The Use of Molecular Markers to Analyze the Inheritance of Morphological and Developmental Traits in Apple

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Abstract. Five morphological and developmental traits (branching habit, vegetative budbreak, reproductive budbreak, bloom time, and root suckering) were analyzed in a family obtained from the apple (*Malus domestica* Borkh) cross 'Rome Beauty' x 'White Angel'. The phenotypic variation in these traits was compared with a selected set of marker loci covering the known genome of each of the parents to locate genes with major effects on the traits. The contrasting branching habits of the two parents appeared to be controlled by at least two loci. One of these, *Tb*, governed the presence or absence of lateral branches, particularly on the lower half of shoots. The locus was heterozygous in 'White Angel' and was mapped to a 5 CM interval on linkage group 6. At least one other locus conditioning spur-type branching appeared to be segregating, but the locus or loci could not be linked to segregating markers. The timing of initial vegetative growth was tightly associated with the chromosomal region in which the *Tb* gene is located and maybe a pleiotropic effect of this gene. Time of reproductive budbreak correlated with segregation at the isozyme marker, *Prx-c*, on linkage group 5. Variation in time of bloom and later stages in flower development appeared to be controlled by different genes not linked to *Prx-c*. The tendency to produce root suckers cosegregated with a marker on 'White Angel' linkage group 1, suggesting control by a single locus, *Rs*. Data from a 'Rome Beauty' x 'Robusta 5' family provided additional information on the inheritance of these traits.

Apple is one of the most important temperate tree fruit in the world (Childers, 1983). Even though apples are not considered a major food crop, ≈40 million tons are in production worldwide (Way et al., 1990). Nevertheless, the development of improved apple cultivars continues to be a slow and laborious task. Generation time from seed to seed may be as long as 8 years due to the juvenility period. In addition, genetic studies in apple are hampered by large growth form, self-incompatibility, and inbreeding depression. Thus, relatively few genes controlling morphological or physiological traits have been described in this crop, and genetic analysis of complex characters such as branching habit and blooming time have not been particularly successful (Brown, 1992).

Modern molecular techniques are being used to circumvent many of the inherent problems in the genetic investigation of apple. Molecular markers, including isozymes, restriction fragment length polymorphisms (RFLPs), and random amplified polymorphic DNA (RAPDs), have been used to develop linkage groups in this species (Hemmat et al., 1994; Manganaris, 1989; Weeden and Lamb, 1987). One important application of a set of markers is the genetic analysis of complex characters. The dissection of polygenic traits into a number of quantitative trait loci (QTL) has been performed successfully in several species (Diers et al., 1992; Doebley and Stec, 1991; Edwards et al., 1987, 1992; Paterson et al., 1988, 1991). However, such analyses require relatively large populations or very large numbers of genetic markers. A large population of a woody perennial requires a substantial commitment of land and materials for an extended

period of time. An alternative approach to QTL mapping, at least at initial stages of analysis, is to determine if apparently complex traits are controlled predominantly by one or a few major loci. Traits such as plant height, time of flowering, and branching pattern usually are considered characters with a complex genetic basis. However, the observed variation in each of these characters has been demonstrated to be under simple genetic control in some species (Gottlieb, 1984). This approach has proven particularly successful in *Pisum sativum*. In a controlled environment, stem height, flowering node, branching pattern, and seed size all appear to be determined by allelic polymorphism at two to six loci (Blixt, 1974; Murfet, 1989; Reid et al., 1983). Although identification of a major gene in one cross does not mean that the gene will be predominant in all crosses, such a finding represents an important advance in the genetic analysis of the character.

The recent development of an extensive set of monogenic markers in apple (Hemmat et al., 1994) provides the opportunity to initiate genetic dissection of some important traits. The objective of this study was to investigate the genetic basis of several morphological and developmental characters segregating in a family produced from a cross between two apple cultivars.

Materials and Methods

Plant material

A family derived from the cross, 'Rome Beauty' x 'White Angel' (family size = 82) was grown at the New York State Agricultural Experiment Station, Geneva, N.Y. The controlled cross was made in 1985, the seeds were germinated in January 1986, and the seedlings were planted in the orchard the following fall. Trees were planted at 1.2-meter spacing and allowed to grow without pruning. Individual trees were scored throughout the 1990 and 1991 growing seasons for several morphological characters. Additional data on budbreak and branching habit were obtained throughout 1992 and Spring 1993. The family also was used to develop linkage maps for both parents (Hemmat et al., 1994).

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The maternal parent, 'Rome Beauty', is a widely grown cultivar (Way et al., 1990) of unknown parentage. It has a standard growth habit. Young trees are upright but later become spreading with slender lateral branches (Beach, 1905). Blind wood (shoots with few or no laterals) with 90-degree branch angles is characteristic of this cultivar. 'Rome Beauty' bears fruit on the terminals of branches with few or no spurs. It has a relatively late bloom season and late vegetative budbreak.

The ornamental crabapple 'White Angel' was used as the pollen parent. Its parentage is unknown, but its pedigree may include *Malus seiboldii* (Simon and Weeden, 1991). The cultivar has a very spurred growth habit with wide branch angles, and it blooms early to mid-season.

A population of 61 individuals from a 'Rome Beauty' x 'Robusta 5' cross also was examined. 'Robusta 5' is an apple rootstock believed to be a hybrid between *Asian Malus* species. This population was used only to examine the effect of a different paternal parent on the segregation of morphological and developmental traits. Segregating molecular markers were not scored in this progeny.

Morphological and developmental traits

Branching habit. The branching pattern of a tree was most conveniently observed during winter when the branches were bare. Individual trees were scored qualitatively as 1) terminal bearing ('Rome Beauty') type, featuring few or no lateral branches on the lower half of the shoots (blind wood) and with most of the fruit buds being borne near the ends of the branches, 2) normal branching, with lateral buds frequently producing leaves and secondary branching, or 3) ambiguous, which referred to those trees that could not be clearly placed in either of the previous categories. Each of these categories was subdivided into trees displaying shoots with spurs, typical of 'White Angel', or trees lacking such spurs.

Vegetative budbreak. The swelling of terminal buds and the initial-expansion of young leaves was examined during Springs 1990, 1991, and 1993. Buds or young leaves were examined from 10 branches for each tree, which was then given a ranking between 0 and 4, with 0 indicating most delayed development (buds unopened) and 4 indicating most advanced development (leaves expanding).

Reproductive bud stages. Flower-bud stages from dormant through fruit set were studied throughout April and May 1990 and 1991 according to the phenological categories of Chapman and Catlin (1976). The key growth stages included dormant, silver tip, green tip, half-inch green, tight cluster, pink, bloom, petal fall, and fruit set. A full pink stage was not described by Chapman and Catlin (1976) but was added to further distinguish the various stages of bloom. Full pink refers to the stage in which the blossom buds have separated, pedicels have elongated, and the blossoms display a bright pink color. Initial budbreak data included the stages of silver tip, green tip, and half-inch green, whereas bloom time included tight cluster, pink, full pink, and bloom.

Root suckering. Root suckers are sprouts from the roots or base of the tree. Trees were scored as possessing or lacking root suckers irrespective of the number of root suckers present. Parental cultivars could not be scored for this trait because they were only available as scions grafted on rootstock.

Linkage analysis

Hemmat et al. (1994) reported the results of a linkage analysis performed on over 400 markers (isozymes, RFLPs, and RAPDs) segregating in the 'Rome Beauty' x 'White Angel' family. Segregation data from 56 individuals were available. Since the population represented a double pseudotestcross format (Weeden, 1993),

a linkage map was generated for each parent. The linkage maps for 'White Angel' and 'Rome Beauty' consist of 24 and 21 linkage groups, respectively. Most markers gave segregation ratios not significantly different from 1:1 or 3:1, indicating that most regions of the two parental genomes were segregating normally. For each parent, the length of the linkage map was ≈ 1000 cM, and 90% of the segregating markers could be assigned to multilocus linkage groups. Most linkage groups were saturated at 10 to 15 cM.

The variation for a particular trait was compared with the segregation of selected markers spaced at ≈ 20 -cM intervals on the maps of both parents. For most traits, the dividing lines between phenotypic classes were arbitrary or difficult to distinguish, and an initial comparison was made using only those individuals at the tails of the distribution or those with a clearly defined phenotype. Trees with intermediate phenotypes were analyzed only after correlations had been identified between one or more marker loci and the initial set of individuals. The total number of comparisons between traits and marker loci was not always the same due to missing data in either the trait or the marker.

Comparisons between marker data and a given trait were performed using the Microsoft Excel macro, QUIKMAP, designed by N.F. Weeden and J. Barnard (available upon request). This program is designed to calculate the frequency of matches between marker loci and morphological traits. The program LINKAGE-1 (Suiter et al., 1983) was used to carry out contingency chi-square tests to analyze the joint segregation between marker loci and the morphological and developmental traits that were in the form of categorical data. When a significant deviation from random assortment was observed between a character and a marker, linkage analysis was performed with all markers on that linkage group to identify the marker displaying the maximum linkage with the character being studied.

Results

Branching habit. Trees displaying the terminal type growth characteristic of 'Rome Beauty' were observed in the 'Rome Beauty' x 'White Angel' family. This phenotype did not appear in the 'Rome Beauty' x 'Robusta 5' population. Of the 82 trees in the former population, 28 were clearly terminal bearing and 26 displayed normal branching. The remaining 28 plants exhibited intermediate ambiguous phenotypes, that usually favored one or the other parental type but showed some characters of the alternate parent. When the branching data (terminal and normal categories) were compared with the segregating molecular markers, the RAPD marker, P98h significantly deviated from random assortment. Seventeen of the 28 terminal bearing plants were scored for P98h. The analysis revealed that 16 of the 17 terminal bearing plants scored for P98h did not show the RAPD fragment, whereas all the normally branched plants scored for this RAPD displayed the fragment. This very strong correlation between habit and the marker suggested that a locus with a major influence on branching habit was closely linked to P98h on linkage group 6 of the 'White Angel' map. When the intermediates were included in the analysis, being assigned to that parental phenotype that each more closely resembled, the correlation between phenotype and markers was maintained. However, there appeared to be a higher proportion of recombinant (Table 1). P27e, a marker mapping ≈ 5 cM from P98h, displayed strong linkage with the terminal branching habit (Table 1). The recombination pattern among the two markers and the branching trait suggested that the locus, here designated *Tb* for terminal bearing, was positioned between the two markers (Table 1, Fig. 1), although a significant number (7) of double crossover

Table 1. Joint segregation analysis for phenotypic traits and marker loci exhibiting significant deviation from random assortment in a 'Rome Beauty' x 'White Angel' family.

Morphological trait	Marker locus	Observed phenotypes ^c				χ^2	P	n ^y
		+/+	+/-	-/+	-/-			
Branch habit ^a	P27e	8	19	21	3	17.63	<0.0001	51
	P98h	20	7	3	22	20.46	<0.0001	52
Vegetative bud break ^w								
25 Apr. 1990	P98h	18	8	3	22	18.10	<0.0001	51
Reproductive bud break ^v								
6 Apr. 1990	<i>Prx-c</i>	14	25	33	10	13.95	0.0001	82
Bloom time ^u	<i>Aat-c</i>	12	7	3	12	6.33	0.012	34
Root suckers ⁱ	P124e	0	11	15	2	23.20	<0.0001	28
	P105i	2	10	16	5	9.79	0.0017	33

^aObserved phenotypes are designated trait/marker loci.

^yNumber of comparisons between trait and marker vary depending on data available.

^aBranch habit: (+) = normal and (-) = terminal bearing. Branching habit data as originally scored without taking into account possible effect of spur habit.

^wVegetative budbreak: (+) = early and (-) = late. Vegetative budbreak classes divided as 0 and 1 = late and 3 and 4 = early

^vReproductive budbreak: (+) = early and (-) = late.

^uBloom time: (+) = early and (-) = late.

ⁱRoot suckers: (+) = presence and (-) = absence.

Fig. 1. Linkage groups 1, 5 and 6 of 'White Angel' (from Hemmat et al., 1994). Arrows identify the approximate positions of chromosomal segments controlling or associated with the variation in reproductive budbreak, bloom time, branching habit, and root suckers. Map distances are measured in centiMorgans (cM).

events between the two markers had to be postulated. This unexpectedly high number of double crossover events could indicate that either the penetrance of the gene is not complete or that one or more additional genes affect the expression of *Tb*.

Spur-type growth habit segregated in the terminal bearing and normal habit categories. Strong spurring was observed both on trees with lateral branches and those trees with the terminal bearing

habit. Similarly, representatives of both branching habits were found that were relatively free of spur branches. However, there were trees that fell into an intermediate category, displaying a moderate amount of spurring, but less than that typical of 'White Angel.' The overall segregation was 35 trees displaying spur-type growth, 37 trees lacking spurs, and 10 with an ambiguous phenotype. Joint segregation analysis with marker loci failed to identify

Table 2. Joint segregation analysis of vegetative budbreak with branching habit in the 'Rome Beauty' x 'White Angel' family'.

Phenotypic class ^y	Branching habit	
	Normal	Terminal bearing
0	0	11
1	2	19
2	13	3
3	8	0
4	20	0

^yData available from 76 individuals.

^yPhenotypic class: 0 = latest to break dormancy, 4 = earliest to break dormancy. Phenotypic classes divided as 0 and 1 = late and 2, 3, and 4 = early.

a significant ($P < 0.001$) correlation between this variation and any molecular marker. However, there appeared to be an interaction between the spurred phenotype and the terminal bearing habit. All seven of the plants classified as normal branching but predicted to be terminal bearing based on marker (P98h and P27e) genotypes were strongly spurred. In contrast, only one of the trees clearly possessing the terminal bearing habit was also scored as being strongly spurred. It is possible that the expression of the strongly spurred phenotype concomitantly induces enough lateral branches to mask the *tb/tb* phenotype. Such a model would place *Tb* very near P98h (3 recombinant in 52 plants) and would eliminate all cases of double crossovers occurring between the markers P98h and P27e.

Vegetative budbreak. Clear and reproducible differences in the relative timing of vegetative bud opening and leaf expansion were observed. These differences were highly correlated with the segregation of P98h and P27e on linkage group 6, as well as with the segregation of the terminal bearing habit. Indeed, the best correlation was with the *Tb* segregation (Table 2). Thus, the vegetative budbreak character maybe a pleiotropic effect of *Tb* and was not assigned a separate locus symbol. However, there was a small overlap (five trees) in the middle range of the time of budbreak data in which three early trees were terminal bearing and two late trees were normal branching. We could not determine whether these recombinant phenotypes represented actual recombination events between two genes or were produced by microenvironmental effects.

Reproductive budbreak. The initial swelling of the flower buds occurred ≈ 6 days earlier in 'White Angel' than 'Rome Beauty'. For the progeny, the reproductive budbreak data for 6 Apr. 1990 gave 40 trees in green tip stage (early) and 42 in silver tip stage (late) (Table 3). A week later, about half the trees in each category had advanced to the next identified category, splitting the population into three groups (Table 3). The budbreak response of all trees was confirmed perfectly by the 1991 data.

For the initial comparison with marker data, the two extreme categories (silver tip and half-inch green) were considered from the 12 Apr. scoring. This comparison revealed a correlation with *Prx-c* segregation (silver tip: 16 *Prx-c'*, 5 *Prx-c*; green tip: 7 *Prx-c'*, 14 *Prx-c*; $\chi^2 = 8.05$). Joint segregation analysis between *Prx-c* and reproductive budbreak for the entire population was performed using the Apr. 6 data (Table 1). The correlation was observed for both dates and was maximal with *Prx-c* compared to that calculated with flanking markers. No other region on either the 'White Angel' or 'Rome Beauty' maps showed significant correlation ($P < 0.001$) with initial budbreak data. These results suggest that there is a dominant allele at a locus, which we designate *Rbb*, that promotes early initiation of reproductive budbreak. 'White Angel' is postulated to be heterozygous at this locus, and 'Rome

Table 3. Summary of phonological growth stage data for apple flower bud development in 'Rome Beauty' x 'White Angel' family, Spring 1990'.

Phonological stage	1 Apr.	6 Apr.	12 Apr.	27 Apr.	4 May
Dormant	82	0	0	0	0
Silver tip	0	42	21	0	0
Green tip	0	40	40	0	0
Half-inch green	0	0	21	0	0
Tight cluster	0	0	0	21	0
Pink	0	0	0	24	5
Full pink	0	0	0	16	22
Bloom	0	0	0	0	40

^yNumber of trees observed varied for sample dates.

Beauty' acts as a homozygous recessive.

Trees displaying recombination between *Prx-c* and the reproductive budbreak phenotype were analyzed as a separate group to test for the possible influence of a second region of the genome. No significant correlations were observed, although the number of individuals (12) in this group was very limited. Examination of the 'Rome Beauty' x 'Robusta 5' family revealed that all trees were early (data not presented), indicating that 'Robusta 5' may be homozygous *Rbb/Rbb* and confirming that 'Rome Beauty' acts as a homozygous recessive.

Bloom time. Two dates were used for the primary analysis of the segregation of blooming time, 27 Apr. 1990 and 4 May (Table 3). The early and late categories from the 27 Apr. data were used initially in the analysis. Reproductive budbreak was weakly associated with *Aat-c* on 'White Angel' linkage group 1 (Table 1, Fig. 1). Field data for 4 May 1990 were analyzed separately. Trees in bloom were considered early and those in pink late. The analysis again showed very weak association with the same regions of the genome, *Aat-c* (5 recombinant out of 15). The total number of comparisons between the trait and corresponding marker was limited due to the number of matches available for both.

Root suckering. In the 'Rome Beauty' x 'White Angel' family, 38 plants possessed root suckers and 44 lacked them, suggesting monogenic inheritance ($\chi^2 = 0.027$; $P = 0.87$ for a 1:1 segregation ratio). The root sucker phenotype was found to be associated with linkage group 1 of 'White Angel' near the RAPD marker P124e (Table 1, Fig. 1). The high correlation suggests that in this cross a single gene, here designated *Rs*, determined the production of root suckers. In the 'Rome Beauty' x 'Robusta 5' family only seven trees out of 61 produced root suckers.

Discussion

We investigated the genetic basis of branching pattern, budbreak, blooming time and root sucker formation in an apple family produced by crossing two highly heterozygous cultivars. Root sucker formation could be scored as a clear presence/absence polymorphism, but each of the other three traits gave a range of phenotypes for which the genetic basis was not immediately obvious. Indeed, it soon became clear that initial vegetative budbreak did not correlate with reproductive budbreak, and the two had to be treated as independent characters in the analysis. The use of an extensive set of Mendelian markers covering ≈ 1000 CM of the linkage map for each parent permitted us to confidently identify at least three genes, each controlling a major portion of the variation in a specific trait.

In the 'Rome Beauty' x 'White Angel' family, the presence of root suckers appeared to be conditioned by a single gene. According to our genetic model, 'Rome Beauty' is homozygous recessive

at *Rs*, whereas 'White Angel' is heterozygous. No previous study of the genetics of this character has been published (Brown, 1992). Our analysis of root sucker segregation in the 'Rome Beauty' x 'Robusta 5' family did not permit confirmation of monogenic inheritance because of the highly skewed ratio observed in this population. As root suckers originate from adventitious buds on the roots (Priestly and Swingle, 1929), only trees on their own roots can be used for genetic analysis. All progeny were seedlings on their own roots, but all available 'Rome Beauty' and 'White Angel' trees were on distinct rootstock and could not be assessed for root suckering under our conditions. However, 'Robusta 5' can produce root suckers (P. Forsline, personal communication). Thus, the appearance in the progeny of trees lacking root suckers indicates that 'Robusta 5' is heterozygous, but the ratio suggests the involvement of more than one locus or some form of selection against the *Rs* region of the genome. The 'Rome Beauty' x 'Robusta 5' population had been selected at an early seedling stage for a number of characters, and linkage between *Rs* and genes being selected might account for the distorted ratio. The location of *Rs* on linkage group 1 also means that this locus is linked to *S*, the self-incompatibility locus (Manganaris and Alston, 1987). Thus, the distorted ratio also could be explained by an incompatibility related selection of genotypes. At present we cannot determine what caused the unexpected low number of sucker-producing genotypes in the 'Rome Beauty' x 'Robusta 5' family.

Tree branching habit is an important character in apple breeding programs. The 90-degree branch angle associated with the 'Rome Beauty' habit is preferred for branch strength and good light interception, but the spur-type habit is desired for its reduced size and good balance between reproductive and vegetative tissues. Trees possessing both characters were identified, but a strong expression of the spur-type growth on terminal bearing trees was low. It appears that spur-type growth modifies the terminal bearing nature of the *tb/tb* genotype, producing a tree with a moderately branched habit. The terminal bearing gene, *tb*, clearly acted as a recessive allele to one of the two alleles in 'White Angel' and to both alleles (which may have been identical) in 'Robusta 5'. Our failure to map the spur-type character maybe due to a multigenic basis to this trait as suggested by Blazek (1983).

An early study by Schmidt (1947) suggested polygenic inheritance for the onset and duration of flowering in apple. Our results are concordant with Schmidt's findings. The higher correlation between the molecular markers and the early flowering data (6 Apr.) suggests that *Rbb* may act at the initiation of reproductive budbreak, and its affect may be quickly diluted by other factors. Similarly, within the bloom time data, the association of 4 May 1990 flowering data (pink to bloom) to *Aat-c* was not as strong as that observed for the 27 Apr. 1990 data (tight cluster to full pink), suggesting that a series of genes may be acting even within these stages. As the relative blooming time for the trees was stable over 2 years, the differences appear to be determined more by the genotype than environment.

This study used one population to examine the correlation between segregating morphological traits and molecular markers. The associations identified may be unique to the parental varieties involved in the cross, and further investigations are necessary to determine if these associations can be generalized to other apple cultivars. Such analyses are currently underway, in our department and as part of several other apple breeding programs (King et al., 1991; S. Gardiner, personal communication). A number of other characters (e.g., fruit size, fruit shape) that appeared to be segregating in the 'Rome Beauty' x 'White Angel' family did not reveal associations with specific regions on the linkage map. A complete

list of characters investigated is given in Hagens (1992), and can be obtained from the authors upon request. Many of these genetically recalcitrant characters may have a polygenic basis and be beyond our analytical techniques. The lack of correlation with any of the segregating markers strongly supports this conclusion. Our results demonstrate the feasibility of mapping regions associated with interesting morphological and physiological traits despite the obvious difficulties of working with a woody perennial crop.

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