

## An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts

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### Summary

Recognizing the enormous potential of DNA markers in plant breeding, many agricultural research centers and plant breeding institutes have adopted the capacity for marker development and marker-assisted selection (MAS). However, due to rapid developments in marker technology, statistical methodology for identifying quantitative trait loci (QTLs) and the jargon used by molecular biologists, the utility of DNA markers in plant breeding may not be clearly understood by non-molecular biologists. This review provides an introduction to DNA markers and the concept of polymorphism, linkage analysis and map construction, the principles of QTL analysis and how markers may be applied in breeding programs using MAS. This review has been specifically written for readers who have only a basic knowledge of molecular biology and/or plant genetics. Its format is therefore ideal for conventional plant breeders, physiologists, pathologists, other plant scientists and students.

**Abbreviations:** AFLP: amplified fragment length polymorphism; BC: backcross; BSA: bulked-segregant analysis; CIM: composite interval mapping; cM: centiMorgan; DH: doubled haploid; EST: expressed sequence tag; SIM: simple interval mapping; LOD: logarithm of odds; LRS: likelihood ratio statistic; MAS: marker-assisted selection; NIL: near isogenic lines; PCR: polymerase chain reaction; QTL: quantitative trait loci; RAPD: random amplified polymorphic DNA; RI: recombinant inbred; RFLP: restriction fragment length polymorphism; SSR: simple sequence repeats (microsatellites); SCAR: sequence characterized amplified region; SNP: single nucleotide polymorphism; STS: sequence tagged site

### Introduction

Many agriculturally important traits such as yield, quality and some forms of disease resistance are controlled by many genes and are known as quantitative traits (also 'polygenic,' 'multifactorial' or 'complex' traits). The regions within genomes that contain genes associated with a particular quantitative trait are known as quantitative trait loci (QTLs). The identification of QTLs based only on conventional phenotypic evaluation is

not possible. A major breakthrough in the characterization of quantitative traits that created opportunities to select for QTLs was initiated by the development of DNA (or molecular) markers in the 1980s.

One of the main uses of DNA markers in agricultural research has been in the construction of linkage maps for diverse crop species. Linkage maps have been utilised for identifying chromosomal regions that contain genes controlling simple traits (controlled by a single gene) and quantitative traits using QTL

analysis (reviewed by Mohan et al., 1997). The process of constructing linkage maps and conducting QTL analysis—to identify genomic regions associated with traits—is known as QTL mapping (also ‘genetic,’ ‘gene’ or ‘genome’ mapping) (McCouch & Doerge, 1995; Mohan et al., 1997; Paterson, 1996a,b). DNA markers that are tightly linked to agronomically important genes (called gene ‘tagging’) may be used as molecular tools for marker-assisted selection (MAS) in plant breeding (Ribaut & Hoisington, 1998). MAS involves using the presence/absence of a marker as a substitute for or to assist in phenotypic selection, in a way which may make it more efficient, effective, reliable and cost-effective compared to the more conventional plant breeding methodology. The use of DNA markers in plant (and animal) breeding has opened a new realm in agriculture called ‘molecular breeding’ (Rafalski & Tingey, 1993).

DNA markers are widely accepted as potentially valuable tools for crop improvement in rice (Mackill et al., 1999; McCouch & Doerge, 1995), wheat (Eagles et al., 2001; Koebner & Summers, 2003; Van Sanford et al., 2001), maize (Stuber et al., 1999; Tuberosa et al., 2003), barley (Thomas, 2003; Williams, 2003), tuber crops (Barone, 2004; Fregene et al., 2001; Gebhardt & Valkonen, 2001), pulses (Kelly et al., 2003; Muehlbauer et al., 1994; Svetleva et al., 2003; Weeden et al., 1994), oilseeds (Snowdon & Friedt, 2004), horticultural crop species (Baird et al., 1996, 1997; Mehlenbacher, 1995) and pasture species (Jahufer et al., 2002). Some studies suggest that DNA markers will play a vital role in enhancing global food production by improving the efficiency of conventional plant breeding programs (Kasha, 1999; Ortiz, 1998). Although there has been some concern that the outcomes of DNA marker technology as proposed by initial studies may not be as effective as first thought, many plant breeding institutions have adopted the capacity for marker development and/or MAS (Eagles et al., 2001; Kelly & Miklas, 1998; Lee, 1995). An understanding of the basic concepts and methodology of DNA marker development and MAS, including some of the terminology used by molecular biologists, will enable plant breeders and researchers working in other relevant disciplines to work together towards a common goal – increasing the efficiency of global food production.

A number of excellent reviews have been written about the construction of linkage maps, QTL analysis and the application of markers in marker-assisted selection (for example: Haley & Andersson, 1997; Jones et al., 1997; Paterson et al., 1991a; Paterson, 1996a,b; Staub et al., 1996; Tanksley, 1993; Young,

1994). However, the authors of these reviews assumed that the reader had an advanced level of knowledge in molecular biology and plant genetics, with the possible exceptions of the reviews by Paterson (1996a,b) and Jones et al. (1997). Our review has been specifically written for readers with only a basic knowledge of molecular biology and/or plant genetics. It will be a useful reference for conventional plant breeders, physiologists, pathologists and other plant scientists, as well as students who are not necessarily engaged in applied molecular biology research but need an understanding of the exciting opportunities offered by this new technology. This review consists of five sections: genetic markers, construction of linkage maps, QTL analysis, towards marker-assisted selection and marker-assisted selection.

## Section I: Genetic markers

### *What are genetic markers?*

Genetic markers represent genetic differences between individual organisms or species. Generally, they do not represent the target genes themselves but act as ‘signs’ or ‘flags’. Genetic markers that are located in close proximity to genes (i.e. tightly linked) may be referred to as gene ‘tags’. Such markers themselves do not affect the phenotype of the trait of interest because they are located only near or ‘linked’ to genes controlling the trait. All genetic markers occupy specific genomic positions within chromosomes (like genes) called ‘loci’ (singular ‘locus’).

There are three major types of genetic markers: (1) morphological (also ‘classical’ or ‘visible’) markers which themselves are phenotypic traits or characters; (2) biochemical markers, which include allelic variants of enzymes called isozymes; and (3) DNA (or molecular) markers, which reveal sites of variation in DNA (Jones et al., 1997; Winter & Kahl, 1995). Morphological markers are usually visually characterized phenotypic characters such as flower colour, seed shape, growth habits or pigmentation. Isozyme markers are differences in enzymes that are detected by electrophoresis and specific staining. The major disadvantages of morphological and biochemical markers are that they may be limited in number and are influenced by environmental factors or the developmental stage of the plant (Winter & Kahl, 1995). However, despite these limitations, morphological and biochemical markers have been extremely useful to

plant breeders (Eagles et al., 2001; Weeden et al., 1994).

DNA markers are the most widely used type of marker predominantly due to their abundance. They arise from different classes of DNA mutations such as substitution mutations (point mutations), rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA (Paterson, 1996a). These markers are selectively neutral because they are usually located in non-coding regions of DNA. Unlike morphological and biochemical markers, DNA markers are practically unlimited in number and are not affected by environmental factors and/or the developmental stage of the plant (Winter & Kahl, 1995). Apart from the use of DNA markers in the construction of linkage maps, they have numerous applications in plant breeding such as assessing the level of genetic diversity within germplasm and cultivar identity (Baird et al., 1997; Henry, 1997; Jahufer et al., 2003; Weising et al., 1995; Winter & Kahl, 1995).

DNA markers may be broadly divided into three classes based on the method of their detection: (1) hybridization-based; (2) polymerase chain reaction (PCR)-based and (3) DNA sequence-based (Gupta et al., 1999; Jones et al., 1997; Joshi et al., 1999; Winter & Kahl, 1995). Essentially, DNA markers may reveal genetic differences that can be visualised by using a technique called gel electrophoresis and staining with chemicals (ethidium bromide or silver) or detection with radioactive or colourimetric probes. DNA markers are particularly useful if they reveal differences between individuals of the same or different species. These markers are called polymorphic markers, whereas markers that do not discriminate between genotypes are called monomorphic markers (Figure 1). Polymorphic markers may also be described as codominant or dominant. This description is based on whether markers can discriminate between homozygotes and heterozygotes (Figure 2). Codominant markers indicate differences in size whereas dominant markers are either present or absent. Strictly speaking, the different forms of a DNA marker (e.g. different sized bands on gels) are called marker 'alleles'. Codominant markers may have many different alleles whereas a dominant marker only has two alleles.

It is beyond the scope of this review to discuss the technical method of how DNA markers are generated. However the advantages and disadvantages of the most commonly used markers are presented in Table 1.

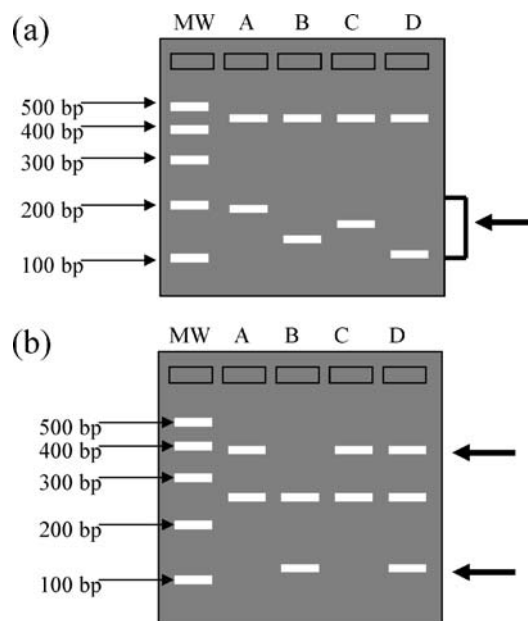


Figure 1. Diagram representing hypothetical DNA markers between genotypes A, B, C and D. Polymorphic markers are indicated by arrows. Markers that do not discriminate between genotypes are called monomorphic markers. (a) Example of SSR markers. The polymorphic marker reveals size differences for the marker alleles of the four genotypes, and represent a single genetic locus. (b) Examples of markers generated by the RAPD technique. Note that these markers are either present or absent. Often, the sizes of these markers in nucleotide base pairs (bp) are also provided; these sizes are estimated from a molecular weight (MW) DNA ladder. For both polymorphic markers, there are only two different marker alleles.

## Section II: Construction of linkage maps

### What are linkage maps?

A linkage map may be thought of as a 'road map' of the chromosomes derived from two different parents (Paterson, 1996a). Linkage maps indicate the position and relative genetic distances between markers along chromosomes, which is analogous to signs or landmarks along a highway. The most important use for linkage maps is to identify chromosomal locations containing genes and QTLs associated with traits of interest; such maps may then be referred to as 'QTL' (or 'genetic') maps. 'QTL mapping' is based on the principle that genes and markers segregate via chromosome recombination (called crossing-over) during meiosis (i.e. sexual reproduction), thus allowing their analysis in the progeny (Paterson, 1996a). Genes or markers that are close together or tightly-linked will be transmitted

Table 1. Advantages and disadvantages of most commonly-used DNA markers for QTL analysis

Molecular marker	Codominant (C) or Dominant (D)	Advantages	Disadvantages	References
Restriction fragment length polymorphism (RFLP)	C	<ul style="list-style-type: none"> <li>• Robust</li> <li>• Reliable</li> <li>• Transferable across populations</li> </ul>	<ul style="list-style-type: none"> <li>• Time-consuming, laborious and expensive</li> <li>• Large amounts of DNA required</li> <li>• Limited polymorphism (especially in related lines)</li> </ul>	Beckmann & Soller (1986), Kochert (1994), Tanksley et al. (1989)
Random amplified polymorphic DNA (RAPD)	D	<ul style="list-style-type: none"> <li>• Quick and simple</li> <li>• Inexpensive</li> <li>• Multiple loci from a single primer possible</li> <li>• Small amounts of DNA required</li> </ul>	<ul style="list-style-type: none"> <li>• Problems with reproducibility</li> <li>• Generally not transferable</li> </ul>	Penner (1996), Welsh & McClelland (1990), Williams et al. (1990)
Simple sequence repeats (SSRs)* or 'microsatellites'	C	<ul style="list-style-type: none"> <li>• Technically simple</li> <li>• Robust and reliable</li> <li>• Transferable between populations</li> </ul>	<ul style="list-style-type: none"> <li>• Large amounts of time and labour required for production of primers</li> <li>• Usually require polyacrylamide electrophoresis</li> </ul>	McCouch et al. (1997), Powell et al. (1996), Taramino & Tingey (1996)
Amplified fragment Length Polymorphism (AFLP)	D	<ul style="list-style-type: none"> <li>• Multiple loci</li> <li>• High levels of polymorphism generated</li> </ul>	<ul style="list-style-type: none"> <li>• Large amounts of DNA required</li> <li>• Complicated methodology</li> </ul>	Vos et al. (1995)

\*SSRs are also known as sequence tagged microsatellite site (STMS) markers (Daviera et al., 2000; Huettel et al., 1999; Mohapatra et al., 2003; Winter et al., 1999).

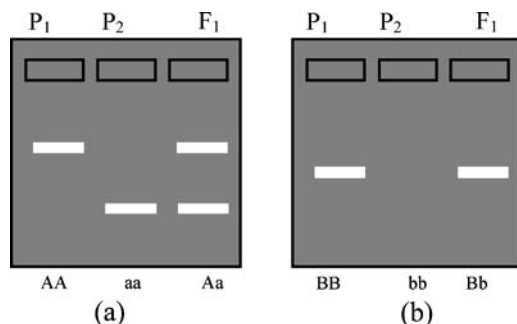


Figure 2. Comparison between (a) codominant and (b) dominant markers. Codominant markers can clearly discriminate between homozygotes and heterozygotes whereas dominant markers do not. Genotypes at two marker loci (A and B) are indicated below the gel diagrams.

together from parent to progeny more frequently than genes or markers that are located further apart (Figure 3). In a segregating population, there is a mixture of parental and recombinant genotypes. The frequency of recombinant genotypes can be used to calculate recombination fractions, which may be used to infer the genetic distance between markers. By analysing the segregation of markers, the relative order and distances between markers can be determined—the lower the frequency of recombination between two markers,

the closer they are situated on a chromosome (conversely, the higher the frequency of recombination between two markers, the further away they are situated on a chromosome). Markers that have a recombination frequency of 50% are described as 'unlinked' and assumed to be located far apart on the same chromosome or on different chromosomes. For a more detailed explanation of genetic linkage, the reader is encouraged to consult basic textbooks on genetics or quantitative genetics (for example, Hartl & Jones, 2001; Kearsey & Pooni, 1996). Mapping functions are used to convert recombination fractions into map units called centiMorgans (cM) (discussed later). Linkage maps are constructed from the analysis of many segregating markers. The three main steps of linkage map construction are: (1) production of a mapping population; (2) identification of polymorphism and (3) linkage analysis of markers.

### Mapping populations

The construction of a linkage map requires a segregating plant population (i.e. a population derived from sexual reproduction). The parents selected for the mapping population will differ for one or more traits of interest. Population sizes used in preliminary genetic mapping studies generally range from 50 to 250 individuals

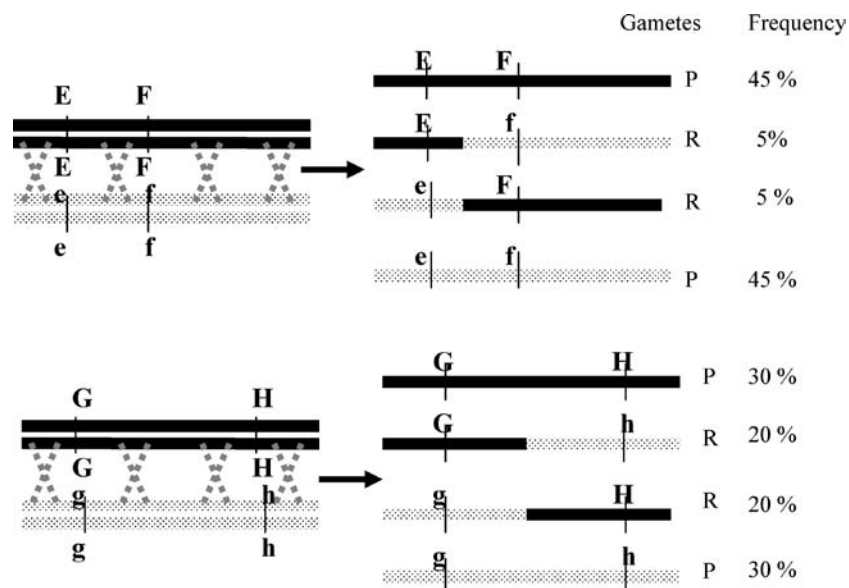


Figure 3. Diagram indicating cross-over or recombination events between homologous chromosomes that occur during meiosis. Gametes that are produced after meiosis are either parental (P) or recombinant (R). The smaller the distance between two markers, the smaller the chance of recombination occurring between the two markers. Therefore, recombination between markers G and H should occur more frequently than recombination between markers E and F. This can be observed in a segregating mapping population. By analysing the number of recombinants in a population, it could be determined that markers E and F are closer together compared to G and H.

(Mohan et al., 1997), however larger populations are required for high-resolution mapping. If the map will be used for QTL studies (which is usually the case), then an important point to note is that the mapping population must be phenotypically evaluated (i.e. trait data must be collected) before subsequent QTL mapping.

Generally in self-pollinating species, mapping populations originate from parents that are both highly homozygous (inbred). In cross pollinating species, the situation is more complicated since most of these species do not tolerate inbreeding. Many cross pollinating plant species are also polyploid (contain several sets of chromosome pairs). Mapping populations used for mapping cross pollinating species may be derived from a cross between a heterozygous parent and a haploid or homozygous parent (Wu et al., 1992). For example, in both the cross pollinating species white clover (*Trifolium repens* L.) and ryegrass (*Lolium perenne* L.), F<sub>1</sub> generation mapping populations were successfully developed by pair crossing heterozygous parental plants that were distinctly different for important traits associated with plant persistence and seed yield (Barrett et al., 2004; Forster et al., 2000).

Several different populations may be utilized for mapping within a given plant species, with each

population type possessing advantages and disadvantages (McCouch & Doerge, 1995; Paterson, 1996a) (Figure 4). F<sub>2</sub> populations, derived from F<sub>1</sub> hybrids, and backcross (BC) populations, derived by crossing the F<sub>1</sub> hybrid to one of the parents, are the simplest types of mapping populations developed for self pollinating species. Their main advantages are that they are easy to construct and require only a short time to produce. Inbreeding from individual F<sub>2</sub> plants allows the construction of recombinant inbred (RI) lines, which consist of a series of homozygous lines, each containing a unique combination of chromosomal segments from the original parents. The length of time needed for producing RI populations is the major disadvantage, because usually six to eight generations are required. Doubled haploid (DH) populations may be produced by regenerating plants by the induction of chromosome doubling from pollen grains, however, the production of DH populations is only possible in species that are amenable to tissue culture (e.g. cereal species such as rice, barley and wheat). The major advantages of RI and DH populations are that they produce homozygous or 'true-breeding' lines that can be multiplied and reproduced without genetic change occurring. This allows for the conduct of replicated

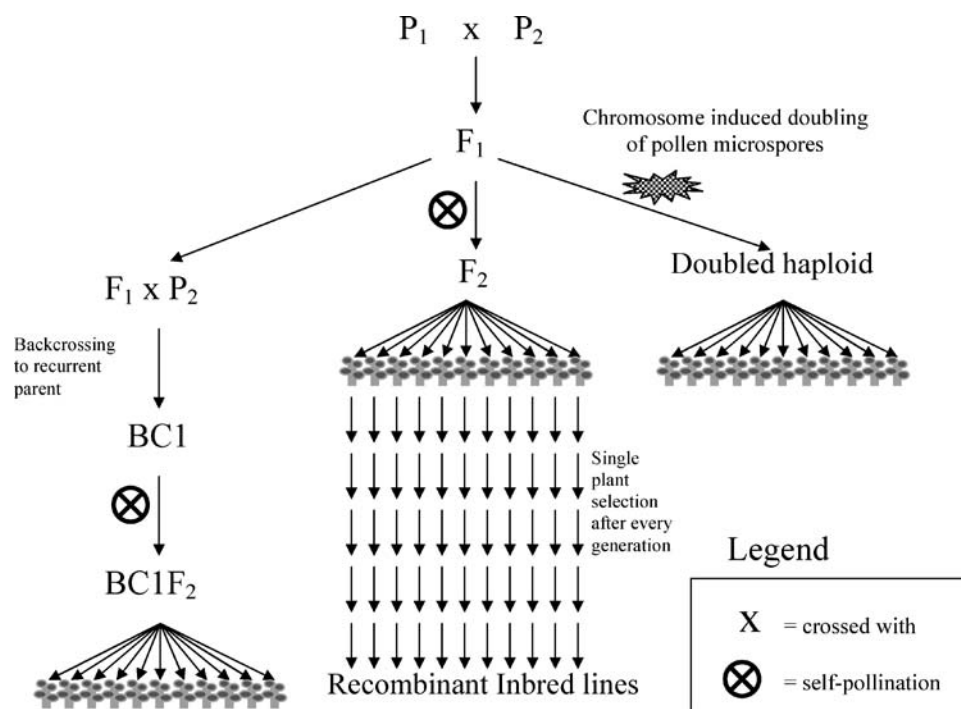


Figure 4. Diagram of main types of mapping populations for self-pollinating species.

trials across different locations and years. Thus both RI and DH populations represent 'eternal' resources for QTL mapping. Furthermore, seed from individual RI or DH lines may be transferred between different laboratories for further linkage analysis and the addition of markers to existing maps, ensuring that all collaborators examine identical material (Paterson, 1996a; Young, 1994).

#### Identification of polymorphism

The second step in the construction of a linkage map is to identify DNA markers that reveal differences between parents (i.e. polymorphic markers). It is critical that sufficient polymorphism exists between parents in order to construct a linkage map (Young, 1994). In general, cross pollinating species possess higher levels of DNA polymorphism compared to inbreeding species; mapping in inbreeding species generally requires the selection of parents that are distantly related. In many cases, parents that provide adequate polymorphism are selected on the basis of the level of genetic diversity between parents (Anderson et al., 1993; Collard et al., 2003; Joshi & Nguyen, 1993; Yu & Nguyen, 1994).

The choice of DNA markers used for mapping may depend on the availability of characterised markers or the appropriateness of particular markers for a particular species.

Once polymorphic markers have been identified, they must be screened across the entire mapping population, including the parents (and  $F_1$  hybrid, if possible). This is known as marker 'genotyping' of the population. Therefore, DNA must be extracted from each individual of the mapping population when DNA markers are used. Examples of DNA markers screened across different populations are shown in Figure 5. The expected segregation ratios for codominant and dominant markers are presented in Table 2. Significant deviations from expected ratios can be analysed using

Table 2. Expected segregation ratios for markers in different population types

Population type	Codominant markers	Dominant markers
$F_2$	1: 2:1 (AA:Aa:aa)	3:1 (B-:bb)
Backcross	1:1 (Cc:cc)	1:1 (Dd:dd)
Recombinant inbred or doubled haploid	1:1 (EE: ee)	1:1 (FF:ff)

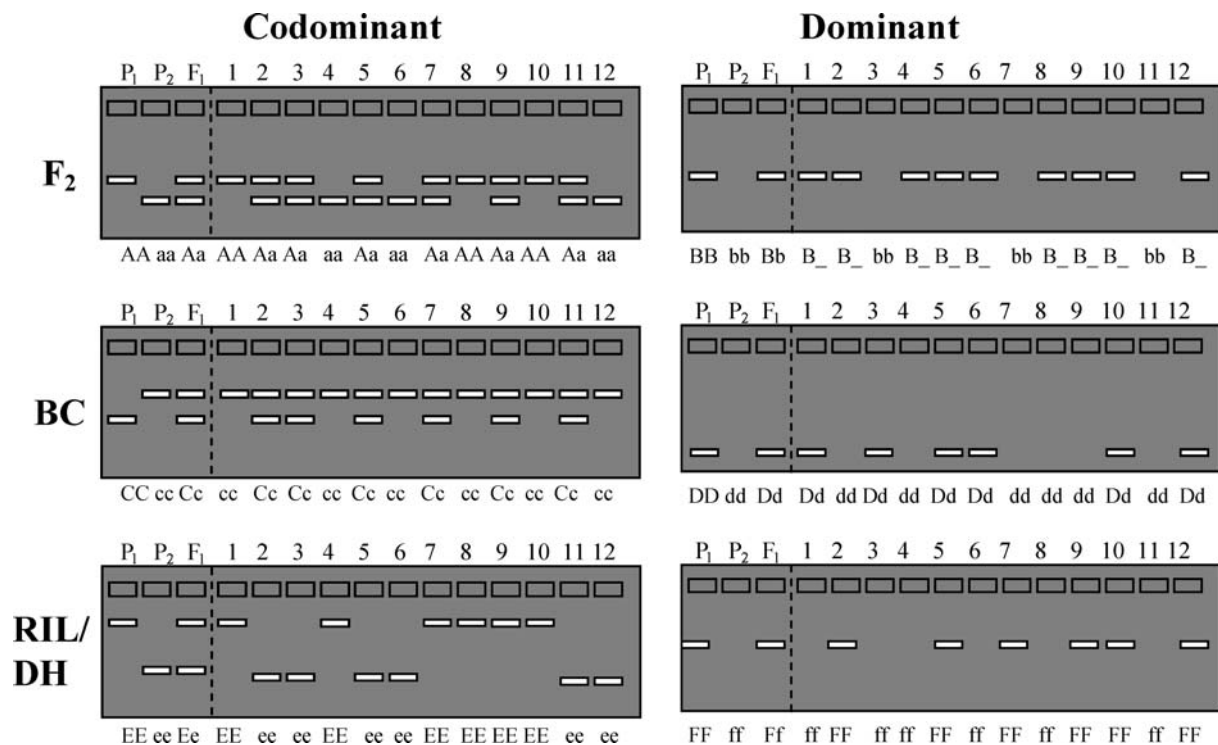


Figure 5. Hypothetical gel photos representing segregating codominant markers (left-hand side) and dominant markers (right-hand side) for typical mapping populations. Codominant markers indicate the complete genotype of a plant. Note that dominant markers cannot discriminate between heterozygotes and one homozygote genotype in F<sub>2</sub> populations. The segregation ratios of markers can be easily understood by using Punnett squares to derive population genotypes.

chi-square tests. Generally, markers will segregate in a Mendelian fashion although distorted segregation ratios may be encountered (Sayed et al., 2002; Xu et al., 1997).

In some polyploid species such as sugarcane, identifying polymorphic markers is more complicated (Ripol et al., 1999). The mapping of diploid relatives of polyploid species can be of great benefit in developing maps for polyploid species. However, diploid relatives do not exist for all polyploid species (Ripol et al., 1999; Wu et al., 1992). A general method for the mapping of polyploid species is based on the use of single-dose restriction fragments (Wu et al., 1992).

#### Linkage analysis of markers

The final step of the construction of a linkage map involves coding data for each DNA marker on each individual of a population and conducting linkage analysis using computer programs (Figure 6). Missing marker data can also be accepted by mapping programs.

Although linkage analysis can be performed manually for a few markers, it is not feasible to manually analyze and determine linkages between large numbers of markers that are used to construct maps; computer programs are required for this purpose. Linkage between markers is usually calculated using odds ratios (i.e. the ratio of linkage versus no linkage). This ratio is more conveniently expressed as the logarithm of the ratio, and is called a logarithm of odds (LOD) value or LOD score (Risch, 1992). LOD values of >3 are typically used to construct linkage maps. A LOD value of 3 between two markers indicates that linkage is 1000 times more likely (i.e. 1000:1) than no linkage (null hypothesis). LOD values may be lowered in order to detect a greater level of linkage or to place additional markers within maps constructed at higher LOD values. Commonly used software programs include Mapmaker/EXP (Lander et al., 1987; Lincoln et al., 1993a) and MapManager QTX (Manly et al., 2001), which are freely available from the internet. JoinMap is another commonly-used program for constructing linkage maps (Stam, 1993).

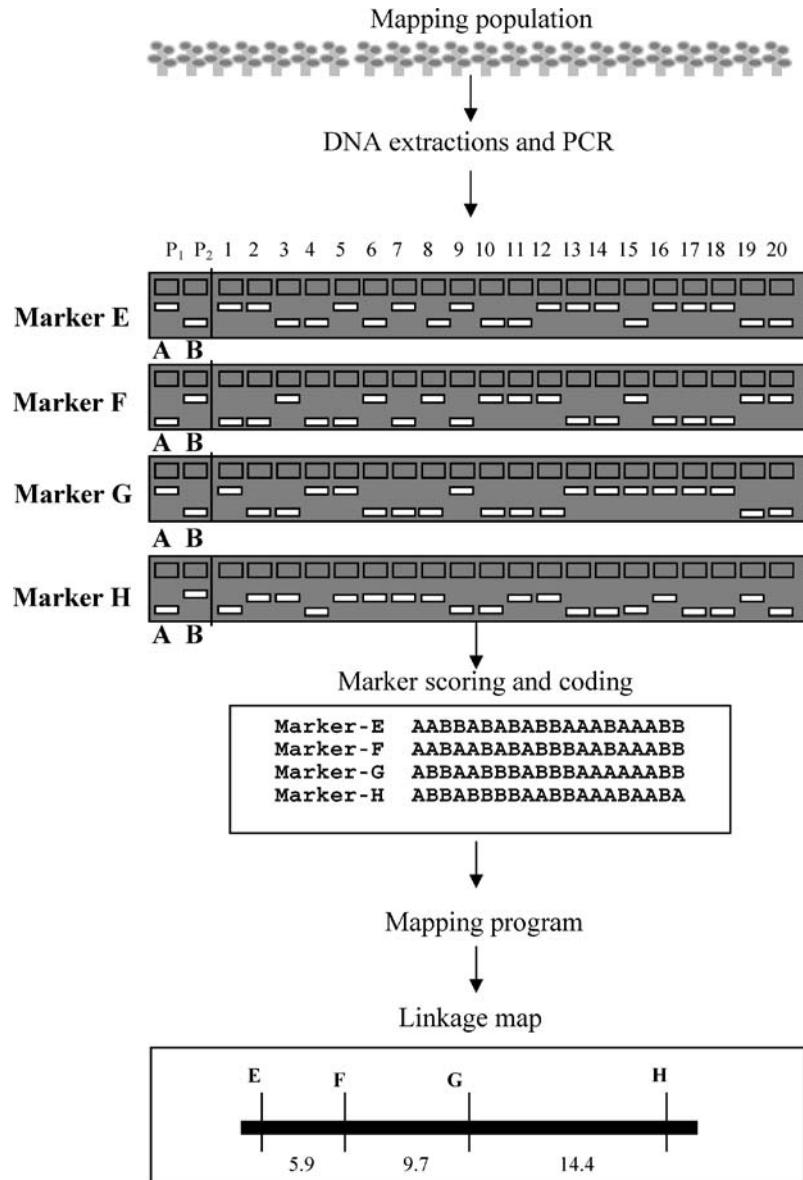


Figure 6. Construction of a linkage map based on a small recombinant inbred population (20 individuals). The first parent (P<sub>1</sub>) is scored as an 'A' whereas the second parent (P<sub>2</sub>) is scored as a 'B'. Coding of marker data varies depending on the type of population used. This linkage map was constructed using Map Manager QTX (Manly et al., 2001) using the Haldane mapping function.

A typical output of a linkage map is shown in Figure 7. Linked markers are grouped together into 'linkage groups,' which represent chromosomal segments or entire chromosomes. Referring to the road map analogy, linkage groups represent roads and markers represent signs or landmarks. A difficulty associated with obtaining an equal number of linkage groups and chromosomes is that the polymorphic markers detected are not necessarily evenly distributed

over the chromosome, but clustered in some regions and absent in others (Paterson, 1996a). In addition to the non-random distribution of markers, the frequency of recombination is not equal along chromosomes (Hartl & Jones, 2001; Young, 1994).

The accuracy of measuring the genetic distance and determining marker order is directly related to the number of individuals studied in the mapping population. Ideally, mapping populations should consist of

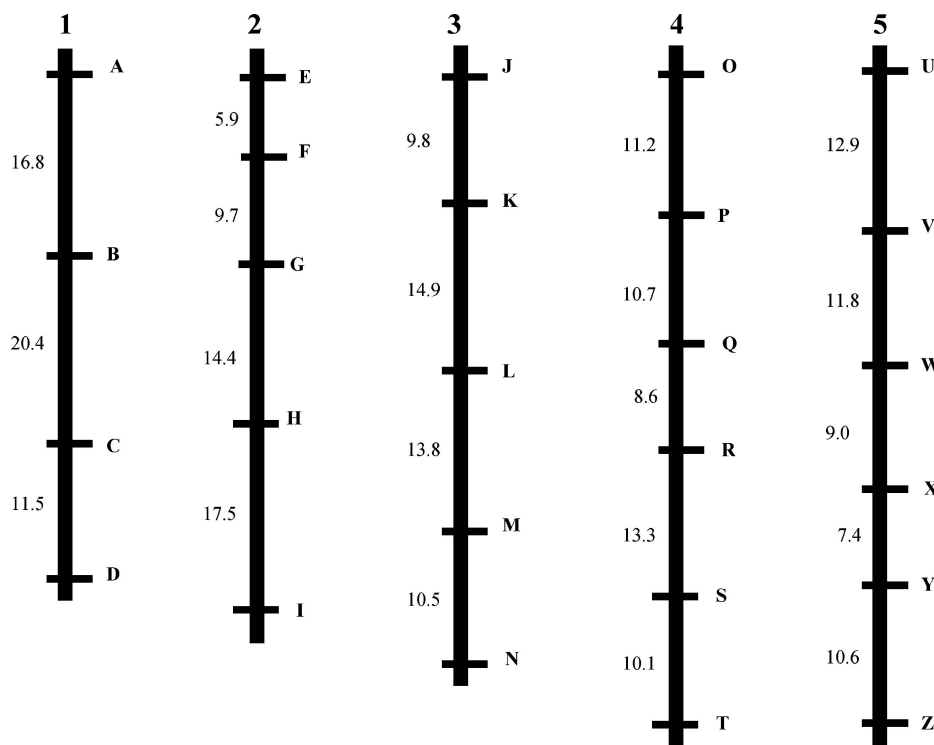


Figure 7. Hypothetical 'framework' linkage map of five chromosomes (represented by linkage groups) and 26 markers. Ideally, a framework map should consist of evenly spaced markers for subsequent QTL analysis. If possible, the framework map should also consist of anchor markers that are present in several maps, so that they can be used to compare regions between maps.

a minimum of 50 individuals for constructing linkage maps (Young, 1994).

#### *Genetic distance and mapping functions*

The importance of the distance between genes and markers has been discussed earlier. The greater the distance between markers, the greater the chance of recombination occurring during meiosis. Distance along a linkage map is measured in terms of the frequency of recombination between genetic markers (Paterson, 1996a). Mapping functions are required to convert recombination fractions into centiMorgans (cM) because recombination frequency and the frequency of crossing-over are not linearly related (Hartl & Jones, 2001; Kearsey & Pooni, 1996). When map distances are small (<10 cM), the map distance equals the recombination frequency. However, this relationship does not apply for map distances that are greater than 10 cM (Hartl & Jones, 2001). Two commonly used mapping functions are the Kosambi mapping function, which assumes that recombination events influence the occurrence of adjacent recombination events, and the

Haldane mapping function, which assumes no interference between crossover events (Hartl & Jones, 2001; Kearsey & Pooni, 1996).

It should be noted that distance on a linkage map is not directly related to the physical distance of DNA between genetic markers, but depends on the genome size of the plant species (Paterson, 1996a). Furthermore, the relationship between genetic and physical distance varies along a chromosome (Kunzel et al., 2000; Tanksley et al., 1992; Young, 1994). For example, there are recombination 'hot spots' and 'cold spots,' which are chromosomal regions in which recombination occurs more frequently or less frequently, respectively (Faris et al., 2000; Ma et al., 2001; Yao et al., 2002).

### **Section III: QTL analysis**

#### *Principle of QTL analysis*

Identifying a gene or QTL within a plant genome is like finding the proverbial needle in a haystack. However, QTL analysis can be used to divide the haystack

in manageable piles and systematically search them. In simple terms, QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured (Tanksley, 1993; Young, 1996). A significant difference between phenotypic means of the groups (either 2 or 3), depending on the marker system and type of population, indicates that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait (Figure 8).

A logical question that may be asked at this point is 'why does a significant  $P$  value obtained for differences between mean trait values indicate linkage between marker and QTL?' The answer is due to recombination. The closer a marker is from a QTL, the lower the chance of recombination occurring between marker and QTL. Therefore, the QTL and marker will be usually be inherited together in the progeny, and the mean of the group with the tightly-linked marker will be significantly dif-

ferent ( $P < 0.05$ ) to the mean of the group without the marker (Figure 9). When a marker is loosely-linked or unlinked to a QTL, there is independent segregation of the marker and QTL. In this situation, there will be no significant difference between means of the genotype groups based on the presence or absence of the loosely-linked marker (Figure 9). Unlinked markers located far apart or on different chromosomes to the QTL are randomly inherited with the QTL; therefore, no significant differences between means of the genotype groups will be detected.

#### Methods to detect QTLs

Three widely-used methods for detecting QTLs are single-marker analysis, simple interval mapping and composite interval mapping (Liu, 1998; Tanksley, 1993). Single-marker analysis (also 'single-point analysis') is the simplest method for detecting QTLs associated with single markers. The statistical methods used for single-marker analysis include  $t$ -tests, analysis of variance (ANOVA) and linear regression. Linear

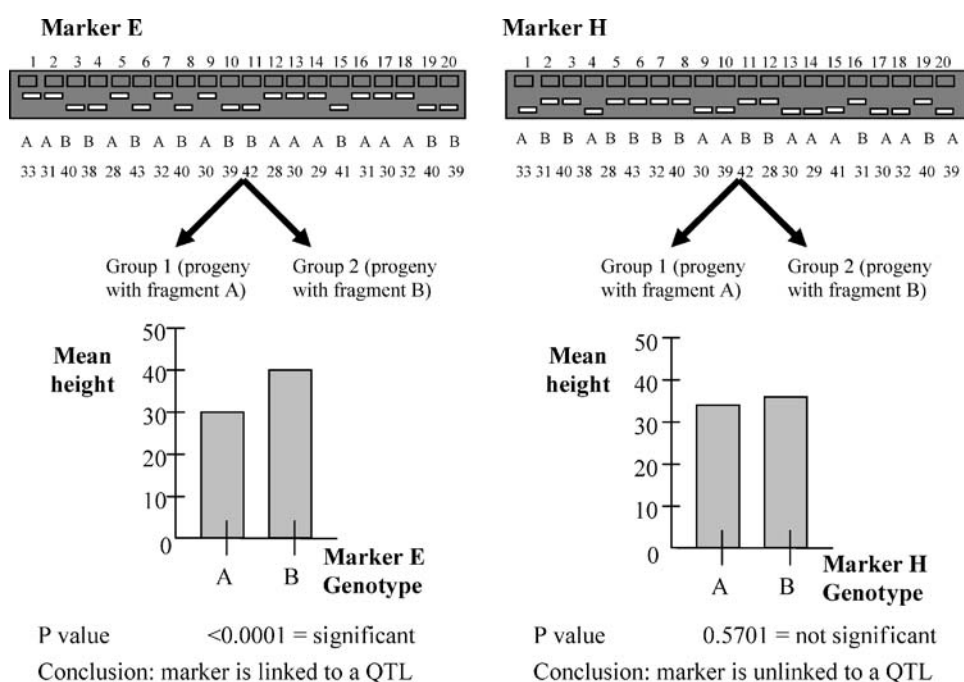


Figure 8. Principle of QTL mapping (adapted from Young, 1996). Markers that are linked to a gene or QTL controlling a particular trait (e.g. plant height) will indicate significant differences when the mapping population is partitioned according to the genotype of the marker. Based on the results in this diagram, Marker E is linked to a QTL because there is a significant difference between means. Marker H is unlinked to a QTL because there is no significant difference between means. The closer the marker is to the QTL of interest, the lower the chance for recombination between marker and QTL.

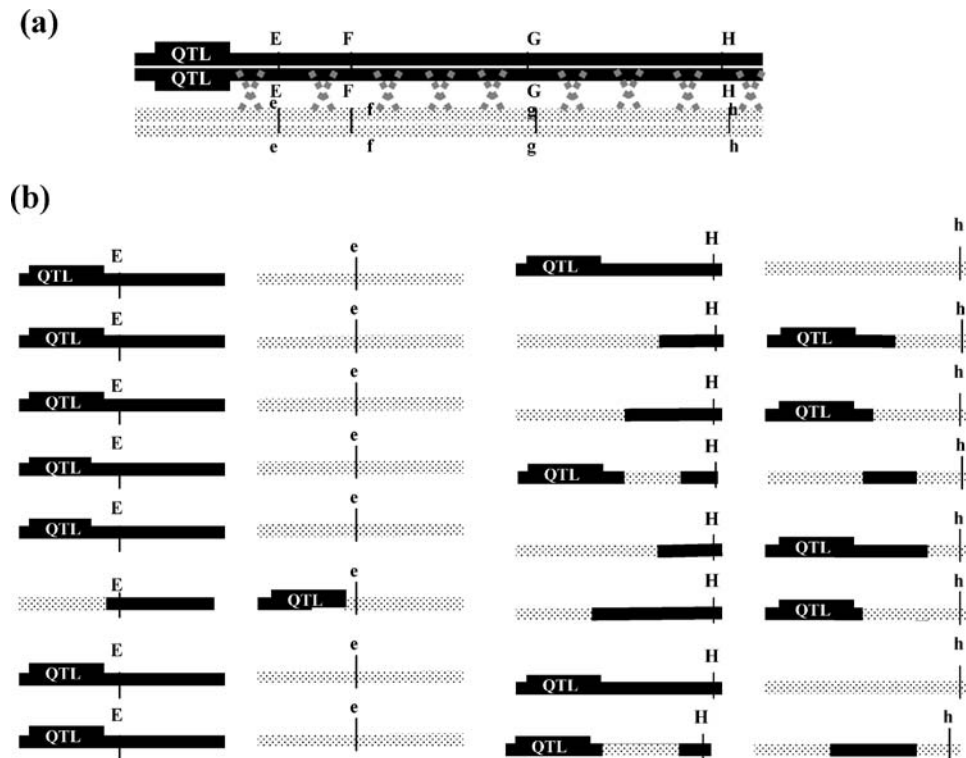


Figure 9. Diagram indicating tight and loose linkage between marker and QTL. (a) Recombination events (indicated by crosses) occurs between QTL and marker loci. (b) Gametes in population. Markers that are tightly-linked to a QTL (Marker E) are usually inherited with the QTL in the progeny. Markers that are only loosely-linked to a QTL (Marker H) are randomly inherited together.

regression is most commonly used because the coefficient of determination ( $R^2$ ) from the marker explains the phenotypic variation arising from the QTL linked to the marker. This method does not require a complete linkage map and can be performed with basic statistical software programs. However, the major disadvantage with this method is that the further a QTL is from a marker, the less likely it will be detected. This is because recombination may occur between the marker and the QTL. This causes the magnitude of the effect of a QTL to be underestimated (Tanksley, 1993). The use of a large number of segregating DNA markers covering the entire genome (usually at intervals less than 15 cM) may minimize both problems (Tanksley, 1993).

The results from single-marker analysis are usually presented in a table, which indicates the chromosome (if known) or linkage group containing the markers, probability values, and the percentage of phenotypic variation explained by the QTL ( $R^2$ ) (Table 3). Sometimes, the allele size of the marker is also reported. QGene and MapManager QTX are commonly used

computer programs to perform single-marker analysis (Manly et al., 2001; Nelson, 1997).

The simple interval mapping (SIM) method makes use of linkage maps and analyses intervals between adjacent pairs of linked markers along chromosomes simultaneously, instead of analyzing single markers (Lander & Botstein, 1989). The use of linked markers for analysis compensates for recombination between the markers and the QTL, and is considered statistically more powerful compared to single-point analysis (Lander & Botstein, 1989; Liu, 1998). Many

Table 3. Single-marker analysis of markers associated with QTLs using QGene (Nelson, 1997)

Marker	Chromosome or linkage group	$P$ value	$R^2$
E	2	<0.0001	91
F	2	0.0001	58
G	2	0.0230	26
H	2	0.5701	2

researchers have used MapMaker/QTL (Lincoln et al., 1993b) and QGene (Nelson, 1997), to conduct SIM.

More recently, composite interval mapping (CIM) has become popular for mapping QTLs. This method combines interval mapping with linear regression and includes additional genetic markers in the statistical model in addition to an adjacent pair of linked markers for interval mapping (Jansen, 1993; Jansen & Stam, 1994; Zeng, 1993, 1994). The main advantage of CIM is that it is more precise and effective at mapping QTLs compared to single-point analysis and interval mapping, especially when linked QTLs are involved. Many researchers have used QTL Cartographer (Basten et al., 1994, 2001), MapManager QTX (Manly et al., 2001) and PLABQTL (Utz & Melchinger, 1996) to perform CIM.

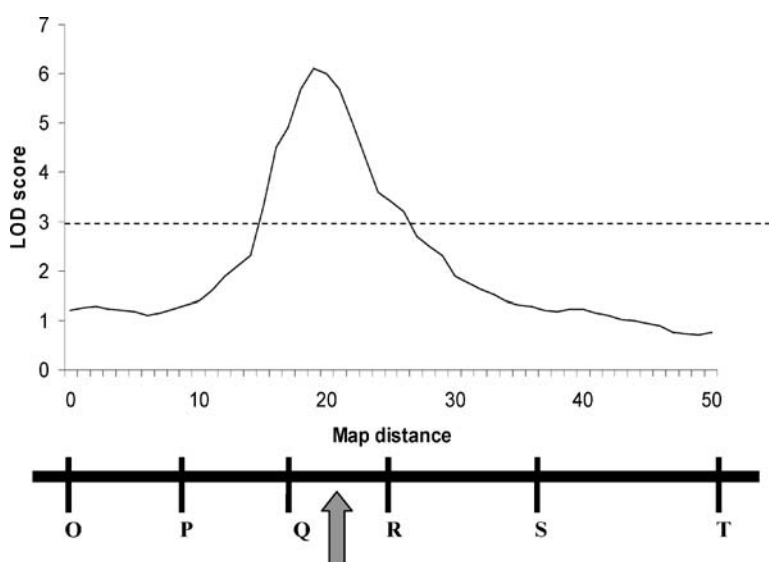
#### *Understanding interval mapping results*

Interval mapping methods produce a profile of the likely sites for a QTL between adjacent linked markers. In other words, QTLs are located with respect to a linkage map. The results of the test statistic for SIM and CIM are typically presented using a logarithmic of odds (LOD) score or likelihood ratio statistic (LRS). There is a direct one-to-one transformation between LOD scores and LRS scores (the conversion can

be calculated by:  $LRS = 4.6 \times LOD$ ) (Liu, 1998). These LOD or LRS profiles are used to identify the most likely position for a QTL in relation to the linkage map, which is the position where the highest LOD value is obtained. A typical output from interval mapping is a graph with markers comprising linkage groups on the *x* axis and the test statistic on the *y* axis (Figure 10).

The peak or maximum must also exceed a specified significance level in order for the QTL to be declared as 'real' (i.e. statistically significant). The determination of significance thresholds is most commonly performed using permutation tests (Churchill & Doerge, 1994). Briefly, the phenotypic values of the population are 'shuffled' whilst the marker genotypic values are held constant (i.e. all marker-trait associations are broken) and QTL analysis is performed to assess the level of false positive marker-trait associations (Churchill & Doerge, 1994; Hackett, 2002; Haley & Andersson, 1997). This process is then repeated (e.g. 500 or 1000 times) and significance levels can then be determined based on the level of false positive marker-trait associations.

Before permutation tests were widely accepted as an appropriate method to determine significance thresholds, a LOD score of between 2.0 to 3.0 (most commonly 3.0) was usually chosen as the significance threshold.



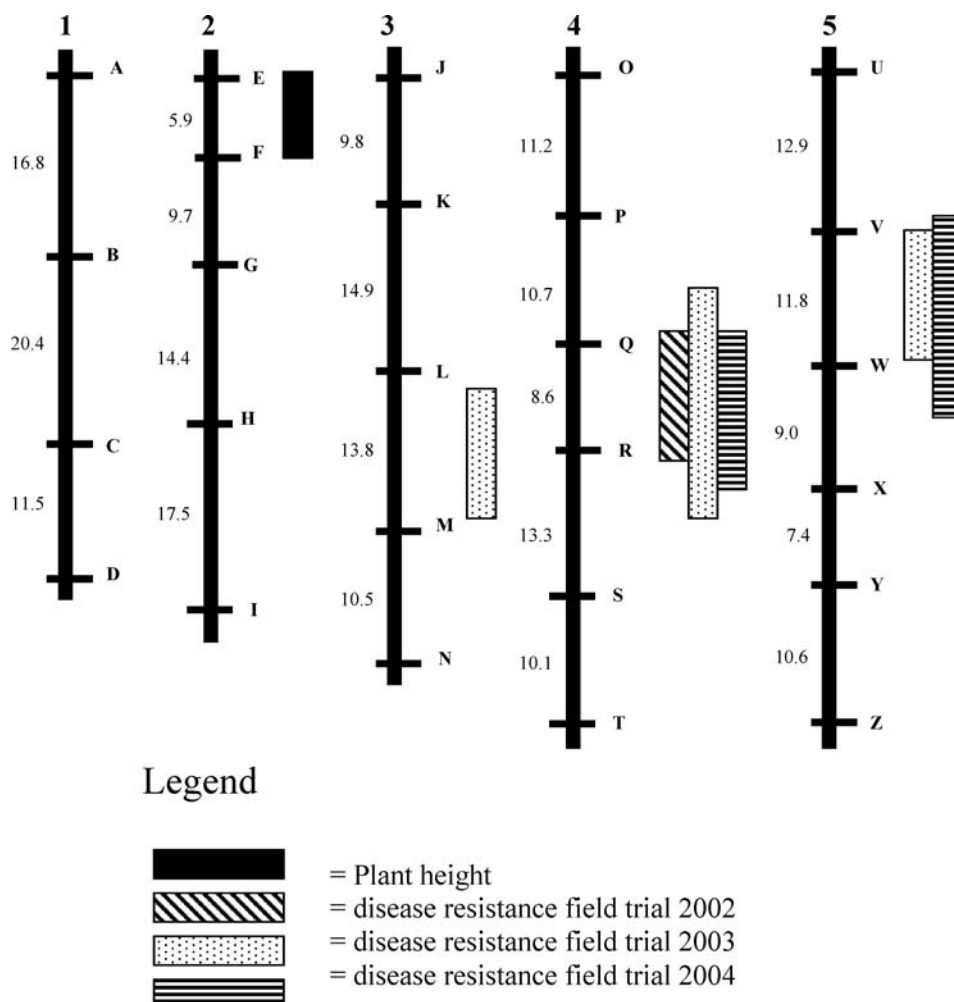
*Figure 10.* Hypothetical output showing a LOD profile for chromosome 4. The dotted line represents the significance threshold determined by permutation tests. The output indicates that the most likely position for the QTL is near marker Q (indicated by an arrow). The best flanking markers for this QTL would be Q and R.

*Reporting and describing QTLs detected from interval mapping*

The most common way of reporting QTLs is by indicating the most closely linked markers in a table and/or as bars (or oval shapes or arrows) on linkage maps (for example: Beattie et al., 2003; George et al., 2003; Hittalmani et al., 2002; Jampatong et al., 2002; McCouch & Doerge, 1995; Pilet-Nayel et al., 2002). The chromosomal regions represented by rectangles are usually the region that exceeds the significance threshold (Figure 11). Usually, a pair of markers – the most tightly-linked markers on each side of a QTL – is also reported in a table; these markers are known as ‘flanking’ markers. The reason for reporting

flanking markers is that selection based on two markers should be more reliable than selection based on a single marker. The reason for the increased reliability is that there is a much lower chance of recombination between two markers and QTL compared to the chance of recombination between a single marker and QTL.

It should also be noted that QTLs can only be detected for traits that segregate between the parents used to construct the mapping population. Therefore, in order to maximize the data obtained from a QTL mapping study, several criteria may be used for phenotypic evaluation of a single trait (Flandez-Galvez et al., 2003a; Paterson et al., 1988; Pilet-Nayel et al., 2002). QTLs that are detected in common regions (based on different



*Figure 11.* QTL mapping of height and disease resistance traits. Hypothetical QTLs were detected on chromosomes 2, 3, 4 and 5. A major QTL for height was detected on chromosome 2. Two major QTLs for disease resistance were detected on chromosomes 4 and 5 whereas a minor QTL was detected on chromosome 3 (adapted from Hartl & Jones, 2001).

criteria for a single trait) are likely to be important QTLs for controlling the trait.

Mapping populations may also be constructed based on parents that segregate for multiple traits. This is advantageous because QTLs controlling the different traits can be located on a single map (Beattie et al., 2003; Khairallah et al., 1998; Marquez-Cedillo et al., 2001; Serquen et al., 1997; Tar'an et al., 2002). However, for many parental genotypes used to construct mapping populations, this is not always possible, because the parents may only segregate for one trait of interest. Furthermore, the same set of lines of the mapping population used for phenotypic evaluation must be available for marker genotyping, and subsequent QTL analysis, which may be difficult with completely or semi-destructive bioassays (e.g. screening for resistance to necrotrophic fungal pathogens).

In general terms, an individual QTL may also be described as 'major' or 'minor'. This definition is based on the proportion of the phenotypic variation explained by a QTL (based on the  $R^2$  value): major QTLs will account for a relatively large amount (e.g. >10%) and minor QTLs will usually account for <10%. Sometimes, major QTLs may refer to QTLs that are stable across environments whereas minor QTLs may refer to QTLs that may be environmentally sensitive, especially for QTLs that are associated with disease resistance (Li et al., 2001; Lindhout, 2002; Pilet-Nayel et al., 2002).

In more formal terms, QTLs may be classified as: (1) suggestive; (2) significant; and (3) highly-significant (Lander & Kruglyak, 1995). Lander & Kruglyak (1995) proposed this classification in order to "avoid a flood of false positive claims" and also ensure that "true hints of linkage" were not missed. Significant and highly-significant QTLs were given significance levels of 5 and 0.1%, respectively, whereas a suggestive QTL is one that would be expected to occur once at random in a QTL mapping study (in other words, there is a warning regarding the reliability of suggestive QTLs). The mapping program MapManager QTX reports QTL mapping results with this classification (Manly et al., 2001).

#### *Confidence intervals for QTLs*

Although the most likely position of a QTL is the map position at which the highest LOD or LRS score is detected, QTLs actually occur within confidence intervals. There are several ways in which confidence intervals can be calculated. The simplest is the 'one-LOD support interval,' which is determined by finding the

region on both sides of a QTL peak that corresponds to a decrease of 1 LOD score (Hackett, 2002; Lander & Botstein, 1989). 'Bootstrapping' – a statistical method for resampling—is another method to determine the confidence interval of QTLs (Liu, 1998; Visscher et al., 1996), and can be easily applied within some mapping software programs such as MapManager QTX (Manly et al., 2001).

#### *Number of markers and marker spacing*

There is no absolute value for the number of DNA markers required for a genetic map, since the number of markers varies with the number and length of chromosomes in the organism. For detection of QTLs, a relatively sparse 'framework' (or 'skeletal' or 'scaffold') map consisting of evenly spaced markers is adequate, and preliminary genetic mapping studies generally contain between 100 and 200 markers (Mohan et al., 1997). However, this depends on the genome size of the species; more markers are required for mapping in species with large genomes. Darvasi et al. (1993) reported that the power of detecting a QTL was virtually the same for a marker spacing of 10 cM as for an infinite number of markers, and only slightly decreased for marker spacing of 20 or even 50 cM.

#### *Making comparisons between maps*

All linkage maps are unique and are a product of the mapping population (derived from two specific parents) and the types of markers used. Even if the same set of markers is used to construct linkage maps, there is no guarantee that all of the markers will be polymorphic between different populations. Therefore, in order to correlate information from one map to another, common markers are required. Common markers that are highly polymorphic in mapping populations are called 'anchor' (also 'core' markers). Anchor markers are typically SSRs or RFLPs (Ablett et al., 2003; Flandez-Galvez et al., 2003b; Gardiner et al., 1993). Specific groups of anchor markers, that are located in close proximity to each other in specific genomic regions, may be referred to as 'bins'. Bins are used to integrate maps, and are defined as '10–20 cM regions along chromosomes; the boundaries of each are defined by a set of core RFLP markers' (Polacco et al., 2002). If common anchor markers have been incorporated into different maps, they can be aligned together to produce 'consensus' maps (Ablett et al., 2003; Karakousis et al., 2003). Consensus maps are produced by combining

or merging different maps, constructed from different genotypes, together. Such consensus maps can be extremely useful for efficiently constructing new maps (with evenly spaced markers) or targeted (or localized) mapping. For example, a consensus map can indicate which markers are located in a specific region containing a QTL, and thus be used to identify more tightly linked markers.

The study of similarities and differences of markers and genes within and between species, genera or higher taxonomic divisions is referred to as comparative mapping (Paterson et al., 1991a). It involves analysing the extent of the conservation between maps of the order in which markers occur (i.e. collinear markers); conserved marker order is referred to as 'synteny'. Comparative mapping may assist in the construction of new linkage maps (or localized maps of specific genomic regions) and in predicting the locations of QTLs in different mapping populations (Young, 1994). Previous linkage maps may provide an indication of which markers are polymorphic, as well as provide an indication of linkage groups and the order of markers within linkage groups. Furthermore, comparative mapping may reveal evolutionary relationships between taxa.

#### *Factors influencing the detection of QTLs*

There are many factors that influence the detection of QTLs segregating in a population (Asins, 2002; Tanksley, 1993). The main ones are genetic properties of QTLs that control traits, environmental effects, population size and experimental error.

Genetic properties of QTLs controlling traits include the magnitude of the effect of individual QTLs. Only QTLs with sufficiently large phenotypic effects will be detected; QTLs with small effects may fall below the significance threshold of detection. Another genetic property is the distance between linked QTLs. QTLs that are closely-linked (approximately 20 cM or less) will usually be detected as a single QTL in typical population sizes (<500) (Tanksley, 1993).

Environmental effects may have a profound influence on the expression of quantitative traits. Experiments that are replicated across sites and over time (e.g. different seasons and years) may enable the researcher to investigate environmental influences on QTLs affecting trait(s) of interest (George et al., 2003; Hittalmani et al., 2002; Jampatong et al., 2002; Lindhout, 2002; Paterson et al., 1991b; Price & Courtois, 1999). As discussed previously, RI or DH populations are ideal for these purposes.

The most important experimental design factor is the size of the population used in the mapping study. The larger the population, the more accurate the mapping study and the more likely it is to allow detection of QTLs with smaller effects (Haley & Andersson, 1997; Tanksley, 1993). An increase in population size provides gains in statistical power, estimates of gene effects and confidence intervals of the locations of QTLs (Beavis, 1998; Darvasi et al., 1993).

The main sources of experimental error are mistakes in marker genotyping and errors in phenotypic evaluation. Genotyping errors and missing data can affect the order and distance between markers within linkage maps (Hackett, 2002). The accuracy of phenotypic evaluation is of the utmost importance for the accuracy of QTL mapping. A reliable QTL map can only be produced from reliable phenotypic data. Replicated phenotypic measurements or the use of clones (via cuttings) can be used to improve the accuracy of QTL mapping by reducing background 'noise' (Danesh et al., 1994; Haley & Andersson, 1997). Some thorough studies include those where phenotypic evaluations have been conducted in both field and glasshouse trials, for ascochyta blight resistance in chickpea (Flandez-Galvez et al., 2003a), bacterial brown spot in common bean (Jung et al., 2003), and downy mildew resistance in pearl millet (Jones et al., 2002).

#### *Confirmation of QTL mapping*

Ideally, due to the factors described above, QTL mapping studies should be independently confirmed or verified (Lander & Kruglyak, 1995). Such confirmation studies (referred to as 'replication studies' by Lander & Kruglyak, 1995) may involve independent populations constructed from the same parental genotypes or closely-related genotypes used in the primary QTL mapping study. Sometimes, larger population sizes may be used. Furthermore, some recent studies have proposed that QTL positions and effects should be evaluated in independent populations, because QTL mapping based on typical population sizes results in a low power of QTL detection and a large bias of QTL effects (Melchinger et al., 1998; Utz et al., 2000). Unfortunately, due to constraints such as lack of research funding and time, and possibly a lack of understanding of the need to confirm results, QTL mapping studies are rarely confirmed. Some notable exceptions are the confirmation of QTLs associated with root-knot nematode resistance (Li et al., 2001) and bud blight resistance in soybean (Fasoula et al., 2003).

Another approach used to confirm QTLs has been to use a specific type of population called near isogenic lines (NILs). NILs are created by crossing a donor parent (e.g. wild parent possessing a specific trait of interest) to a recurrent parent (e.g. an elite cultivar). The F<sub>1</sub> hybrid is then backcrossed to the recurrent parent to produce a first backcross generation (BC1). The BC1 is then repeatedly backcrossed to the recurrent parent for a number of generations (e.g. 7). The final BC7 will contain practically all of the recurrent parent genome except for the small chromosomal region containing a gene or QTL of interest. Homozygous F<sub>2</sub> lines can be obtained by selfing the BC7 plant. It should be noted that in order to produce a NIL containing a target gene, the gene has to be selected for during each round of backcrossing. By genotyping NILs with important markers, and comparing mean trait values of particular NIL lines with the recurrent parent, the effects of QTLs can be confirmed. Examples of studies utilizing NILs to confirm QTLs include agronomic traits in tomato (Bernacchi et al., 1998), leaf rust resistance in barley (Van Berloo et al., 2001), nematode resistance in soybean (Glover et al., 2004) and phosphorus uptake in rice (Wissuwa & Ae, 2001).

#### *Short cuts for gene tagging*

The construction of linkage maps and QTL analysis takes a considerable amount of time and effort, and may be very expensive. Therefore, alternative methods that can save time and money would be extremely useful, especially if resources are limited. Two 'short-cut' methods used to identify markers that tag QTLs are bulked segregant analysis (BSA) and selective genotyping. Both methods require mapping populations.

BSA is a method used to detect markers located in specific chromosomal regions (Michelmore et al., 1991). Briefly, two pools or 'bulks' of DNA samples are combined from 10–20 individual plants from a segregating population; these two bulks should differ for a trait of interest (e.g. resistant vs. susceptible to a particular disease). By making DNA bulks, all loci are randomised, except for the region containing the gene of interest. Markers are screened across the two bulks. Polymorphic markers may represent markers that are linked to a gene or QTL of interest (Figure 12). The entire population is then genotyped with these polymorphic markers and a localized linkage map may be generated. This enables QTL analysis to be performed and the position of a QTL to be determined (Ford et al., 1999).

BSA is generally used to tag genes controlling simple traits, but the method may also be used to identify markers linked to major QTLs (Wang & Paterson, 1994). 'High-throughput' or 'high-volume' marker techniques such as RAPD or AFLP, that can generate multiple markers from a single DNA preparation, are generally preferred for BSA.

Selective genotyping (also known as 'distribution extreme analysis' or 'trait-based marker analysis') involves selecting individuals from a population that represent the phenotypic extremes or tails of the trait being analysed (Foolad & Jones, 1993; Lander & Botstein, 1989; Zhang et al., 2003). Linkage map construction and QTL analysis is performed using only the individuals with extreme phenotypes (Figure 13). By genotyping a subsample of the population, the costs of a mapping study can be significantly reduced. Selective genotyping is typically used when growing and phenotyping individuals in a mapping population is easier and/or cheaper than genotyping using DNA marker assays. The disadvantages of this method are that it is not efficient in determining the effects of QTLs and that only one trait can be tested at a time (because the individuals selected for extreme phenotypic values will usually not represent extreme phenotypic values for other traits) (Tanksley, 1993). Furthermore, single-point analysis cannot be used for QTL detection, because the phenotypic effects would be grossly overestimated; interval mapping methods must be used (Lander & Botstein, 1989).

#### **Section IV: Towards marker-assisted selection**

Marker-assisted selection (MAS) is a method whereby a phenotype is selected on the the genotype of a marker (see Section V). However, the markers identified in preliminary genetic mapping studies are seldom suitable for marker-assisted selection without further testing and possibly further development. Markers that are not adequately tested before use in MAS programs may not be reliable for predicting phenotype, and will therefore be useless. Generally, the steps required for the development of markers for use in MAS includes: high-resolution mapping, validation of markers and possibly marker conversion (discussed below).

#### *High-resolution mapping of QTLs*

The preliminary aim of QTL mapping is to produce a comprehensive 'framework' (also 'skeletal' or 'scaffold' linkage map) that covers all chromosomes

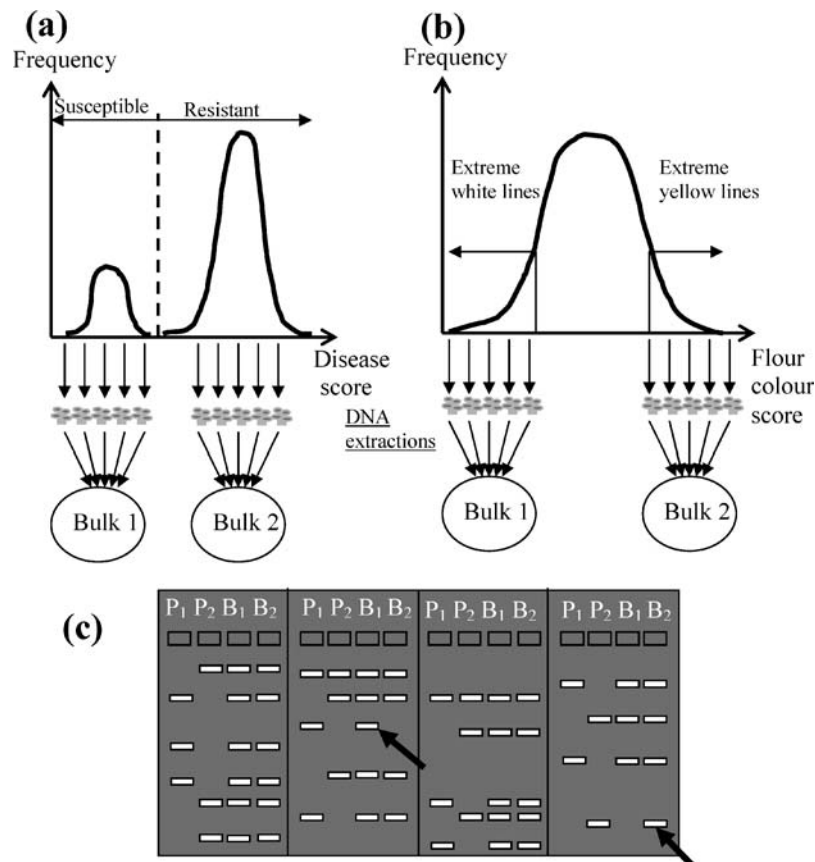


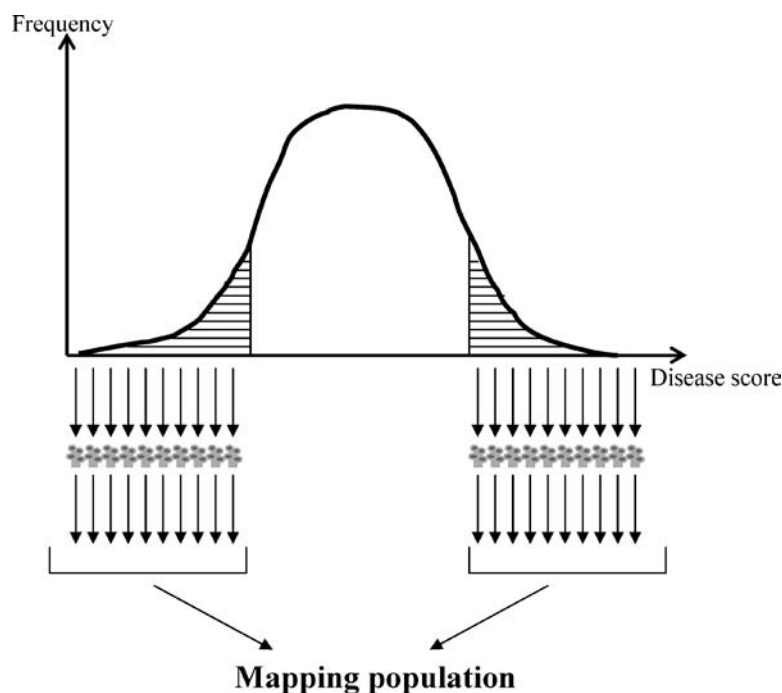
Figure 12. The preparation of DNA bulks for a simple disease resistance trait (a) and a quantitative quality trait (flower colour) (b). In both cases, two bulks (B<sub>1</sub> and B<sub>2</sub>) are made from individuals displaying extreme phenotypic scores. (c) Polymorphic markers (indicated by arrows) that are identified between bulks may represent markers that are linked to genes or QTLs controlling the traits. Such markers are then used to genotype the entire mapping population and QTL analysis performed. (Adapted from Langridge et al., 2001; Tanksley et al., 1995.)

evenly in order to identify markers flanking those QTLs that control traits of interest. There are several more steps required, because even the closest markers flanking a QTL may not be tightly linked to a gene of interest (Michelmore, 1995). This means that recombination can occur between a marker and QTL, thus reducing the reliability and usefulness of the marker. By using larger population sizes and a greater number of markers, more tightly-linked markers can be identified; this process is termed 'high-resolution mapping' (also 'fine mapping'). Therefore, high-resolution mapping of QTLs may be used to develop reliable markers for marker-assisted selection (at least <5 cM but ideally <1 cM away from the gene) and also to discriminate between a single gene or several linked genes (Michelmore, 1995; Mohan et al., 1997).

There is no universal number for the appropriate population size required for high-resolution mapping.

However, population sizes that have been used for high-resolution mapping have consisted of >1000 individuals to resolve QTLs to distances between flanking markers of <1 cM (Blair et al., 2003; Chunwongse et al., 1997; Li et al., 2003).

The mapping of additional markers may saturate framework maps. High-throughput marker techniques that generate multiple loci per primer combination (e.g. AFLP) are usually preferred for increasing marker density (Figure 14). Bulked-segregant analysis may also be used to identify additional markers linked to specific chromosomal regions (Campbell et al., 2001; Giovannoni et al., 1991). However, the extent to which framework maps can be saturated depends on the size of the population used to construct the map. In many cases, the sizes of segregating populations being used are too small to permit high-resolution mapping, since smaller populations have



*Figure 13.* Selective genotyping. The entire population is phenotypically evaluated for a particular trait (e.g. disease resistance). However, only individuals representing the phenotypic extremes from the mapping population are selected for marker genotyping, and subsequent linkage and QTL analysis.

fewer recombinants than larger populations (Tanksley, 1993).

High-resolution maps of specific chromosomal regions may also be constructed by using NILs (Blair et al., 2003). Markers that are polymorphic between NILs and the recurrent parent should represent markers that are linked to the target gene and can be incorporated into a high-resolution map.

#### *Validation of markers*

Generally, markers should be validated by testing their effectiveness in determining the target phenotype in independent populations and different genetic backgrounds, which is referred to as 'marker validation' (Cakir et al., 2003; Collins et al., 2003; Jung et al., 1999; Langridge et al., 2001; Li et al., 2001; Sharp et al., 2001). In other words, marker validation involves testing the reliability of markers to predict phenotype. This indicates whether or not a marker could be used in routine screening for MAS (Ogbonnaya et al., 2001; Sharp et al., 2001).

Markers should also be validated by testing for the presence of the marker on a range of cultivars and other

important genotypes (Sharp et al., 2001; Spielmeier et al., 2003). Some studies have warned of the danger of assuming that marker-QTL linkages will remain in different genetic backgrounds or in different testing environments, especially for complex traits such as yield (Reyna & Sneller, 2001). Even when a single gene controls a particular trait, there is no guarantee that DNA markers identified in one population will be useful in different populations, especially when the populations originate from distantly related germplasm (Yu et al., 2000). For markers to be most useful in breeding programs, they should reveal polymorphism in different populations derived from a wide range of different parental genotypes (Langridge et al., 2001).

#### *Marker conversion*

There are two instances where markers may need to be converted into other types of markers: when there are problems of reproducibility (e.g. RAPDs) and when the marker technique is complicated, time-consuming or expensive (e.g. RFLPs or AFLPs). The problem of reproducibility may be overcome by the development

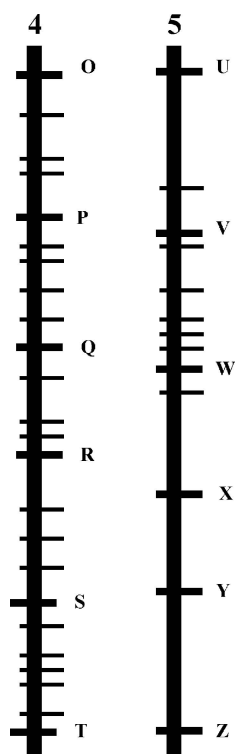


Figure 14. High-resolution linkage mapping. Additional markers have been utilised to 'fill in the gaps' between the anchor markers. The identification of additional markers in the vicinity of QTLs (e.g. between Q and R on chromosome 4) could be useful for MAS. A bulked-segregant analysis approach can be applied to target specific chromosomal regions as shown on chromosome 5 (between V and W).

of sequence characterised amplified regions (SCARs) or sequence-tagged sites (STSs) derived by cloning and sequencing specific RAPD markers (Jung et al., 1999; Paran & Michelmore, 1993). SCAR markers are robust and reliable. They detect a single locus and may be codominant (Paran & Michelmore, 1993). RFLP and AFLP markers may also be converted into SCAR or STS markers (Lehmensiek et al., 2001; Shan et al., 1999). The use of PCR-based markers that are converted from RFLP or AFLP markers is technically simpler, less time-consuming and cheaper. STS markers may also be transferable to related species (Brondani et al., 2003; Lem & Lallemand, 2003).

### Section V: Marker-assisted selection

Selecting plants in a segregating progeny that contain appropriate combinations of genes is a critical component of plant breeding (Ribaut & Betran, 1999; Weeden

et al., 1994). Moreover, plant breeders typically work with hundreds or even thousands of populations, which often contain large numbers (Ribaut & Betran, 1999; Witcombe & Virk, 2001). 'Marker-assisted selection' (also 'marker-assisted breeding' or 'marker-aided selection') may greatly increase the efficiency and effectiveness in plant breeding compared to conventional breeding methods. Once markers that are tightly linked to genes or QTLs of interest have been identified, prior to field evaluation of large numbers of plants, breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTLs (Figure 15) (Michelmore, 1995; Ribaut et al., 1997; Young, 1996). The advantages of MAS include:

- time saving from the substitution of complex field trials (that need to be conducted at particular times of year or at specific locations, or are technically complicated) with molecular tests;
- elimination of unreliable phenotypic evaluation associated with field trials due to environmental effects;
- selection of genotypes at seedling stage;
- gene 'pyramiding' or combining multiple genes simultaneously;
- avoid the transfer of undesirable or deleterious genes ('linkage drag'; this is of particular relevance when the introgression of genes from wild species is involved).
- selecting for traits with low heritability
- testing for specific traits where phenotypic evaluation is not feasible (e.g. quarantine restrictions may prevent exotic pathogens to be used for screening).

#### *Selection of QTLs for MAS*

One commonly asked question is that "since quantitative traits are controlled by at least several QTLs, how many QTLs are typically selected for MAS?" Theoretically, all markers that are tightly linked to QTLs could be used for MAS. However, due to the cost of utilizing several QTLs, only markers that are tightly linked to no more than three QTLs are typically used (Ribaut & Betran, 1999), although there have been reports of up to 5 QTLs being introgressed into tomato via MAS (Lecomte et al., 2004). Even selecting for a single QTL via MAS can be beneficial in plant breeding; such a QTL should account for the largest proportion of phenotypic variance for the trait (Ribaut & Betran, 1999; Tanksley, 1993). Furthermore, all QTLs selected for MAS should be stable across

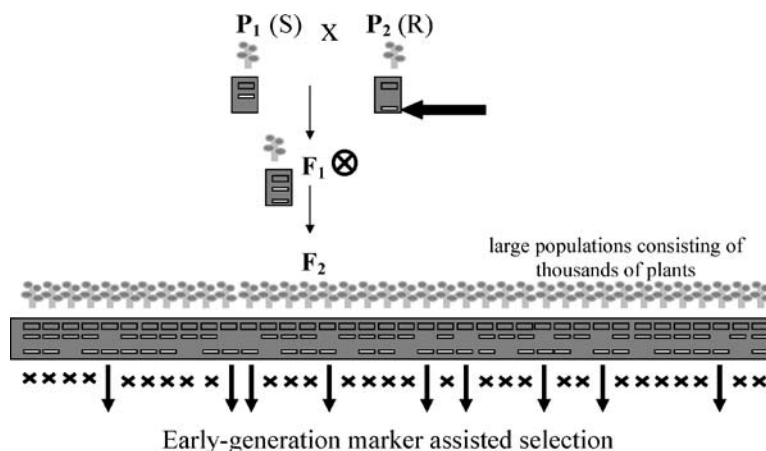


Figure 15. MAS scheme for early generation selection in a typical breeding program for disease resistance (adapted from Ribaut & Hoisington, 1998; Ribaut & Betran, 1999). A susceptible (S) parent is crossed with a resistant (R) parent and the F<sub>1</sub> plant is self-pollinated to produce a F<sub>2</sub> population. In this diagram, a robust marker has been developed for a major QTL controlling disease resistance (indicated by the arrow). By using a marker to assist selection, plant breeders may substitute large field trials and eliminate many unwanted genotypes (indicated by crosses) and retain only those plants possessing the desirable genotypes (indicated by arrows). Note that 75% of plants may be eliminated after one cycle of MAS. This is important because plant breeders typically use very large populations (e.g. 2000 F<sub>2</sub> plants) derived from a single cross and may use populations derived from hundreds or even thousands of crosses in a single year.

environments (Hittalmani et al., 2002; Ribaut & Betran, 1999).

#### Cost/benefit analysis of MAS

The cost of using 'tools' in breeding programs is a major consideration. The cost of using MAS compared to conventional plant breeding varies considerably between studies. Dreher et al. (2003) indicated that the cost-effectiveness needs to be considered on a case by case basis. Factors that influence the cost of utilizing markers include: inheritance of the trait, method of phenotypic evaluation, field/glasshouse and labour costs, and the cost of resources.

In some cases, phenotypic screening is cheaper compared to marker-assisted selection (Bohn et al., 2001; Dreher et al., 2003). However, in other cases, phenotypic screening may require time-consuming and expensive assays, and the use of markers will then be preferable. Some studies involving markers for disease resistance have shown that once markers have been developed for MAS, it is cheaper than conventional methods (Yu et al., 2000). In other situations, phenotypic evaluation may be time-consuming and/or difficult and therefore using markers may be cheaper and preferable (Dreher et al., 2003; Young, 1999; Yu et al., 2000). An important consideration for MAS, often not reported, is that while markers may be cheaper to use, there is a

large initial cost in their development. An estimate for the cost to develop a single marker was AUD \$ 100,040 (Langridge et al., 2001).

#### Marker-assisted backcrossing

Using conventional breeding methods, it typically takes 6–8 backcrosses to fully recover the recurrent parent genome. The theoretical proportion of the recurrent parent genome after  $n$  generations of backcrossing is given by:  $(2^{n+1} - 1)/2^{n+1}$  (where  $n$  = number of backcrosses; assuming an infinite population size). The percentages of recurrent parent recovery after each backcross generation are presented in Table 4. The percentages shown in Table 4 are only achieved with large populations; the percentages are usually lower in

Table 4. Percentage of recurrent parent genome after backcrossing

Generation	Recurrent parent genome (%)
BC1	75.0
BC2	87.5
BC3	93.8
BC4	96.9
BC5	98.4
BC6	99.2

smaller population sizes that are typically used in actual plant breeding programs.

Although the average percentage of the recurrent parent genome is 75% (for the entire BC1 population), some individuals possess more of the recurrent parent genome than others. Therefore, if tightly-linked markers flanking QTLs and evenly spaced markers from other chromosomes (i.e. unlinked to QTLs) of the recurrent parent are used for selection, the introgression of QTLs and recovery of the recurrent parent may be accelerated. This process is called marker-assisted backcrossing. The use of additional markers to accelerate cultivar development is sometimes referred to as 'full MAS' or 'complete line conversion' (Morris et al., 2003; Ribaut et al., 2002).

Simulation studies using PLABSIM—a computer program that simulates recombination during meiosis—indicate that efficiency of recurrent parent recovery using markers is far greater compared to conventional backcrossing (Figure 16) (Frisch et al., 1999, 2000). Therefore, considerable time savings can be made by using markers compared to conventional backcrossing.

Although the initial cost of marker-assisted backcrossing would be more expensive compared to conventional breeding in the short term, the time savings could lead to economic benefits. This is an important consideration for plant breeders because the accelerated release of an improved variety may trans-

late into more rapid profits by the release of new cultivars in the medium to long-term (Morris et al., 2003).

#### Future trends

Although there have been numerous QTL mapping studies for a wide range of traits in diverse crop species, relatively few markers have actually been implemented in plant breeding programs (Young, 1999). The main reason for this lack of adoption is that the markers used have not been reliable in predicting the desired phenotype. In many cases, this would be attributable to a low accuracy of QTL mapping studies or inadequate validation (Sharp et al., 2001; Young, 1999).

However, despite the lack of examples of MAS being practiced, there is a 'cautious optimism' regarding the role of MAS in the future by leading researchers (Young, 1999). Young (1999) cited two main reasons for this 'cautious optimism': improvements in mapping software using more statistically powerful methods; and more innovative and efficient strategies to incorporate MAS within plant breeding programs. Statistical methodology is a vital component of mapping studies, and in the last 15 years considerable progress has been made in the development of more powerful statistical methods (Doerge, 2002). An important study concerning the more effective integration of MAS and plant breeding was the proposal of 'advanced backcross

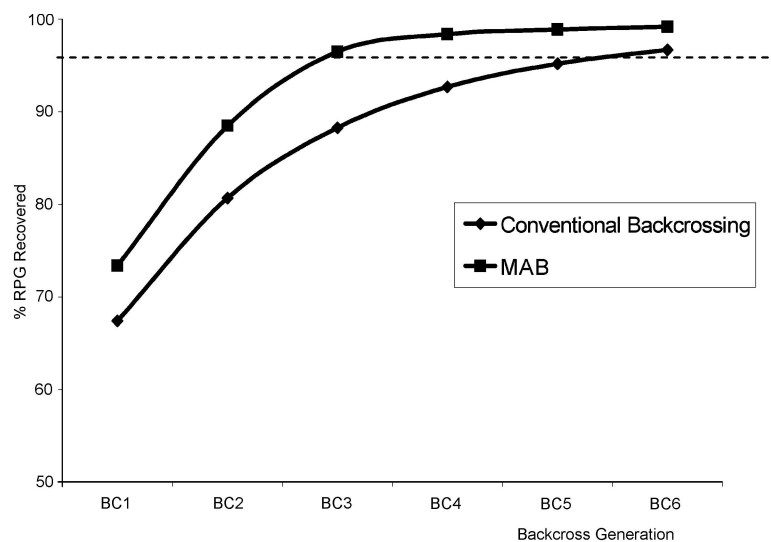


Figure 16. Graph of PLABSIM (Frisch et al., 2000) computer simulations of recurrent parent genome (RPG) recovery using marker-assisted backcrossing (MAB) and conventional backcrossing. The use of markers can reduce the number of generations required to achieve the desired proportion of the recurrent parent genome (indicated by the dotted line).

QTL analysis,' which combined QTL analysis with variety development simultaneously (Tanksley & Nelson, 1996; Tanksley et al., 1996).

Valuable lessons learnt from past research are likely to encourage more researchers to develop reliable markers and plant breeders to adopt MAS. However, Young (1999) emphasized that scientists must realise the necessity of using larger population sizes, more accurate phenotypic data, different genetic backgrounds and independent verification, in order to develop reliable markers for MAS.

We believe that several other factors will greatly affect the efficiency and effectiveness of QTL mapping and MAS research in the future: New developments and improvements in marker technology, the integration of functional genomics with QTL mapping, and the availability of more high-density maps.

New types of markers and high-throughput marker techniques should play an important role in the construction of second-generation maps, provided that these methods are not too expensive. Due to the abundance of single nucleotide polymorphisms (SNPs) and development of sophisticated high-throughput SNP detection systems, it has recently been proposed that SNP markers will have a great influence on future mapping research studies and MAS (Rafalski, 2002; Koebner & Summers, 2003). At present, methods for detection and analysis of widely-used markers are becoming faster and more sophisticated, and many of these methods are automated (Ablett et al., 2003; Hori et al., 2003; Rampling et al., 2001; Warburton et al., 2002). One example of an improvement in the efficiency of marker analysis is multiplex PCR, which enables multiple marker loci to be tested simultaneously (Ablett et al., 2003; Donini et al., 1998; Masi et al., 2003). Furthermore, commonly used marker techniques are constantly being simplified in innovative ways (Gu et al., 1995).

Currently, the cost of utilizing markers is possibly the most important factor that limits the implementation of MAS. However, it is anticipated that in the future, novel applications and technology improvements will result in a reduction in the cost of markers, which will subsequently lead to a greater adoption of markers in plant breeding (Dreher et al., 2003).

The latest trends are to combine QTL mapping with methods in functional genomics, developed for the study of gene expression. These techniques include expressed sequence tag (EST) and microarray analysis, which can be utilized to develop markers from genes themselves (Gupta et al., 2001; Morgante & Salamini, 2003). The use of gene sequences derived from ESTs

or gene analogues, described as the 'candidate gene approach,' holds much promise in identifying the actual genes that control the desired traits (Cato et al., 2001; Pflieger et al., 2001; Yamamoto & Sasaki, 1997). These methods can also be utilized to identify SNPs (Rafalski, 2002). EST-derived and SNP markers are usually integrated into existing maps that have already determined the locations of QTLs (Hayashi et al., 2004; Ishimaru et al., 2001; Skiba et al., 2004; Wang et al., 2001; Zhang et al., 2004). Furthermore, the number of EST and genomic sequences available in databases is growing rapidly (especially from genome sequencing projects e.g. rice), and the accumulation of these sequences will be extremely useful for the discovery of SNPs and data mining for new markers in the future (Gupta et al., 2001; Kantety et al., 2002).

The development of more high-density (or 'saturated') maps that incorporate SNPs, EST-derived markers, and STSs will provide researchers with a greater arsenal of tools for QTL mapping and MAS. More importantly, comparative mapping may become more widely practiced (Laurie & Devos, 2002). The availability of high-density consensus maps greatly facilitates the construction of new maps and mapping specific chromosomal regions (Chalmers et al., 2001; Harker et al., 2001; Karakousis et al., 2003; Lefebvre et al., 2002; Lombard & Delourme, 2001). Comparative mapping could be used to make considerable progress in QTL mapping between related species. For example, rice markers may be used to identify new syntenic markers in barley (Han et al., 1998; Perovic et al., 2004) and wheat (Liu & Anderson, 2003). Furthermore, this approach could be used to improve neglected or 'orphan' crops such as pearl millet (Gale & Devos, 1998).

It is expected that the development of high-resolution maps will also facilitate the isolation of actual genes (rather than markers) via 'map-based cloning' (also 'positional cloning'). Map-based cloning involves the use of tightly linked markers to isolate target genes by using the marker as a 'probe' to screen a genomic library (Tanksley et al., 1995; Meyer et al., 1996). The identification of genes controlling important traits will enable plant scientists to predict gene function, isolate homologues and conduct transgenic experiments. To enhance the efficiency of MAS, knowledge of the DNA sequence of the gene enables the design of 'perfect' or 'diagnostic' markers, which are located within the actual gene sequence, thus eliminating the possibility of recombination between marker and gene (Ellis et al., 2002; Ogonnaya et al., 2001). However, DNA sequences for the majority of genes

controlling agronomically important traits remain unknown, and most probably, will remain unknown for sometime. In the meantime, plant scientists will continue to use QTL maps and markers that tag genes of interest for many years to come.

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