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Application of Molecular Markers in Sugar Beet Breeding

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"Ἡ δὲ τοῦ τευτλίου μία μέν μακρά καί παχεῖα καί ὀρθή, καθάπερ ἡ τῶν ῾ραφανίδων, ἀποφύσεις δέ ἔχει παχείας ὁτέ μέν δύο ὁτέ δέ καί τρείς ὁτέ δέ καί μίαν, τάς δέ μικράς ἐκ τούτων. σαρκώδης δέ ἡ ῾ρίζα καί τῇ γεύσει γλυκεῖα καί ἡδεῖα, δι᾽ ὅ καί ὠμήν ἐσθίουσί τινες."

"The beet has a single long stout straight root like that of the radish, and has stout out-growths, sometimes two, sometimes three, sometimes only one, and the small ones are attached to these. The root is fleshy and sweet and pleasant to the taste, wherefore some even eat it raw."

THEOPHRASTUS (370-287 B.C)
ENQUIRY INTO PLANTS, Book VII, II. 6

List of papers

This thesis is based on the following scientific publications:

Study I. Hjerdin A., Säll T., Tuvesson S. and Hallden C. (1994). RFLP markers in the genus *Beta:* characterization of DNA sequences from a *Beta vulgaris* library. *Genetica* **92:** 91-99.

Study II. Hjerdin A., Säll T., Nilsson N.-O., Bornman C. H. and Hallden C. (1994). Genetic variation among wild and cultivated beets of the section *Beta* as revealed by RFLP analysis. *J Sugar Beet Res* **31:** 59-67.

Study III. Halldén, C., Hjerdin A., Rading I.M., Säll T., Fridlundh B., Johannisdottir G., Tuvesson S., Åkesson C. and Nilsson N.-O. (1996). A high density linkage map of sugar beet. *Genome* **39:** 634-645.

Study IV. Halldén C., Säll T., Olsson K., Nilsson N.-O., and Hjerdin A. (1997). The use of bulked segregant analysis to accumulate RAPD markers near a locus for beet cyst nematode resistance in *Beta vulgaris*. *Plant Breeding* **116:** 18-22.

Study V. Nilsson, N.-O., Hansen M., Panagopoulos A.H., Tuvesson S., Eldhe M., Christiansson M., Rading I.M., Rissler M., and Kraft T. (1999). QTL analysis of *Cercospora* leaf spot resistance in sugar beet. *Plant Breeding* **118:** 327-334.

Study VI. Hjerdin-Panagopoulos A., Kraft T., Rading I. M., Tuvesson S., and Nilsson N.-O. (2002). Three QTL regions for restoration of Owen CMS in sugar beet. *Crop Science* **42:** 540-544.

Summary in Swedish

Runt 1750 upptäckte en tysk kemist vid namn Marggraf att det var möjligt att utvinna socker från betor. Ett par decennier senare började en av hans studenter, Archard, att selektera betor utifrån deras sockerhalt och öppnade också världens första sockerbetsfabrik år 1801. Mängden utvunnet socker per rot låg runt 4 %. Sedan dess har förädling och förbättrade jordbruksmetoder ökat mängden utvunnet socker per rot till runt 18 %. Sockerhalten är fortfarande en av de viktigaste egenskaperna, men i takt med att den samlade kunskapen och förståelsen för andra egenskaper ökar, ökar också möjligheterna till förädling för dessa egenskaper. Dagens förädlare står således inför uppgiften att försöka kombinera många, olika, mer eller mindre komplexa egenskaper på ett optimalt sätt.

De molekylära markörteknikerna har tillfört den traditionella förädlingen ett mycket kraftfullt redskap, och de har i många fall ökat effektiviteten i urvalsprocessen. Genom att använda molekylära markörer, små spårbara bitar av arvsmassan, har det blivit möjligt att förädla med en större precision och med en större grad av kontroll över vad som verkligen händer när växter korsas. De har också gjort det möjligt att bena upp komplexa egenskaper i några av deras bidragande faktorer, samt att studera arvsmassan och relationer mellan mer eller mindre besläktade arvsmassor på ett sätt som förut inte varit möjligt.

Studierna i avhandlingen har samtliga genomförts med avseende att använda molekylära markörer i förädling av sockerbeta. De tre första studierna utgör basen för markör-användningen i förädlingsprojekt och de följande tre studierna utgör exempel på användningsområden. I den första studien utvärderas en samling DNA-sekvenser från sockerbeta för att se hur användbara dessa skulle vara som markörer i förädlingssammanhang och för evolutionära studier. Eftersom sockerbeta är en relativt ung gröda med ett snävt ursprung, studerades och jämfördes den genetiska variationen bland förädlingslinjer med variationen bland vilda betor i den andra studien. I den tredje studien gjordes en stor RFLP-markörkarta över sockerbetans arvsmassa, vilket behövs för att kunna använda markörer i återkorsningsprojekt och för att lokalisera viktiga egenskaper. Samtidigt kopplades markörer till två resistensegenskaper. I den fjärde studien användes tekniken bulkad segregant analys för att ackumulera markörer och slutligen hitta en markör mycket nära en resistens mot sockerbetsnematoder. I den femte studien studerades den kvantitativa nedärvningen av

resistens mot svampsjukdomen Cercospora för att kartera de viktigaste faktorerna som påverkar egenskapen. I den sjätte studien undersöktes slutligen den kvantitativa egenskapen för återställandet av fertilitet hos cytoplasmatiskt hansterila plantor.

Eftersom avhandlingen baseras på studier som genomförts med avseende att använda molekylära markörer i förädling av sockerbeta, ägnas en stor del av den åt att beskriva hur sockerbetor förädlas, hur molekylära markörer kan användas för att underlätta förädlingsprocessen, samt vad som bör beaktas vid användning av markörer. Då de flesta studierna baseras på RFLP-markörer beskrivs endast denna markörtyp.

Introduction

Around 1750 the German chemist Marggraf discovered it to be possible to extract sugar crystals from beet juice. A few decades later, a student of his, Archard, began selecting beets on the basis of their sugar content and in 1801 established the world's first beet sugar factory. The amount of sugar extracted per root fresh weight was around 4% (Winner, 1993). Since then, breeding and improved agricultural practices have increased the concentration of sucrose per root fresh weight to around 18% (Elliott & Weston, 1993). Sugar content is still one of the most important characters in sugar beet breeding, but as the overall knowledge and understanding of other characters increase, so does the possibilities of breeding efforts for those characters. Breeders of today are thus faced with many more characters, many of a complex nature, and with problems of trying to combine them in an optimal way.

The introduction of molecular marker techniques has added a very powerful tool to traditional breeding techniques. Through use of molecular markers it has become possible to breed with higher precision and with a higher degree of control of what is really happening when plants are crossed. The abundance of molecular markers, as compared with phenotypic characters has enabled genomes and relationships between partly related genomes to be studied in a way not possible earlier. Molecular markers have also enabled complex traits to be dissected into some of their contributing factors, which can increase the efficiency of the selection process.

The studies included in the thesis have all been carried out for the purpose of finding and investigating molecular markers useful in sugar beet breeding. The first three studies formed the basis for the use of markers in breeding applications, whereas the three studies thereafter are examples of marker applications. In study I, DNA sequences from a *Beta vulgaris* library were characterized to evaluate the usefulness of such sequences as markers in backcrossing and in comparative mapping. Since sugar beet is a crop of recent and narrow origin the variation within its breeding pool as compared with that of wild beets was of interest. This was investigated in study II. A high-density RFLP linkage map of sugar beet was constructed in study III to create a basis for backcrossing and localisation of factors that influence important traits. During the mapping procedure markers were also linked to two resistance traits. In study IV, bulked segregant analysis was used to accumulate markers near a locus for resistance to beet cyst nematodes so as to generate a marker closer to the gene responsible for

the trait and develop a simple trait selection assay for the trait. In study V, the inheritance of resistance to the fungal disease Cercospora leaf spot was investigated by means of QTL analysis in order to map some of the most important factors influencing the trait. Finally, in study VI the genetic basis for the restoration of Owen cytoplasm male sterility was investigated by means of QTL analysis.

Since the thesis is based on work carried out for the purpose of employing molecular markers in the breeding of sugar beet, large parts of it are concerned with describing how sugar beets are bred, how molecular markers can assist in this process and what should be taken account of in marker applications. Since most of the studies are based on the use of RFLP-markers, only this type of marker will be described.

Sugar beet breeding

"The objectives of sugar beet breeding programmes are to create stable dependable varieties, which give the highest possible yield of white sugar per unit area in relation to cost of production, and which meet various other requirements of the growers and the sugar beet factories" (Bosemark, 1993).

Breeding objectives

Sugar beet accounts for 27% of the world sucrose supplies (Francis & Luterbacher, 2003) and is the second most important source of sucrose - sugar cane, *Saccharum officinarum,* being the most important. Sugar beets are essentially grown in the temperate regions between 30° and 60°N, from the southern Mediterranean region to southern Finland (Winner, 1993). As a source of sucrose, sugar beet has its origin in central Europe in the early parts of the nineteenth century and has since then spread throughout the world. The cultivated beets all belong to *Beta vulgaris* and are divided into four groups: foliage or leaf beets, garden beets, fodder beets, and sugar beets (Gill & Vear, 1958). Most of the varieties involved are diploid and have a haploid chromosome number of nine, although some breeding varieties are triploid resulting from crosses between male-sterile diploid females and tetraploid pollinators (Bosemark, 1993).

In most countries today, the various sugar beet varieties are hybrids. This means that in breeding, desirable characteristics are combined in two different types of lines, in this case cytoplasmic male sterile mother lines and male pollinator lines. Subsequently, these two types of lines are crossed to form hybrid seed. The design of new varieties depends on several different factors, such as the policy of the sugar industry in the geographical market intended, and the climate and soil conditions of the region, with its specific disease pressure. The performance of different varieties in a specific area can vary considerably due to such factors as the disease pressure of the area and the climate and soil conditions.

Sugar beet has a number of features that make breeding of it complex. One is that it is a biennial plant. The first year, leaves are produced and the root size increases and accumulates sucrose. The second year, after a period of cold-induced vernalisation, the accumulated sucrose is used as energy to produce the elongated flowering stem and subsequently to form seeds (Elliott & Weston, 1993). Breeders want beets that extend their flowering stem and flower easily to produce seed. This extension of the flowering stem and of the flowering is a process which in sugar beet breeding is termed bolting. Farmers, on the other hand, want beets that do not bolt at all, regardless fluctuations in the weather, since it is the root produced the first year that is harvested in late autumn to extract the sugar that has accumulated.

Another challenging feature of sugar beet is that it is rather sensitive to inbreeding (Bosemark, 1993). This is at least partly due to self-incompatibility. There are a number of self-incompatibility loci in sugar beet that prevent fertilisation if an allele at one of these loci is shared by the two gametes involved (Larsen, 1977; Owen, 1942b). This self-incompatibility can also be affected by environmental conditions such as temperature. At low temperatures (around 15°C) and at high (around 35°C), self-incompatible plants can set a certain amount of seed (Bosemark, 1993). Because of the difficulties connected with inbreeding, sugar beet lines are usually not inbred for more than three generations.

A dominant self-fertility gene has been identified and been introduced into some breeding materials (Savitsky, 1954). Plants carrying the dominant allele of this gene set 90-95% selfed seed even in the presence of large amounts of unrelated pollen (Bosemark, 1993). The self-fertility gene in a material makes inbreeding of such materials very efficient, since the plants do not have to be isolated in order to be selfed.

For a long period, the growing of sugar beet was very labour-intensive. Up until 1957 in the USA and until the mid-1960s in western Europe, sugar beet varieties were multigerm. As a result, several shoots emerged from each seed ball and extra shoots had to be removed. The advent of the monogerm seed, which resulted in a single shoot per seed ball and the development of monogerm varieties, removed the labour-intensive thinning of the rows. In combination with use of mechanical harvesting machines, precision drills and selective pesticides, this reduced the labour costs dramatically (Winner, 1993). Thus, an essential feature of the commercial sugar beet seed is its monogerm character, which is conditioned by a single recessive gene in the mother genotype (Savitsky, 1952). Monogerm sugar beets produce one seed per seed ball instead of three to four, this character being fully introduced into parts of the breeding program today.

Desirable characters

The desirable characters can be divided into traits of interest for the actual breeding process and those of interest to the growers and in connection with processing in the sugar factories. Some of the characters most important in the breeding process are bolting resistance, monogermity, self-incompatibility, cytoplasmic male sterility (CMS), and maintainer character (O-type). Sugar beet plants are vernalised in order to bolt and be used in crosses. How long a vernalisation period is needed for a plant to bolt is determined by the resistance level of the plant to bolting. Once the breeding process is terminated and a particular variety exists, it is important that it does not bolt in the field despite an occasional cooler period. There is also an annual trait available that can be used in speeding up the breeding process. However, before a variety is released, the annual trait needs to be removed. CMS and fertility restoration are discussed further in a later chapter.

The most important parameters for growers and for processing in the factories are sugar content and root weight. However, there is a strong negative correlation between high root yield and high sugar content, and breeding for a combination of the two is thus very difficult. A beet should also have good sugar extractability. This is affected by impurities, such as the levels of sodium, potassium and α-amino nitrogen in the roots. Higher levels of these result in reduced extractability and a lower yield white sugar.

Disease resistance

Resistances to various pathogens are desirable, mainly because of their ability to protect the plant itself, and also because they can reduce the need for pesticides. In breeding programs, wild species are valuable as sources of genes of interest, such as those conferring resistance to nematodes, viruses and fungi, and sources of resistance to pathogens are frequently found in wild *Beta* species (Asher *et al.*, 2000).

Taxonomically sugar beet belongs to the genus *Beta vulgaris* subsp. *vulgaris* L. within the *Chenopodiaceae* family. The genus *Beta* L. is divided into four sections, *Beta, Corrolinae* (Ulbrich), *Nanae* (Ulbrich), and *Procumbentes* (Ulbrich) (Barocka, 1985). Sugar beet is sexually compatible with many of the wild species from the section Beta, but is less compatible with species from the other sections (Van Geyt *et al.*, 1990). The section *Beta* includes all cultivated beets as well as many wild maritime beets. Beets in this section grow in the entire Mediterranean region and can be found as far to the east as in western India, in Scandinavia to the north, and in England and the Canary Islands to the west. Section *Corrolinae* (Ulbrich) grows mainly inland in Asia Minor at an elevation above 300 meters, and has an extension from eastern Europe in the west to Iran in the east. Section *Nanae* (Ulbrich) has only been found on certain mountains in Olympus, Parnassos and Taiyetos in Greece. Beets from the section *Procumbentes* (Ulbrich) grow in the western Mediterranean region, on the coast of North Africa and on the Canary Islands (Winner, 1993).

The most important and destructive of the beet diseases is rhizomania, caused by the beet necrotic yellow vein virus, BNYVV (Scholten & Lange, 2000). It can result in losses as high as 100% in fields with high infestation. It is transmitted by the soil-borne fungus *Polymyxa betae* Keskin. Plants that are affected have underdeveloped roots with proliferation of lateral rootlets on the main taproot, thus the name rhizomania, Greek for root madness (Duffus, 1986). There are various sources of resistance to rhizomania (Scholten & Lange, 2000), several factors being involved. Two linked genes conferring high levels of resistance to rhizomania have been mapped, *Rz1* (Lewellen *et al.*, 1987; Pelsy & Merdinoglu, 1996) and *Rz2* (Scholten *et al.*, 1999). In addition, complete resistance to the vector *P. betae* has been found in wild relatives of the sections *Procumbentes* and *Corrolinae* (Fujisawa & Sugimoto, 1979; Paul *et al.*, 1992). It has been shown that a combination of resistance to the virus and to the vector results in much lower virus levels (Asher *et al.*, 1997).

Another important disease is Cercospora leaf spot, which occurs wherever beets are grown. It is a disease caused by the *Cercospora beticola* Sacc, and is one of the most widespread and destructive fungal diseases affecting beets. It is favoured by high humidity and by high temperatures, in which temperatures at night are above 16°C. It is spread by wind and splashing rain. The affected leaves have nearly circular spots that eventually merge, and tissue that is heavily infected finally becomes necrotic (Ruppel, 1986). Resistance to Cercospora leaf spot is polygenic in nature (Bilgen *et al.*, 1969; Mesbach *et al.*, 1997; Smith & Gaskill, 1970; Smith & Ruppel, 1974). There are several molecular marker studies supporting this polygenic inheritance (Nilsson *et al.*, 1999; Schäfer-Pregl *et al.*, 1999; Setiawan *et al.*, 2000).

The sugar beet cyst nematode, *Heterodera schachtii*, can affect whole fields or defined areas of a field. The nematodes are spread by infected plants and soil. The plants affected have reduced growth, with underdeveloped storage roots and an excessive formation of fibrous roots (Steele, 1986). Resistance to sugar beet cyst nematodes does not occur naturally in sugar beet, although it has been found in wild relatives from the section *Procumbentes*, tolerance to sugar beet cyst nematodes also having been found in the wild relative *Beta vulgaris* subsp. *maritima* (Asher *et al.*, 2000). The resistance of the section *Procumbentes* has been introduced into the sugar beet genome by interspecific hybridization (Dejong *et al.*, 1986; Heijbroek *et al.*, 1988; Jung *et al.*, 1992).

Genes from wild relatives can be difficult to introduce, the difficulties depending on how distantly related they are. Another problem of genes from wild species that are introduced into breeding lines is that of the undesirable traits associated with such genes, the linkage drag. These undesirable traits can be very difficult to eliminate. The degree of difficulty depends on how closely linked the gene for the desired trait and the genes responsible for the undesired traits are. The closer the linkage is between the genes involved, the more difficult it is to separate them. Desired traits involving several genes are more difficult to work with. One method of ridding a breeding line of such unwanted linkage drag is through continuous backcrosses in combination with recurrent selection for the desired trait.

Male sterility

Male sterility, MS, is a condition involving a plant's inability to produce functional pollen. It is a character that is very useful in seed production since MS- plants do not produce any functional pollen. MS is used in breeding as a tool to facilitate crosses by omitting the emasculation step and ensuring that self-fertilisation does not occur. This allows crosses to be performed easily by MS-plants being placed together with plants that produce pollen, so-called pollinators. Seed harvested from an MS-mother plant has to be the result of a cross between this plant and a pollinator. There are two types of MS, cytoplasmic male sterility, CMS, and nuclear male sterility, NMS.

CMS is commonly found in natural plant populations. It is most frequently caused by mitochondrial genes (Schnable & Wise, 1998) and is thus maternally inherited via the cytoplasm. CMS is very useful in hybrid seed production since it is possible to produce CMS-lines which allow large-scale crosses involving many CMS-plants to be made. However, since CMS-lines do not produce any functional pollen, the breeding of them requires the development of male fertile equivalents that carry nuclear genetic material identical to the CMS-line but having fertile cytoplasm, so-called maintainer lines or O-types.

Nuclear restorer genes have evolved in many species in response to CMS, the sugar beet being no exception. Owen (1942b; 1945) reported the occurrence of CMS and associated restorers in the genus *Beta.* He suggested a model in which two unlinked nuclear genes, *X* and *Z*, interact with a sterilising cytoplasmic component, the S-cytoplasm. To be completely male sterile, a plant carrying the sterile S-cytoplasm has to be homozygous for the non-restoring allele at both loci (*xx zz*), whereas plants containing the normal N-cytoplasm are fertile regardless of the genotype found at those loci (Table 1). In study VI (Hjerdin-Panagopoulos *et al.*, 2002), the genetic basis for restoration was investigated using molecular markers, three map regions for the restoration of the Owen CMS in sugar beet being identified. The regions were found on chromosomes III and IV of the Butterfass (1964) terminology. Two of the regions were located approximately 15 cM apart on chromosome IV. To be able to use the CMS in breeding, all the breeding material involved, the CMS-lines and the maintainer lines, have to be homozygous for the non-restoring alleles at the loci capable of restoring the fertility of the CMS-plants. A single restoring allele could easily put the CMS out of function.

Table 1: **Effect on the fertility of the interaction between restorer genes in the nucleus and mitochondrial genes.**

Nucleus	*Mitochondria*	*Fertility*
non-restorer alleles	fertile allele	fertile
non-restorer alleles	sterile allele	male sterile
restorer alleles	sterile allele	fertile

NMS is due to a recessive gene in the nucleus (Owen, 1952). It is used in breeding to create new variation by facilitating crosses. However, for commercial hybrid production it has been more practical to use CMS, since NMS-plants have to be identified in lines in which the gene segregates. The progeny of crosses involving NMS-plants are either homozygous or heterozygous for the recessive allele. If only fertile plants are selected in the following generations the recessive gene is ultimately lost.

Hybrid production

Currently, most sugar beet varieties are hybrids produced by pollinating a diploid mother line with pollen from a diploid multigerm father line. Hybrids can be produced in several ways. The following are examples: a single cross between two lines A and B; a three way cross between a cross between lines A and B, and a line C; and a double cross between lines A and B, and lines C and D. The lines involved in such crosses should preferably be inbred to a certain extent; ideally, homozygous lines should be used since this reduces the variation in the hybrid. The lines should also be as complementary to each other as possible in terms of desirable alleles, since both lines carry some favourable and some unfavourable alleles in homozygous form. When two different homozygous inbred lines are crossed with each other, many unfavourable alleles will become masked in the progeny by more favourable alleles from the opposite parent making the progeny more fit than the parental lines. For some characters, the heterozygote is more fit than the two homozygotes, a phenomenon termed overdominance. The better performance of the progeny of two inbred lines, as an effect of heterozygosity and overdominance, is generally referred to as the heterosis effect and forms the basis for hybrid production.

In producing a hybrid, the mother line can either be a CMS-line or a CMS-hybrid, so-called F1MS, produced by a cross between a CMS-line and a fertile equivalent of a CMS-line of different origin. The advantage of using a CMS-line is that both lines involved in the cross

can be inbred resulting in a more stable and reliable hybrid. However, plants from an inbred CMS-line often produce less seed and are mediocre as seed producers. F1MS-plants, on the other hand, are good seed producers. The consequence of using an F1MS is that for some favourable alleles originating from the F1MS the hybrid segregates 1:1, which can affect the performance.

Breeding CMS-lines and pollinators

In a typical breeding program, new variation is created by crosses being made between plants having different characters which, in combination with each other have the potential to produce a progeny superior to the parents. Individuals from such a progeny are allowed to self-pollinate, their progeny being subjected to selection based on phenotypic or genotypic selection. To preserve the variation created and reduce the within-line variation to a minimum, the individuals selected are inbred for a number of additional generations. In each generation, a few individuals are selected to be used in the next inbreeding step. Having considerable variation between lines is thus desirable allowing new combinations of characters to be created by different lines being crossed. At the same time, little within-line variation is desired since this creates unstable and undependable varieties. In sugar beet, the breeding for CMS-lines and pollinator lines is done in separate breeding programs. During the process of breeding, the programs interact several times so that the performance of the hybrids between the CMS-lines and the pollinator lines can be evaluated.

In the breeding program for the CMS-lines, new variation is introduced into the maintainer lines. In sugar beet breeding, new variation is normally created by crosses between existing maintainer lines that are free of restorer alleles. However, new variation can also come from other sources, such as broad populations or pollinator lines which may contain restorer alleles. Thus, the new maintainer line has to be tested for the presence of such restoring alleles before an equivalent CMS-line is developed. This is done by crosses being made between single individuals from the new line and single individuals that carry an annual-CMS (Bosemark, 1993). The reason for using annual CMS-plants is to shorten the generation time. If the progeny of such a cross remains sterile, the plant that was tested had no restoring alleles and was of the maintainer type. Depending upon the degree of variation within the maintainer line, it can be subjected to further inbreeding for a few generations, after which its performance is evaluated in line selections and yield trials. An equivalent CMS-line is

developed either in parallel to the development of the maintainer line or once the latter has been developed. The CMS-line is developed by repeated backcrosses of the new maintainer line with an existing CMS-line, preferably the CMS-equivalent of the most closely related maintainer line.

The breeding program for the pollinator lines is more straightforward. There is no CMS to take into consideration here. In addition a pollinator can be either monogerm or multigerm, and either diploid or tetraploid. Multigerm pollinators are also in many cases more vigorous than monogerm ones. When a hybrid is produced, the genotype of the father at the mono/multigerm locus has no impact, since it is the genotype of the mother, in the case of sugar beet a monogerm CMS-line, which determines the number of seeds per seed ball. The genotype of the hybrid offspring is of no importance, since it is the first-year root that is harvested and not the seed.

Line selections and yield trials

The performance of new maintainer and pollinator lines is evaluated in line selections and yield trials. In line selections, lines are evaluated for such characters as disease resistance, sugar content, bolting resistance, and level of impurities. In yield trials, it is mainly the performance of the hybrid combination that is evaluated in terms of such characters as sugar content, root yield, and levels of impurities. To test the performance of a maintainer line as part of a hybrid combination, it is first crossed to a well-known CMS-test line to produce an F1MS. The F1MS is then crossed with a set of pollinators, the performance of the progeny being evaluated in yield trials. The performance of a pollinator line is tested by crossing it with a set of F1MS-test lines to produce hybrids which are evaluated in yield trials. The most promising maintainer and pollinator lines are then further evaluated in additional combinations and are tested more extensively, being compared to existing varieties. Hybrids that perform better than existing varieties are produced in large quantities and are subjected to official testing by variety testing authorities (Bosemark, 1993). A potential new variety needs to be tested for two to four years before receiving approval by these authorities.

Molecular marker analysis

Choice of marker system

Molecular DNA-markers detect sites of variation, or polymorphisms, at the DNA-sequence level. These polymorphisms usually have no phenotypic consequences and represent nothing more than a single nucleotide difference, an insertion or deletion, or a piece of repetitive DNA. The great advance they provide is that they are much more numerous than morphological markers and do not disturb the physiology of the organism. Furthermore, such markers are independent of the developmental stage of the organism and of the tissue studied.

The polymorphisms that molecular DNA-markers reveal can be hybridization-based, as in the case of Restriction Fragment Length Polymorphism, RFLP. They can also be PCR-based, as in the case of the following; Amplified Fragment Length Polymorphism, AFLP; Random Amplified Polymorphic DNA, RAPD; Simple Sequence Repeats, SSR; Sequence Tagged Sites, STS; Cleaved Amplified Polymorphic Sequences, CAPS; as well as others. (Kumar, 1999; Mohan *et al.*, 1997). Marker systems are either dominant, detecting only one allele, or co-dominant, detecting both alleles. In the cases of RAPD, AFLP and STS, the markers are dominant, whereas in the cases of RFLP, SSR and CAPS they are co-dominant. The detection of both alleles makes it possible to discriminate between the two homozygote classes and the heterozygote class, whereas detection of only one of the alleles makes it impossible to discriminate between one of the homozygote classes and the heterozygote class. Marker systems also differ with respect to the number of loci they can detect simultaneously. Some marker systems, such as RAPD and AFLP, detect multiple loci, whereas others such as RFLP, SSR and STS, detect essentially single loci. The level of polymorphisms differs between marker systems. SSR are highly polymorphic, whereas AFLP and CAPS have comparatively lower levels of polymorphisms, and RFLP, STS and RAPD fall in between.

The choice of markers depends on many factors, one being the experimental setup. In backcross experiments, only two genotypic classes can be expected, one of the homozygote classes and the heterozygote class, and dominant markers would work just as well as co-dominant ones. In experiments with materials segregating into more than two genotypic classes, co-dominant markers are clearly advantageous. The reliability of different marker

systems is important, as well as the costs of development and the costs per data point. The reliability of most marker systems is high, with the exception of RAPD-markers, which are associated with an increased risk of genotyping errors due, to a phenomenon termed competition (Halldén *et al.*, 1996a; Hansen *et al.*, 1998; Heun & Helentjaris, 1993). The developmental costs of STS, CAPS and SSR-markers are relatively high, whereas for RAPD-markers they are low. However, once markers have been developed, the costs per data point are comparable to those for RAPD and AFLP, and less than those for RFLP-markers.

Another matter to take into consideration in choosing a marker system is the technical difficulties of the system and of the process logistics surrounding each process. It is difficult to automate a technically difficult process and adapt it to a high throughput. AFLP and RFLP are processes that are technically complex, whereas CAPS, STS, SSR and RAPD are simpler and are thus easier to automate and scale up. Nevertheless, the throughput of most systems can be increased considerably through efficiency measurements such as all samples being handled in the same format, such as the microtiter format (8×12 or 16×24) throughout the process. Such measurements also increase the overall control essentially (Halldén et al, 1996).

RFLP-markers

Restriction fragment length polymorphisms, or RFLP, were first used in the genetic mapping of temperature-sensitive mutants of adenovirus serotypes at Cold Spring Harbor in the mid-1970s (Grodzicker *et al.*, 1974; Grodzicker *et al.*, 1975; Sambrook *et al.*, 1975). Subsequently, RFLP have been used to construct a genetic linkage map in human (Botstein *et al.*, 1980). Since then, impressive progress has taken place in the use of RFLP and other molecular markers to construct genetic maps and to map inherited characters in various organisms.

The molecular basis for RFLP-markers is the detection of DNA-variations through digestion by a restriction endonuclease. Restriction endonucleases are enzymes that digest DNA at defined sequences. For example, the restriction endonuclease *Eco*RI, isolated from *Echerichia coli*, always cuts double stranded DNA at a specific site, whereas *Pst*I, isolated from *Providencia stuartii*, cuts double stranded DNA at a different site (Table 2). A restriction endonuclease with a six base pair recognition site should on average cut DNA once every $4^6/2 = 2048$ base pair. Thus, a sugar beet genome with a haploid size of approximately 1.15×10^9

base pairs (Bennett & Smith, 1976) would result in 560 000 restriction fragments being produced.

Table 2: **Recognition sequences and digestion points (▼/▲) for restriction endonucleases *Eco*RI and *Pst*I.**

*Eco*RI	*Pst*I
5´-G▼A A T T C-3´	5´-C T G C A▼G-3´
3´-C T T A A▲T-5´	3´-G▲A C G T C-5´

The origin of a polymorphism is always a mutation. A single nucleotide alteration can create a new restriction site or destroy an existing one, and deletions and insertions can alter the length of a restriction fragment. Thus, polymorphisms between individuals can be detected by the length of the DNA-fragments between the cutting sites, resulting in RFLPs.

Digestion of DNA-molecules from different individuals does not always produce the same set of restriction fragments. This is due to variations, or polymorphisms, in the number and the position of the restriction endonuclease recognition sites. The digestion of eukaryotic DNA by a particular restriction endonuclease generates a whole range of fragments in terms of size. These fragments can be size-separated by electrophoresis through agarose gels. Stained by ethidium bromide they will appear as a smear containing large fragments close to the negative pole and small fragments close to the positive pole. In order to identify an RFLP, it is necessary to distinguish a fragment that is being studied against a background of many irrelevant fragments. Southern (1975) described a method for transferring DNA from agarose gels to solid supports such as nitrocellulose or nylon membranes. Once immobilized on a membrane, the DNA can be made single-stranded and can be incubated together with a previously isolated radioactively labelled single-stranded DNA fragment, or probe. If the probe is complementary to the whole or to a part of one of the fragments in the smear, it anneals to the DNA and bands will appear on film exposed to the membrane with the attached probe.

In RFLP-analysis, the DNA that is investigated is digested by one or more restriction endonucleases, is size-separated on a gel and is blotted onto membranes. Then one or more probes are labelled and are hybridized individually to the blots containing DNA. The segregating RFLP-band patterns for each probe-restriction endonuclease combination are then recorded.

RFLP-probes

Probes can be prepared from genomic DNA or from complementary DNA, cDNA, either as homologous probes from the species studied, or as heterologous probes from a closely related species, and are arranged in DNA-libraries. A typical genomic library, serving as a source for RFLP-probes is produced by digesting DNA by a restriction endonuclease recognizing a six-base-pair site, such as *Pst*I. The use of the methylation-sensitive *Pst*I for construction of a plant genomic library has been reported to enrich the library for single-copy sequences and minimize the number of repetitive sequences that are cloned (Burr *et al.*, 1988; Helentjaris, 1987; Murray *et al.*, 1989). Gel electrophoresis is used to size-fractionate the DNA-fragments. Fragments in the range of 500-2000 base pairs are recovered, cloned into plasmids, and arranged in genomic DNA-libraries. To identify clones containing repetitive DNA, labelled genomic DNA can be hybridized to dot blots of the DNA-clones. Weak signals reveal clones that contain low or single-copy sequences. The cloned single-copy DNA-fragments are used as probes to detect RFLP-fragments.

A typical cDNA-library serving as a source of RFLP-probes is produced by the isolation of RNA and reverse transcription of it to cDNA. Gel electrophoresis is then used to size-fractionate the cDNA-fragments. Fragments in the range of 500-2000 base pairs are recovered, cloned into plasmids and arranged in genomic DNA-libraries. To identify redundant clones, labelled cDNA can be hybridized to dot blots of the cDNA-clones. The cloned cDNA-fragments are used as probes to detect RFLP-fragments.

Both probe types have advantages and disadvantages. cDNA-clones guarantee that the probes used are really genes, whereas genomic DNA-clones are sampled randomly from the genome. cDNA, on the other hand, originate from certain RNA-populations expressed under particular circumstances, and all the existing genes are not likely to be expressed at the sampling occasions in question. Due to the expression levels, the RNA-populations also contain differing amounts of the various RNA-types. Genomic DNA-clones are sampled from one and the same sample, regardless of the stage of the sample, and there will be repetitive sequences that will occur more often than single-copy sequences. The best coverage of a genome is probably attained by a combination of the two types of probes.

Aspects on choice of marker system

Molecular markers have proven to be a powerful tool for studies of both basic and applied genetics (Kumar, 1999; Mohan *et al.*, 1997). Many matters need to be considered in choosing a marker system. Earlier marker systems and routines often affect the choice of a new system. In general, a combination of systems is often the best choice. A reliable co-dominant marker system, such as RFLP, SSR or CAPS forms a good basis and can be extended with other markers or marker systems. Such markers can be used for most breeding applications, especially for linkage analysis, screening for homozygote individuals and studies of variation. For backcross breeding, however, a reliable, dominant marker system detecting multiple loci, such as AFLP, could be a good choice. For evolutionary studies and studies comparing species, RFLP-markers have a unique characteristic of being hybridization-based. The hybridization conditions can be set so that homologous but not identical loci in partly related species can be detected and be studied in a relatively simple and robust way. Comparable studies with PCR-based markers are much more difficult.

Segregating populations

A prerequisite for marker linkage analysis is to create a segregating population of good quality. The quality is largely dependent on the choice of parents. In plants, the parents are often collected from two inbred lines that are each homozygous at as many loci as possible, yet are genetically divergent enough to exhibit sufficient polymorphisms. Two individual plants, each representing one inbred line, are crossed, resulting in an F1-progeny. The F1-progeny inherits one intact set of chromosomes from each of the two parents and is thus very heterozygous.

The type of individual that the F1-progeny is crossed with determines the type of segregating population. There are four types of mapping populations, backcross populations, F2-populations, double haploid lines, and recombinant inbred lines (Kumar, 1999). A backcross population, BC-population, is produced by crossing an F1-individual with one of its parents resulting in a segregating population. An F2-population is produced by selfing an F1-individual, or crossing two F1-individuals from the same cross. Double haploid lines are produced from the gametes of an F1-individual and represent a direct sample of the segregating F1-gametes. Double haploid lines have fixed genotypes and can be propagated

through sexual means. Recombinant inbred lines, RIL, are produced by inbreeding individuals of an F2-population. Each RIL has a characteristic combination of genes with a different pattern of alternative alleles at multiple loci.

When a segregating F2-population is produced by crossing two F1-individuals, the progeny of this cross inherit one chromosome set from each F1-individual. If an F1-individual is selfed, the progeny inherit both sets from the same F1-parent. The inherited chromosome set can be an intact set from one of the two parents, or can be a mosaic of the two parental sets of chromosomes, with respect both to intact chromosomes and parts of chromosomes.

Mosaics are the results of recombination during meiosis in the F1-individual. Recombination is the occurrence of new combination of alleles other than those present in the parents. It is due to the processes of independent assortment and crossing over. In the process of independent assortment, new combinations of whole intact parental chromosomes are produced. This only affects loci on different chromosomes. In the process of crossing over, homologous chromosomes exchange genetic material. This only affects loci on the same chromosome, producing chromosomes carrying genetic material from both parental chromosomes.

In sugar beet, the production of mapping populations from both double haploid lines and RIL involves difficulties. The production of double haploids in sugar beet has proven to be laborious and time consuming. The best results have been achieved using egg cells rather than pollen cells (Wremerth Weich and Levall, 2003). This makes the production of segregating populations that originate from a single individual difficult since isolation of the egg cells is difficult. Since sugar beet has a rather long generation time, use of RIL as mapping populations is too costly to develop, and the risk of problems of self-incompatibility being encountered during the inbreeding also being high.

We have successfully used both BC-populations and F2-populations in mapping the sugar beet genome. In species in which homozygous parents can be used, all individuals from a given parental line are identical, all F1-individuals thus being identical as well. However, since sugar beet lines are often not inbred for many generations, they remain heterogeneous to a certain extent. This affects the way in which a mapping population is produced. To minimize the effects of heterogeneous parents, it is important to use single F1-individuals,

since each F1-individual is unique. In sugar beet, the F2-populations used in mapping studies are thus produced by selfing single F1-individuals.

In the case of a BC-population, the parent with which the F1-individual is backcrossed should ideally be one of the actual parental individuals or at least a progeny of one of the actual parental individuals having been selfed. Keeping the actual parent alive is almost impossible, however, and to self it often results in problems such as timing the flowering of the plants involved in a cross. In practice, parental lines that are as homogeneous as possible are used. To avoid analysing loci that segregate due to polymorphisms within the recurrent parent used at different crossing stages and to enable the origin of the alleles to be determined, the original parents and F1-individual should be included at some stage of the analysis.

Screen for polymorphic markers

Linkage analysis is often carried out in two steps. In a first step, the parental DNA is screened by means of markers so as to detect polymorphisms and to thus avoid spending resources on non-informative markers, a step which is sometimes omitted. For markers such as RFLP, CAPS, STS and SSR, mapping information from previous projects can be used directly to select markers that represent the genome at defined intervals. In sugar beet, a first screen of five to seven previously mapped markers per linkage group is sufficient to cover most parts of the genome. In a second step, the polymorphic markers selected are used to analyse all the individuals in the segregating population. If the first step of screening is omitted, it is important to include parents and F1-individuals in this analysis, so as to be able to trace the origin of the alleles.

In the case of RAPD- or AFLP-markers, each position on a map is unique for a given project and cannot be translated to unrelated projects. There are several reasons for this. A primer or primer combination amplifies in many loci simultaneously, generating many bands of differing size. A unique collection of bands is generated for each population, making it difficult to compare populations in terms of specific bands. Also, two genotypes might differ in the size of the markers a given locus generates. For these marker systems, having a parental screen to select primer combinations is thus not particularly important, since each primer combination detects a large number of marker loci amplified from different parts of the genome, and there will always be some polymorphic markers. When setting up such a marker

system, however, it is best to screen several different materials with many primer combinations in order to select the five to ten most reliable and polymorphic primer combinations, which can be used in subsequent projects. In sugar beet, six to eight AFLP-primer combinations produce a sufficient number of markers to cover most parts of the genome.

Linkage analysis

Once all the data has been gathered, the actual linkage analysis can begin. It starts with the recombination frequencies and their standard errors being estimated for all pair-wise comparisons between loci by use of the maximum likelihood method (Allard, 1956). The recombination frequency between two given markers is the number of gametes having recombination events between those two markers divided by the total number of gametes. After that, LOD-scores are calculated for all pairs of marker loci. LOD is the logarithm of the ratio of the maximum likelihood to the likelihood of a recombination frequency of 0.5 (Morton, 1955). Thus, LOD-score is the ratio of the probability of two markers being linked, to the probability of their being unlinked, expressed as a logarithm to the base10. High LOD-scores reflect tight linkage, a LOD-score of 3 corresponding, for example, to 1000:1 odds that the loci will be linked.

The likelihood of markers being linked to each other can be tested at different LOD thresholds. This result in groups of markers directly linked to each other or linked to each other via other markers. At low LOD thresholds, most markers are linked to each other, few linkage groups being observed, therefore, whereas at higher thresholds markers will split up into a greater number of linkage groups. The most likely number of linkage groups in a project can often be observed as being the number of linkage groups that remain stable over a wide range of intermediate LOD thresholds (Halldén *et al.*, 1996a). Ideally, this number corresponds to the number of chromosomes found in the species that is investigated.

The next step is to produce a map of each linkage group (Figure 1). A genetic linkage map represents graphically the arrangement of numerous loci, including morphological, isozyme and DNA-markers, along the chromosomes (Paterson *et al.*, 1991).

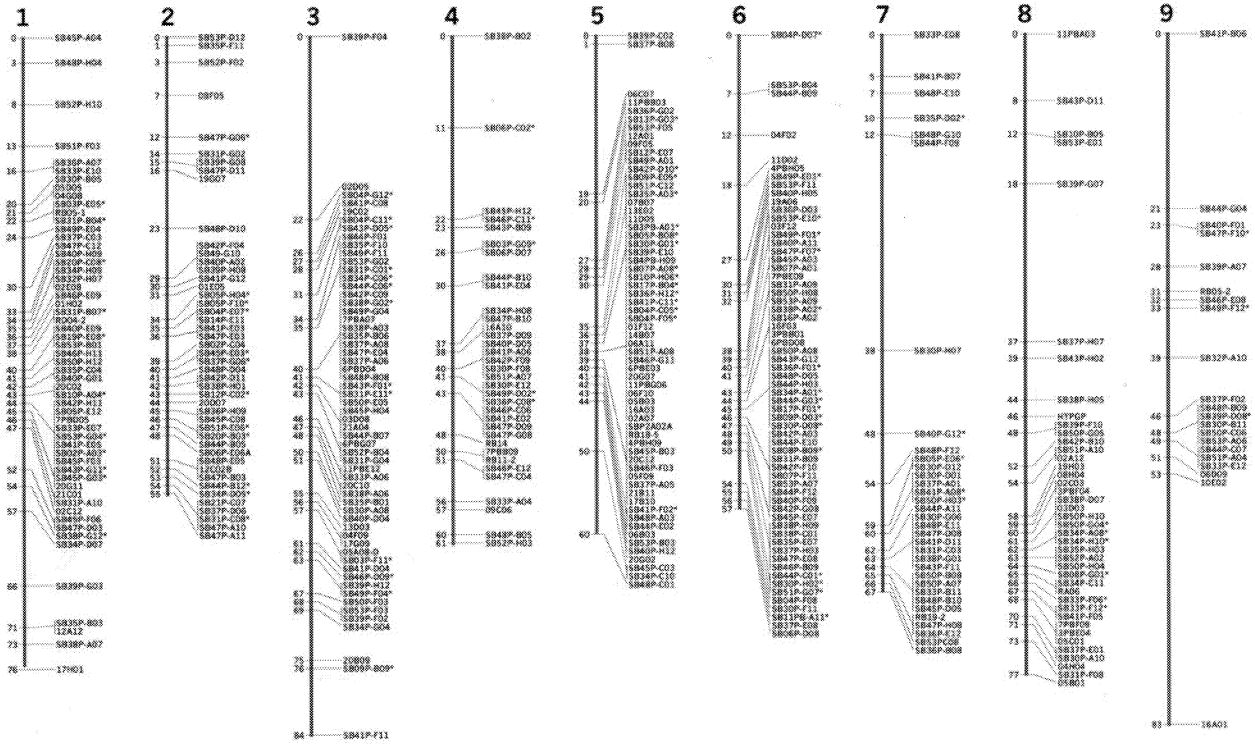


Figure 1: RFLP map of the sugar beet genome with nine linkage groups (Study III). Markers are listed on the right and map distances in centiMorgan on the left.

A difficulty with recombination frequencies is that they are not additive measures, since an even number of recombination events in a marker interval generates no recombination between the two markers that flank the interval. Therefore, a mapping function is used to convert the recombination frequency estimates for all pairs of loci to map distances, which are additive measures. Map distances are reported in centiMorgans (cM), one cM corresponding to one crossover per hundred gametes. It should be noted that there is no direct relationship between genetic map distances and physical map distances. The two mapping functions most commonly used are the Haldane mapping function (Haldane, 1919), which assumes there to be no interference, and the Kosambi mapping function (Kosambi, 1944), which assumes the interference to be proportional to the distance between the crossovers. Interference is when crossovers interact so that one crossover event reduces the likelihood of having another crossover event occurring in its vicinity. The Kosambi mapping function has been used in many mapping studies of sugar beet (El-Mezawy *et al.*, 2002; Halldén *et al.*, 1996b; Nilsson *et al.*, 1999; Pillen *et al.*, 1992; Schäfer-Pregl *et al.*, 1999).

As the number of markers, n, increases, the number of possible marker orders, $n!/2$, increases as well. When there are three markers on a linkage group, 3 different orders of the marker are possible, for five markers there are 60 marker orders which are possible, and for 10 markers 1.8 million marker orders are possible. Since mapping projects most often include more than five markers per linkage group and dense linkage maps can have more than 50 markers in a linkage group, computer software such as MAPMAKER and JOINMAP has been developed to facilitate the construction of linkage maps (Lander et al 1987; Stam, 1993).

Map variation

The rate of recombination varies along the chromosomes. In some regions markers cluster due to low recombination rates, whereas in other regions markers are located far apart due to high recombination rates. In regions of low recombination rates it can be difficult to separate markers and to assign the correct order to them. To resolve such marker clusters, larger mapping populations are needed.

The total map size also varies between species. This is because species differ in their genome sizes and in their rates of recombination. Map sizes can also vary between sexes, although in most species the differences are small. In plant breeding, a map of an F2-population is a sex

average map, since the same individual is used both as father and as mother, and since the recombination events that took place during the meioses of both sexes are involved. If a backcross population is employed, the type of map depends on the use of an F1-individual. If an F1-individual is used as a father, the map reflects the recombination rates of the male genome, whereas if an F1-individual is used as a mother the map reflects the recombination rates of the female genome. In sugar beet, it is more convenient to use an F1-individual as a father because several mother plants can then be pollinated. This approach generates a larger number of progeny and if one cross fails, a backup is available. There being only few progeny or a cross failing when an F1-individual is employed as a mother plant is fatal for a mapping project.

Map sizes also vary between mapping populations of the same species. However, although the rate of recombination varies between populations, the marker order is usually the same, except in regions involving small distances that are difficult to resolve. The choice of parents in a mapping project is important so as to assure maximum marker coverage of the genome. The parents should be as polymorphic as possible so as to maximize the heterozygosity of the F1-individual used to create the mapping population. After all, no markers can be mapped in a region that does not segregate. If a larger population is used, a greater number of markers will are resolved in dense marker regions. The size of a map is affected by the number of markers used and the marker coverage of the genome. If the coverage is poor, it can become difficult to find all the linkage groups and to link fragments of linkage groups with each other. This results in a fragmented map that is often difficult to use in marker-assisted applications.

Genotyping errors

The introduction of errors generally inflates the estimates of genetic distance. The higher the error rate, the greater the inflation and the longer the map is (Buetow, 1991; Goldstein *et al.*, 1997). The effect on a marker associated with errors is that it appears to have higher recombination rates with all the other markers than is actually the case. This often positions such a marker at the end of a linkage group, if it is in fact mapped at all. Thus, genotype errors affect the accuracy of the marker map and the localization of traits. Although errors in genotyping data can clearly lead to severe errors in the analysis, there is no adequate consensus concerning how genotyping errors can best be identified and what steps to minimize these errors are most appropriate. In general, all large genotype data sets have errors. The

range of error types that can be introduced during the steps associated with the genotyping process includes human errors and evaluation errors, as well as equipment and reagent failures. Strategies have been proposed for decreasing these errors or identifying them. These include the extreme suggestion of genotyping in duplicate and comparing both sets of data, and that of having all data evaluated independently by two people and then comparing the scoring of the alleles.

One type of errors, is that associated with the quality of the DNA-samples that are analysed. The quality of the DNA-samples is essential, DNA of lower quality often resulting in genotyping errors. The consequences of low DNA quality differ between markers of different types. The consequence for PCR-based markers is often a failed reaction or a smear for a given individual and for several markers. Such an individual will lack data for several markers and is usually excluded from the analysis.

For RFLP-markers, where restriction endonucleases are employed, partially digested or over-digested DNA can result in a misinterpretation of the genotypes of individuals of lower DNA quality. Partial digestion is the result of an endonuclease not cutting all available sites, whereas over-digestion is the result of an endonuclease recognising a site which is shorter than its recognition site, thus cutting more frequently than it is supposed to. Over-digested samples are easy to spot, since they often contain ladders of fragments shorter than the alleles that are expected. It is more difficult to identify the errors involving partially digested DNA. However, one way of identifying erroneous individuals, independent of the marker system used, is to calculate the number of crossover points and to study the graphical genotypes, which are graphical descriptions of the chromosomal composition of each individual. Erroneous individuals are characterised by an improbably large number of crossover points in adjacent intervals and can be excluded from the analysis. A better understanding of what constitutes an error enables appropriate identification to be made and the number of errors to be reduced, improving the overall quality of the marker data used for linkage analysis.

Segregation distortion

The degree to which segregation is distorted fluctuates along the chromosomes continuously. Naturally occurring segregation distortion has been observed in many plant species, including sugar beet (Pillen *et al.*, 1993). In our mapping populations we find genomic regions with

distorted segregation frequently. Significant distortion from the expected Mendelian segregation of markers can arise by chance, linked markers nearby also showing the same distortion in such cases. This involves whole genomic regions. The selection at a locus also causes segregation distortion at that locus and at linked loci nearby. Whether segregation distortion by chance is due to selection of some kind which is unknown to us is difficult to know. In sugar beet, where several self-incompatibility loci segregate (Larsen, 1977; Owen, 1942b), selection for certain genotypes at these loci could generate distortion of this type.

In a BC-population, markers with significant segregation distortion can be detected by χ^2-analysis of the 1:1 segregation of homo- and heterozygotes. In an F2-population, distorted markers can be detected by χ^2-analysis of the 1:1 segregation of homo- and heterozygotes and by the 1:1 segregation of the two parental homozygous types. In many cases, distorted segregation has natural causes. However, markers potentially associated with errors show distorted segregation without linked markers nearby showing distortion or show non-distorted segregation in a distorted region. Inadequate interpretation of markers or markers being of low reliability can results in distorted segregation of these markers. Distorted markers are often excluded from an analysis, although it is difficult in many cases to determine whether a distorted marker is located in a distorted region or not, since this depends on the marker density of the region in which the marker is located.

Localising the genes influencing a trait

One application of genetic linkage maps has been the tagging of economically important genes by means of molecular markers (Kumar, 1999; Mohan *et al.*, 1997). Breeding efforts can be greatly facilitated by detailed knowledge of the genes that influence important traits. Knowledge of this type includes the number of genes influencing the trait, their chromosomal location, the phenotypic effects of each gene, and the gene dosage, as well as pleiotropic, environmental and epistatic effects of each of the genes involved (Paterson *et al.*, 1991). The quality of the phenotypic evaluation made is crucial for the subsequent linkage analysis and the positioning on a map. The development of reliable phenotypic tests for old and for new traits will always be important.

The chromosomal location of a gene that influences a trait, either simple or complex, is determined by identifying nearby markers, since these are usually co-transmitted with the

gene from the parent to the progeny (Paterson *et al.*, 1991). Once a tight marker-trait linkage has been established, the marker can be used as a tag for the gene, as an alternative to regular phenotypic tests, so as to distinguish the genotype at a very early stage. The allelic forms of the marker will represent the allelic forms of the gene, and in most cases a marker assay, compared with a phenotypic test for a trait, is both cheaper and faster. Should a crossover between the gene and the marker occur, however, this will result in the selection of an incorrect genotype. It is therefore important, to confirm the linkage between the marker alleles and the trait alleles regularly.

Mapping simple traits

Simple traits, segregating in a Mendelian fashion, are often easy to position on a map, provided reliable phenotypic data is available. These are traits that usually involve one or two genes whose allelic forms provide qualitatively distinct phenotypes.

Phenotype tests can be made in the same generation as the marker analysis if they provide reliable data, whereas if there are any doubts about the reliability of the data the tests should be made on selfed offspring of the plants used in the marker analysis. Since time is gained if the phenotypic analysis can be made in the same generation as the marker analysis, an analysis is sometimes repeated to obtain more reliable phenotypic values. In some cases a test affects the continuous development of the plants. Examples are tests for diseases or pests not present in the geographical test region, the material involved needing to be tested under quarantine conditions and then discarded.

The expression of the phenotype, as well as the type of population used in the mapping, is also important for a phenotypic analysis. A phenotype test of a dominant trait in a BC1-population discriminates between homo- and heterozygote individuals, whereas the same test in an F2-population does not discriminate between the dominant homozygote and heterozygote individuals. If it is difficult or impossible to discriminate between one of the homozygote classes and the heterozygote class, selfed offspring testing is an option.

Examples of simple traits that have been mapped in sugar beet are annuality (*B*-gene) (Boudry *et al.*, 1994; El-Mezawy *et al.*, 2002); hypocotyl colour (*R*-gene) and mono/multigermity (*M*-gene) (Barzen *et al.*, 1992); and resistances to several pests such as beet cyst nematodes,

Heterodera schachtii, (Halldén *et al.*, 1997; Jung *et al.*, 1992) and rhizomania (*Rz1*) caused by the beet necrotic yellow vein virus (Barzen *et al.*, 1992).

Mapping quantitative traits

Several agronomical and economically important traits such as size, shape, yield, and stress tolerance are influenced by a variety of different genes. Such traits are designated as complex or quantitative traits since the progeny do not fall into discrete classes but show a continuous range of phenotypes, depending upon the cumulative action of multiple genes, each gene making its own, either positive or negative contribution to phenotypic expression (Kumar, 1999). A genetic locus identified through the statistical analysis of complex traits is termed QTL, or quantitative trait locus (Doerge, 2001). Development of a reliable phenotypic test and of a molecular approach tends to be more difficult the more complex the underlying genetics is. Inheritance can be further complicated by epistatic effects, low penetrance and environmental effects, and the efficiency of phenotypic selection for such genes frequently being low as well.

Although dense molecular marker maps can be used to resolve the complex quantitative characters into some of their contributing QTL (Jones *et al.*, 1997), QTL cannot be mapped in the same way as single marker loci or simple traits. The positions of the QTL can be determined by associations between the inheritance of the marker loci and the trait in question (Jones *et al.*, 1997). The principles for detecting QTL were developed eighty years ago by Sax (1923) analysing genes affecting the quantitative seed-size character in bean, through study of the morphological pattern and pigment markers. The idea of using two markers to bracket a region for detecting a QTL was proposed by Thoday (1961). The basic idea of Sax and of Thoday for detecting associations between a QTL and a marker is based on the comparison of the trait means of different marker classes. The ability to detect an association depends upon the magnitude of the QTL effect on the trait, the size of the population being studied and the recombination frequency between the marker and the QTL. The chances of mapping a QTL to a narrow region depends on the size of the mapping population, since the larger its size, the more opportunities for recombination events there are.

It is virtually impossible to detect all the QTL that influence a complex trait on the basis of a single segregating population, and the number of QTL that affect a trait are probably

underestimated for the most part in QTL mapping (Barton & Keightley, 2002). One reason for this is that the effect of a QTL needs to attain a certain level for it to be detected at all, and small QTL with minor effects are difficult to detect. Another reason is that significant association between a chromosomal region and a trait does not distinguish between a QTL of strong effect and multiple QTL, each with only a weak effect (Asins, 2002). It is even more difficult is to detect closely linked QTL with weak effects in opposite directions, since few recombination events occur between the QTL and because the effects tend to eliminate each other (Barton & Keightley, 2002). Furthermore, if the marker and the QTL are located far apart, a QTL may not be detected with statistical significance or its effect may be underestimated (Kumar, 1999). The environment can also influence the number of QTL detected in such a way that different QTL may be detected in different environments. Last but not least, in the population used all the QTL may not be segregating.

In the last decade and a half, the availability of DNA-markers and of powerful biometric tools has led to considerable progress in QTL mapping in plants (Asins, 2002), and QTL have been mapped for various traits in a number of different species (Kumar, 1999). To study the underlying causes of a QTL, and eventually clone these, it is necessary to fine-map individual QTL. In tomato, a QTL for fruit sugar content, *Brix9-2-5*, was fine-mapped and the gene responsible for the variation was identified (Fridman *et al.*, 2000). From originally being mapped to a 9 cM segment, the QTL region was narrowed down to an 18 kb region on a bacterial artificial chromosome (BAC), and finally to a 1 kb region. The 1 kb region was sequenced for the parental types and recombinant individuals, and the QTL could then be located to a 484 base pair interval. This interval turned out to be part of a fruit-specific apoplastic invertase gene (*Lin5*). In another study, a QTL for tomato fruit size, *fw2.2*, was fine-mapped, the gene responsible being identified (Frary *et al.*, 2000). In this study, a yeast artificial chromosome (YAC) containing the QTL was isolated and was used to isolate four unique clones from a cDNA-library. The cDNAs were used to isolate cosmids that were used for complementation analyses in transgenic plants. When transformed into large fruit cultivars, one of the cosmids reduced fruit weight significantly, indicating that complementation had been achieved. Sequence analysis showed that a single gene, *ORFX*, expressed in early floral development, was responsible for the QTL.

In sugar beet, QTL have been mapped for resistance to Cercospora leaf spot (Nilsson *et al.*, 1999; Schäfer-Pregl *et al.*, 1999; Setiawan *et al.*, 2000), restoration of CMS (Hjerdin-

Panagopoulos *et al.*, 2002), sucrose content, yield and such qualitative traits as α-amino nitrogen, potassium, and sodium content, as well as ion balance (Schneider *et al.*, 2002). In study V, the inheritance of resistance to Cercospora leaf spot was investigated and five QTL on four linkage groups were detected, together explaining 63% of the phenotypic variation (Nilsson *et al.*, 1999). In study VI, three QTL on two linkage groups were detected for the restoration of CMS, together explaining 79% of the phenotypic variation (Hjerdin-Panagopoulos *et al.*, 2002).

Marker applications

Efforts to improve the performance of breeding lines and develop superior hybrids have led to the development of sophisticated testing and selection strategies. Prior to the introduction of molecular markers, however, all selection was made on the basis of the observable phenotypes. Molecular marker techniques have made it possible to construct genetic linkage maps of genomes, to map traits onto these maps, to use markers in different selection strategies, to analyse the genetic structure of populations, and to conduct comparative studies between genomes. Use of markers in applied plant genetics and of marker-assisted selection has become possible both for traits controlled by identified genes and for traits controlled by QTL (Ribaut & Hoisington, 1998). In sugar beet breeding, molecular markers are routinely used in such applications as single-trait selection, bulked segregant analysis, fine-mapping QTL, backcrossing using markers, and quantitative trait selection, as well as in fingerprinting and quality control.

Single-trait selection

Single-trait selection using molecular markers involves markers that are closely linked to a trait, making it possible to make a selection directly on the marker genotype rather than on the trait phenotype. The application is best suited for simple traits that segregate in a Mendelian fashion whose allelic forms give qualitatively distinct phenotypes. Molecular markers can be used there to assure the transfer of one or more genes from one breeding generation to the next. Single-trait selection is effectively used in breeding programs to screen large amounts of offspring from many crosses at an early plant, saving both resources and time (Figure 2).

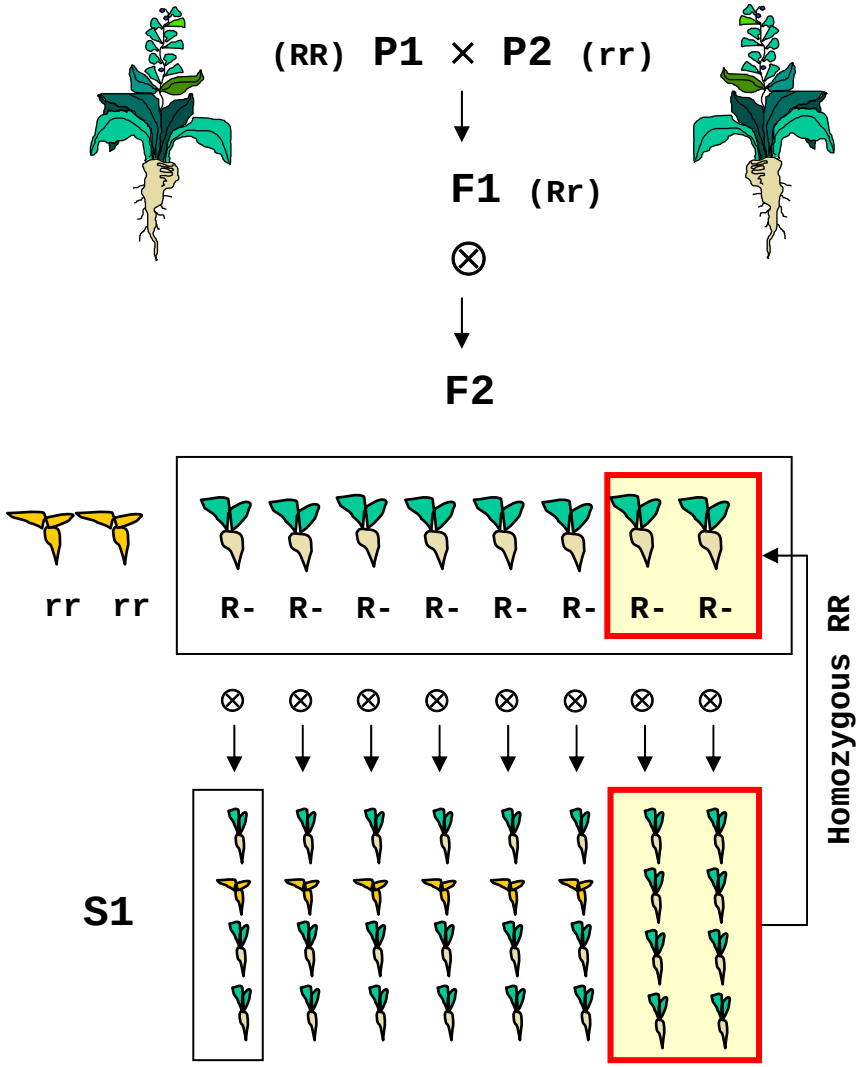


Figure 2: Single trait selection of individuals homozygous for a dominant character. A parent 1 (P1) carrying an interesting dominant character (R) in homozygous form is crossed (×) to a parent 2 (P2), not carrying the character (rr), generating an F1-generation being heterozygous for the character. One F1-individual is selfed (⊗) and an F2-generation is produced in which the character segregates. Individuals not carrying the character (rr) are easily distinguished with a phenotypic test, whereas to be able to distinguish between individuals carrying the character in homozygous or heterozygous form, F2-individuals carrying the character (R-) will have to be selfed and the phenotypes of the S1-lines has to be evaluated. F2-individuals homozygous for the character will result in S1-lines homozygous for the character, and F2-individuals heterozygous for the character will result in segregating S1-lines. With markers linked to the character a molecular test can be made on individuals at an early stage in the F2-generation, one whole generation earlier than with a traditional approach.

Before selection can begin, it is necessary to ensure the correct relationship between the alleles of the marker and of the gene. This is done by phenotypic and genotypic screening of the parents of the offspring in which selection is to be made. Only offspring with parents in which the relationship between the marker and the gene alleles is correct can be part of the molecular selection program. Nevertheless, a molecular marker being linked to a gene that is responsible for a character is not an absolute test for the gene, since there being a distance between the marker and the gene creates the possibility of a crossover between the marker and the gene in any generation. Such a crossover reverses the linkage between the marker alleles and the alleles of the gene, which leads to selection of the correct marker allele resulting in selection of the wrong trait allele. For the breeding material in a single-trait selection program, offspring tests to assure the correct relationship between the alleles of the marker and of the gene are carried out regularly, a few individuals from each cross being selfed and the resulting phenotypes being evaluated. If the linkage has been reversed in a material, i.e. if the individuals are homozygous for the marker allele but segregate for the phenotype, the material can no longer be used in the single-trait selection program using that marker.

Since a phenotypic test is often just as easily performed and as reliable as a molecular test, the apparent advantages of a molecular approach should be carefully considered and be weighed against those of a traditional phenotypic approach. In sugar beet, transgene herbicide resistance is an example of a trait whose phenotype is easily evaluated by spraying plants with the herbicide, this being done at a plant stage similar to that used in sampling for molecular analysis. When a reliable phenotypic test is difficult to establish, however, or when quarantine regulations requiring the destruction of infected material makes phenotypic selection impossible, a molecular test is an attractive alternative (Young, 1999). Many disease resistance genes fall into this category. A good example of this in sugar beet is resistance to rhizomania (Barzen *et al.*, 1992). Molecular tests are also attractive as an alternative to phenotypic selection, when the phenotype is manifested late in development and when the generation times are long.

Bulked segregant analysis

There are different methods of identifying markers linked to traits of interest for use in single-trait selection. One is to map a gene directly by creating a linkage map. A drawback of this is that there is no assurance that a marker will map within 1-2 cM of the gene of interest, which

is what is desired for use in single-trait selection. Another method is to perform a bulked segregant analysis, BSA, which is a rapid procedure for identifying markers in specific regions of the genome (Churchill *et al.*, 1993; Michelmore *et al.*, 1991). It can be used to link markers both to single Mendelian traits (Foisset *et al.*, 1995; Somers *et al.*, 2002) and to QTL (Bryan *et al.*, 2002; Schuster *et al.*, 2001), although the latter is much more difficult to carry out and requires very reliable phenotypic data. BSA has the advantage that no prior knowledge other than the data concerning the phenotype is needed.

This method, in which pooled DNA-samples are compared, involves a number of steps. First, individuals are scored for an interesting trait and are divided into pools on the basis of their phenotypes. If a sufficient number of individuals are present in each pool, the pools differ genetically in the selected region only and are genetically variable at all other loci. A large number of markers of unknown position are then tested on the pools. If the pools differ genetically in the selected region only, any polymorphism should originate from that region. Before a marker identified by BSA can be used to screen for a trait, linkage to the trait needs to be confirmed on individuals from the segregating population from which the bulks were generated, since it is difficult to assure that the pools differed genetically in the selected target region only and were genetically variable at all other loci. This is done by mapping the polymorphic markers in the population used for the original mapping.

How the pools are created is very important and there are limitations to the number of individuals that can be included in each pool. The limit is determined by the type of marker system employed and its ability to detect a rare allele in a background of more frequent alleles. One way of circumventing this limitation is to create several pools of each pool type. In sugar beet, a pool size of five individuals, equivalent to ten alleles, is suited for marker systems such as RFLP, RAPD, AFLP, and SSR (unpublished data). With this pool size, the probability of detecting a single rare allele is very high, whereas for pools of larger size, the chances of detecting a rare allele decrease. Since many markers are tested in a BSA, multiplex marker systems, such as the dominant RAPD- and AFLP-markers, are very efficient. In these systems, a single primer combination amplifies sequences from several loci in the genome in one and the same reaction. However, these markers of this type are not very well suited for large-scale single-trait selection. AFLP is very laborious, and neither marker system is easily automated.

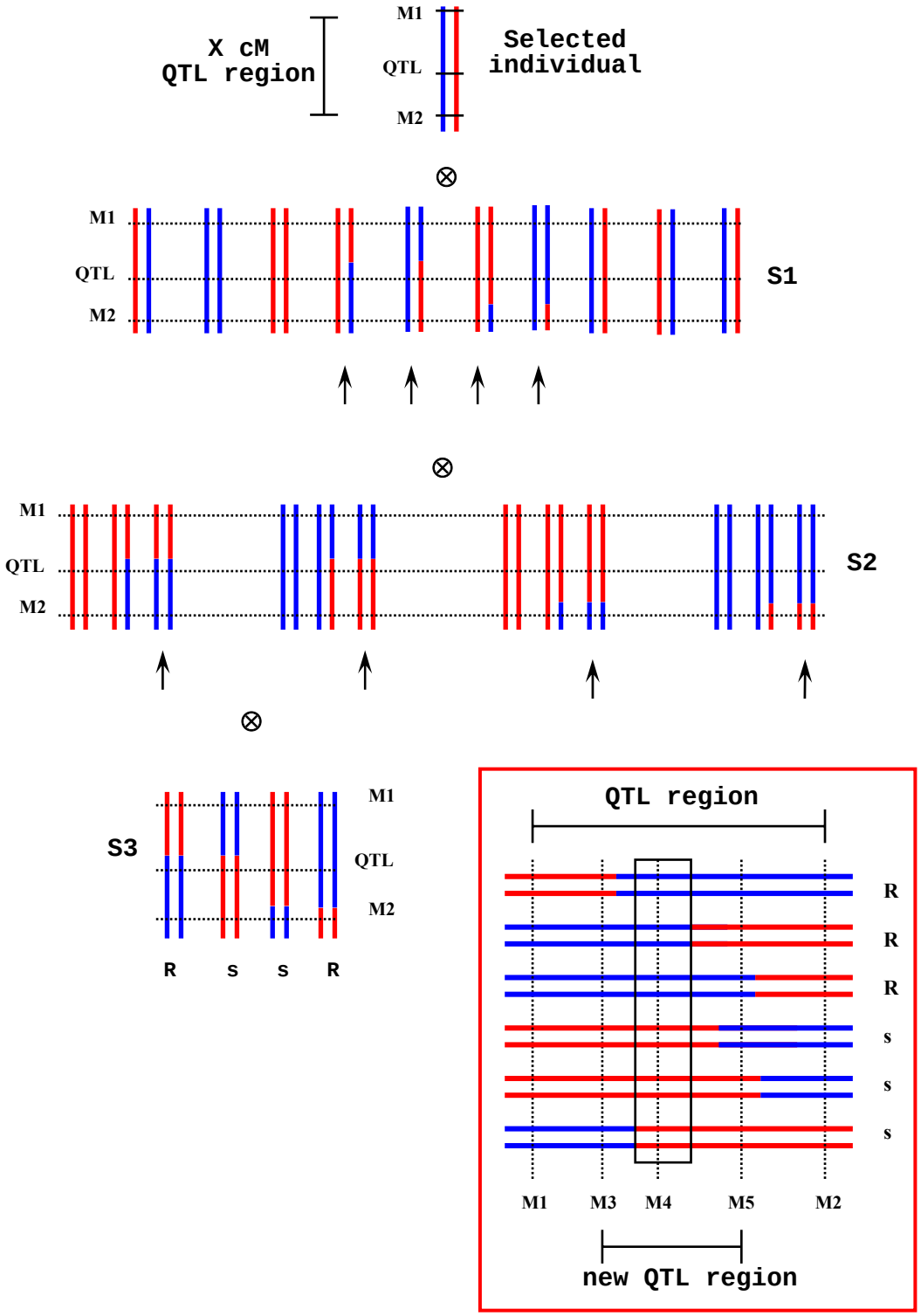
Once a reliable marker has been found and the linkage has been confirmed, the marker should thus be converted to one that is better suited for use in large-scale single-trait selection, such as a CAPS or an STS marker.

BSA can also be used to identify markers close to mapped genes (El-Mezawy *et al.*, 2002; Halldén *et al.*, 1997), through the use of a combination of genotype and phenotype data. Individuals with recombination events occurring in the target region, i.e. the region between the two markers flanking the target locus or the closest marker and the target itself, are selected on the basis of their graphical genotypes. With use of phenotype data, the selected individuals can then be divided into two classes and be used to create bulks.

Fine-mapping QTL

The procedure for developing closely linked markers for QTL regions that have been mapped is more complicated. The markers have to be developed for each QTL region separately and the phenotypic data are often less distinct due to the polygenic nature of the trait. A QTL is always mapped to a region spanning several cM, there usually being several markers mapped to the region in question.

The first step is to identify individuals that are variable for only one QTL. To do this, markers flanking the QTL regions are used to identify individuals in which one target QTL region is heterozygous and all other QTL regions are homozygous. These individuals are selfed and the progeny is analysed using the markers that flank the target QTL region. Individuals with recombination events that occur in the target region are selected on the basis of their graphical genotypes. The individuals that are selected are selfed and a marker analysis is conducted to select homozygous recombinant individuals and to map other markers that are available in the region. The target QTL can then be positioned in a marker interval narrower than the one before on the basis of the positions of the various recombination events in the different lines involved and of phenotype data from the selfed offspring, (Figure 3).

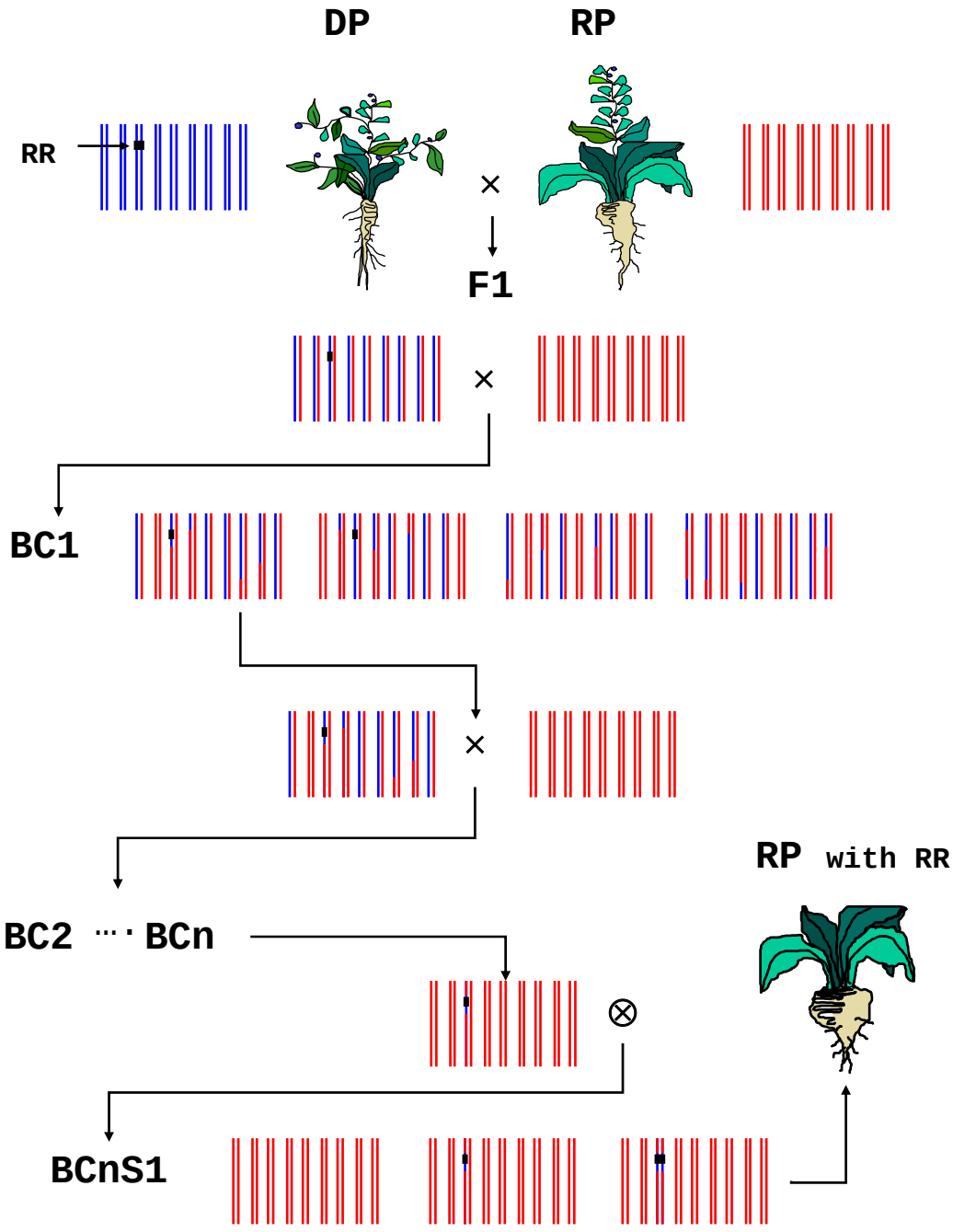

X cM
QTL region
M1
QTL
M2
Selected
individual
S1
S2
S3
R s s R
QTL region
R
R
R
s
s
s
M1 M3 M4 M5 M2
new QTL region

Backcrossing using markers

Backcrossing is a method of transferring one or more chromosome segments from a donor genotype to a recipient genotype through continuous backcrosses in combination with recurrent selection for the desired trait (Figure 4). In traditional backcrossing, a donor parent carrying a trait of interest, the target trait, is crossed with a recipient parent, a recurrent parent, not carrying the trait. The hybrid is crossed with the recurrent parent and the progeny is screened for the target trait. Individuals carrying the target trait are again crossed with the recurrent parent and the process is repeated. After four to five backcross generations a progeny that is identical to the recurrent parent except for the target trait, for which it is heterozygous, is obtained (Tanksley *et al.*, 1989). Thereafter, this progeny can be selfed to fix the desired genotype in the genome.

Molecular markers can be used to monitor the transfer of the donor segment to the recipient genome during backcrossing. This is one of the best examples of efficient use of molecular markers (Dekkers & Hospital, 2002). It has several advantages and both dominant and co-dominant marker systems can be used to support backcrossing. To fix the desired genotype, however, co-dominant molecular markers are especially valuable. A desired target gene is often associated with certain undesired genes, the linkage drag, which is co-transferred to the recurrent parent. In traditional backcross programs, this linkage drag usually remains for many generations and can be very difficult to eliminate because of there being no effective way of selecting for recombination events close to the target gene (Tanksley *et al.*, 1989). The use of markers makes it possible to use graphical genotypes to select individuals having crossover events that are close to the gene of interest as well as a high proportion of the recurrent parent genome.

Figure 3: Marker assisted fine mapping of one QTL region for a trait R. The QTL is mapped to a X cM region, flanked by markers M1 and M2. One individual being heterozygous for M1 and M2, but homozygous for all other QTL regions, is selected and selfed (⊗). The S1-generation is analysed with M1 and M2 and individuals with recombination events within the QTL region (blue-red genome borders) are selected (↑) and selfed. The S2-lines are analysed with M1 and M2, as well as with additional markers known to be located between M1 and M2, to select individuals with fixed recombination events. The selected S2-individuals are selfed and the resulting S3-lines are evaluated on their phenotype for the QTL (R or s). Finally, the phenotype data for the S3-lines exists and the graphical genotypes of S2-individuals with recombination events within the QTL region can be compared (red box). In this case the QTL is most likely localized in the interval between markers M3 and M5, close to marker M4, with the blue genome carrying the R-allele and the red genome the s-allele.


DP
RP
RR
F1
BC1
BC2 ···· BCn
RP with RR
BCnS1

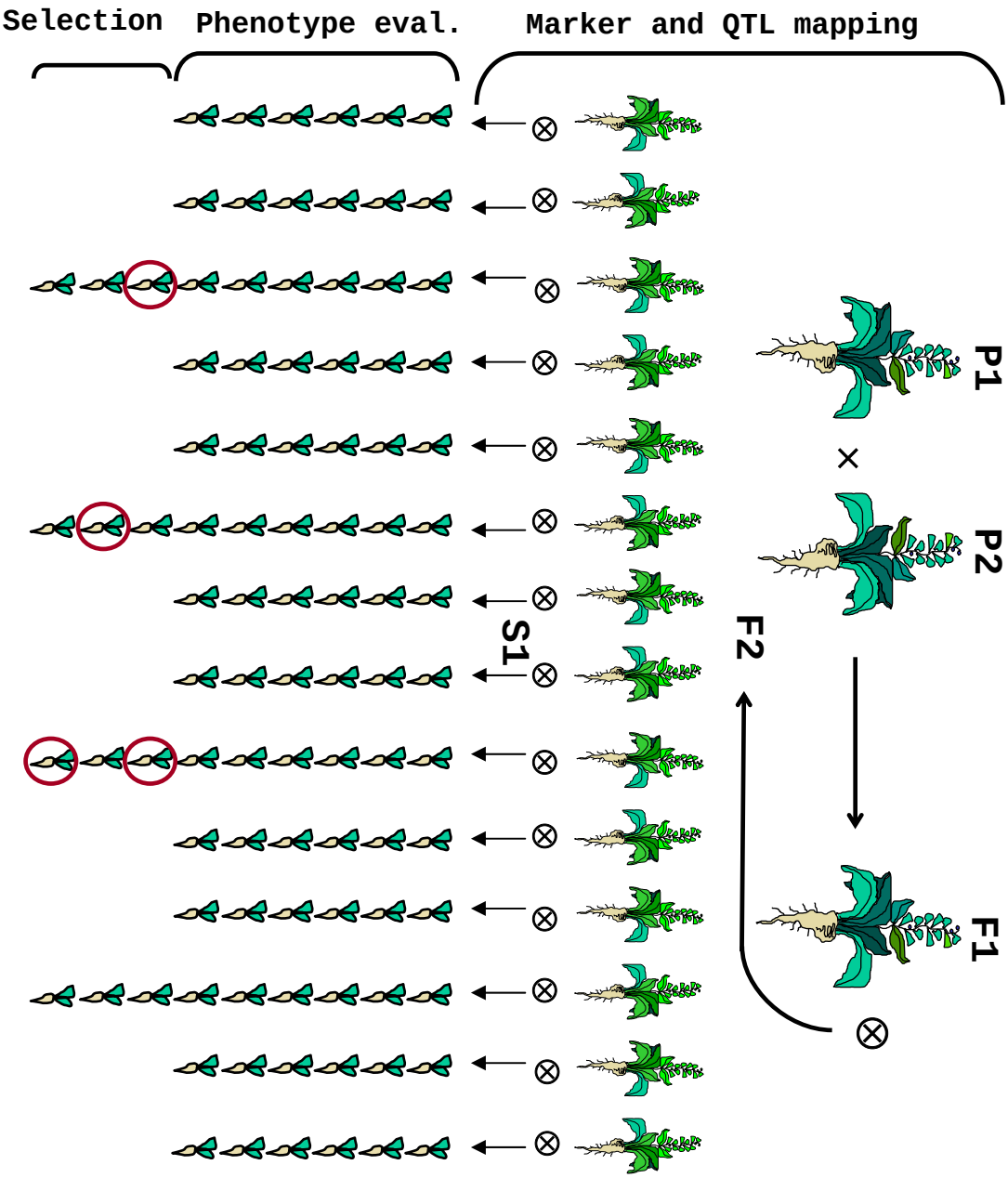
The number of generations needed to transfer one or more chromosome segments from a donor genotype to a recipient inbred genotype can also be reduced in this way (Young & Tanksley, 1989). Computer simulations in sugar beet have shown that with use of markers it is possible to achieve the same amount of recurrent parent genome in 2-3 backcross generations as would have required six or more backcross generations if no markers had been used (Engkvist, 2002).

Sugar beet lines are often quite heterogeneous (Bosemark, 1993). This has two consequences when markers are used to support backcrossing. First, the possible number of backcross generations required may become limited if there are problems of self-incompatibility within the recurrent parent. Secondly, a backcross program using markers may result in a progeny that is more inbred with respect to the recurrent parent genome than the original recurrent parent was.

Quantitative trait selection

Molecular markers can be used in quantitative trait selection to map QTL regions. This map information can then be used to select and combine individuals during the development of new inbred lines. The selection can be based on one or more traits involving differing numbers of QTL regions, and is much more difficult than the types of selection described earlier. Selection for simple traits involves one or two genome regions and backcrossing involves one or a few genome regions of equal importance. Quantitative trait selection involves several genome regions that vary in their effect on the phenotype and can involve several different traits.

Figure 4: Efficient backcrossing with markers representing the genome at defined intervals, making it possible to "paint" the genome according to origin of alleles. The result is a graphical description of the chromosomal compositions of each individual, so-called graphical genotype. A donor parent (DP, blue genome) carrying a trait of interest in homozygous form (R, black) is crossed (×) with a recipient parent (RP, red genome) not carrying the trait. An F1-individual, being heterozygous for the genome, is backcrossed to the RP and the progeny is screened with markers for the trait and for the rest of the genome. The graphical genotypes of the first backcross progeny (BC1) are used to select an individual with a crossover event (blue-red genome border) close to the R and a high proportion of RP genome (red). The selected individual is backcrossed to the RP and the selection process is repeated. After a few backcross generations, BC2-BCn, a BCn individual that is identical to the RP except for R, for which it is heterozygous, can be selected. Subsequently, this individual can be selfed (⊗) (BCnS1), to fix R in the genome and a new RP homozygous for R has been obtained.


Selection
Phenotype eval.
Marker and QTL mapping
P1
P2
F1
F2
S1

In a genome region in which a QTL for a particular trait has been located, there may also be a QTL for a completely different trait, and the desired allele for a trait in a QTL region may be linked to an undesired allele for another trait having a QTL in the same region.

The aim of marker-assisted quantitative trait selection projects in sugar beet is to obtain new lines which outperform the original parental lines through the use of markers to combine the best QTL from both parents. The process starts with two parental lines with different characters being crossed. A single F1-individual from the cross is selfed, resulting in an F2-progeny. Plant material from each F2-individual is sampled and the DNA is analysed with molecular markers resulting in a recombination map of the genome. Meanwhile, each F2-individual is selfed, resulting in S1-progeny. Part of the S1-progeny is used directly in line selections and part of it is crossed with a number of testers to produce hybrids that are evaluated on their performance in yield trials. The average of the phenotypic evaluation of an S1-line and a hybrid of this line is a measure of the phenotype of the parental F2-individual for the character being evaluated.

Once all the phenotypic data has been collected, the marker data, the map data and the phenotypic data for the F2-individuals are analysed and QTL for the analysed phenotypic traits are mapped. It is then possible, on the basis of the locations of the QTL on the map and the effect and origin of the alleles at each QTL, to outline an ideal genotype. This information, together with the marker data for all F2-individuals, is analysed to select those S1-lines in which the chances of obtaining individuals that match the ideal genotype are highest.

Figure 5: Marker assisted quantitative trait selection in sugar beet to obtain new lines which outperforms the original parental lines by using markers to combine the best QTL from both parents. Two parental lines, P1 and P2, with different characters are crossed (×). One F1-individual from the cross is selfed (⊗) resulting in an F2-progeny. Marker analysis is made in the F2-generation, resulting in a recombination map. Each F2-individual is selfed resulting in S1-progeny. The phenotypes of the S1-lines for a number of traits are evaluated in line selections as well as in yield trials. The marker data, map data and phenotypic data for the F2-individuals are used to map QTL for the analysed phenotypic traits. Based on the locations of the QTL on the map, and the effect and origin of the alleles at each QTL, an ideal genotype is outlined. This information together with the marker data for all F2-individuals is analysed to select S1-lines, in which the chances of finding individuals matching the ideal genotype are highest. Selected S1-lines are analysed with the mapped markers and individuals which, selfed or in crosses with each other, have the highest potential to produce individuals matching the ideal genotype are selected (circled individuals). The selected individuals are selfed or crossed in pairs and their progeny is again analysed with the mapped markers and new individuals are selected and crossed. The marker analysis and selection procedure is repeated until an individual matching the ideal genotype is obtained.

Stored S1-progeny for the lines selected are analysed with use of the mapped markers, individuals which either selfed or in crosses with each other have the highest potential of producing individuals that match the ideal genotype being selected. The individuals selected are selfed or crossed in pairs, their progeny again being analysed with the use of the mapped markers, and new individuals are selected and crossed. This procedure is repeated until an individual which matches the ideal genotype is obtained (Figure 5).

Parental lines are selected because of their combined characters being thought to complement each other. Due to the heterogeneity of sugar beet lines, however, there is a certain variation between different F1-individuals, no given individual representing all the parental alleles represented in the line. A single F1-individual, therefore, does not represent the entire potential of a cross. This makes it difficult to predict the genotypic and phenotypic outcome of crosses.

Fingerprinting and quality control

Once a good line has been developed, it is used in the production of commercial hybrid seed, and thus needs to be propagated regularly. The propagation of lines and hybrids take place in open-pollinated fields and there is always a slight risk of contamination by genetic material from such sources as other sugar beets, wild weed beets and garden beets. Although contamination is usually not a big problem, a line that is moderately self-sterile can be very susceptible to contaminating pollen from outside. In addition, during all the steps in commercial seed production it is important to monitor the quality so that no important characters, such as those concerning resistance to various pathogens as well as yield performance, are lost. The allele frequencies should therefore be kept as constant as possible from one generation to the next, and not change due to genetic drift that occurs when the alleles that are sampled in the parental generation fail to represent the allele frequencies found in that generation. The heterogeneity of sugar beet lines makes genetic drift possible, whereas in a completely inbred line the allele frequencies would not change.

Genetic constancy in the production of new seed can be effectively monitored by use of a fixed selection of molecular markers that represent the entire genome. Such a selection of markers can also be used to identify and characterise individuals, lines and groups of lines, as well as populations, each individual, line, group of line, and population that is analysed being

given its own characteristic "fingerprint". These fingerprints can then be used to separate lines or populations from each other, to study the relatedness of different lines and populations, to group lines and populations into larger groups on the basis of their relationship, and to study genetic drift in a line or a population. However, depending on the number of individuals tested, contamination or genetic drift of low frequency may go undetected. To guarantee a contamination level of less than 1%, each line needs to be tested for the presence of its specific characters. In the cases in which molecular markers are available, a molecular analysis can be carried out.

New challenges and applications

During the last two decades, molecular markers, genetic mapping and applications of markers and maps have developed to a tremendous extent. Many different marker technologies have been developed, and laboratory procedures have been standardized and simplified. The size of the samples needed for an analysis has been scaled down, whereas the number of samples employed has increased, high throughput equipment and tools to analyse ever increasing amounts of genetic data having been developed. In sugar beet breeding, genetic maps are now routinely being produced to support the development of new breeding lines and to link markers to traits of interest, both to those that are simple and to those that are genetically more complex. However, markers linked to traits have the disadvantage that the linkage between the marker and the trait can be broken, and it would be preferable to have a marker located in the gene responsible for the trait. To achieve this, it is necessary to identify the gene corresponding to a mapped locus. For polygenic characters involving several QTL in different regions of the genome, identifying all the QTL is a challenge in itself. Having access to QTL data from many different independent populations and from different environments makes it possible to pinpoint the strongest and most frequently occurring QTL. Once such a QTL has been identified, it can be subjected to high resolution mapping to narrow down the QTL region and to further characterise it in terms of being one or several QTL.

Genes responsible for traits can be identified in different ways, depending in part on the crop in question. Information on coding sequences in model species such as the sequenced rice, *Oryza sativa* (Karlowski et al. 2003; Yuan et al. 2003) and *Arabidopsis thaliana* (*Arabidopsis* Genome Initiative, 2000) can be applied to other species so as to search for genome regions

with homologous genes and partly conserved gene orders. Such regions can be entire chromosomes or parts of these. Once a trait has been mapped and a region of interest has been identified in a crop, information on the functions of all of the genes found in the homologous region in the model species can be extracted. This information can be used to identify candidate genes and can eventually lead to the identification of the gene responsible for the crop trait located in that region. The more closely related a crop is to a model species, the easier it is to use such information and to identify homologous regions. In cereals and other grasses, both the chromosomal gene content and the order have been found to be very similar (Phillips & Freeling, 1998). A gene found in one species can be expected at a similar location in another species. This makes studies of the development of genomes from an evolutionary perspective possible, and it has been shown that the genomes of many cereals such as wheat, maize and sugar cane can be derived from the smaller genome of rice (Bennetzen and Ma, 2003; Ware and Stein, 2003).

For sugar beet, the most closely related model species is *Arabidopsis*, yet the two species come from completely different families. This makes a direct comparison between the two genomes, in search of larger homologous regions, difficult. However, this does not prevent the use of information on coding sequences in searching for genes responsible for traits mapped to a given region. Sequence information on genes in *Arabidopsis*, with functions related to a mapped trait, can be used to identify the homologous genes in sugar beet. This can be done by designing degenerated primers for the genes and using these on the sugar beet genome itself to amplify and sequence the homologous genes. The sequence information from *Arabidopsis* can also be used to search for homologues among the sequences in expressed-sequence-tag (EST) libraries of sugar beet. Once sugar beet homologues have been identified, specific primers can be designed and be used to map the genes. If a gene maps to the same region as a trait, the gene is considered a candidate and can be submitted to further testing to confirm its being responsible for the trait.

In most cases, the observable phenotype is the result of interactions, however, both between the genetic components themselves, and between the genetic components and the environment in which they act. In many cases, therefore, identification of the genes responsible for a trait and the development of assays for these genes will be unable to replace a phenotypic test. A major challenge will be to try to discover such interactions and identify the genetic and environmental components involved, which these are and how they act. From

a breeding perspective such knowledge would facilitate the development of varieties specially designed for specific environments.

This thesis

The studies included in the thesis were all carried out for the purpose of employing molecular markers in the breeding of sugar beet. In the following section, each of the studies is summarised briefly.

Study I. Characterising DNA-sequences from a Beta vulgaris library

In this study DNA sequences from a *Beta vulgaris* library were characterized to evaluate the feasibility of using such genomic sugar beet DNA sequences as RFLP-markers in marker-assisted backcrossing and comparative mapping.

A total of 32 sugar beet DNA sequences were analysed for their ability to cross-hybridize at two different hybridization stringencies to DNA from 40 accessions representing 13 different species and subspecies. Of the accessions used, 34 were wild or cultivated beets from the same family (*Chenopodiaceae*), genus (*Beta*) and section (*Beta*) as sugar beet, two were from the same family and genus as sugar beet but from another section (*Procumbentes*), two were from genus *Atriplex* within the same family as sugar beet, and two of the accessions were from completely unrelated genera (*Zea mays* and *Arabidopsis thaliana*). The DNA-sequences selected had been categorized as being single-copy non-polymorphic (4), single-copy polymorphic (17), low-copy repeated (8), or high-copy repeated (3).

It was found that at the medium hybridization stringency, 12 of the sequences cross-hybridized exclusively to the accessions within the section *Beta*, 16 sequences also cross-hybridizing to accessions within the section *Procumbentes*, and 13 sequences also cross-hybridizing to *Atriplex* accessions. At the lower hybridization stringency, 94% of the single-copy polymorphic sequences cross-hybridized to the *Beta* and *Procumbentes* beets. The high levels of polymorphisms found among the wild and cultivated beets and the considerable amount of variation among the *Procumbentes* and *Atriplex* accessions indicated marker-assisted backcrossing and comparative mapping to be feasible when using genomic sugar beet

DNA-sequences as RFLP-markers. A total of 87 genomic sugar beet DNA-sequences, 58 of which had been characterized as being single-copy polymorphic, were also used in a Norhern analysis on RNA from a tissue at one particular developmental stage to estimate the proportion of transcribed sequences in the *Beta vulgaris* library used. Altogether, 17 of these clones detected transcripts.

Study II. Investigating the genetic variation among wild and cultivated beets

In this study, wild and cultivated beets of the section *Beta* were compared in terms of genetic variation to investigate whether genetic variation was less within the breeding pool, due to the recent and narrow origin of sugar beet as a crop.

A total of 32 DNA-sequences were used for this purpose in RFLP-analysis. The DNA-sequences were chosen on the basis of a previous polymorphic screen, 21 of which were single-copy polymorphic, 8 low-copy repeated, and 3 high-copy repeated.

Altogether, 351 bands were scored over all accessions and this data was used to calculate genetic distances between all pairs of accessions. These distance estimates were then used in a cluster analysis to produce a dendrogram of genetic distances. The analysis separated the cultivated beets from the wild ones, and defined the fodder beets as a cluster within the sugar beets. It also revealed a considerable amount of variation among the sugar beet breeding lines as compared with the wild beets accessions.

Study III. Constructing a high-density RFLP-linkage map

In this study, a high-density RFLP linkage map of sugar beet was constructed for the purpose of forming a basis for backcrossing and the localisation of genes that influence important traits, as well as for most of the applications described above. During the mapping process, markers to two resistance traits were also linked.

Two different F2-populations, containing 222 and 133 individuals respectively, were used to map over 400 RFLP-markers. The two data sets obtained were integrated into a single map using the JOINMAP computer software (Stam, 1993), 90 markers being common to both data sets. The common markers were also used to investigate how often markers were mapped in

the same order in both populations. For markers separated by more than two cM the marker order was found to be highly reliable. The error rate of the overall process was estimated to be 0.3% by independent repetition of the analysis for 41 of the markers.

The integrated map consisted of 413 markers distributed over nine linkage groups containing between 21 and 62 markers. The total map length was 621 cM and the average distance between markers was 1.5 cM. The map shows an exceptional clustering of markers along the linkage groups, 44% of the markers being located in areas having a marker density of more than five markers per two cM. Only four gaps larger than 20 cM were found, the largest gap being 29.6 cM.

Study IV. Accumulating RAPD-markers near a locus for beet cyst nematode resistance

In this study, bulked segregant analysis was used to accumulate RAPD-markers near a locus for resistance to beet cyst nematodes in sugar beet, so as to generate a marker close to the gene responsible for the trait and develop a simple trait selection assay for the trait.

Graphical genotype information from study III was used to select individuals having recombination events close to a beet cyst nematode resistance gene mapped to one end of linkage group 2. Four pools of DNA were created, each containing five unique individuals. Two of the DNA pools contained individuals homozygous for the resistant allele, the other two pools containing individuals homozygous for the susceptible allele. The pools were created in such a way that only the target region was represented exclusively by the one or the other of the parental genotypes.

The four pools were analysed with 668 RAPD-primers and a total of 44 candidate markers that were easy to score and in which polymorphisms between the two pool types were evident, were selected for confirmation of linkage. To confirm linkage to the target region, the corresponding primers were assayed on the DNA from 14 individuals in which the recombination events were located at different distances from the target region. Linkage was confirmed for 19 bands or 17 markers (in two cases two bands displayed a co-dominant inheritance and were considered to be a single marker). Four of the markers were mapped. Three of them were in coupling phase with the resistant allele, whereas one was in coupling phase with the susceptible allele. The markers extended the existing map by 3 cM, the most

closely linked marker being mapped 0.2 cM from the beet cyst nematode resistance locus.

Study V. Investigating the inheritance of Cercospora leaf spot resistance

In this study, the inheritance of Cercospora leaf spot resistance in sugar beet was investigated by means of QTL analysis so as to map some of the most important factors influencing the trait and, if possible, to develop marker assays for the most important factors.

A segregating F2-population of 237 individuals was used to map 622 AFLP- and 48 RFLP-markers. For the QTL analysis genotypic data for 221 AFLP- and 46 RFLP-markers, and phenotypic data for 204 individuals was employed. The reason for the drastic reduction in the number of AFLP-markers used in the QTL analysis is that there was a strong clustering of the markers. Thus, many markers were found to be redundant when the scaffold of markers for the QTL analysis was selected.

In the QTL analysis, interval mapping identified QTL on four of the linkage groups, 1, 2, 3 and 9. Further analysis using composite interval mapping identified a QTL at one end of linkage group 3, and also revealed the presence of additional peaks. RFLP-markers closely linked to the QTL on linkage groups 1, 2 and 9, as well as to the QTL at the end of linkage group 3, were used as variables in a multiple regression model. Interval mapping of the residuals obtained from the model identified one additional peak 35 cM from the first, on linkage group 3, permutation analysis showing this additional peak to be highly significant. The markers most closely linked to the QTL that were identified were used to estimate the variance components of the QTL. Each locus accounted for 7-18% of the total phenotypic variation, leaving approximately 37% unexplained.

Study VI. Investigating the inheritance of restoration of Owen cytoplasmic male sterility

In this study, the genetic basis for the restoration of Owen cytoplasm male sterility was investigated by means of QTL analysis.

Three segregating populations were employed: two large populations, A and B, and one small, C. The phenotypes were evaluated on offspring from crosses between F2 individuals and an annual male sterile line. In the marker analysis, all the RFLP-markers used were

selected from an existing map (Halldén *et al.*, 1996a). The markers were selected as scaffolds of evenly distributed markers. Denser marker scaffolds were selected for linkage groups III and IV corresponding to linkage groups III and I, respectively, in the map of Halldén et al. (1996a). The reason for this was that previously published results had located restorer loci on the corresponding chromosomes (Schondelmaier & Jung, 1997). In one case, the marker analysis was carried out on F2-individuals, represented by population B, whereas in the other cases it was carried out on bulks of individual F3-families.

Population A, consisting of 187 F3 families, was analysed using 51 RFLP-markers. Following the initial mapping, QTL analysis using composite interval mapping detected a QTL on linkage group IV. Further analysis revealed another QTL on that linkage group, 15 cM from the first. Together, the two QTL explained 79% of the phenotypic variation. There was also a weak signal from one end of linkage group III, but this peak was not significant.

Population B, consisting of 339 F2-individuals, was analysed using 42 RFLP-markers. Following the initial mapping, mapping, QTL analysis using composite interval mapping detected a very strong QTL on linkage group III, one which explained 72% of the phenotypic variation. Further analysis revealed a weak signal from linkage group IV, although this peak was not significant.

Population C, consisting of 63 F3 families, was analysed using 6 RFLP-markers which covered the regions on linkage groups III and IV in which QTL had been detected in populations A and B. In the QTL analysis, linear regression was used for single marker analysis, detecting a QTL on linkage group IV, which explained 57% of the phenotypic variation. Further analysis revealed a weak signal from linkage group III, although this peak was not significant.

In summary, the QTL analyses detected QTL for restorer loci on linkage groups III and IV. In addition, the insignificant peaks that were detected all corresponded to the positions of a QTL that was detected in one of the other populations. The terminal position of the QTL on linkage group III corresponded well to the position of the restorer gene *X* mapped by Pillen et al. (1993). One or both of the QTL on linkage group IV could then correspond to the other restorer gene in Owens model, restorer gene *Z* (Owen, 1942a; Owen, 1942b; Owen, 1945).

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