	GGCCAAGTTAGGTC AAGACG	122	122-126		17		17				
SAmsCN49 0324	AG (16)	ATAGAGAGGTAGAG GACTGG	TTCGCCCAGTGTAAC ATTGG	230-232	223-232		17		17	17			
SAmsCN93 8125	TTC (13.7)	GCCTTCATCCCCCCT TGA	GGTGTATAGGAATCT TGGAG	338-345	345-352	340-354	17	17	17		17	17	
SAmsCN91 0036	CTT (13.7)	GAGAAACCGTTTGA TTACAGC	CTCCATCCCCAATCA CACC	235-241	232-235	220-241	17	17	17		17	17	
SAmsCN85 5917	AAT (15.3)	CTCTTTCTTCTCCCT TCTCC	GATGAGATCCAAAT CCGTAGT	149-174	159-174	146-165	17		17				
SAmsCN92 9037	TA (13.5)	AGTTGACTACCTCC TCCGC	GTGGTTCTCACGGTA CACG	218-225	218-239	218-220	17	17	17		17	17	

Table 3 Parameters associated with the QTL for time of initial vegetative budbreak (IVB) identified on LG 9 of the consensus map used for population A and population B, using multiple QTL mapping (MQM)

Year	**LOD[a]**	**mu_ac{00}[b]**	**mu_bc{00}[b]**	**mu_ad{00}[b]**	**mu_bd{00}[b]**	**% Expl.[c]**
Population A: 'Golden Delicious' x 'Anna'						
Average	6.07 (6.8)	271	271	247	252	36.7
1999	3.1 (5.4)	309	311	285	292	25.4
2000	3.91 (8.9)	307	312	277	302	4.8
2002	5.7 (4.4)	282	279	269	271	23.6
2004	7 (4.8)	254	254	226	235	40.1
Population B: 'Sharpe's Early' x 'Anna'—adult trees						
Average	8.65 (7.0)	252	262	219	219	41.6
1996	9.52 (8.6)	258	267	221	222	44.6
1997	8.04 (6.1)	254	262	224	222	39
1998	6.83 (5.3)	245	256	212	212	38.2
Population B: 'Sharpe's Early' x 'Anna'—juvenile trees						
Average	4.68 (4.9)	246	246	226	225	17.6
1997	2.56 (4.5)	260	256	245	242	11.9
1998	4.44 (4.6)	254	255	235	232	20.9
1999	4.49 (5.8)	251	253	230	226	17.9

[a]Maximum LOD score with considered threshold in parentheses

[b]Estimated mean of the distribution of time of IVB associated with each genotypic class with alleles "a" and "b" inherited from the parental cultivars 'Golden Delicious' and 'Sharpe's Early', respectively, and alleles "c" and "d" inherited from the cultivar 'Anna'

[c]Percentage of the variance explained by the QTL