Towards efficient improvement of greenhouse grown roses: genetic analysis of vigour and powdery mildew resistance

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Chapter 1

General introduction

Roses and their uses

Rose has been admired for its beauty and fragrance since its first cultivation 5000 years ago by ancient civilizations of China, Western Asia and Northern Africa (Gudin 2000). After selection and breeding for thousand years, especially after the first Hybrid tea roses were bred, rose has become one of the most economically important ornamental crops. It is cultivated today in gardens and alongside roads for decoration, in open fields for rose oil and hip production, and in greenhouses for production of cut and pot flowers.

The genus *Rosa* comprises more than hundred botanical (wild) species, of which only about ten (Crespel and Mouchotte 2003) contributed to the development of cultivated roses: *R. chinensis, R. foetida, R. gallica, R. gigantean, R. moschata, R. multiflora, R. phoenicea, R. rugosa, R. wichurana* and *R. rubra*. Most of the roses grown today are not true species but are derivatives of interspecific hybridization (Zhang 2003), leading to a wide diversity among cultivated roses.

A diploid plant of the genus *Rosa* contains seven pairs of chromosomes. In this genus polyploidy occurs frequently in wild as well as in cultivated roses. The majority of the wild species are diploid, whereas most cultivated roses are tetraploid or triploid (Crane and Byrne 2003). The rose genome consists of fairly small chromosomes and has an average DNA content of 1.1pg/2C in diploid roses (Yokoya et al. 2000). The genome is about four times larger in size than that of *Arabidopsis thaliana* (Debener and Mattiesch 1999; Rajapakse et al. 2001).

Cultivation and economic aspects

Roses are cultivated outdoors and indoors on all continents except Antarctica. The current world production under protected cultivation is annually about 8500 ha with 15-18 billion stems for cut rose (Blom and Tsujita 2003) and 60-80 million pots for pot rose (Pemberton et al. 2003). The main production of cut roses and pot roses is concentrated in areas with a suitable climate and the availability of sufficient skilled labour. In recent years cut rose and pot rose production has shifted from developed countries, for example The Netherlands and USA, to developing countries, for example Kenya, Zimbabwe and Ecuador, where costs of facilities and labour are lower. The light availability in those countries is greater especially during the winter and spring seasons in Europe

when the demand for roses is relatively high (Pemberton 2003). However, in Northern European countries such as The Netherlands and Denmark, the cut roses and pot roses produced in greenhouses still are the main source of rose products present on the market. The production area of roses in greenhouses in The Netherlands was 853 hectares in 2003, which is 15 % of the greenhouse area in use for cultivation of floricultural crops (Anonymous 2004).

Breeding of roses

Rose breeding is mainly performed through conventional cross breeding supported by technologies such as mutation induction, embryo rescue, ploidy level manipulation, gene transformation and molecular markers. About 25-30 highly competitive international companies and many amateur breeders are involved in the development of new rose cultivars (Gudin 2003). More than 20,000 modern cultivars have been registered today (Crespel and Mouchotte 2003; Leus et al. 2004).

In breeding programmes, different breeding goals, breeding strategies and gene pools are used to develop cultivars for specific uses (Guidin 2003), for example for cut roses (Chaanin 2003), pot roses (De Vries 2003), scent roses (Verhoeven et al. 2003), garden roses and rootstocks (Guidin 2003). The emphasis in breeding in the past used to be on ornamental characters like flower colour, scent and morphology, recurrent blooming and plant habit. In recent years criteria like disease resistance against the major pathogens and pests, frost tolerance in garden roses, productivity and vase life for cut roses, and shelf life and plant habit for pot roses have become increasingly important.

Some studies have been conducted to reveal the inheritance of traits like flower morphology, moss character, dwarf phenotype, prickles and important disease resistances (Table 1). These results have been helpful for rose breeding. However, some results need verification since some studies are based on low numbers of offspring and a lack of repeated experiments and statistical analysis (Debener 2003).

Molecular markers and their use

The advent of molecular markers made it possible to detect specific genes or chromosome regions controlling important traits. This helps to understand the structural organization and function of the genes, and provides information for marker-assistant selection in rose breeding. A variety of molecular markers are available in roses: RFLPs (restriction fragment length polymorphisms), RAPDs (randomly amplified polymorphic DNAs), AFLPs (amplified fragment length polymorphisms), SSRs (simple sequence repeat or microsatellites) and SCARs (sequence characterized amplified regions), etc. (reviewed by Rajapakse 2003a).

Genetic studies of roses are limited due to their open-pollinating mating system and difference in ploidy level. Inbred lines that could serve as parents of a classical mapping population are not

easily obtained. This complication is solved through “pseudo-testcross” strategy (Grattapaglia and Sederoff 1994), in which unrelated parents with a high degree of heterozygosity are crossed. The resulting mapping population is suitable for mapping of genes for the traits of interest. Theoretically, segregation of up to four alleles for diploid roses and up to eight alleles for tetraploid roses per locus is possible in the respective populations.

Molecular markers have been used in roses for genetic studies (Debener and Mattiesch 1999; Rajapakse et al. 2001; Crespel et al. 2002; Linde et al. 2004), cultivar identification (Esselink et al. 2003; Leus et al. 2004) and genetic diversity or phylogenetic studies (reviewed by Debener 2002). However, molecular marker –assisted breeding in rose still is in its infancy. Molecular genetic study of a trait of interest comprises phenotypic evaluation of the trait in a mapping population, construction of a genetic map for this population based on polymorphic molecular markers, mapping of quantitative trait loci (QTLs) for the trait, and possibly identification and cloning the genes underlying the QTLs.

Genetic mapping

The construction of a marker linkage map provides a tool to analyse the genetic variation for the

Table 1 Inheritance of traits in roses

Trait	Inheritance	Reference
Prickles on petioles	Single recessive	Rajapakse et al. 2001
Recurrent flowering	Single recessive	De Vries and Dubois 1984; Debener 1999; Crespel et al. 2002
Corolla	Single dominant	Debener and Mattiesch 1999; Crespel et al. 2002
Double flowers	Single dominant	Debener and Mattiesch 1999
Dwarf phenotype	Single dominant	Dubois and De Vries 1987
Moss phenotype	Single dominant	De Vries and Dubois 1984
Prickles on stems	Single dominant	Debener and Mattiesch 1999; Rajapakse et al. 2001
Resistance to black spot	Single dominant	Malek and Debener 1998
Resistance to powdery mildew	Single dominant	Linde and Debener 2003
	Quantitative	Zhang 2003
Yellow flower color	Single dominant	De Vries and Dubois 1984
Pink flower color	Single codominant	Debener and Mattiesch 1999
Prickle density	A major and a minor QTL	Crespel et al. 2002
Flower color	Quantitative	Zhang 2003
Leaf size	Quantitative	Zhang 2003
Petal number	Quantitative	Debener 2003; Zhang 2003
Prickle size	Quantitative	Zhang 2003
Winter hardiness	Quantitative	Svejda 1979

traits of interest present in the underlying mapping population. Markers on the same chromosomes may be linked while markers on different chromosomes show independent segregation. The relative distance between two markers is expressed by the recombination rate. A good map for a diploid rose contains seven linkage groups, each corresponding to one of the seven chromosomes, and ideally has good genome coverage with evenly distributed markers. Some genetic maps of rose have been constructed using different kinds of mapping populations and molecular markers: diploid rose with RAPD and AFLP markers (Debener and Mattiesch 1999; Crespel et al. 2002), tetraploid roses with AFLP and SSR markers (Rajapakse et al. 2001; Zhang 2003).

Mapping of QTLs

Mapping QTLs for a trait is looking for associations between quantitative variation of the trait in a mapping population and segregating markers. The finding of associations implies that the genomic regions in the vicinity of those markers harbour genes involved in the trait. QTL mapping provides the most likely position for each QTL, together with estimation for the effect of an allele substitution and confidence intervals for each QTL. In rose, QTL mapping has been performed for thorn quantity (Crespel et al. 2002), flower colour, number of flower petals, leaf size, prickle size and resistance to powdery mildew (Zhang 2003).

Critical problems in greenhouse production

From an economical and environmental point of view, a high energy input and control of powdery mildew are the most critical problems to deal with in the production of cut and pot roses in greenhouses in Northern Europe.

High energy use

Rose production in greenhouses requires a large amount of fossil fuel to heat and ventilate greenhouses and to provide light for plant growth. This is costly and results in a large contribution to the emission of CO_2, a greenhouse gas causing global warming. Therefore, the Dutch government agreed in 1997 to stimulate research to improve the energy efficiency of crop production in greenhouses. The goal set was a reduction by 65 % in 2010 compared to the energy used in 1980 (Korner 2003) by technical improvement of greenhouse production systems and genetic improvement of crops.

More efficient energy use in greenhouse production can be achieved through increase of plant production with the same energy input and/or decrease in energy consumption with the same production (Korner 2003). Greenhouse temperature is one of the important factors determining plant growth and development (Rijsdijk and Vogelezang 2000). Generally the development rate increases linearly with temperature from 10 to 25 ^{o}C and the optimal temperature is about 22 ^{o}C

(Blom and Tsujita 2003). The objective for plant breeding is the development of rose cultivars with a vigorous growth at lower temperatures to achieve the above-mentioned goal and to save energy. However, vigour is a complex plant characteristic that still is poorly understood. Increase of knowledge on the genetic variation and the heridity of vigour and its components will be of paramount importance for breeding energy-efficient rose cultivars.

Diseases

Several fungal pathogens adversely affect rose production in greenhouses. The most serious and widespread fungal disease is powdery mildew (*Podosphaera pannosa* var.*rosae*, syn. *Sphaerotheca pannosa*) (Linde and Shishkoff 2003). Other diseases such as *Botrytis* (*Botrytis cinerea*), black spot (*Diplocarpon rosae*) and downy mildew (*Peronospora sparsa*) also influence rose production negatively (Horst et al. 2003). Powdery mildew can infect the whole young, above-ground, parts of a plant giving white to grey white patches of powdery fungus. Powdery mildew affects the normal growth of rose plants, reduces the quality of flowers and even destroys plants.

Powdery mildew is an obligate parasite, i.e. it can only survive on a living host. It lives epiphytically on the outer surface of host plants with whitish hyphae (mycelium) that form pegs to penetrate epidermal cell walls and produce haustoria to absorb nutrients from leaf tissue. Vegetative hyphae produce chains (conidia) of spores at their tips, giving a powdery appearance to the infected leaves. Sexual spores are occasionally produced in colonies (Linde and Shishkoff 2003).

Wind and air condition are mainly responsible for the spreading of spores to other plants. Environmental conditions influence the germination of spores. The optimal conditions are a temperature of 22 ^{o}C and a relative humidity of 90 % (Xu 1999). Under moist condition conidia can withstand a long period of low temperature. Both spores and mycelia are sensitive to extreme heat and direct sunlight. Some reports mentioned that water might damage the viability of conidia and thus reduces infection by the pathogen (Wheeler 1973; Linde and Shishkoff 2003). However, leaf wetness in the first six hours after infection does not inhibit the germination of conidia (Linde and Shishkoff 2003).

The pathogenicity of isolates collected from outdoor-grown roses in different geographic regions (Leus et al. 2002, 2003) as well as isolates produced from single spore of the pathogen (Linde and Debener 2003) has been studied. The pathogenic races of powdery mildew are highly diverse worldwide (Mence and Hildebrandt 1966; Bender and Coyier 1984; Leus et al. 2002; Linde and Debener 2003).

In practice, rose is frequently treated with fungicide to control powdery mildew. About 40 % of all fungicides sprayed on roses are for this purpose (Linde and Shishkoff 2003), which is environmentally unfriendly and increases production cost. As there is an increasing public awareness of the negative impact of such chemicals, any reduction of use is welcome. The use of resistant cultivars is the best way. Several studies for this pathogen have shown that both horizontal

and vertical resistance exist in rose. Linde and Shishkoff (2003) reported that one or two major genetic factors as well as a few minor ones likely control resistance. One major genetic factor was found and denoted as *Rpp1* (Linde and Debener 2003; Linde et al. 2004). QTL analysis, however, showed that the resistance picture is far from clear (Zhang 2003). Molecular study of powdery mildew resistance will improve our understanding of the inheritance of the resistance of roses.

Scope of the thesis

The objectives of the research described in this thesis were to get insight into the inheritance of vigour and powdery mildew resistance in roses and to identify genes or QTLs for the traits of interest in order to enable marker-assisted selection in rose breeding.

To address the objectives, a diploid population (94/1) derived from the wild species *R. multiflora*, one of the ancestors of cultivated tetraploid roses, was used to study vigour. This population was in part molecularly genotyped (Debener and Mattiesch 1999). It was shown in a pilot study that the population harbour a large variation for growth vigour.

Genetic variation for powdery mildew was studied in cut rose at the tetraploid level, using a segregating population specifically made for this purpose. The two parents of the population were both partially resistant to powdery mildew. The use of the tetraploid population has potential difficulties in genetic mapping and QTL analysis due to the nature of tetrasomic inheritance.

The diploid population was molecularly characterized to produce dense genetic maps as a tool for dissecting the genetic variation for vigour (Chapter 2). In parallel, a screening method for assessment of various vigour-related traits was designed and tested in a pilot experiment. The method was subsequently used in greenhouse studies of the mapping population in Denmark and The Netherlands under suboptimal growth conditions to establish the genotypic variation present in the diploid population for each of the vigour components (Chapter 3). The integrated map and genotypic data were used for the analysis of the quantitative variation and the identification of quantitative trait loci (QTLs) for vigour and its components (Chapter 4).

The investigation into the genetic variation for powdery mildew resistance was performed on the tetraploid mapping population using two monospore isolates of the fungus. An inoculation method using spore suspensions was worked out and employed for monitoring the variation for resistance (Chapter 5). At the same time, the mapping population was molecularly genotyped. Multiple regression analyses of the resistance on marker genotypes were conducted to find markers linked to genes for resistance (Chapter 6). Impact of the overall results on rose breeding as well as some other aspects of the present study are addressed in Chapter 7.

Chapter 2

Construction of an integrated map of rose with AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers

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Abstract

A high-density genetic map with a number of anchor markers has been created to be used as a tool to dissect genetic variation in rose. Linkage maps for the diploid 94/1 population consisting of 88 individuals were constructed using a total of 520 molecular markers including AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers. Seven linkage groups, putatively corresponding to the seven haploid rose chromosomes, were identified for each parent spanning 487 cM and 490 cM, respectively. The average length of 70 cM may cover more than 90 % of the rose genome. An integrated map was constructed by incorporating the homologous parental linkage groups, resulting in seven linkage groups with a total length of 545 cM. The present linkage map is currently the most advanced map in rose with regard to marker density, genome coverage and with robust markers, giving good perspectives for QTL mapping and marker-assisted breeding in rose. The SSR markers, together with RFLP markers, provide good anchor points for future map alignment studies in rose and related species. Codominantly scored AFLP markers were helpful in the integration of the parental maps.

Introduction

Rose (*Rosa*) is the most important ornamental crop in the floriculture industry. The genus *Rosa* belonging to the Rosaceae family, includes more than 150 species and thousands of cultivars (Gudin 2000). Most modern roses do not belong to a simple rose species but are complex hybrids derived from about ten species (Gudin 2000; Zhang 2003). Wild species are often diploids ($2n = 2x = 14$) while almost all cultivated roses are tetraploids ($2n = 4x = 28$). Rose chromosomes are fairly small with an average DNA content of 1.1 pg/2C for diploid roses (Yokoya et al. 2000). The genome size is estimated to be about four times larger than that of *Arabidopsis thaliana* (Debener and Mattiesch 1999; Rajapakse et al. 2001). Despite the low chromosome number and small genome size, little is known on the genetics of rose (De Vries and Dubois 1996; Gudin 2000). This is largely due to characteristics like a high degree of heterozygosity, varying ploidy levels between species, difficulty in sexual reproduction, low seed set and poor seed germination. However, current advances in molecular genetic mapping have enhanced the understanding of rose genetics and the genes controlling important traits, including resistance to fungal diseases (Debener 2003; Rajapakse et al. 2001; Crespel et al. 2002; Von Malek et al. 2000; Kaufmann et al. 2003). Furthermore, the future availability of dense genetic maps will facilitate the identification of quantitative trait loci (QTLs), and provide markers for marker-assisted breeding, map-based cloning of genes and the introgression of beneficial genes from wild species into modern cultivars (Liebhard et al. 2003; Rajapakse 2003b).

The first molecular genetic linkage map for rose covering over 300 markers was published by Debener and Mattiesch (1999) using a diploid population derived from *Rosa multiflora* hybrids. Seven pairs of homologous linkage groups were identified with RAPD (randomly amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) markers. Genes controlling pink flower colour (*Blfa*) and double flower (*Blfo*) were localised. Debener and co-workers (2001a) extended their map with additional AFLP, SSR (simple sequence repeat or microsatellite), RFLP (restriction fragment length polymorphism) and SCAR (sequence specific amplified region) markers, and were able to map a resistance gene (*Rdr*1) to blackspot (*Diplocarpon rosae*). Rajapakse et al. (2001) developed two genetic maps based on a tetraploid population and identified genes for prickles and the enzyme malate dehydrogenase. Crespel et al. (2002) published an AFLP map based on a diploid population and localised genes controlling the number of prickles, double corolla and recurrent blooming. These maps so far have a medium marker density and provided initial tools for genetic research and marker-assisted breeding of roses (Rajapakse 2003b). For better comparison of different maps an advanced map for roses is needed with full genome coverage and also including a wide set of polymorphic PCR-based anchor markers. Codominant markers, such as SSRs and RFLPs, would allow alignment of homologous linkage groups between maps, and facilitate marker transfer across populations as well as across

related species.

AFLP markers have widely been used for map construction and map saturation in rose-related species such as peach (Sosinski et al. 1998) and apple (Maliepaard et al. 1998; Liebhard et al. 2003) as well as other crops (Haanstra et al. 1999; Chagne et al. 2002). The utility of AFLP markers was improved by the possibility to score them codominantly (Castiglioni et al. 1999; Piepho and Kogh 2000). Sequence-based markers, such as resistance gene analogues (RGA) markers that are based on the conserved sequences of leucine-rich repeats (LRRs) and nucleotide-binding sites (NBS), most likely lead to target genes for disease resistance. Sequences based on protein kinase (PK) motifs lead specifically to this class of genes which are involved in signal transduction processes in plants (Bent 1996; Van der Linden et al. 2004). The mapping of RGA and PK markers on linkage maps has been used as a candidate gene approach to identify genes and pseudo-genes with a possible role in the resistance mechanisms to various pathogens (Foolad et al. 2002; Quint et al. 2002; Mohler et al. 2002; Donald et al. 2002; Di Gaspero and Cipriani 2003).

The linkage maps for diploid rose presented in this chapter are composed of AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers. A number of markers, especially the developed SSRs, provide good anchor points on the maps for the alignment of diploid and tetraploid rose maps. The maps serve as an essential step towards a reference map of rose. This chapter gives a detailed description and discussion on the approach we took for construction of the map and the alignment of the parental and integrated maps.

Material and methods

Mapping population

A diploid rose population, 94/1, derived from a cross between 93/1-119 (P119) and 93/1-117 (P117) (Debener and Mattiesch 1999) and consisting of 88 individual genotypes, was used to generate the present genetic map. The parents are half sibs resulting from open pollination of a diploid genotype 81/42-15, originating from a cross between *Rosa multiflora* and unidentified garden roses. Genomic DNA of each genotype of the population was extracted from young leaves according to Esselink et al. (2003).

Marker analysis

AFLP marker analysis was performed as described by Vos et al. (1995) with some minor modifications using two restriction enzyme combinations, *Eco*RI/*Mse*I (E-M) and *Pst*I/*Mse*I (P-M). A total of 500 ng genomic DNA was used for each sample. Pre-amplification was performed with the E01/M02 and P01/M02 primers each containing one additional base (E01:-A, P01:-A and M02:-C). Selective amplification was carried out with primers that contained two (only in the *Pst*I

primers) or three (in the *Pst*I, *Eco*RI and *Mse*I primers) additional selective nucleotides. Forward *Pst*I and *EcoR*I primers were labelled with fluorophores (6FAM, HEX, NED) at the 5' ends. All PCRs were performed on a Perkin Elmer 9600 thermocycler (Perkin Elmer/Applied Biosystems) under standard conditions. Electrophoresis was performed on an ABI Prism 377 DNA Sequencer (Perkin Elmer) with 5 % denaturing polyacrylamide gels.

Semi-automated scoring of the amplified fragments was performed with the programs GeneScan® 3.1.2 and Genotyper® 2.5 (Perkin Elmer/Applied Biosystems). Polymorphic AFLP fragments with a clear segregation pattern, i.e. discriminative at ± 0.5 bp within a size range of 50-500 bp and a peak height (intensity of fragment) of more than 100, were selected, labelled and either dominantly scored as 0 (fragment absent) or 1 (fragment present), or codominantly scored as 0 (fragment absent) or 1 (homozygous fragment present) or 2 (heterozygous fragment present). In the case of codominant scoring, the peak heights of the segregating markers were scored, taking into account the peak heights of the flanking non-segregating markers.

Rose genomic DNA libraries enriched for dinucleotide and trinucleotide SSRs were constructed (Esselink et al. 2003). The clones were sequenced and primers were generated according to Esselink et al. (2003). The "Rh" SSR primers used in this study are available upon request from Plant Research International, The Netherlands. The "MicD" and "RMS" SSRs are available from the Federal Centre for Breeding Research on Cultivated Plants, Institute for Ornamental Plant Breeding, Germany.

Protein kinase profiling was performed according to the protocol described in Van der Linden et al. (2004) with some modifications. In brief, 400 ng genomic DNA of each sample was digested with *Mse*I then adapter ligated, followed by amplification of PK-specific fragments using a two-step PCR procedure. PCR products were radioactively labelled by primer extension using the [γ-^{33}P]ATP-labelled protein kinase primer and an adapter primer and separated on a 6 % polyacrylamide sequencing gel. Marker patterns were visualized by autoradiography.

A rose RGA library was established containing expressed and genomic rose RGAs according to Pan et al. (2000) with different degenerate primers based on conserved motives of NBS-LRR resistance genes. The clones were sequenced and specific primers for the RGAs were designed and used for genotyping of the population. PCR amplifications were performed with 25 ng genomic DNA in 25 µl assays containing 0.1 mM dNTPs, 0.5 µM of each primer (MWG Biotech AG, Ebersberg, Germany) and 0.5 U Taq DNA polymerase, in a buffer consisting of 10 mM Tris pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ and 0.01 % gelatine. The following PCR program was used: 5 min at 95 °C, then 35 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C, and 30 min at 72 °C for the final extension. Polymorphism of the PCR products was visualised by SSCP analysis on 0.5 x MDE gels (Slabaugh et al. 1997).

A total of 51 previously generated markers (Debener and Mattiesch 1999; Debener et al. 2001a) were chosen as bridge markers: 26 AFLP markers (coded as "AFLP", 2 markers on each parental

linkage group except on A6), 4 SSR markers (coded as “MicD”), 11 RFLP markers (coded as “RGF” or “BMA”), 6 SCAR markers (coded as “PAS”) and 4 morphological markers including pink flower colour (*Blfa*), double flower (*Blfo*), resistance to black spot (*Rdr*1) and powdery mildew (M*ehltau*).

Marker segregation type

Markers were divided into uni-parental markers, being markers heterozygous in either the female or the male parent, and bi-parental markers that showed heterozygosity in both parents. The coding of the marker segregation types is indicated in Table 1. Segregation types 1, 2 and 3 were scored dominantly while marker types 4, 5 and 6 were scored codominantly.

Map construction

JoinMap® version 3.0 (Stam 1993; Van Ooijen and Voorrips 2001) was used for linkage analysis and map calculations. After all the marker data had been imported, different sets of marker data were set up with a selection of marker loci by using the “excluding” function.

Parental maps were separately constructed by using different sets of marker data. Each parental linkage map was constructed using its uni-parental and common bi-parental markers. As for the bi-parental markers, separate data sets with and without type 3 markers were employed in order to enable the comparison of the marker orders before the less informative type 3 markers were added. JoinMap® used the defined marker data to perform a stepwise building of the map by adding one marker at a time, and to estimate the recombination frequencies between a given pair of markers that were then used to determine the linear arrangement of markers by minimising the number of recombination events in the data set (Stam 1993). The marker order in a linkage group was determined by calculation of the goodness-of-fit criterion to find the best fitting order and simultaneously calculating the map positions corresponding to that order (Stam 1993; Van Ooijen and Voorrips 2001). Linkage groups were determined using a LOD threshold of 5.0 and map construction was performed using the Kosambi mapping function with JoinMap parameter settings as follows: Rec=0.45, LOD=1.0, Jump=5, “first run” and “second run”. The “first run” option resulted in a stepwise build-up of a map by adding markers one by one with best “goodness-of-fit” for all markers. Subsequently, the “second run” option was applied in an attempt to add previously omitted markers to the map (Van Ooijen and Voorrips 2001). A “third round” option that enforces the mapping of problematic markers was not employed. Mapped markers were then inspected and some of the markers were removed when they showed insufficient linkage and conflict with other markers, e.g. those having low “pair count”, a high chi-square contribution ($\chi^2 \geq 3.0$) and markers evenly distributed per linkage group was employed prior to adding type 3 markers.

Integrated linkage groups were built up by “merging” the pair-wise marker data from homologous parental linkage groups having common anchor markers. The same parameters as

mentioned above were employed to test the robustness of the linkages. The resulting marker order was compared to that of the homologous parental linkage groups. In cases where the resulting order in the integrated map was clearly conflicting with that in either of the parental maps, the order in one parental linkage group was taken as a fixed (reference) order. The resulting linkage maps were drawn, the comparison of the integrated and the parental maps was performed by using MapChart software (Voorrips 2001).

Nomenclature of markers and linkage groups

Newly developed AFLP markers were coded according to Keygene's nomination system comprising a letter code for restriction enzyme, followed by a figure for the combination of selective nucleotides (Haanstra et al. 1999) and the size of the fragment in base pairs. SSR markers start with "Rh", or "RMS"; PK markers with "PK" and RGA markers with "RGA". The last digit of the marker represents the code of the segregation type (Table 1). The coding and orientation of linkage groups follows those of Debener and Mattiesch (1999).

Estimation of genome coverage

The proportion of the diploid rose genome covered by each of the parental maps was calculated (Stam, unpublished program) by repeated sampling of markers from the maps without replacement. The average map length covered by a single marker sample of a given size was first calculated. The average coverage of the maps was based on 20,000 samples. The asymptotic upper limit was estimated by increasing the sample size up to the actual number of markers in the maps and by fitting an exponential curve to the relation between sample size and average map length covered. The validity of this procedure has been verified extensively using simulated mapping data (Stam, unpublished results).

Results

Segregating markers

The mapping study comprised a total of 469 newly generated AFLP, SSR, PK and RGA markers, which are grouped in Table 1. AFLP analysis, based on 56 AFLP primer pairs, including 33 E-M and 23 P-M, resulted in 320 polymorphic markers (Table 1). Out of these markers 220 were uni-parental and 100 bi-parental. From the bi-parental markers 11 were codominantly scored.

From the enriched SSR libraries, 149 new clones were sequenced and primers were designed based on their flanking sequences. Of these primers 58 showed correct amplification and were added to the "Rh" SSR primer database at Plant Research International, The Netherlands. A set of 42 primer pairs from the database and 16 "RMS" SSR primer pairs showed polymorphisms in the

present population and resulted in 74 polymorphic markers (Table 1), 26 of which could be scored codominantly. The size of the alleles found for a subset of the mapped SSR markers in the present population as well as the primers needed to generate the markers are shown in Table 2. The numbers of alleles detected in the present population ranged from two to four.

Protein kinase profiling resulted in 24 PK markers, using a gene-specific primer based on a protein kinase specific variant. A total of 51 RGA markers were generated with 32 RGA primer pairs. Both PK and RGA markers were dominantly scored.

Different segregation types were assigned for the markers (Table 1). Among the 469 markers, 328 markers were uni-parental, of which 160 (34 %) were derived from parent P117 (type 1) and 168 (36 %) from parent P119 (type 2). The remaining 141 markers showed a bi-parental inheritance, of which 104 (22 %) were dominantly scored (type 3) and 37 (8 %) were codominantly scored (types 4, 5 and 6).

Before the map was constructed all the markers were subjected to a chi-square test using expected segregation ratios given by the JoinMap® program. Of all markers, 101 (22 %) showed distorted segregation ($P \leq 0.05$, chi-square test). Of these markers, 64 were derived from P117, 14 from P119 and 23 from both parents, of which 15 were type 3 markers, indicating that the distorted markers mainly originated from parent P117. These distorted markers were included in the linkage analysis since the segregation distribution hardly effects the estimation of recombination frequency. The remaining markers with distorted segregation are labelled with asterisks on the map (Figure 1).

Table 1 Markers grouped by marker type and segregation type. Segragation type: *ab*x*aa* (type 1) for markers segregating only in paternal P117 with two alleles; *aa*x*ab* (type 2) for markers segregating only in maternal P119 with two alleles; *ab*x*ab* (type 3) for markers segregating in both parents with two alleles; *ab*x*cd* (type 4) for markers segregating in both parents with four alleles; *ab*x*ac* (type 5) for markers segregating in both parents with three alleles and *cd*x*cd* (type 6) for markers segregating in both parents with two alleles. Segregation types 1, 2 and 3 are dominantly scored markers, the rest are codominantly scored markers. PRI: Plant Research International B.V., Wageningen University and Research Centre, The Netherlands. BAFZ: Institute for Ornamental Plant Breeding, Ahrensburg, Germany.

Marker type	Uni-parental		Bi-parental markers				Total	Source
	abxaa	*aaxab*	*abxab*	*abxcd*	*abxac*	*cdxcd*		
	1	2	3	4	5	6		
AFLP	107	113	89	0	0	11	320	PRI
PK	8	7	9	0	0	0	24	PRI
SSR	18	24	6	4	18	4	74	PRI/BAFZ
RGA	27	24	0	0	0	0	51	BAFZ
Total	160	168	104	4	18	15	469	

Parental maps

A total of 520 markers including 469 newly generated AFLP, SSR, PK, RGA and 51 previously developed AFLP, SSR, RFLP, SCAR and morphologic markers were employed for the construction of the genetic linkage maps. Parental maps were first generated with uni- and (codominantly scored) bi-parental markers. Each map consisted of seven linkage groups, putatively corresponding to the seven rose chromosomes. Subsequently, the type 3 markers were added to the parental maps a second mapping effort. Fixed marker orders based on 5-7 markers per linkage group from the first mapping attempt were used to give extra weight to the most informative markers. For the P119 map, 27 markers were eliminated and 44 were not assigned to linkage groups. Out of the segregating markers from P117, a total of 17 markers were excluded from mapping and 39 remained unmapped. The resulting parental maps covered a total length of 490 and 487 cM in the paternal P117 (A) and maternal P119 (B), respectively, with an average chromosome length of ~ 70 cM (Table 3 and Figure 1).

Table 2 A selection of the SSR markers used for mapping. The sizes of DNA fragments (in base pairs) as well as chromosome location (LG) of the SSR markers are indicated. The annealing temperature was 50 ^{o}C for all the primers. Additional SSR information is available upon request from the authors.

SSR	Forward (5'--3')	Reverse (5'--3')	Size (bp)	LG
Rh79	ttcttcttgctcgccattttgatt	gaacgtccaccaccacccactctg	135, 147, 149	1
RhAB9-2	gtcaatttgtgcataagctc	gtgagaacagatgagaaatg	101, 108, 124	1
Rh48	gatagtttctctgtaccccaccta	ttgaccagctgcaacaaaattaga	99, 107	2
Rh80	catgccaaacgaaatgagtta	ttatctaaagggctgctgtaagtt	134, 148	2
Rh96	gccgatggatgccctgctc	agattccctgcgacattcacattc	267, 276, 294	2
RhB510	aaacgataggtgaatctgtgggt	cactcaaccttgtccactcctaat	159, 161	2
Rh50	tgatgaaatcatccgagtgtcag	tcactttcattggaatgccagaat	343	3
Rh58	acaatttagtgcggatagaacaac	ggaaagcccgaaagcgtaagc	269	3
Rh59	cgcggatgaagctagtgaatcagt	ctagcccatctcagtatccctcacc	197, 200, 216	3
RhABT12	caagtttgtctccttggacc	catagatgattatcctagagcc	166, 172, 180	4
Rh65	agtacgccgacgcagatccagtga	acggcgttgtaggtcgtcattctc	128, 130, 132	4
Rh78	aaagaaacgcgaaatctatgatgc	tctggatgggatttaaaagacagg	216, 250	4
Rh77	caactgaaaggaacaaatggatgt	ggaatggcttgtaaatttgtgatt	262	5
Rh93	gctttgctgcatggttaggttg	ttctttttgtcgttctgggatgtg	251, 273, 275	5
RhAB38	gaggtggtcgattccatgtc	ttaccgttctacctaagtgactaac	149,173,190	5
Rh60	tctcttttcacggccaccact	tgaatccaaggccgtatagttaga	234, 240, 252	6
Rh85	acttttgggcgttcatcgcattacac	ggctatatgggctcaagtctagacaa	221	6
Rh98	ggcctctagagtttgggatagcag	acgacgtcaataactccatcagtc	121	6
Rh72	ccaaaagacgcaaccctaccataa	tcaaaacgcatgatgcttccactg	285	7
Rh73	ggttagacgggtggaagaag	actgccgatagaagtatttcatca	160, 162, 172	7
RhAB28	gcagatgttattcatgttaa	ccaagtattttagtttcttc	171, 175	7

Integrated map

Homologous linkage groups were identified with the help of common markers and integrated using the JoinMap® program. Markers on the linkage map were checked and excluded when they did not meet the same criteria as those used for construction of the parental maps. The markers mapped in the integrated map tend to have the same marker order as in corresponding parental maps. Only a few cases of conflicting marker order were found, like those in linkage group 2. This was solved by using a "fixed (marker) order". The final outcome was a map with seven integrated linkage groups (Figure 2) having a total length of 545 cM and an average chromosome length of 78 cM.

Distribution of markers

All parental linkage groups contained uni- and bi-parental markers, except for A6 which had only bi-parental markers. For the P117 map, 323 markers were employed in the linkage analysis and 271 (84 %) of them could well be assigned to seven linkage groups, which had 14 to 56 markers, a chromosome length ranging from 51 to 91 cM and a marker density of 0.3 to 0.9 markers per cM (Table 3). For the P119 map, 338 markers were used and finally 273 (81 %) of these could be assigned to seven linkage groups, each containing 24 to 52 markers, with a map length of 58 to 90 cM and a marker density of 0.4 to 0.7 markers per cM (Table 3).

Markers were randomly distributed with high and moderate density on the 14 parental linkage groups, but with a number of gaps ranging from 5.7 to 14.9 cM (Table 3). The clustering of markers was most prominent in the centre of the linkage groups, especially on linkage groups A1, A2, A4, A7, B1, B2, B4, B6 and B7. These locations presumably coincide with the centromeric regions. Bi-parental (common) markers co-localized well on the homologous parental linkage maps except small variations in the order of markers, especially in the marker-dense regions.

Table 3 Distribution of markers on parental maps (A and B) and linkage group statistics

Marker type	Linkage group														Total
	A1	B1	A2	B2	A3	B3	A4	B4	A5	B5	A6	B6	A7	B7	
AFLP	26	35	36	26	13	17	38	33	23	13	12	23	33	32	360
PK	0	0	2	4	2	4	3	1	6	4	0	0	0	0	26
SSR	5	3	9	17	5	3	10	5	7	5	2	7	4	6	88
RGA	3	6	2	1	0	0	2	1	4	0	0	1	5	4	29
Others	6	6	3	4	3	3	3	2	2	2	0	2	2	3	41
Total	40	50	52	52	23	27	56	42	42	24	14	31	44	45	542
Length (cM)	64	67	84	90	54	58	65	65	91	62	51	77	81	68	977
Marker density (markers/cM)	0.6	0.7	0.6	0.6	0.4	0.5	0.9	0.6	0.5	0.4	0.3	0.4	0.5	0.7	
Average distance between markers (cM)	1.6	1.3	1.6	1.7	2.3	2.1	1.2	1.5	2.2	2.6	3.6	2.5	1.8	1.5	
Largest gap between markers (cM)	7.3	14.9	11.6	8.4	7.9	5.9	12.3	7.9	8	6.9	7.9	9.1	10.4	5.7	

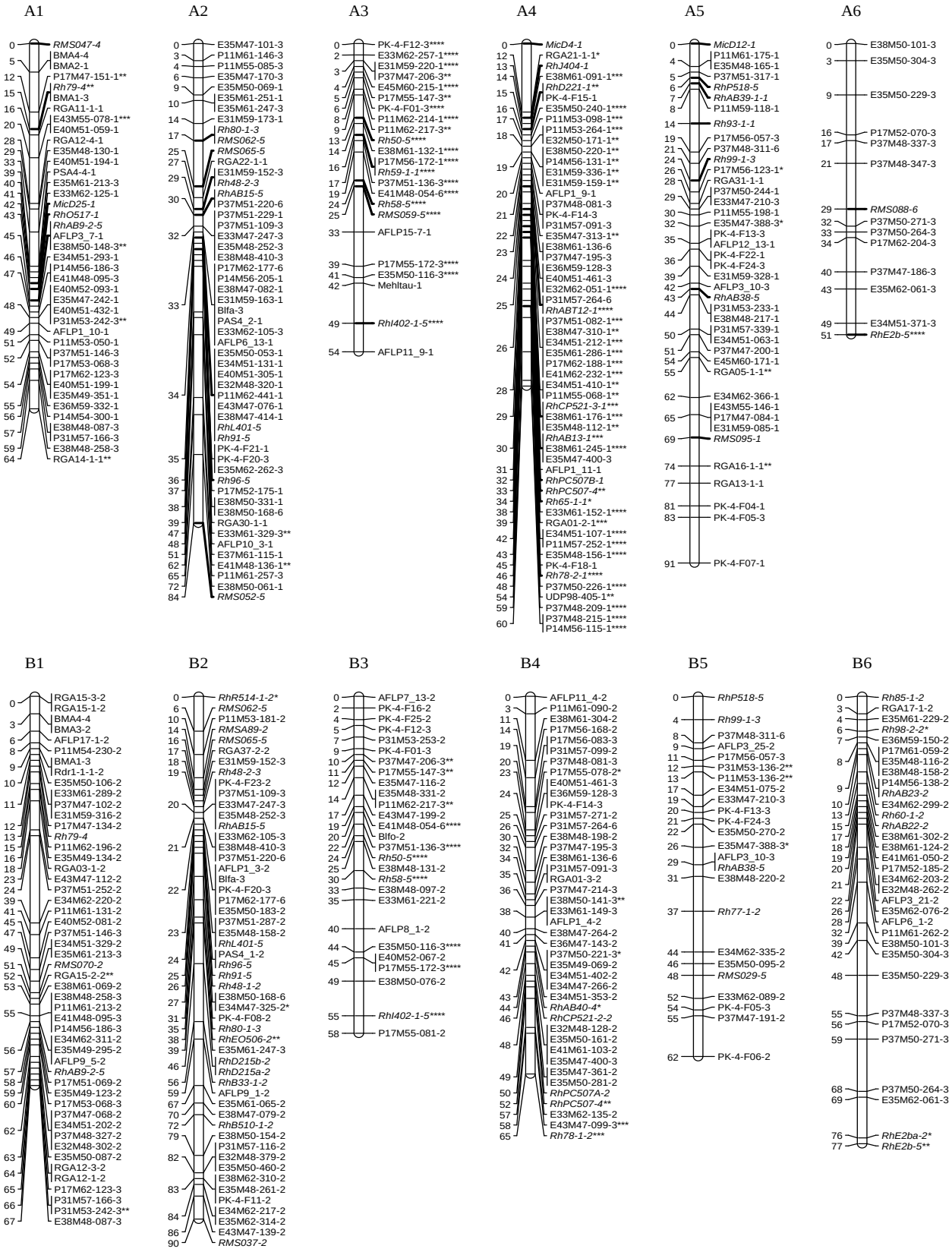
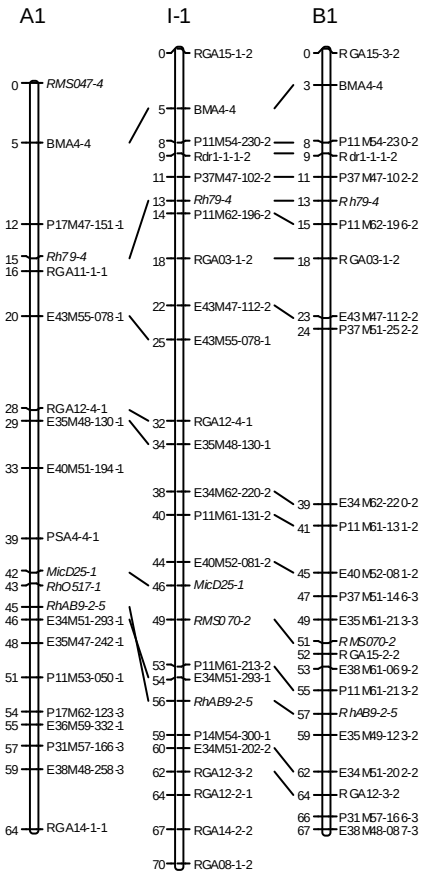


Figure 1 Genetic linkage map of diploid rose progeny of parents P117 (A) and P119 (B). Linkage groups are numbered from A1 to A7 and from B1 to B7 according to Debener and Mattiesch (1999). Marker names are indicated at the right of each linkage group. Distances are in cM at the left of each linkage group. SSR markers are printed in italic. Segregation distortion is indicated with significance of chi-square test: * ≤ 0.05, ** ≤ 0.01, *** ≤0.005, **** ≤ 0.001.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B1
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16 RGA11-1-1
20 E43M55-078-1
28 RGA12-4-1
29 E35M48-130-1
33 E40M51-194-1
39 PSA4-4-1
42 MicD25-1
43 RhO517-1
45 RhAB9-2-5
46 E34M51-293-1
48 E35M47-242-1
51 P11M53-050-1
54 P17M62-123-3
55 E36M59-332-1
57 P31M57-166-3
59 E38M48-258-3
64 RGA14-1-1
0 RGA15-1-2
5 BMA4-4
8 P11M54-230-2
9 Rdr1-1-1-2
11 P37M47-102-2
13 Rh79-4
14 P11M62-196-2
18 RGA03-1-2
22 E43M47-112-2
25 E43M55-078-1
32 RGA12-4-1
34 E35M48-130-1
38 E34M62-220-2
40 P11M61-131-2
44 E40M52-081-2
46 MicD25-1
49 RMS070-2
53 P11M61-213-2
54 E34M51-293-1
56 RhAB9-2-5
59 P14M54-300-1
60 E34M51-202-2
62 RGA12-3-2
64 RGA12-2-1
67 RGA14-2-2
70 RGA08-1-2
0 RGA15-3-2
3 BMA4-4
8 P11M54-230-2
9 Rdr1-1-1-2
11 P37M47-102-2
13 Rh79-4
15 P11M62-196-2
18 RGA03-1-2
23 E43M47-112-2
24 P37M51-252-2
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45 E40M52-081-2
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49 E35M61-213-3
51 RMS070-2
52 RGA15-2-2
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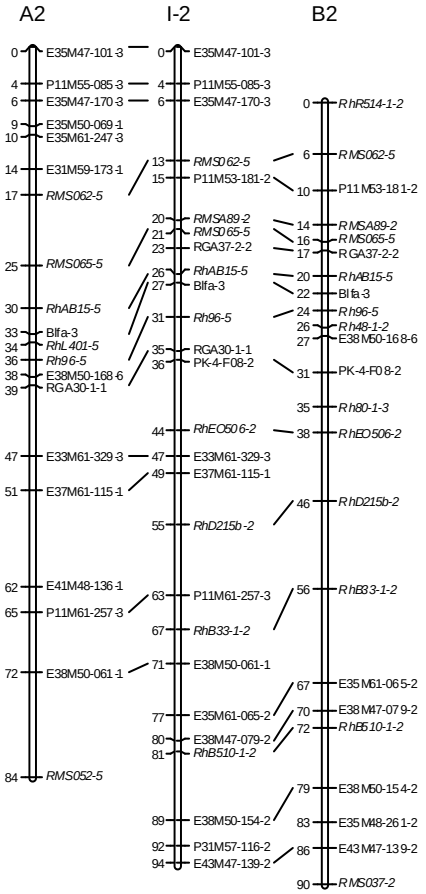
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9 E35M50-069-1
10 E35M61-247-3
14 E31M59-173-1
17 RMS062-5
25 RMS065-5
30 RhAB15-5
33 Blfa-3
34 RhL401-5
36 Rh96-5
38 E38M50-168-6
39 RGA30-1-1
47 E33M61-329-3
51 E37M61-115-1
62 E41M48-136-1
65 P11M61-257-3
72 E38M50-061-1
84 RMS052-5
0 E35M47-101-3
4 P11M55-085-3
6 E35M47-170-3
13 RMS062-5
15 P11M53-181-2
20 RMSA89-2
21 RMS065-5
23 RGA37-2-2
26 RhAB15-5
27 Blfa-3
31 Rh96-5
35 RGA30-1-1
36 PK-4-F08-2
44 RhEO506-2
47 E33M61-329-3
49 E37M61-115-1
55 RhD215b-2
63 P11M61-257-3
67 RhB33-1-2
71 E38M50-061-1
77 E35M61-065-2
80 E38M47-079-2
81 RhB510-1-2
89 E38M50-154-2
92 P31M57-116-2
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6 RMS062-5
10 P11M53-181-2
14 RMSA89-2
16 RMS065-5
17 RGA37-2-2
20 RhAB15-5
22 Blfa-3
24 Rh96-5
26 Rh48-1-2
27 E38M50-168-6
31 PK-4-F08-2
35 Rh80-1-3
38 RhEO506-2
46 RhD215b-2
56 RhB33-1-2
67 E35M61-065-2
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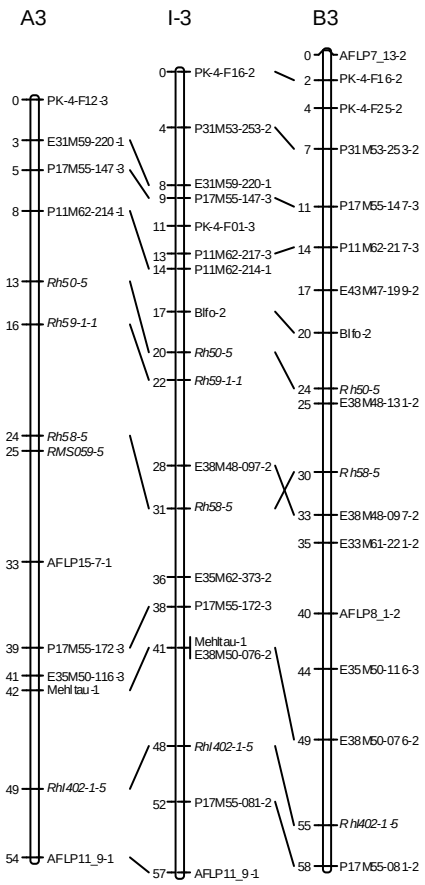
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B3
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16 Rh59-1-1
24 Rh58-5
25 RMS059-5
33 AFLP15-7-1
39 P17M55-172-3
41 E35M50-116-3
42 Mehltau-1
49 Rhl402-1-5
54 AFLP11_9-1
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4 P31M53-253-2
8 E31M59-220-1
9 P17M55-147-3
11 PK-4-F01-3
13 P11M62-217-3
14 P11M62-214-1
17 Blfo-2
20 Rh50-5
22 Rh59-1-1
28 E38M48-097-2
31 Rh58-5
36 E35M62-373-2
38 P17M55-172-3
41 Mehltau-1
E38M50-076-2
48 Rhl402-1-5
52 P17M55-081-2
57 AFLP11_9-1
0 AFLP7_13-2
2 PK-4-F16-2
4 PK-4-F25-2
7 P31M53-253-2
11 P17M55-147-3
14 P11M62-217-3
17 E43M47-199-2
20 Blfo-2
24 Rh50-5
25 E38M48-131-2
30 Rh58-5
33 E38M48-097-2
35 E33M61-221-2
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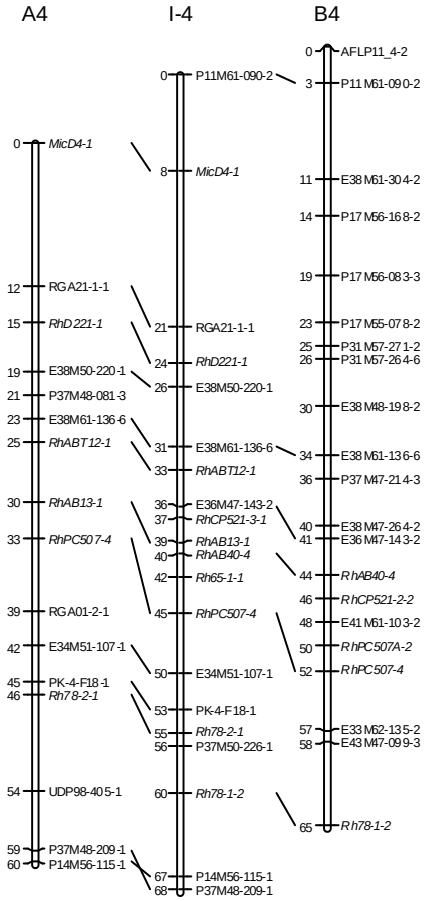
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25 RhABT12-1
30 RhAB13-1
33 RhPC507-4
39 RGA01-2-1
42 E34M51-107-1
45 PK-4-F18-1
46 Rh78-2-1
54 UDP98-405-1
59 P37M48-209-1
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0 P11M61-090-2
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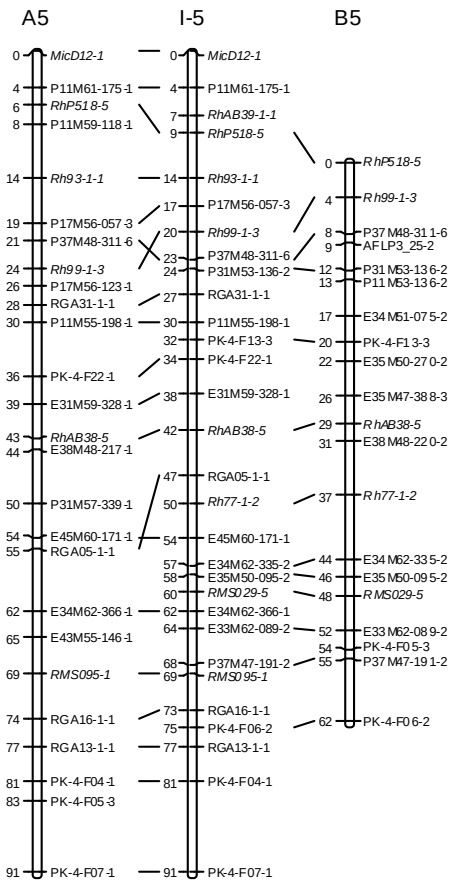

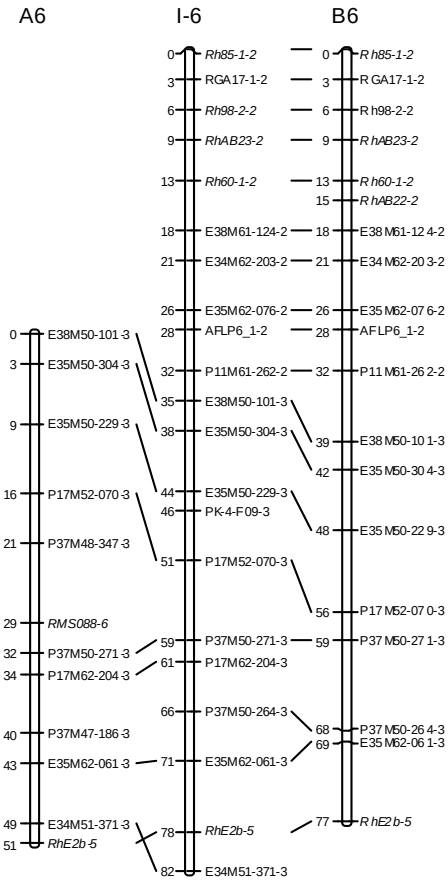

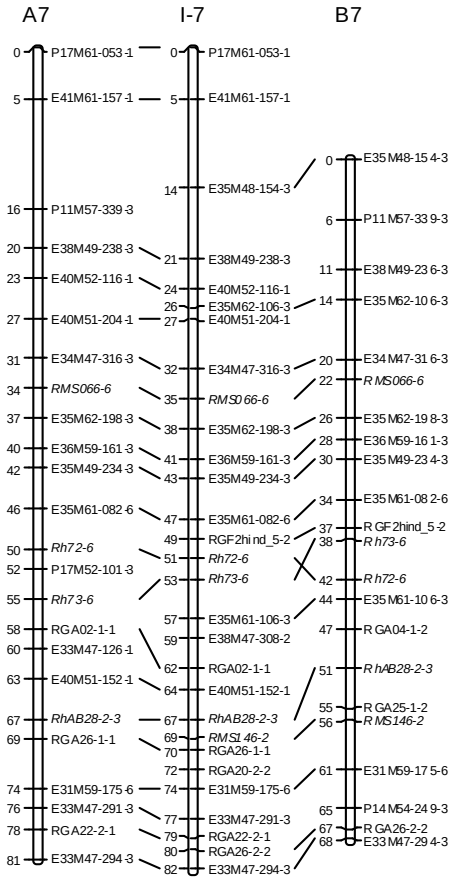

Figure 2 Alignment of parental (A and B) and integrated (I) maps. For ease of survey only the markers at a distance of ~ 2 cM are shown on each linkage group. Marker names are indicated at the right of each linkage group. For details of marker nomenclature see “Materials and Methods”. Distances are given in Kosambi cM at the left of each linkage group. SSR markers are printed in *italic*. Segregation distortion is indicated for the significance level of the chi-square test: * ≤ 0.05, ** ≤ 0.01, *** ≤0.005, **** ≤ 0.001. Corresponding markers are indicated by solid lines between maps.

Nearly 85 % of the AFLP markers were mapped on the parental maps. The distribution of the AFLP markers generated with the different enzyme combinations on the linkage maps was well spread and quite similar (Figure 1). The codominantly scored AFLPs were assigned to all linkage groups except on groups 1 and 6. More than 80 % of the SSR markers could be mapped and were distributed over all parental chromosomes. About 75 % of the PK markers were assigned to linkage groups 2, 3, 4 and 5. Half of the RGA markers mapped on linkage groups 1, 2, 4, 5 and 7. Some clustering of the PK and RGA markers was observed on some regions of linkage groups A2, A5, A7, B1, B3 and B5. Most RFLP and SCAR markers mapped on linkage groups 1, 2, 3 and 7. The morphological marker *Rdr*1 was localised on B1, *Blfa* on A2 and B2, *Mehltau* on A3 and *Blfo* on B3, which was in line with the chromosomal locations on the maps published by Debener and co-workers (1999, 2001a). Markers with distorted segregation ($P \leq 0.05$) were assigned to most of the linkage groups with the majority on A3, A4 and B3 (Figure 1)

Alignment of the maps

The integrated maps were aligned with the parental maps and presented with markers at about 2 cM intervals (Figure 2). A majority of the common markers, especially the SSRs showed colinearity between the maps, indicating a high reliability of the constructed maps. The set of 21 SSR markers (Table 2) are well spread across the seven chromosomes (Figures 1 and 2) and can be used as anchor points to enable alignment of the present map with other rose maps. Using the AFLP bridge markers, the present parental maps aligned well with the core maps of Debener and Mattiesch (1999) (data not shown).

Genome coverage

Statistical estimation of the genome coverage was performed with repeated sampling of the markers mapped on both parental maps. The asymptotic upper limit was approximately 500 cM for both parental maps (Figure 3), indicating that the total length of the rose genome was estimated to be about 500 cM. Both of the present parental maps would cover more than 95 % of the diploid rose genome.

Discussion

Marker analysis

AFLP technology is considered to be an efficient marker platform due to its high multiplex ratio of markers, reliability, reproducibility and locus specificity (Pejic et al. 1998; Haanstra et al. 1999). However, in most cases AFLP markers can only be scored dominantly, often making them less informative and limiting their use as anchor points for map alignment. Codominant scoring of

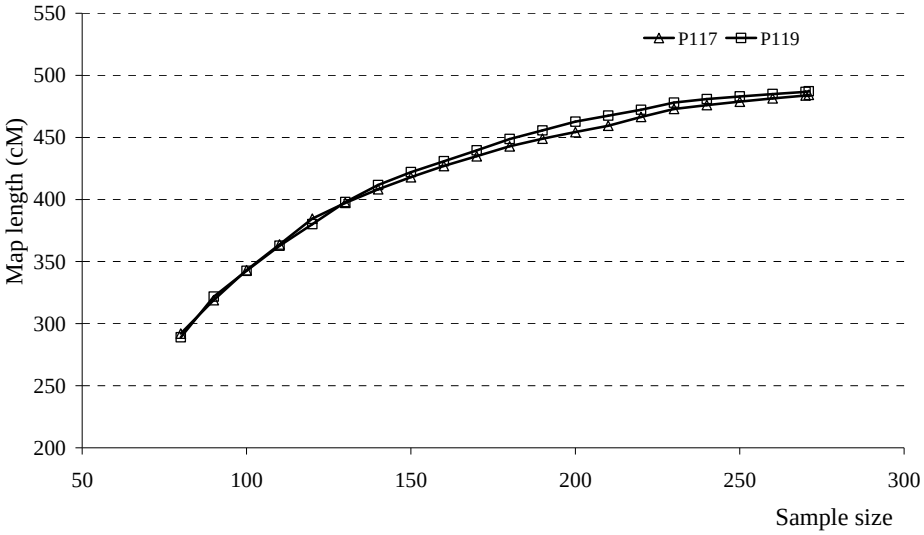

Figure 3 Estimation of genome coverage with the parental maps. Genome coverage with parental maps (P119 and P117) was estimated by repeated sampling of markers from the maps without replacement.

AFLP markers, nevertheless, can be achieved based on quantitative assessment of the optical density of bands on a gel or from the fluorescence level in a gel-free marker assay (Piepho and Koch 2000; Jansen et al. 2001; Geerlings et al. 2003), but literature references on their use for genetic mapping are still scarce (Castiglioni et al. 1999; Bradeen et al. 2001). In the present study 320 reliable AFLP markers were generated by using 56 different primer combinations. A large proportion of the markers (31 %) was found to be bi-parental; only 11 markers could be scored codominantly. Scoring of these markers should be maximised by taking special precautions, e.g. the normalized peak heights of the heterozygous individuals should be similar and about half the peak heights of the flanking monomorphic markers. The mapping of codominant AFLP markers in this study yielded additional anchor markers for the alignment of the parental maps. Therefore, codominant scoring may be considered as a good procedure to obtain more of this kind of markers for mapping studies, taking into account map position and suitability to score the markers, even though the analysis is time-consuming and perhaps error prone. The latter can, however, be minimized by dominant scoring of certain plants in case of doubt.

The SSR analysis performed in this study revealed a high fraction (57 %) of SSRs with only two marker alleles. In contrast, SSR markers in similar studies in rose-related species usually yielded at least three alleles. For example, more than 75 % of the SSRs in apple (Maliepaard et al. 1998; Liebhard et al. 2002) and 73 % in *Prunus* (Aranzana et al. 2003) were multi-allelic. The reason for the lower number of multi-allelic SSR markers is most likely due to the fact that the parents are half sibs. This implies that both parents theoretically have one quarter of all alleles in common (Debener and Mattiesch 1999). The common ancestor of the population also explains the

presence of a large proportion (22 %) of type 3 markers (Table 1).

Nearly 22 % of the present markers showed distorted segregation ($P \leq 0.05$), of which the larger part was contributed by the loci from the male parent P117. The linkage analysis showed that a high proportion of markers with distorted segregation were found on linkage groups 3 and 4, especially on A3, B3 and A4. A high frequency of markers showing distorted segregation is common in outcrossing species like pine (Kubisiak et al. 1995), willow (Hanley et al. 2002), peach (Dettori et al. 2001) and apple (Liebhard et al. 2002). The common origin as well as the distortion pattern of the markers indicates the presence of gametophytic selection for “sub-lethal genes”, i.e. coding factors controlling the viability of pollen, zygote or seedlings, putatively located on one or more of these chromosomes. This is in line with the observation that reciprocal crosses with the parents of this population and backcrosses of individual plants to both parents indicated the presence of a self-incompatibility system (data not shown).

Parental maps

In comparison with the rose maps published to date (Debener and Mattiesch 1999; Rajapakse et al. 2001; Crespel et al. 2002), the presented parental maps are quite dense and are well covered with different types of markers. The rose genome is small. A mean chiasma frequency of 1.4 per bivalent (Lata 1982) and an average nuclear DNA content of 1.1 pg/2C (Yokoya et al. 2000) observed in diploid rose species would suggest that the average chromosome length is only about 70-80 cM with a total of about 500 cM. This estimation is in agreement with the result of statistical simulation by using the markers mapped on the present parental maps, i.e. the asymptotic upper limit is about 500 cM for both parental maps. This implies that the present maps, having an average length of 70.4 cM, may cover more than 90 % of the rose genome.

Reliability of the integrated map

The integrated map presented here was generated with a two-step strategy. Parental linkage groups were first constructed using different types of segregation markers and then homologous linkage groups were “merged “using the JoinMap® program. These maps, resulting from pre-grouping of uni-parental markers, are more reliable than maps generated with mixed markers since the estimates of the recombination rate and the determination of marker linkage phase for dominantly scored bi-parental markers were less accurate (data not shown).

The accessibility of common markers and especially codominant ones allows not only the identification of homologous linkage groups but also the integration of both parental maps (Qi et al. 1996). For most of the linkage groups, the order of the markers on the integrated map was consistent with the marker orders observed in the individual parental linkage groups, apart from some minor differences on some linkage groups, for example on two regions of linkage group 2. Inconsistencies like these are, however, not alarming since usually a number of almost equivalent

marker orders exists, i.e. orders that fit the data equally well. As the algorithm for marker ordering of the JoinMap® program does not guarantee the best solution to make integrated maps (Stam 1993), the "fixed order" option was used to solve the differences. As all the markers were mapped with high LOD scores (LOD ≥ 5.0), the chi-square value was low ($\chi^2 \leq 3.0$) and the orders of the majority of common markers were similar in all maps, we conclude that the integrated map has a high reliability.

Prospects for the maps

Genotyping of the mapping population with new markers and the integration of these with existing markers (Debener and Mattiesch 1999; Debener et al. 2001a) in both parental and integrated maps in this study have led to a dense and reliable map of rose, which aligns well with the maps published by Debener and Mattiesch (1999). Both parental and integrated maps are useful for genetic analysis in rose. The parental maps will facilitate separate QTL analysis of the variation present in individual parents, while the integrated map allows a simultaneous analysis of QTLs from both parents (Maliepaard and Van Ooijen 1994; Hanley et al. 2002).

In the well-documented species *Arabidopsis thaliana*, protein kinases and RGAs are large gene families involved in many biological processes in plants (*Arabidopsis* genome Initiative 2000). For example, protein kinases play a crucial role in the self-incompatibility systems of plants (Nasrallah et al. 1994), plant hormone activation (Machida et al. 1997) and incompatible plant-pathogen interactions (Vallad et al. 2001). Therefore, the mapped PK and RGA markers in the vicinity of genes or QTLs for plant growth and defence especially for resistance to pathogens (Van der Linden 2004) are interesting candidate markers for use in marker-assisted selection.

Currently, a co-operative effort is being made with several research groups acting in rose genetics to establish a consensus map for rose by future integration of the ongoing mapping studies using common SSR markers as anchor points. Finally, the addition of gene-based markers to the present map may provide a good starting point for comparative mapping with other rose related species like *Prunus, Malus, Fragaria* and well-documented species like *Arabidopsis*. This facilitates the research of genes associated with traits of interest for rose breeding and allows the identification of useful universal genes.

Acknowledgements

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Chapter 3

Vigour evaluation for genetics and breeding in rose

Euphytica (2005), accepted with minor modification

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Abstract

Breeding of cut and pot rose cultivars for efficient production under low energy conditions in greenhouses will be facilitated by understanding the inheritance of vigour. To get insight into the genetic variation of vigour-related traits, a diploid rose population was employed for an evaluation study in greenhouses in The Netherlands and Denmark. For all the traits investigated the population showed a continuous quantitative variation as well as a considerable transgression. For most of the traits, the genetic variation found among the tested entries was highly significant and tended to be large in comparison to the effects of genotype by environment interaction. The heritability based on means of the traits was high and ranged from 68 to 92 %. Strong simple correlations (r = 0.65 to 0.95) were found among the traits shoot length, leaf area, leaf dry weight, stem dry weight, total dry weight and growth rate. The total dry weight and leaf area are suggested to be good parameters for early selection of rose genotypes with vigorous growth under suboptimal growth conditions.

Introduction

Energy is a significant factor in the production costs for cut and pot rose producers in Northern Europe, where the crops are cultivated in greenhouses all the year around and require, especially in the winter season, supplementary heat and light to reach optimal growth and flower production. In addition, growers are confronted with an increasing political pressure to reduce the CO_2- emission resulting from greenhouse production. For example, in 1997 an agreement was reached between Dutch growers and the Dutch government to improve the efficiency of energy use in greenhouse production by 65 % in 2010 compared to 1980 (Korner 2003). To reach this goal, both technical improvements of greenhouse production systems and genetic crop improvements are pursued. The development of new cultivars with a higher production per unit energy input requires criteria for selection to facilitate breeding. This implies that such criteria should be simple to assess and should comprise all or at least most of relevant component traits of vigour.

The choice of a selection strategy in breeding for crop improvement requires at least some knowledge of the inheritance of the major target traits (Debener 2003). In rose, however, the genetic knowledge is still limited and research certainly does not match its economical importance. This is partly due to the complex genetic nature of rose cultivars, including polyploidy, self-incompatibility, low seed set, poor seed germination and a high degree of heterozygosity. Nevertheless, rose geneticists have started to unravel the inheritance of some morphological and physiological traits. As reviewed by Gudin (2000) and Debener (2003), monogenic inheritance was found for traits such as recurrent flowering, prickles on stems and petioles, flower colours yellow and pink, double flowers, double corolla, dwarfing, moss phenotype, resistance to black spot and powdery mildew. In addition, polygenic inheritance was found for winter hardiness, number of petals, and thorn density on shoots (Crespel et al. 2002). Little, however, is known to date on characteristics that determine the productivity of rose under suboptimal conditions, like vigour and adaptation to a low energy environment (De Vries et al. 1982; De Vries and Dubois 1996).

Vigour is a poorly defined and complex trait that is likely to be controlled by numerous elementary genetic factors, and therefore a direct genetic analysis is difficult and usually not very rewarding. A common strategy to circumvent this is to dissect a complex quantitative trait into its underlying components and study their genetics component by component (Rami et al. 1998; Xu 2001). The basic assumption is that the component traits are easier to be determined and have a relatively simple inheritance (Xu 2001).

The objective of the current study was to elaborate a simple procedure for testing vigour of roses and to use this screening method for the evaluation of a variable diploid rose population for traits related to vigour under suboptimal growth conditions. The testing method developed was based on re-growth of single secondary shoots on rooted cuttings, and its efficiency as well as the importance of genotype by environment interactions was evaluated in the experiments under

controlled conditions at different geographic locations. The implications of the results for early selection in breeding genotypes with a higher production per unit energy input will be discussed.

Materials and methods

Plant materials

The genotypes used in this study consisted of 88 diploid rose plants of population 94/1, and its two parents, 93/1-119 (P119) and 93/1-117 (P117), which were derived from *R. multiflora* (Debener & Mattiesch, 1999). The same population has been used previously to develop a fairly dense genetic map of rose (Chapter 2). Rooted cuttings of each genotype were produced under commercial conditions from mother plants of the same age. The number of cuttings per entry was in excess to allow some selection for uniform starting materials. Each cutting was allowed to produce a single shoot from one axillary bud and the others were removed. When the shoots were about 5 cm in length, the cuttings were transplanted from trays into 10 cm^2 pots with commercial potting soil, and transferred to the testing rooms with conditions designed for the experiment. When the first shoot had reached the stage of a visible flower bud, the shoot was cut back to the first internodes, leaving again only one basal axillary bud to form a second shoot. A final selection of uniform plants was performed before evaluation at the stage that the second shoots had reached a length of about 5 cm.

Pilot experiment

Eleven genotypes differing in vigour were selected from the diploid rose population and were used, together with both parents of the population, to conduct a pilot experiment for vigour. The experiment was carried out in two phytotron rooms (Smeets 1978) set at 16 ^oC and 20 ^oC, respectively. The photoperiod was set at 20 h light/4 h dark and light intensity at canopy level in both rooms was about 120 μmol m^{-2} s^{-1}. Relative humidity was kept between 60 and 70 %. Just after removal of the first shoots, the plants were placed according to a randomized block design with 3 replications and 5 pots as an experimental unit (plot).

Population evaluation

The entire population was evaluated in greenhouse experiments in Fredensburg, Denmark, in October 2002 (DK) and in Wageningen, The Netherlands, in March 2003 (NL). Only one temperature condition was used. Growth conditions were set as temperature 20 ^oC and lowered by 2 ^oC during the dark period, and light intensity 120 μmol m^{-2} s^{-1} with a period of 16 h per day. The experimental design was also similar to that of the pilot experiment.

Vigour-related traits

In the pilot and population studies, ten vigour-related traits were measured on the shoot of

individual plants when the flower buds of the shoot reached a length of 0.6 cm. The traits were: number of internodes, shoot length, stem thickness, chlorophyll content, shoot leaf area, leaf dry weight, stem dry weight, total dry weight, specific leaf area and absolute growth rate. The codes used for the traits are indicated in Table 1. Chlorophyll content (in SPAD values) was measured on the 3 lowest leaves with a Minolta SPAD-520 chlorophyll meter (Minolta, Ramsey, NJ, USA). Leaf area was measured on collected leaves of the shoot with a leaf area meter (Li-Cor 3100, NEB, Lincoln, USA). Leaf and stem dry weights were determined after drying for 24 h in an oven at 80 ^{o}C. Specific leaf area was the ratio of leaf area and leaf dry weight. Growth rate was calculated by dividing the total dry weight of the second shoot at harvest by the growth period of the shoot (number of days from the moment the first shoot was cut until the second shoot was harvested).

Data analysis

For each trait and each experiment the distribution of mean trait values were inspected. GenStat version 6.1 (Payne et al. 2002) was used to perform analyses of variance for the pilot study, the two evaluation studies of the population, as well as a combined analysis of the latter studies. Mean comparison, the computation of broad-sense heritability and correlation coefficients between the traits were conducted. Heritability of the trait was calculated based on plot means by $h^2 = \sigma_g^2/(\sigma_g^2+\sigma_e^2/r)$ for an individual experiment, and by $h^2 = \sigma_g^2/(\sigma_g^2+ \sigma_{gl}^2/l +\sigma_e^2/rl)$ for joint data from both experiments, where, σ_g^2 represents the genetic variance, σ_{gl}^2 the variance due to genotype by experiment interaction (G x E), σ_e^2 the error variance, r the number of replications and l the number of experiments.

Results

Pilot experiment

In the pilot experiment, vigour was studied under two growth conditions, i.e. at 16 ^{o}C and 20 ^{o}C. The results are summarized in Table 2. Highly significant genotypic differences (P<0.01) were found for all the traits except for SLA (P<0.05). Interactions between genotype and growth environment were also found to be significant, indicating that relative performance of entries was temperature dependent. However, the magnitude of the interaction component was small compared to the genetic variance.

The parents of the mapping population had a stronger growth at 20 ^{o}C than at 16 ^{o}C, whereas they also differed considerably from each other as well as from the selected offspring for most of the traits. In general, P119 performed better in growth than P117 under both conditions. For all the traits, the range of means of the tested progeny was much broader at 16 ^{o}C than at 20 ^{o}C (Table 2). However, the performance of each genotype at both temperatures was highly correlated (data not

shown). At 16 °C, plants had longer growth periods, and thus ended up with lower growth rates (Table 2).

Greenhouse evaluation of the population

The frequency distribution of the mean performance and the population entries are shown trait by trait for each location (Figure 1). All traits showed a more or less normal and continuous

Table 1 Description of vigour-related traits.

Vigour-related trait	Code	Unit	Description of measurement
Number of internodes	NI		Number of extended internodes at harvest.
Stem thickness	ST	mm	Diameter of the stem at middle of the 2nd internodes from shoot.
Shoot length	SL	cm	Length from the top to the shoot basis.
Chlorophyll content	CC	mg/l	Measured on the lowest three leaves of a plant with a meter
Leaf area	LA	cm^2	Area of all shoot leaves including petiolule and leafstalks.
Specific leaf area	SLA	cm^2/g	Ratio between leaf area and leaf dry weight.
Leaf dry weight	LDW	g	Dry weight of leaves including petiolule and leafstalks.
Stem dry weight	SDW	g	Dry weight of stem, excluding leaves, petiolule and leafstalks.
Total dry weight	TDW	g	Sum of leaf dry weight and stem dry weight.
Growth rate	GR	g/day	Ratio between total dry weight and growth period.

Table 2 Variance analysis and means of entries in pilot experiment. Codes of traits are given in Table 1. Significance of variances among genotypes (G) and genotype by environment interaction (G x E) are indicated with *, ** at the 0.05 and 0.01 levels of probability, respectively.

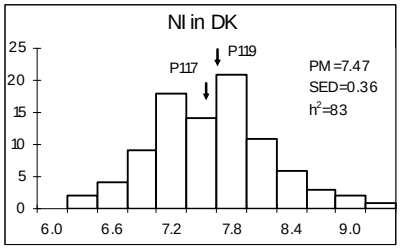
Trait	Mean at 16 °C			Mean at 20 °C			F value	
	P119	P117	Range progeny	P119	P117	Range progeny	Genotype	GxE
NI	7.47	7.33	4.93-9.07	7.92	7.85	6.05-9.02	37.11**	6.11**
ST	2.10	1.96	1.57-2.53	2.30	2.10	1.64-2.46	33.46**	4.29**
SL	22.21	22.57	11.59-30.37	23.34	23.57	15.77-25.39	61.94**	6.94**
CC	40.24	38.51	36.77-46.69	42.17	42.55	35.87-44.15	14.05**	2.34*
LA	155.40	118.00	67.90-189.50	162.40	140.60	78.70-169.00	24.59**	3.06**
SLA	366.10	251.40	254.00-386.80	306.50	314.90	297.60-332.70	2.73*	3.15**
LDW	0.66	0.52	0.17-0.91	0.83	0.61	0.34-0.78	24.37**	4.42**
SDW	0.27	0.28	0.07-0.44	0.35	0.25	0.17-0.46	20.82**	5.03**
TDW	0.94	0.81	0.21-1.33	1.19	0.79	0.52-1.16	20.32**	4.02**
GR	0.04	0.03	0.02-0.04	0.05	0.03	0.01-0.05	13.53**	3.14**

distribution. The variation range among entries was much wider than the difference found between the parents, indicating the presence of transgressive segregation. The distributions of the population for different traits in NL and DK were quite similar and had an approximately equal range. However, the population means varied from trait to trait, for example, a higher population mean for ST, SL, SLA, SDW, TDW and GR was recorded in the DK experiment, indicating the growing conditions were somewhat better in the Danish experiment.

Means and variance components were also obtained from the analyses of variance using the combined data from the two greenhouse experiments (Table 3). The parents differed much for all traits, except for NI and SLA (Figure 1; Table 3). The means of P119 were generally higher than those of P117, similarly as observed in the pilot study. In most cases, the overall means of the population was between the parental values (Figure 1; Table 3). The analyses of the separate experiments as well as the overall analyses showed highly significant differences among entries (P<0.01) for all the traits. The G x E interaction was also significant for all the traits except for SLA (Table 3). In all cases, however, its magnitude was much smaller than that of the factor genotype. The variation for most traits assessed in the experiments was highly heritable. The broad-sense heritability estimates based on plot means ranged from 68 to 92 % in individual experiments (Figure 1). The estimates based on the entry means over experiments ranged from 48 to 72 %

Table 3 Estimates of means, variance components and heritabilities of vigour-related traits with combined data of the experiments carried out in Denmark and The Netherlands. Codes of traits are given in Table 1. SED indicates standard error of difference of means. *, ** indicate significance at the 0.05 and 0.01 levels of probability, respectively. σ^2_g, σ^2_{gl} and σ^2_e are variance components for genotype, genotype by experiment interaction and error, respectively. Broad-sense heritability (h^2, %) was estimated on plot means by $h^2 = \sigma^2_g/(\sigma^2_g + \sigma^2_{gl}/2 + \sigma^2_e/6)$.

Trait	Means					Variance components			
	P119	P117	Population	Range	SED	σ^2_g	σ^2_{gl}	σ^2_e	h^2 (%)
NI	7.67	7.50	7.44	6.21-9.10	0.25	0.223**	0.121**	0.186	71
ST	2.09	1.85	1.91	1.61-2.33	0.06	0.011**	0.005**	0.011	71
SL	18.69	20.45	17.87	12.86-25.04	0.89	4.377**	2.663**	2.39	72
CC	33.97	34.69	34.92	29.88-38.84	0.88	1.900**	1.651**	2.32	61
LA	157.9	122.2	128.9	88.8-175.4	8.30	209.00**	180.80**	207.3	63
SLA	298.4	300.9	307.9	267.9-364.6	13.20	290.00**	68.79ns	628.8	67
LDW	0.54	0.42	0.43	0.29-0.65	0.03	0.0034**	0.0025**	0.004	65
SDW	0.17	0.17	0.16	0.11-0.23	0.02	0.00052**	0.00043**	0.0007	62
TDW	0.71	0.59	0.59	0.40-0.87	0.05	0.0060**	0.0047**	0.007	63
GR	0.03	0.02	0.03	0.018-0.036	0.003	0.000007**	0.000015**	0.00002	48


NI in DK
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P119
PM =7.47
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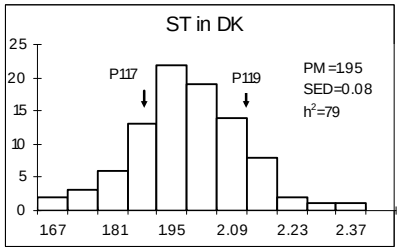
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P117
P119
PM =1.95
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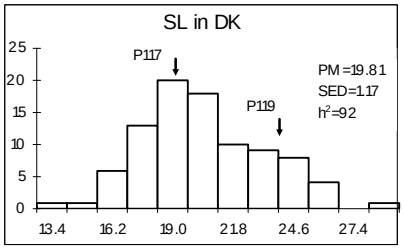
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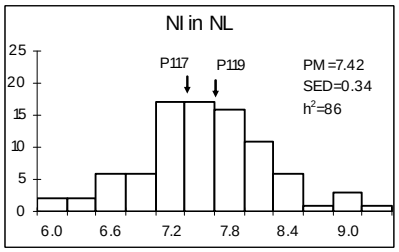
13.4 16.2 19.0 21.8 24.6 27.4


NI in NL
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P119
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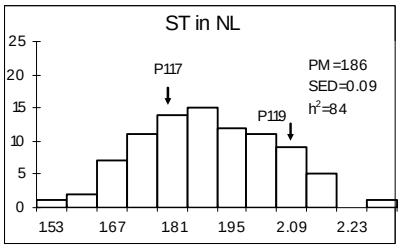
6.0 6.6 7.2 7.8 8.4 9.0


ST in NL
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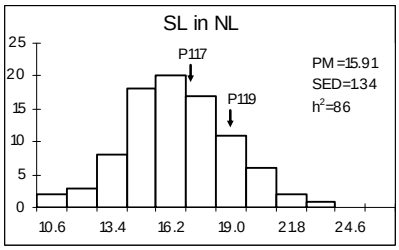
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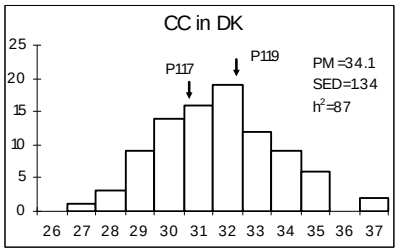
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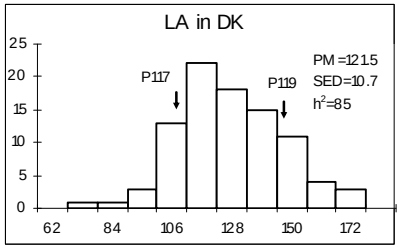
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P117
P119
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h²=85
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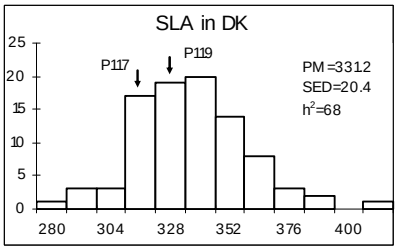
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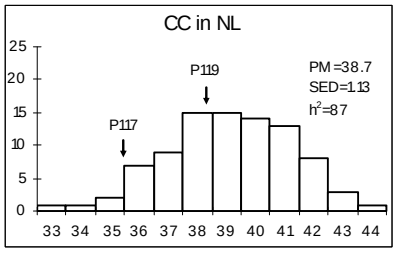
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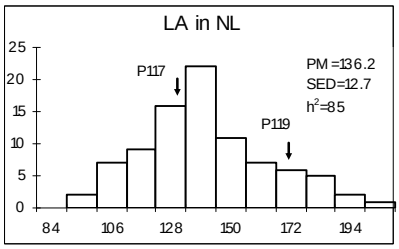
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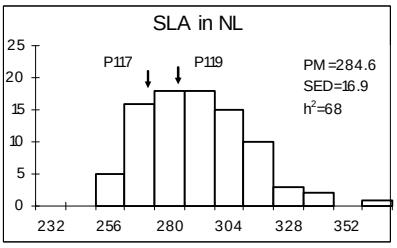
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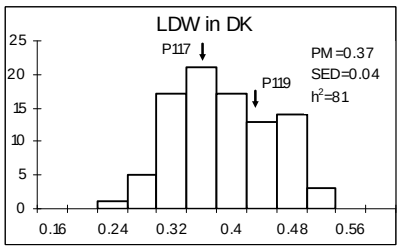
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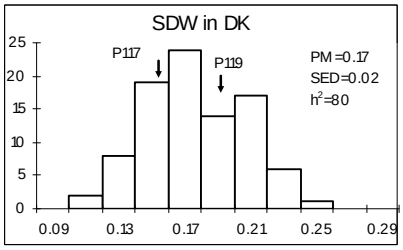
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P119
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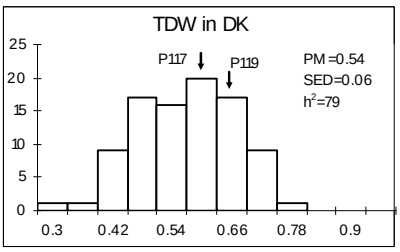
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P119
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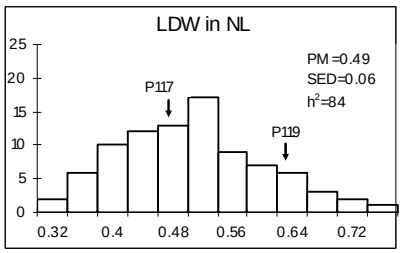
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P119
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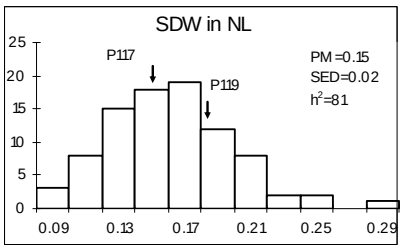
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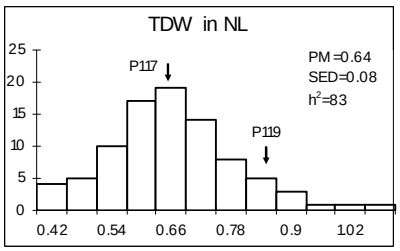
TDW in NL
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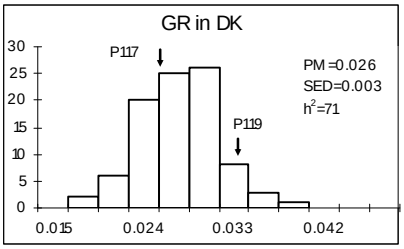

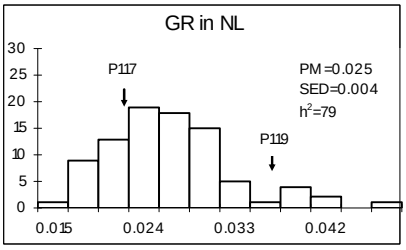

Figure 1 Distribution of vigour-related traits measured in the experiments carried out in Denmark (DK) and The Netherlands (NL). The means of parents P119 and P117 are indicated by arrows. Population mean (PM), standard error of difference of means (SED) and broad-sense heritability (h^2, %) are shown. Codes for traits are given in Table 1.

Table 4 Correlation coefficients of vigour-related traits evaluated in the experiments carried out in Denmark (DK; upper left) and The Netherlands (NL; lower right).

Trait	GR	TDW	SDW	LDW	SLA	LA	CC	SL	ST	NI
NI	0.35	0.49	0.53	0.41	-0.06	0.46	0.18	0.59	0.54	-
ST	0.56	0.6	0.61	0.52	-0.13	0.55	0.12	0.52	-	0.61
SL	0.5	0.67	0.81	0.53	-0.13	0.57	0.22	-	0.66	0.63
CC	0.2	0.32	0.29	0.32	-0.38	0.18	-	0.32	0.15	0.16
LA	0.76	0.84	0.66	0.85	-0.1	-	0.25	0.77	0.72	0.63
SLA	-0.38	-0.43	-0.24	-0.5	-	-0.13	-0.26	-0.28	-0.25	-0.22
LDW	0.86	0.94	0.67	-	-0.48	0.9	0.33	0.78	0.72	0.62
SDW	0.65	0.83	-	0.85	-0.34	0.79	0.3	0.84	0.69	0.6
TDW	0.85	-	0.92	0.95	-0.46	0.9	0.33	0.82	0.74	0.63
GR	-	0.95	0.91	0.93	-0.35	0.87	0.27	0.83	0.71	0.57

(Table 3). The estimates from the combined analyses tended to be somewhat lower than those from the corresponding separate analyses. However, the three estimates with different sets of data for a trait showed the same tendency.

Simple correlation coefficients were calculated for all pair-wise combinations of trait means for both experiments (Table 4). The estimates for corresponding combinations in the two experiments were fairly similar (Table 4). The morphological traits NI, ST and SL were moderately correlated (r = 0.52 to 0.66). Strong positive correlations (r = 0.67 to 0.95) were observed for the traits describing the dry matter allocation, i.e. LDW, SDW and TDW. The photosynthesis-related traits CC, LA and SLA, on the other hand, showed a weak relation (r = -0.10 to 0.25). The morphological traits were moderately related with other traits except SLA and CC. The relationship of SLA with

other traits was practically absent to medium (r = -0.06 to -0.50). Trait CC had positive low and intermediate relations with other traits except SLA (r = 0.12 to 0.33). High correlations (r = 0.65 to 0.95) were found among LA, LDW, SDW, TDW and GR.

Discussion

Vigour is an important agronomic trait in crop improvement. Genetic studies on vigour and related traits have been performed in a wide range of plant species, like sorghum (Cisse and Ejeta 2003), wheat (Regan et al. 1992), rice (Redona and Mackill 1996; Cui et al. 2002), maize (Revilla et al. 1999), chickpea (Sabaghpour et al. 2003) and willow (Tsarouhas et al. 2002). Vigour is a complex plant characteristic that usually is reflected in the variation of plant traits, such as leaf number, leaf size, leaf area, leaf weight, plant height, plant weight, root weight, etc. The present study was focussed on the development of a simple procedure to test plant vigour using one single growing shoot per cutting. According to breeders, the evaluation on second shoots would yield the most useful results since in cut rose production these secondary shoots form part of the backbone of the plants and produce the first saleable flowering lateral shoots. Cuttings for pot rose production are also cut back twice before allowing them to flower. In the testing method developed, ten vigour-related components were evaluated on the second shoot of rooted rose cuttings. Random variation was minimized by the procedures followed during pre-treatment of plant materials, standardization of the starting plant materials at the onset of an experiment and the design of the experiments. The studies in this way showed that data for vigour-related components can be collected in a fairly short period of time, for example, about one month in the present study.

For the evaluation of the testing method, a diploid population derived from the wild species *R. multiflora*, one of the ancestors of cultivated tetraploid roses, was used. This offered the opportunity to study the genetic variation of the vigour-related traits investigated, as well as the importance of genotype by environment interactions. The reason to employ a diploid population instead of a tetraploid population for the present study was to prevent the complexity of tetrasomic inheritance in the molecular marker studies envisaged. This approach to obtain the required knowledge on evaluation and inheritance of growth vigour will pave the road towards marker-assisted selection for vigour at the tetraploid level in rose breeding. Genetic studies in polyploid species like potato and alfalfa are often performed at the diploid level and turned out to be quite rewarding (Bonierbale et al. 1988; Gebhardt and Valkonen 2001; Bryan et al. 2002; Echt et al. 1993). The present population derived from *Rosa multiflora* may harbour some valuable genes or alleles for vigour that can improve modern roses. A direct transfer of valuable genes from diploid to tetraploid genotypes can be achieved by doubling the chromosome number of some selected genotypes from the

mapping population, followed by crossing with pot or cut rose genotypes. Subsequently, the progeny of such crosses can be used as starting materials for marker-assisted selection.

A small scale pilot experiment under two different growth conditions and two large scale experiments in greenhouses at one temperature revealed significant genetic differences among tested entries of the population for vigour-related traits as well as the presence of G x E interactions. However, the magnitude of the latter was much smaller than the genetic variation. The large genetic variation for vigour-related traits was indicated by differences in performance of the parents for most of the traits and by the observed transgression in the population. A continuous frequency distribution of the entry means for all vigour-related traits together with a transgressive segregation was observed, suggesting a polygenetic inheritance of the traits (Hartl 1980).

The current study demonstrated that total shoot dry weight, an important part of biomass production, is largely dependent on leaf dry weight (r = 0.94 to 0.95), stem dry weight (r = 0.83 to 0.92), leaf area (r = 0.84 to 0.90) and partly dependent on number of internodes (r = 0.49 to 0.63), shoot thickness (r = 0.60 to 0.74) and shoot length (r = 0.67 to 0.82). A similar magnitude of relationship existed for growth rate with the above-mentioned vigour components. It is obvious that leaf area, rather than specific leaf area (leaf thickness) and chlorophyll content, contributed most to biomass accumulation, suggesting that leaf area, total dry weight and growth rate are key traits to examine vigour in rose breeding programs. However, growth rate has a relatively low heritability in the present study. Therefore, total dry weight and leaf area are suggested to be good parameters for early selection of genotypes with vigorous growth under suboptimal growth conditions. Further studies are needed to find out whether the observed prominent correlations are due to ploitropy or linkage. A validation of the findings in the present study also needs to be performed in a tetraploid population.

Due to practical limitations, neither root production nor branching capacity of the genotypes was evaluated in this study, although both characteristics were shown to influence rose flower production (Fuchs 1994; De Vries 1993), and are possibly correlated to vigour. A sound root system usually is a prerequisite for a strong shoot formation and a large flower production (De Vries 1993; Kool 1996). Physiological studies on rose have revealed close relationships between root dry weight, shoot dry weight and flower production (Hu 2001; Costa 2002).

A large amount of genetic variation together with a high heritability was found for most of the examined vigour-related traits. Based on these results, a breeding program for vigour, based on selection via molecular markers for vigour QTLs, is proposed as an appropriate strategy. As a next step in this process, QTLs for the ten vigour-related traits described in this chapter will be identified on the molecular linkage map of the used population (Chapter 2). The localization of these QTLs will offer the possibility to select separately for individual components of vigour in rose. In addition, it will facilitate the introgression of favourable alleles from wild species into cultivated rose cultivars (Tanksley and McCouch 1997; Stam 2003).

Chapter 4

QTL analysis of variation for vigour in rose

Theoretical and Applied Genetics (2005), submitted

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Abstract

The improvement of energy efficiency in the greenhouse production of cut rose and pot rose can be achieved through the use of rose cultivars having vigorous growth. A better understanding of the inheritance of vigour and its related traits will assist the breeding activities. QTL analyses were performed with the help of an integrated linkage map of a diploid rose population for ten vigour-related traits evaluated in two greenhouse experiments under suboptimal growing conditions. We identified ten chromosomal regions, scattered over the seven linkage groups, containing QTLs for these traits. Considering each trait separately, we detected a total of 42 QTLs. Among these QTLs, 24 were found in both of the experiments, 8 and 10 were specific to either of the two experiments. The number of QTLs for individual traits varied from three to five with a respective contribution to the phenotypic variation from 12 % to 35 %. QTLs for highly correlated traits frequently co-localized, indicating a common genetic basis. Clustering of QTLs for different traits was noted in some chromosome regions, for instance, one on chromosome 2 included major QTLs for eight out of the ten traits under study, suggesting co-localization of several separate genes or/and the occurrence of various genes having pleiotropic effects. The markers associated to QTL regions provide an initial step towards marker-assisted selection for vigour improvement in rose.

Introduction

The production of cut rose and pot rose (*Rosa*) in greenhouses in Northern Europe requires much energy input, which leads to high production costs of marketable flowers. The use of fossil energy also contributes to environmental problems like global warming. The improvement of the energy efficiency of rose cultivation in greenhouses can be achieved through the use of rose cultivars having vigorous growth. A better understanding of the inheritance of vigour will help to set up an adequate strategy for selection and speed up the breeding of new cultivars. Vigour is, in general, a complex plant trait that is difficult to evaluate. However, vigour can be decomposed into component traits, making it easier to be measured and handled (Xu 2001). We developed a simple standardised procedure for evaluation of vigour in rose (Chapter 3), in which only one single shoot per cutting was allowed to form. At suboptimal growing conditions the shoots were evaluated for the following ten vigour-related traits: number of internodes, stem thickness, shoot length, chlorophyll content, leaf area, leaf dry weight, stem dry weight, total dry weight, specific leaf area and growth rate. Statistical analysis indicated that all vigour-related traits inherited in a quantitative way. The differences between parental phenotypic values were large for most of the traits. The heritabilities for most of the traits were high. A majority of the traits also showed close relationships, such as leaf area, leaf dry weight, stem dry weight, total dry weight and growth rate (Chapter 3). Therefore, there are good prospects for improvement of vigour breeding using a subset of the traits evaluated. Nevertheless, breeding for vigour is time-consuming and also costly.

The advent of molecular markers has provided novel opportunities for the analysis of qualitative and quantitative variation. Through a marker-trait association study, quantitative trait locus (QTL) analysis facilitates the detection of genetic factors influencing a quantitative trait and localizes them to specific regions of the genome. Such a study further results in estimates of the magnitude of the effects of QTLs and their contribution to the variation (Melchinger et al. 2004), insight in the stability of QTL effects across environments (Piepho 2000) as well as the relationships between QTLs underlying different physiological processes (Tsarouhas et al. 2002). The use of molecular markers will provide information on the relevance of the application of marker-assisted selection in rose breeding as well as marker-guided introgression (Stam 2003) of valuable genes from wild species into elite cultivars (Bouchez et al. 2002).

An essential tool for a QTL analysis is a molecular linkage map. Several maps of roses have been published (Debener and Mattiesch 1999; Debener et al. 2001a; Rajapakse et al. 2001; Crespel et al. 2002; Chapter 2) and some maps were used to identify either genes controlling qualitative traits, for example, flower colour and type (Debener and Mattiesch 1999), petal number and resistance to black spot (Debener et al. 2001b), prickles on petioles (Rajapakse et al. 2001; Debener et al. 2001a), recurrent blooming and double corolla (Crespel et al. 2002) or genes for quantitative traits, for example, the density of thorns (prickles) on shoots (Crespel et al. 2002). Markers

associated with these traits can be used to accelerate selection in rose breeding.

In a previous study we evaluated the variation for vigour-related traits in a diploid population (Chapter 3). In this chapter we report the outcome of the molecular analysis of the variation for the corresponding traits, i.e. the chromosomal positions and the contributions of putative QTLs affecting vigour and related traits in the population. Its implication for rose breeding is discussed.

Materials and methods

Mapping population and genetic map

The mapping population used to map QTLs for vigour-related traits was the diploid rose 94/1 population developed by Debener and Mattiesch (1999). To this end, two parental maps and one integrated map were constructed by using different types of molecular markers including AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers (Chapter 2). The integrated map, incorporating the seven homologous parental linkage groups (LGs) of the two parental maps, was used for QTL analyses.

Evaluation of the population for vigour

The population under study was evaluated for ten vigour-related traits in replicated experiments carried out in greenhouses at two locations, i.e. Fredensburg, Denmark in October 2002 (DK) and Wageningen, The Netherlands in March 2003 (NL), as described in detail in Chapter 3. The traits are: number of internodes (NI), stem thickness (ST), shoot length (SL), chlorophyll content (CC), leaf area (LA), leaf dry weight (LDW), stem dry weight (SDW), total dry weight (TDW), specific leaf area (SLA) and growth rate (GR). The entry means from the two experiments were subjected to a QTL analysis, trait by trait.

Mapping QTLs

MapQTL® 4.0 (Van Ooijen et al. 2002) was employed to perform QTL analyses of the genetic variation for each of the vigour-related traits. The statistical tools, each implemented in MapQTL, used to detect candidate QTLs were, in their order of application, Kruskal-Wallis test (KW) (Lehmann 1975), interval mapping (IM) (Stam 1993; Jansen and Stam 1994) and the restricted multiple QTL method (MQM) (Van Ooijen et al. 2002). The analysis started with the non-parametric KW test to get a rough idea of the presence and locations of QTLs. The next step was an IM analysis to get a better positioning of QTLs on the map. Markers located in the vicinity of QTLs, preferably the codominantly scored ones, were put in an initial set of cofactors. Restricted MQM analysis was then performed to precisely locate QTLs after selection of an adequate set of cofactors choosing from the initial set of cofactors. Map intervals of 5 cM were used for IM and

restricted MQM analyses. Log of odds (LOD) 2.5 was used as a significance threshold for the presence of a candidate QTL. The chromosomal location with the maximum LOD score was considered to be the most likely position of a QTL. For each QTL, a 2-LOD support interval as well as its contribution to the variation was calculated. QTLs for a trait with a similar map position in both experiments got the same code only when the 2-LOD support intervals did overlap by more than 10 cM. A QTL code is a combination of an abbreviation of the trait name and a serial number. Graphics were produced by MapChart software (Voorrips 2001).

Results

Number of QTLs

Using the means of each genotype in the population, QTL analysis was separately performed on ten vigour-related traits in each of the two experiments. A total of 42 QTLs with LOD scores above 2.5 were identified and their detailed QTL information is presented in Table 1. Among the detected QTLs, 24 were found in both experiments (in common), 8 only in the DK experiment (DK-specific) and 10 only in the NL experiment (NL-specific). The number of QTLs for the traits differed from three to five: three QTLs for SL, four QTLs for NI, ST, CC, LA, LFW, SDW and SLA, respectively, and five QTLs for LDW, TDW and GR, respectively. Individual QTLs explained from 12 to 35 % of the phenotypic variation (Table 1).

Genomic location of QTLs

The chromosomal locations of the detected 42 QTLs are shown in Figure1. The QTLs were not randomly distributed over the genome, but clustered in some rather small regions of the seven rose chromosomes. The QTLs could be grouped into ten different small regions if they were less than 10 cM apart on a chromosome. One region was found on LGs 1, 3, 4, and 7, respectively; and two regions on LGs 2, 5 and 6, respectively. Among the ten regions, a total of eight QTL clusters were found.

The clustering involved QTLs for two or more traits. For instance, a high concentration of QTLs for eight of the ten traits studied was found on one region (15-45cM) on LG 2 and similar hot

Table 1 (on next page) QTLs for vigour-related traits detected in the population evaluations in Denmark (DK) and The Netherlands (NL). A QTL name consists of the code of a trait and a serial number. For each QTL the linkage group (LG), peak location (cM), the LOD value and the percentage of phenotypic variation accounted for (var %) are presented. QTLs not detected in an experiment are indicated as “-“.

Trait	QTL	LG	Performance in DK			Performance in NL		
			cM	LOD	Var %	cM	LOD	Var %
No of internodes (NI)	*NI1*	2	62.6	4.8	22	62.6	5.2	23
	NI2	3	28.3	3.9	20	31.2	3.0	13
	NI3	6	18.3	3.6	19	8.8	3.0	13
	NI4	7	18.6	2.9	13	32.2	3.5	15
					74			64
Stem thickness (ST)	*ST1*	1	-	-	-	49.2	3.8	20
	ST2	2	55.1	3.6	20	55.1	3.2	16
	ST3	5	20.4	3.1	16	-	-	-
	ST4	6	42.6	6.9	31	75.9	3.4	19
					67			55
Shoot length (SL)	*SL1*	1	56.1	3.4	16	-	-	-
	SL2	2	25.8	9.5	35	34.9	5.5	28
	SL3	5	46.9	3.0	13	42.2	2.5	13
					64			41
Chlorophyll content (CC)	*CC1*	2	21.3	4.4	21	21.3	7.0	31
	CC2	3	14.4	2.6	15	14.4	3.8	20
	CC3	6	-	-	-	70.9	3.4	18
	CC4	7	21.4	5.7	29	-	-	-
					65			69
Leaf area (LA)	*LA1*	1	-	-	-	45.7	3.5	17
	LA2	2	30.7	4.2	18	30.7	3.6	18
	LA3	4	20.9	5.4	26	20.9	3.0	15
	LA4	7	53.0	3.6	17	40.6	2.6	14
					61			64
Specific leaf area (SLA)	*SLA1*	1	45.7	3.2	15	-	-	-
	SLA2	2	21.3	4.1	18	21.3	6.1	28
	SLA3	6	61.3	4.0	20	61.1	4.1	22
	SLA4	7	5.3	2.7	12	-	-	-
					65			50
Leaf dry weight (LDW)	*LDW1*	1	-	-	-	49.2	3.5	17
	LDW2	2	19.8	5.3	23	21.3	4.2	20
	LDW3	4	20.9	3.3	15	33.4	2.6	13
	LDW4	6	61.1	2.8	12	-	-	-
	LDW5	7	18.6	5.7	26	-	-	-
					76			50
Stem dry weight (SDW)	*SDW1*	2	19.8	5.1	24	21.3	3.2	18
	SDW2	5	20.5	2.9	15	17.2	2.8	15
	SDW3	6	-	-	-	70.9	2.5	13
	SDW4	7	5.3	3.0	16	-	-	-
					55			46
Total dry weight (TDW)	*TDW1*	1	-	-	-	49.2	2.9	14
	TDW2	2	19.8	4.4	21	21.3	4.2	21
	TDW3	4	20.9	3.2	15	26.5	2.6	12
	TDW4	6	61.1	4.0	17	66.3	3.0	15
	TDW5	7	10.3	4.1	19	27.2	2.5	12
					72			73
Growth rate (GR)	*GR1*	2	20.9	2.5	13	34.9	3.0	17
	GR2	4	20.9	2.5	13	-	-	-
	GR3	5	-	-	-	17.2	2.6	15
	GR4	6	61.1	3.8	18	-	-	-
	GR5	7	10.3	3.0	15	-	-	-
					59			32

spots for QTLs were also found on LGs 1, 4, 6 and 7 (Figure 1). It is striking that QTLs for highly correlated traits frequently co-localized, for example, the QTLs for LA, LDW, STW, TDW and GR found on LGs 2, 4, 6 and 7 (Figure 1).

Characterization of QTLs for different traits

Number of internodes

Four QTLs for number of internodes were detected in both experiments. The QTL, *NI1*, on LG 2 was found to have the largest effect. In total, the QTLs for this trait explained 74 % of phenotypic variation in DK and 64 % in NL (Table 1). The QTLs were located on LGs 2, 3, 6 and 7, which coincided with the QTLs for physiological and morphological traits, such as stem thickness, chlorophyll content and leaf area.

Stem thickness

A total of four QTLs for stem thickness were identified, of which *ST2* and *ST4* were detected in the two experiments, *ST1* only in DK and *ST3* only in NL (Table 1). *ST4* was found to have the largest contribution to the phenotypic variation. The four QTLs were situated on LGs 1, 2, 5 and 6, which coincided with the QTLs for traits describing dry-matter production and allocation, for example, shoot dry weight, leaf dry weight, total dry weight and growth rate.

Shoot length

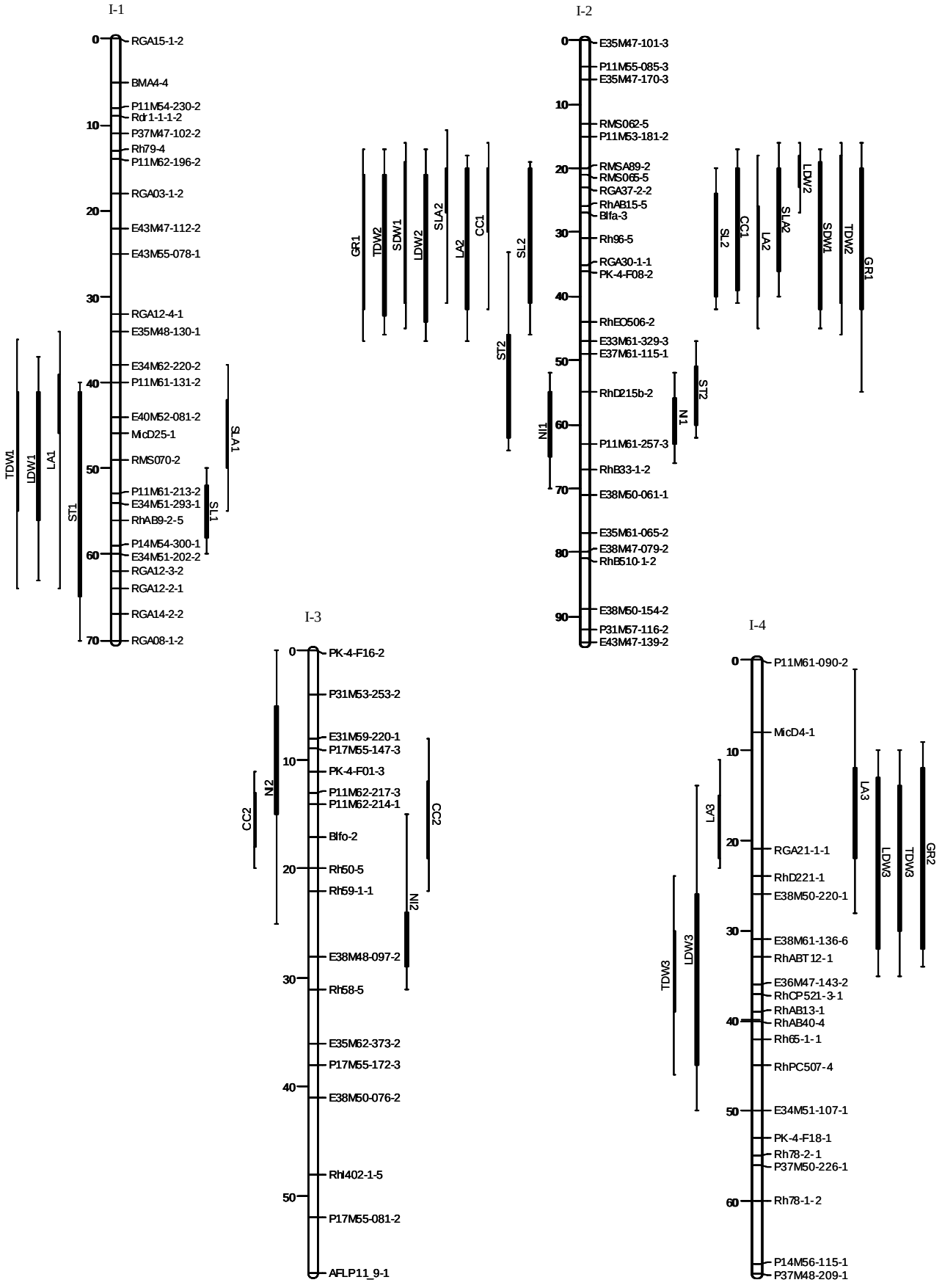
The analysis of shoot length showed three QTLs, of which two were present in both experiments and one was DK-specific. The QTL, *SL2*, with the largest contribution to the variation of shoot length, i.e. 35 %, was located on LG 2 and coincided with QTLs for most of other traits.

Chlorophyll content

Four QTLs for chlorophyll content were identified on LGs 2, 3, 6 and 7, of which two were found in both experiments and two were experiment-specific. Most of the QTLs coincided with QTLs for the dry-matter production and allocation traits, and in part with the QTLs for photosynthesis-related traits like leaf area.

Leaf area

A total of four QTLs for leaf area were detected on LGs 1, 2, 4, and 7, respectively, three of which were present in both experiments and one (*LA1*) was NL-specific (Table 1). These QTLs were associated with QTLs affecting dry-matter allocation, explaining the high positive correlations between these traits and LA (Chapter 3).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Rh79-4
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RGA03-1-2
E43M47-112-2
E43M55-078-1
RGA12-4-1
E35M48-130-1
E34M62-220-2
P11M61-131-2
E40M52-081-2
MicD25-1
RMS070-2
P11M61-213-2
E34M51-293-1
RhAB9-2-5
P14M54-300-1
E34M51-202-2
RGA12-3-2
RGA12-2-1
RGA14-2-2
RGA08-1-2
TDW1
LDW1
LA1
ST1
SL1
SLA1
I-2
E35M47-101-3
P11M55-085-3
E35M47-170-3
RMS062-5
P11M53-181-2
RMSA89-2
RMS065-5
RGA37-2-2
RhAB15-5
Blfa-3
Rh96-5
RGA30-1-1
PK-4-F08-2
RhEO506-2
E33M61-329-3
E37M61-115-1
RhD215b-2
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P31M57-116-2
E43M47-139-2
GR1
TDW2
SDW1
LDW2
SLA2
LA2
CC1
SL2
ST2
NI1
N1
ST2
SL2
CC1
LA2
SLA2
LDW2
SDW1
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Blfo-2
Rh50-5
Rh59-1-1
E38M48-097-2
Rh58-5
E35M62-373-2
P17M55-172-3
E38M50-076-2
Rh1402-1-5
P17M55-081-2
AFLP11_9-1
CC2
N2
CC2
NI2
I-4
P11M61-090-2
MicD4-1
RGA21-1-1
RhD221-1
E38M50-220-1
E38M61-136-6
RhABT12-1
E36M47-143-2
RhCP521-3-1
RhAB13-1
RhAB40-4
Rh65-1-1
RhPC507-4
E34M51-107-1
PK-4-F18-1
Rh78-2-1
P37M50-226-1
Rh78-1-2
P14M56-115-1
P37M48-209-1
LA4
TDW3
LDW3
LA3
LDW3
TDW3
GR2

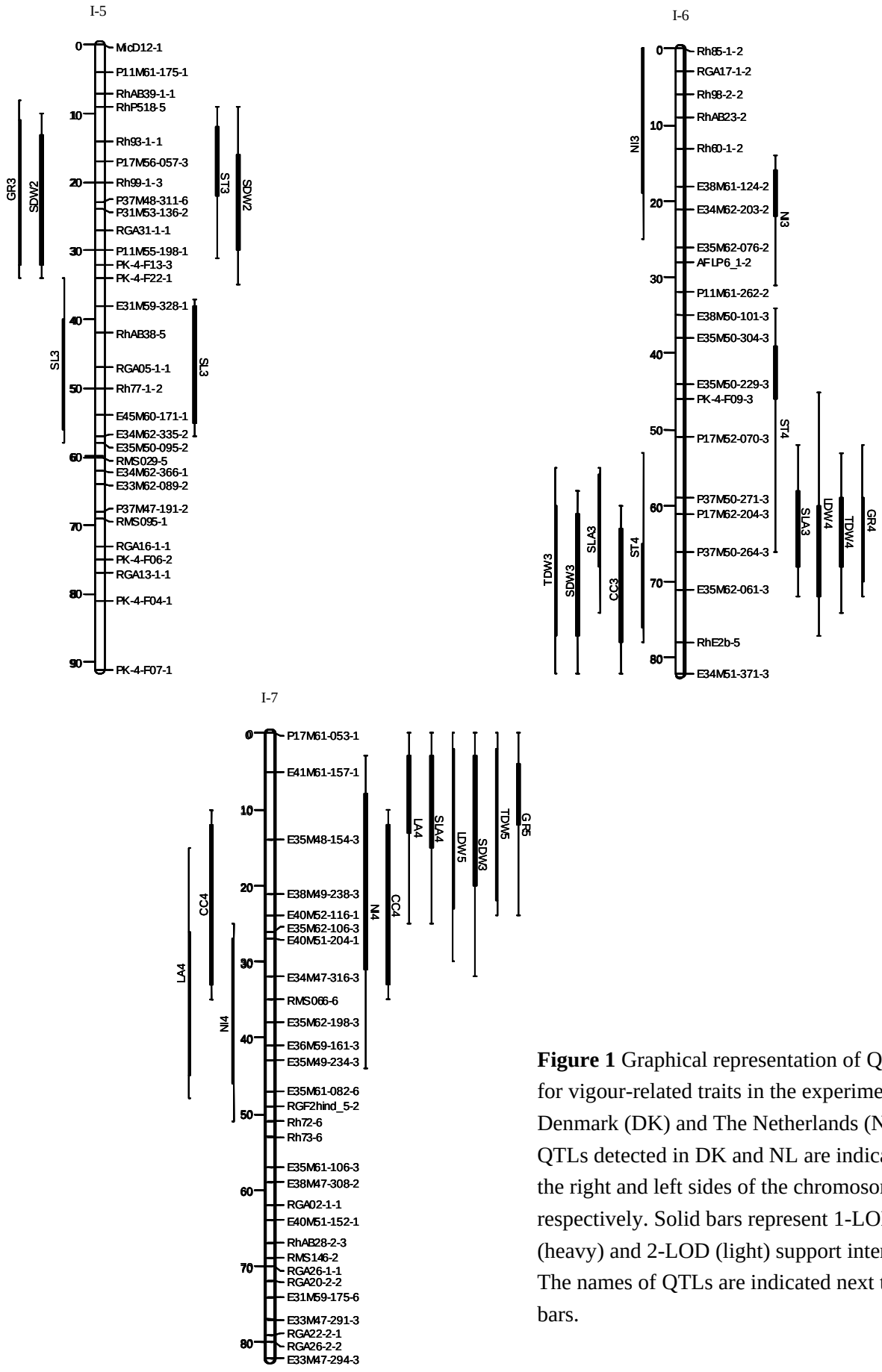

Figure 1 Graphical representation of QTLs for vigour-related traits in the experiments in Denmark (DK) and The Netherlands (NL). QTLs detected in DK and NL are indicated at the right and left sides of the chromosomes, respectively. Solid bars represent 1-LOD (heavy) and 2-LOD (light) support intervals. The names of QTLs are indicated next to the bars.

Leaf dry weight

Five QTLs for leaf dry weight were identified, of which two (*LA2* and *LA3*) were present in both experiments, one, *LA1*, was DK-specific and two, *LA4* and *LA5*, were NL-specific. The QTLs for this trait located on LGs 1, 2, 4, 6 and 7 largely coincided with QTLs for leaf area, stem dry weight, total dry weight and growth rate, indicating that these traits have a common genetic basis.

Stem dry weight

Four QTLs were found to determine the variation of stem dry weight, of which two were observed in both experiments; one was DK-specific and one NL-specific. The QTLs located on LGs 2, 5, 6 and 7 coincided with some QTLs affecting dry-matter allocation, indicating a common genetic basis.

Total dry weight

For total dry weight a total of five QTLs were identified, all of which were present in both experiments except for one, which was only found in the DK experiment. The QTLs accounted for 70 % of the variation. The QTL, *TDW2*, with the largest contribution to the variation was found on LG 2. Most of the QTLs were found in QTL clusters, including QTLs for other closely correlated traits, for example, leaf area, leaf dry weight, stem dry weight and growth rate.

Specific leaf area

Four QTLs for specific leaf area were identified. The two experiments had two QTLs in common and two QTLs were DK-specific (Table 1). The QTL, *SLA2*, with largest effect was found on LG 2, the others on LGs 1, 6 and 7, which were co-localized in clusters with QTLs for leaf area and dry-matter allocation traits.

Growth rate

Five QTLs for growth rate were found, of which only one was found in the two experiments, three were DK-specific and one was NL-specific. The QTLs were located on LGs 2, 4, 5, 6 and 7, which were mapped in the region where QTLs affecting dry-matter allocation were present. Each of the QTLs for growth rate contributed relatively little to the phenotypic variance, indicating that growth rate is genetically a very complex trait.

Consistency of QTLs across experiments

Separate QTL analyses for the different traits demonstrated the presence of 35 QTLs in experiment DK and 31 in NL (Table 1). About 57 % (24 out of 42) of the QTLs were detected in both experiments, among which one locus for GR; two loci for SL, ST, CC, LDW, SDW and SLA, respectively; three loci for LA and four loci for NI and TDW. In most cases, the support intervals

for QTLs occurring in both experiments showed a high degree of correspondence (Figure 1), indicating that the variation for the traits has at least in part a solid and fairly simple genetic basis.

Discussion

In the present study we did identify many robust and stable QTLs scattered all over the rose genome that affect plant growth, confirming the complexity of the inheritance of the vigour-related traits (Hund et al. 2004). The results also show that the variation for the traits is controlled by a limited number of genes having a major effect. The true number of QTLs controlling vigour-related traits, however, is likely to be different from what we have detected since both over- and under-estimation of QTLs may have occurred. It is likely that only QTLs having moderate to large effects, e.g. from 12 % to 35 % of phenotypic variance, could be detected. The size of the present population with 88 individuals also limits the discovery of QTLs, especially the ones with small genetic effects (Kearsey and Farquhar 1998; Melchinger et al. 2004). Among the reported ten vigour-related traits, SLA, TDW and GR were not directly measured but derived from their components; therefore, identical QTLs for the component and derived traits may be found. Some of the vigour-related traits are highly correlated and the 2-LOD support intervals of some QTLs for those traits overlap, indicating that the relationships are in part based on the action of the same genes or sets of clustered genes.

A striking finding was the co-localization of QTLs for different traits in a limited number of chromosomal regions. Taking total shoot dry weight as an example, the trait-specific QTL regions coincided with those of its closely related traits such as leaf area, leaf dry weight, stem dry weight and growth rate on all linkage groups except LG3 and LG5. The coincidence of QTLs for closely related traits provides evidence that the traits are genetically interrelated. Several authors (e.g. El-Lithy et al. 2004) have suggested that the localization of the QTLs for related traits at a similar chromosomal position may indicate that a single gene controls the variation for these traits. In our case this implies that some common genes affect leaf area and traits related to the allocation of dry matter, like leaf dry weight and stem dry weight. However, the resolution of our QTL analyses does not allow the distinction between pleiotropic effects of genes or the effect of different linked genes controlling these traits. Another striking finding was that three out of four QTLs for chlorophyll content were located in the same clusters of QTLs for other traits, which is not in line with the very weak correlations of CC with the other traits (r = 0.15-0.32). This might due to the combination of the related positive and /or negative effect of the alleles. Nevertheless, the hot spot QTL regions found in this study, especially the one on LG2, provide attractive targets for the development of simple selection markers to be used in marker-assisted selection for vigour.

A dedicated mapping population is needed to estimate the effects of QTL alleles. Good examples are diploid populations derived from crosses between inbred lines contrasting for the trait

of interest, such as F_2 and BC_1, etc. The linkage disequilibrium between QTL and marker alleles is high and the number of alleles for each locus is restricted to two. If sufficient genotypic mapping data of parents are available, the deduction of linkage phase of the markers will be easy. Such a strategy is not possible in an outbreeding species like rose, which does not allow selfing and the production of inbred lines. Therefore, the pseudo-testcross strategy of Grattapaglia and Sederoff (1994) was used to generate the mapping population used in this study. The parents are two highly heterozygous individuals and thus the maximum number of alleles per locus is four. The complex nature of the population highly restricts the possibilities for estimating the effects of QTL alleles. The linkage phase between marker and QTL alleles in the parent study is unknown and can only be deduced from the segregation data (Kearsey 1998, Mackay 2001). Only parents that are heterozygous for both marker and linked QTL provide linkage information; however, parents may differ in QTL-marker linkage phase (Mackay 2001). The variation due to a QTL may either be a consequence of the heterozygosity in one of the parents or in both parents. All the complications hampering the assignment of the linkage phase between marker and QTL alleles stopped us from estimating the effects of QTL alleles.

The detection of QTL by environment interaction requires evaluation of traits in multiple environments (Piepho 2000; Mackay 2001), which allows not only the identification of common QTLs but also environment-specific QTLs. The two experiments of the present study were similar in design. However, the actual growing conditions were somewhat different, largely due to the fact that the experiments were performed in greenhouses in different geographic locations and seasons. Therefore, the two experiments can be seen as two different environments. In general, the evaluation showed a significant G x E interaction for some traits (Chapter 3). However, the interaction effects were small, which is in agreement with the finding that most QTLs were detected in both experiments. Nevertheless, for some traits the analyses of the individual experiments showed environment-specific QTLs. The slight differences in locations of QTLs for some traits found in the two experiments may due to some genotypes having higher G x E interactions. The level of QTL consistency across the experiments was generally related to the heritability of the traits. For example, growth rate that showed a fairly low heritability had only one QTL showing up in both experiments. In contrast, number of internodes, a trait that showed a high heritability had four QTLs in common.

In this study, we detected a total of ten genome regions distributed over all seven chromosomes with putative genes for vigour. The primary QTL mapping studies on vigour in the present research represent only the initial step towards a full understanding the genetics that underlies the variation in vigour and the genetic relations among its component traits. Nevertheless, markers associated with QTLs, especially those from hot-spot QTL regions, are valuable as tools for marker-assisted selection and marker-guided introgression to generate rose cultivars with vigorous growth.

Acknowledgements

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Chapter 5

Assessment of partial resistance to powdery mildew (*Podosphaera pannosa*) in a tetraploid rose population using a spore-suspension inoculation method

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Abstract

Powdery mildew (*Podosphaera pannosa*) is the most important fungal disease in greenhouse roses and is in practice controlled by fungicides. The creation of novel cultivars with durable resistance to powdery mildew is highly desirable. To understand the inheritance of mildew resistance, a tetraploid rose population with a size of 181 genotypes was obtained by crossing two tetraploid cultivars each having partial resistance to powdery mildew. The population and its parents were tested under greenhouse conditions with two well-defined monospore isolates (2 and F1) using artificial inoculation of spore suspensions. Disease score at 11 days post inoculation, latency period and rate of symptom development were used to describe the resistance of the population. The tests for both isolates exhibited a wide and significant variation among genotypes for resistance. The distribution of the genotypic means of the disease scores was continuous and showed a considerable transgression. Statistical analysis, scatter plot of disease scores for the isolates, and correlation analyses indicated that the two isolates differed in pathogenicity. The outcome of the tests showed that the inoculation assay with spore suspensions was a reliable and effective way to screen large numbers of genotypes under greenhouse conditions for genetic and breeding studies. This is the first report on spore-suspension inoculation to be successfully used in rose.

Introduction

Powdery mildew caused by the obligate biotrophic pathogen *Podosphaera pannosa* (Wallr.: Fr.) de Bary (syn. *Sphaerotheca pannosa*) is the most important disease in greenhouse roses (Linde and Shishkoff 2003). It causes severe yield and quality reductions due to the formation of white powdery pustules that appear on the leaves, stems and flowers. A recurrent use of chemical fungicides is needed to prevent and control the outbreaks of the disease. As there is an increasing attention for the reduction of the use of chemicals in horticulture worldwide, it becomes increasingly desirable to create novel cultivars with resistance to powdery mildew. The development of cultivars with improved resistance has been a major breeding challenge for a long time, but successes are scarce. This is due to the lack of highly resistant genetic sources in cultivated roses, a limited knowledge of resistance mechanisms and a large diversity in the pathogen species. There is also a need for more reliable assessment methods for screening.

Evaluations of powdery mildew resistance have been conducted in many rose species, cultivars and hybrids. Genotypes with different degrees of resistance were found and several defence mechanisms of plants against the pathogen have been described. Morphological structures like the vacuolization of epidermal cells (Temmen et al. 1980), physical barriers such as leaf cuticle thickness (Ferrero et al. 2001) and the formation of papillae in plant cells (Mence and Hildebrandt 1966) were found to influence fungal penetration. Hypersensitive response (HR) (Conti et al. 1985), simple monogenic control (De Vries and Dubois 2001), a single dominant gene (Linde and Debener 2003) and horizontal resistance (Schlosser 1990; De Vries and Dubois 2001) were identified to confer powdery mildew resistance. It seems that vertical resistance (race-specific, HR or monogenic resistance) is very common in roses. For durable resistance, however, horizontal resistance (race non-specific, partial or polygenic resistance) is desirable. This type of resistance delays the infection, growth and reproduction of powdery mildew and tends to be much longer effective (Temmen et al. 1980; Schlosser 1990).

The pathogen species shows a lot of variation in pathogenicity. The races of the pathogen are traditionally defined by differences in virulence of individual isolates on a so-called differential set of host genotypes. Mence and Hildebrandt (1966) reported two races differing in host range and growth of conidia. Bender and Coyier (1984) identified five races in nine samples from Oregon (USA). Leus et al. (2002; 2003) studied eight isolates collected in Belgium and showed a differential host response, indicating difference in virulence among isolates. Linde and Debener (2003) recently classified eight different races in Northern Germany and concluded that the pathogen harbours a high diversity of virulence genes.

Homogeneous inoculation of the pathogen is essential for an accurate screening of genotypes for resistance and race identification in *P. pannosa*. Inoculation methods such as leaf-to-leaf contact, dusting with dry conidia and dispersal of the spores over the test plants with a blower are widely

used in breeding and research programs. These methods, however, often result in deposition of groups of conidia at the inoculation site. This implies that these methods give fairly variable results and are not very accurate (Francisco et al. 1988). An improvement of the blowing method using a vacuum-setting tower (Francisco et al. 1988; Linde and Debener 2003) has made it possible to tackle the above-mentioned problem but the inoculation method is not adequate for screening large numbers of plants, especially in field and greenhouse tests. The use of spore suspension in water has been extensively employed for the inoculation of powdery mildew in cucumber (Zijlstra et al. 1995), tomato (Bai et al. 2003) and pepper (Lefebvre et al. 2003), but has never been used in rose since it has long been thought that water may damage the viability and infectivity of the conidia (Yarwood 1939; Wheeler 1973).

The objective of this study was to get insight into the genetic variation in powdery mildew resistance in cultivated rose. To this end, we have made a tetraploid population by crossing two cultivars each having partial resistance against powdery mildew. Two well-defined monospore isolates have been used to screen the population for resistance. An assay with spore-suspension inoculation was tested and proved to be a rapid and reliable quantitative technique suitable for large-scale screening of rose genotypes for mildew resistance in genetic and breeding studies. In this chapter the results of the greenhouse tests and statistical analyses are presented.

Materials and methods

Plant materials and experimental design

A tetraploid (K5) population of 181 individuals was obtained from a cross between two tetraploid rose cultivars, P540 and P867, each being partially resistant to powdery mildew. Cuttings from each of the individuals and the parents were made from mother plants of the same age and rooted in plastic trays with commercial potting soil. A randomised block design with three replications was employed for the experiments. Two-week-old cuttings of uniform growth were selected from each genotype and placed in plastic trays according to the design. More cuttings of the parents were distributed among the plants to be tested as well as at the borders of the plots to check the uniformity of spore deposition. As a control, about fifty cuttings were randomly selected and kept in a separate greenhouse compartment without inoculation to test whether the source materials were free of mildew.

Inoculation and evaluation

Two well-defined monospore isolates, isolate 2 from Ahrensburg, Germany (Linde and Debener, 2003) and isolate F1 from Lesdain, Belgium (Leus et al. 2002), were kindly provided by the authors. The monospore isolates were maintained *in vitro* as described by Linde and Debener (2003). To obtain sufficient inoculum of the isolates, fresh cultures of the pathogen were made three weeks

before inoculation on clean susceptible plants in a small growth cabinet with conditions set at 22 ^{o}C (day) / 18 ^{o}C (night), ~ 75 % humidity and ~ 200 µmol m^{-2} s^{-1} light intensity for 16 h.

To test the powdery mildew resistance of the population, separate experiments with isolates 2 and F1 were conducted in March and October 2003, respectively, in temperature-controlled greenhouse compartments at Wageningen, The Netherlands. Artificial inoculations were performed when the plants on average had four unfolded leaves. For inoculation, a spore suspension with a concentration of 10^{3}-10^{4} conidia ml^{-1} was quickly made by rinsing infected leaves with tap water and immediately sprayed on the plants in a dose of 60 ml m^{-2}. The temperature in the compartment was increased from 22 ^{o}C to ~28 ^{o}C prior to spraying and maintained for about 15 min in order to stimulate the evaporation of water from the inoculum droplets on the leaves. The temperature was then lowered to 22 ^{o}C again. Growth conditions in the greenhouses were set at 22 ^{o}C (day) / 18 ^{o}C (night), ~ 75 % humidity and ~ 200 µmol m^{-2} s^{-1} light intensity for 16 h.

A 0-6 disease score was used to describe the development of the symptoms on the four unfolded leaves of a plant present at inoculation. The basis for the score is the percentage of total area of the four leaves covered with mycelium. The scores given were 0: no symptoms; 1: very small necrotic lesions with <1 % leaf area covered with mycelium; 2: 1-5 % leaf area with mycelium; 3: 6-20 % leaf area with mycelium; 4: 21-40 % leaf area with mycelium; 5: 41-60 % leaf area with mycelium and 6: >61 % leaf area with mycelium. Evaluation was conducted daily from the first day when symptoms became visible until the moment that almost all susceptible plants were heavily infected.

For further data analysis and interpretation, the two component traits, i.e. latency period (LP) and rate of symptom development (RSD), were calculated based on the time course of disease scores of individual plants during the first 11 days post inoculation (dpi). The LP is defined as the number of days from inoculation to the day of the first visual appearance of the disease. The RSD indicates the ratio of the disease score at 11 dpi and the time interval (in days) from the appearance of the first visible symptom to 11 dpi (i.e. 11-LP).

Statistical analysis

For the traits of disease score at 11 dpi, latency period and rate of symptom development, separate variance analyses were performed with GenStat® (Payne et al. 2002) using (1) the two separate data sets of the tests with individual isolates and (2) combined data of the tests. Broad-sense heritability for each trait was calculated by: $h^2 = \sigma_g^2/(\sigma_g^2+\sigma_e^2/r)$ for the separate tests with individual isolates, where, σ_g^2 represents the genetic variance, σ_e^2 the residual variance and r the number of replications.

To describe genetic differences in response to the isolates, two contrasting subsets of genotypes were composed based on the disease scores selected at 11 dpi. One subset included the 30 most resistant genotypes and the other set the 30 most susceptible ones. The disease scores of the selected genotypes at each evaluation time point were then used to calculate the average scores of the classes.

Disease progress curves of the contrasting classes for each isolate were plotted against evaluation time points.

Results

Inoculation and assessment methods

To tackle the common problems of artificial inoculation of plants, such as even spore distribution among the plants to be tested and the laboriousness of inoculating hundreds of plants simultaneously, we adapted an inoculation assay based on spore suspension of powdery mildew that has not been described before in rose. The assay was successfully used in the two resistance tests, giving sufficient and evenly distributed infections. In contrast, the non-inoculated cuttings kept in an adjacent compartment did not show infection (data not shown), indicating that the cuttings used in the tests were free from mildew at the start of the experiments.

The first symptoms of powdery mildew on the susceptible cuttings became visible at 5 dpi. From this moment the plants were scored daily for about ten days. At the end almost all the susceptible plants were heavily infected (scoring rating 6). The frequency distributions of the mean disease scores of genotypes at the various monitoring times were continuous and changed from negatively skewed to more or less normal, to positively skewed for both tests (data not shown). At 10-11 dpi, the distribution of the disease scores was approximately normal (Figure 1, A). Variance analysis of the scores at this time point showed the largest genetic variation among genotypes and a normal distribution of residuals (data not shown).

Variation among rose genotypes

Disease score

Significant genetic differences for disease scores at 11 dpi were found among the genotypes in both isolate tests (data not shown). The analysis of the combined data from both tests collected at 11 dpi showed highly significant genotypic variation ($P<0.001$) for resistance (Table 1).

The population showed a continuous normal distribution for the mean disease scores of the genotypes at 11 dpi in both resistance tests (Figure 1, A). A transgressive segregation of the resistance was observed. The two parents showed partial resistance to both isolates 2 and F1. However, a significant difference between the parents was only found with isolate F1. Nevertheless, parent P867 appeared to be more resistant than parent P540 (Figure 1, A).

Comparing the tests with two isolates, differences in the ranges of variation and distributions of the disease scores existed although the population means were similar. The population mean with isolate 2 was smaller than the parental means, whereas that with isolate F1 was in between the parental ones (Table 1). The estimates of broad-sense heritabilities of the disease score were high to different isolates, being 57 % for isolate 2 and 62 % for isolate F1 (Figure 1, A).

Latency period

Significant (P<0.001) genetic differences for the duration of the latency periods were observed in the tests with individual isolates (data not shown) as well as from the combined data (Table 1). The frequency distributions for the duration of latency periods for the two different isolates are illustrated in Figure 1 (B). To both isolates, a majority of genotypes had a latency period of about 6 days. A wide range in latency period, i.e. 5-11 days for isolate 2 as well as for isolate F1, was found among genotypes (Figure 1, B). The two parents had the same latency period to a specific isolate but differed with different isolates, i.e. both parents having a latency period of 5.0 days in the test with isolate 2 and 5.7 days with isolate F1 (Figure 1, B). The estimates of the heritabilities for latency period were 54 % for isolate 2 and 56 % for isolate F1 (Figure 1, B).

Rate of symptom development

The rate of symptom development is a measure of the speed at which an epidemic develops. The genetic differences for rate of symptom development were also found to be significant (P<0.001) in the tests with isolates 2 and F1 (data not shown) as well as within combined data of the tests (Table 1). The frequency distributions of the rate of symptom development of the genotypes are presented in Figure 1 (C) with both isolates. The heritability estimates for rate of symptom development were 48 % for isolate 2 and 55 % for isolate F1 (Figure 1, C).

Table 1 Combined ANOVA for resistance tests with isolates 2 and F1. The traits analysed are disease score at 11 days post inoculation (dpi), latency period and rate of visual symptom development. The numbers of missing data are in brackets in the “DF” column. *** indicates P<0.001.

Source of variation	DF	SS	MS	F
Disease rating				
Genotypes (G)	182	677.46	3.72	3.36***
Isolates (I)	1	15.49	15.49	13.98***
G x I	179(3)	370.30	2.07	1.87***
Residual	702(28)	777.69	1.11	
Latency period				
Genotypes	182	859.62	4.92	2.51***
Isolates	1	42.76	42.76	21.77***
G x I	179(3)	583.69	3.26	1.66***
Residual	704(26)	1382.88	1.96	
Rate of symptom development				
Genotypes	182	23.08	0.13	1.45***
Isolates	1	0.19	0.19	2.13***
G x I	179(3)	26.07	0.15	1.67***
Residual	704(26)	61.49	0.09	

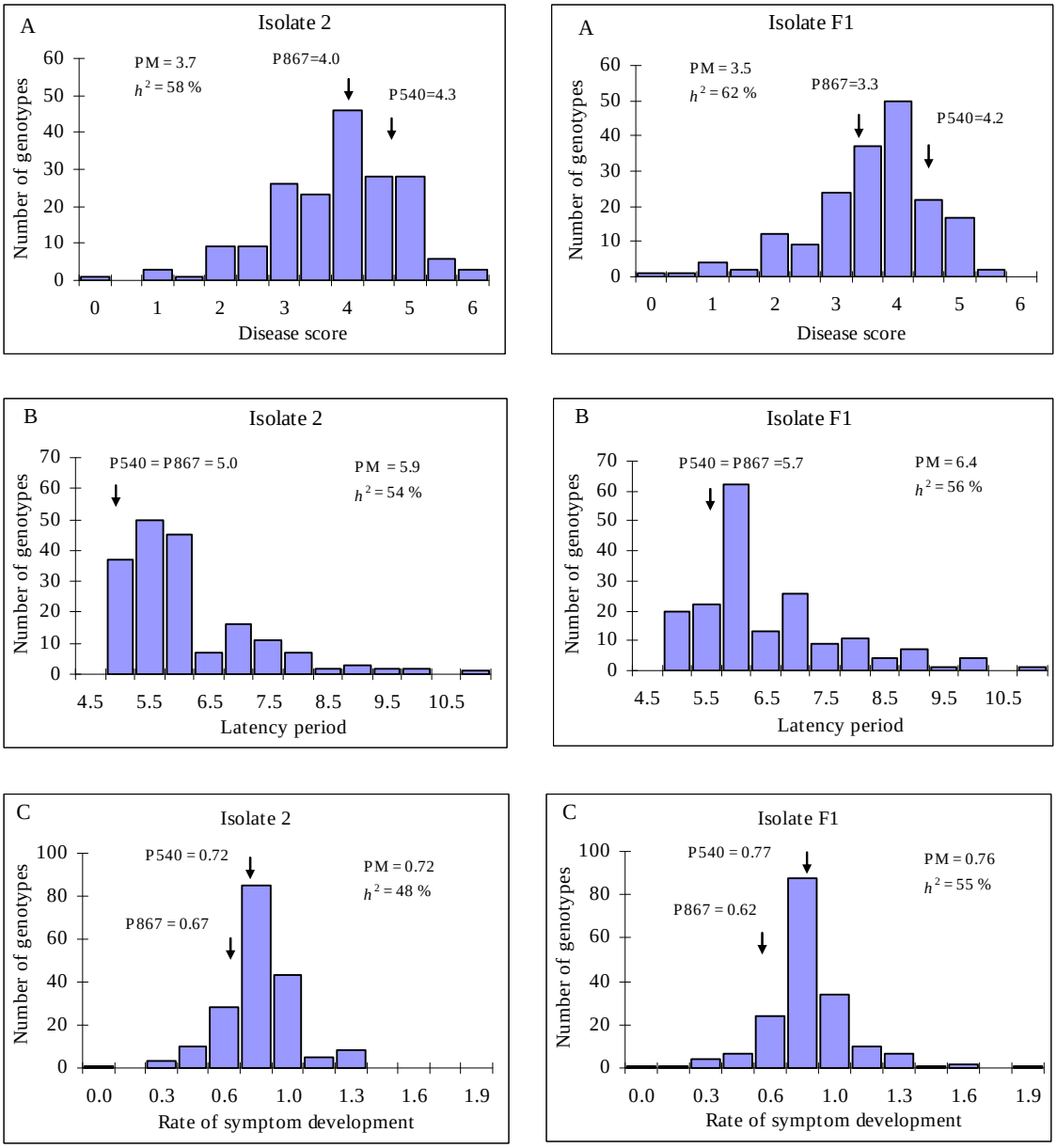


Figure 1 Frequency distributions of disease scores at 11 dpi (A), latency period (B) and rate of symptom development (C) of the population after inoculation with isolates 2 and F1. The means of parents P540 and P867, population mean (PM) and broad-sense heritability (h^2) are presented.

Relationship of the isolates

A scatter distribution of the mean disease scores at 11 dpi with isolates 2 and F1 is plotted in Figure 2. No strong relationship was found between the two isolates. The coefficient of correlation between the disease scores for the two isolates was low (r = 0.19, Figure 2). A weak relationship was also found for latency period (r = 0.21) and rate of symptom development (r = 0.16). The variance analyses of combined data sets for the three traits showed that the two isolates differed in somewhat in pathogenicity (Table 1).

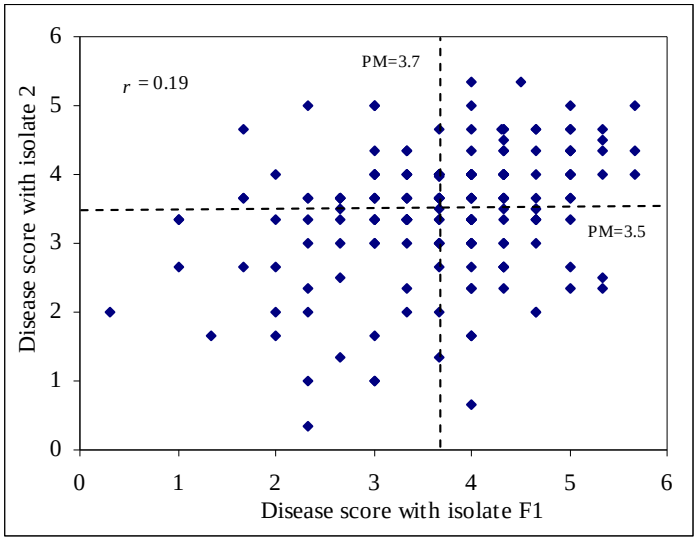


Figure 2 Scatter plot describing the mean disease scores at 11 dpi with isolates 2 and F1. The coefficient of correlation (*r*) between the disease scores for the two isolates and population mean (PM) are presented.

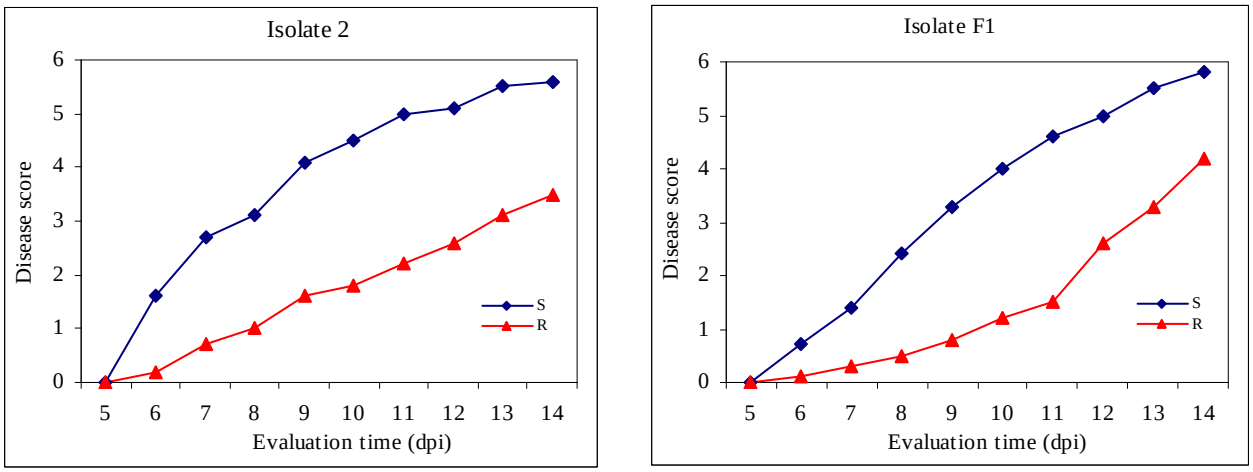


Figure 3 Time courses of the disease in two classes of rose genotypes contrasting for resistance. At each evaluation time point, mean disease scores of the 30 most resistant (R) and the 30 most susceptible (S) individuals chosen from the population at 11 dpi were used.

Interaction between rose genotype and isolate

The interaction of rose genotype and pathogen isolate was highly significant for all traits (Table 1). This interaction for disease scores at 11 dpi is illustrated in Figure 2. Some genotypes responded more to isolate 2, some responded more to isolate F1 and others had a similar response to both isolates.

In Figure 3, the disease progress for two contrasting classes of resistance to each isolate is presented. A clear difference in disease development was found between the classes of resistance for both isolates. As expected, the resistant class showed a relatively slow increase of the mean

disease scores with time and the susceptible a fast increase. The largest difference between classes was found at 10-11 dpi for both resistance tests. After that time point the disease score of the resistant class also increased quickly (Figure 3). Compared to isolate F1, isolate 2 showed a quicker progress after 11 dpi.

Discussion

A tetraploid population of rose was evaluated for resistance to two different mono-spore isolates of powdery mildew that were obtained from Belgium and Germany. The novel inoculation method with a spore suspension gave consistent results with both isolates. The satisfactory results were largely due to the even distribution of the spores and the easy applicability of the method. The homogeneous distribution of the spores is essential for the evaluation of plant diseases having a quantitative inheritance. It is a key factor for genetic studies to measure the contribution of minor genes for resistance (Lindhout 2002; Linde and Debener 2003). This is the first report on the use of spore-suspension inoculation of powdery mildew in rose. Prior to the current experiments, a pilot study was carried out with the *in vitro* bioassay according to Linde and Debener (2003). This assay makes use of detached leaves placed on water agar that are inoculated with dry spores using a vacuum-setting tower. Using this assay we encountered many problems like uneven distribution of the spores and infections with unwanted fungi like *Botrytis* during the incubation period. All of those negatively affected the repeatability and reliability of the assay. In addition, this type of laboratory assay would be very laborious and time consuming taking into account the size of the population to be evaluated in this study (183 genotypes x 3 replications x 2 mildew isolates).

The inoculation method with spore suspensions is based on the methods for powdery mildew inoculation used in cucumber (Zijlstra et al. 1995), tomato (Bai et al. 2003) and pepper (Lefebvre et al. 2003). In our hands the use of spore suspensions of *P. pannosa* to test for resistance in rose proved to be a reliable and easy-to-perform method of inoculation. It is essential that the spore suspension is prepared quickly. Furthermore, a temperature-controlled greenhouse compartment or climate room in which the temperature can be shifted quickly from 22 ^{o}C to about 28 ^{o}C is a necessity to evaporate the water from the fine droplets of inoculum after dispersion on plant leaves as quickly as possible. This inoculation assay is suitable for resistance screening of large sets of genotypes under growth conditions similar to those found in commercial greenhouses.

The present population showed a considerable quantitative variation for resistance to both isolates. The transgressive segregation observed for resistance indicates that each parent is heterozygous for one or more resistance genes (Falak et al. 1999). In earlier resistance studies in rose a major dominant gene (*Rpp1*) for race-specific resistance to powdery mildew has been identified (Linde and Debener 2003; Linde et al. 2004). However, it is quite likely that, in addition, rose as well as other species may have several genes contributing to the overall resistance against

this pathogen. For example, both qualitative and quantitative resistances to powdery mildew have been found in species such as barley (Jorgensen 1994; Williams 2003), wheat (Mingeot et al. 2002), tomato (Bai 2004) and *Arabidopsis thaliana* (Schiff et al. 2001). Therefore, studies including other resistant rose genotypes are needed to get a complete picture of the inheritance of powdery mildew resistance in cultivated roses.

The largest differences in resistance among genotypes were found at about 11 dpi, which was indicated by genetic analysis as well as by the disease progress for different classes of resistance. This time point is preceded by a 5-6 day latency period, which is in line with 5 days for other genotypes at optimal conditions (Frinking and Verweij 1989; Xu 1999) and a 6-day period with mild symptom development. The symptoms increased dramatically after 11 dpi when a second infection cycle became evident. Therefore, the resistance differences among plant genotypes became less pronounced as infection pressure increased.

Variance analyses, disease progress curves and correlation analyses indicated that the two isolates are most likely to be different in pathogenicity. It is obvious, as shown in Figure 2, that different host ranges existed between isolates or, in another words, race-specific responses among plant genotypes. The large diversity of responses of the genotypes, the significant genetic variation and the relatively high heritability of resistance found in the present population may facilitate the selection of highly resistant genotypes. Hopefully, a QTL analysis in which isolate-specific and non isolate-specific QTLs for resistance can be identified will shed more light on the genetics of resistance as well as on the differential responses. In addition, such a QTL analysis will facilitate pyramiding of resistance genes from both parents, preferably effective against both isolates as well as others.

In conclusion, using a new method of spore-suspension inoculation, partial resistance was found in the present tetraploid rose population to two different isolates of powdery mildew. The continuous distribution with transgressive segregation found for its resistance to the isolates tested indicates a polygenic nature of the resistance. However, the occurrence of dosage effects for single resistance genes segregating in the tetraploid population cannot be ruled out completely and needs to be clarified. A molecular marker analysis of the data obtained for this population will be used to elucidate the inheritance of powdery mildew resistance further. This is expected to result in molecular tools for the breeding of new cultivars with enhanced durable resistance to this important disease.

Acknowledgements

We thank Dr. L. Leus, Ghent University, Ghent, Belgium; Prof. dr. T. Debener and Dr. M. Linde, Hannover University, Hannover, Germany, for providing the isolates used in the present study. Mr. R. Groenwold is acknowledged for his support in powdery mildew tests.

Chapter 6

Polygenic powdery mildew (*Podosphaera pannosa*) resistance revealed by marker-trait association analysis in tetraploid rose

Molecular Breeding, ready for submission

Authors: Z. Yan, T. W. Prins, P. B. Visser, P. Stam, O. Dolstra

Abstract

Powdery mildew (*Podosphaera pannosa*) is an important disease in the greenhouse production of cut and pot roses. To get insight into the genetics of powdery mildew resistance in cultivated roses, a tetraploid population derived from two partially resistant tetraploid cultivars was tested with two monospore isolates. The component traits of resistance, i.e. disease score at 11 days post inoculation, latency period and rate of symptom development, were used to describe the variation in resistance within the population. In addition, the population was characterized with AFLP and SSR markers. Uni-parental as well as bi-parental simplex markers were used to construct parental maps with 23 and 17 linkage groups, respectively. The length of the respective maps was 695 and 697 cM. Multi-allelic SSR markers were successfully used to assign most of the linkage groups to one of the seven rose chromosomes of a diploid reference map. Marker-trait association analyses were performed to identify marker loci associated with genes for the three component traits. A number of marker loci were identified that accounted for a moderate to minor part of the variation observed for the different components. Multiple regression analyses revealed some isolate-specific markers differed in resistance. Markers associated with resistance were found on different chromosomes, indicating a polygenic nature of the resistance in this population. The markers found in this study can be used for pyramiding resistance genes into rose genotypes through marker-assisted selection in the future.

Introduction

The majority of cut and pot rose (*Rosa*) cultivars are highly heterozygous tetraploids ($2n = 4x = 28$), which originate from a wide variety of tetraploid and diploid species. Their genome constitution is, therefore, obscure but likely consists of seven groups of four homologous chromosomes. Molecular studies in rose were mainly done at the diploid chromosome level to avoid complications due to the uncertainties and the complexity of tetrasomic inheritance. Genetic analyses took advantage of diploid linkage maps with RFLP, RAPD, AFLP and SSR markers to elucidate the variation for a number of agronomic traits, for example, flower colour and type (Debener and Mattiesch 1999), petal number, prickles on petioles and resistance to black spot (Debener et al. 2001b), recurrent blooming, double corolla and the density of thorns (prickles) on shoots (Crespel et al. 2002) and vigour-related traits (Chapters 3 and 4). Rajapakse et al. (2001), however, used a tetraploid population to study the genetics of the formation of prickles on petioles.

Molecular studies on tetraploid roses can be more challenging than that of diploids. The genome constitution of the rose is complex but random chromosome pairing is more common than preferential pairing (Lata 1982; Ma et al. 2000), implying that tetraploid roses most likely have a tetrasomic inheritance as in autotetraploid species. Generating a map having in total 56 linkage groups, i.e. four homologous groups of seven chromosomes for each parent of a tetraploid rose, with sufficient coverage of markers is tedious. A consequence of tetrasomic inheritance is that in the offspring of a cross for each locus a large number of combinations of alleles are possible. Theoretically, segregation of up to eight different alleles is possible, resulting in at most 36 genotypic classes (Meyer et al. 1998; Debener 2003). This high number makes a genetic analysis of a quantitative trait very complicated. A second complication is the occurrence of double reduction, which causes the production of partly homozygous gametes and may influence marker segregation (Julier et al. 2003).

Knowledge of the inheritance of important traits in tetraploid rose is relevant to breeding since most of breeding activities are performed at the tetraploid level. Various strategies have been used to construct linkage maps and to perform genetic analyses of target traits in polyploids. Uni-parental simplex markers were used in rose (Rajapakse et al. 2001) and in octoploid sugarcane (Sobral and Honeycutt 1993). Bi-parental simplex markers, duplex markers as well as triplex markers were employed in tetraploid sugarcane (Da Silva et al. 1993), alfalfa (Yu and Pauls 1993) and potato (Meyer et al. 1998) to identify and merge homologous linkage groups. However, the most reliable strategy is to use simplex (single dose) markers (Wu et al. 1992). Segregation ratios and recombination rates for simplex markers in coupling phase are equivalent to those observed in diploid mapping studies. Therefore, mapping software developed for diploid mapping population can also be used to construct linkage maps in autotetraploids.

Powdery mildew is a severe disease that may occur in cut and pot rose greenhouse production

and for which no complete resistance is known in cultivars that are currently on the market. To get insight into the inheritance of powdery mildew resistance in cultivated roses, a tetraploid offspring population derived from two partially resistant tetraploid cultivars was tested with two monospore isolates under greenhouse conditions. The methodology and evaluation results are presented in Chapter 5. The traits: disease score at 11 days post inoculation (dpi), latency period and the rate of symptom development were employed to describe the variation in resistance within the population. The variation found for those resistance traits was continuous and heritable.

The current study aims to identify genetic loci controlling variation of resistance observed in the tetraploid population (Chapter 5) by using molecular markers. However, a genome scan for quantitative trait loci (QTLs) using interval mapping (Stam and Van Ooijen 1995; Van Ooijen et al. 2002) is not possible for autotetraploid populations due to a lack of suitable software. Instead, a marker-trait association analysis comprising marker by marker ANOVAs (Groover et al. 1994), followed by a stepwise multiple-marker regression analysis with only relevant markers (Kumar et al. 2000), can be done to find those markers best describing the phenotypic variation for resistance.

The objectives of the present study were to explore the feasibility of performing linkage analysis, to find molecular markers linked to genes for powdery mildew resistance in tetraploid rose and to get to know the inheritance of powdery mildew resistance in cultivated rose. Here we describe the results of the genetic studies.

Materials and methods

Plant materials and resistance tests

The tetraploid population K5 with a size of 181 genotypes derived from a cross between two tetraploid cultivars, P540 and P867, was used for the present study (Chapter 5). In a previous study, the same population and its parents were evaluated for resistance to powdery mildew under greenhouse conditions, using replicated tests with two different monospore isolates, designated as isolates 2 and F1. The disease was monitored daily using a 0-6 disease score for a period of 14 days. The phenotypic means for the traits, i.e. disease score at 11 dpi, latency period and rate of symptom development, were used in the present analyses.

Molecular markers

Genomic DNA was extracted from young leaves of the genotypes in the mapping population and its parents as described by Esselink et al. (2003). The quantity and quality of DNA were measured with a Biophotometer (Eppendorf AG, Hamburg, Germany). The genotypes were characterized with AFLP and SSR markers.

AFLP markers were generated as described by Vos et al. (1995) with some minor modifications

(Chapter 2) using two restriction enzyme combinations, i.e. *Eco*RI/*Mse*I (E-M) and *Pst*I/*Mse*I (P-M). The pre-amplification and selective amplification were performed using the same protocol as in the previous study (Chapter 2). A prescreening for polymorphisms with different primer combinations, having either two (some *Pst*I primers) or three (some *Pst*I and all *Eco*RI and *Mse*I primers) selective nucleotides, was done using DNA of the parents and a few K5 genotypes. Only the primer combinations giving a relatively high number of polymorphic markers were used for genotyping the K5 population. PCR amplification of DNA fragments was performed on a Perkin Elmer 9600 thermocycler (Perkin Elmer/Applied Biosystems) and electrophoresis on an ABI Prism 377 DNA Sequencer (Perkin Elmer) using fluorescent-dye technology for marker detection and 5 % denaturing polyacrylamide gels. Polymorphic markers were dominantly scored as described in Chapter 2.

A set of selected rose SSR primer pairs from our previous study (Chapter 2) was used to generate SSR markers. The PCR conditions and other procedures were similar to the ones used in that study. The presence of SSR alleles was scored allele by allele. No attempt was made to score the allele dosage.

Coding of markers was the same as in Chapter 2. AFLP markers were coded according to Keygene's nomination system comprising a letter code for the restriction enzyme combination, followed by figures for the combination of selective nucleotides and the size of the corresponding DNA fragment in base pairs. The name of an SSR marker starts with "Rh". For all markers, the last digit of the marker name refers to the segregation type: types 1, 2 and 3 for markers from P540, P867 and both parents, respectively.

Analysis of marker segregation ratios

In autotetraploids, different dosages of marker alleles may be present. Since no allele dosage data were collected for a marker in present study, it is necessary to deduce the genotype from the segregation ratios. Markers originating from just one parent (uni-parental) were accepted as present in single- (simplex) or double- (duplex) dose if their observed segregation ratio did not differ significantly from 1:1 or 5:1, respectively, as tested by a χ^2 goodness of fit at 5 % significant level. The markers with a 5:1 ratio were also tested to see whether the ratios fit 3:1 as expected with disomic inheritance at two loci. Markers originating from both parents (bi-parental) were accepted as present in single-dose if their segregation ratio was not significantly different from 3:1 ratio at 5 % level. Similar tests were performed to identify triplex (segregation ratio 11:1) and quadruplex (ratio 35:1) markers.

Map construction

JoinMap® 3.0 (Van Ooijen and Voorrips, 2001) was used to perform the construction of genetic maps. Criteria for grouping were similar to those in our previous study (Chapter 2). A two-step

approach was followed. Uni-parental simplex markers were first used to generate the parental maps. Bi-parental markers were subsequently mapped. To this end, fixed marker orders comprising the same markers from the initial maps were employed. Allelic SSR and common markers were used to align and name homologous groups. The basis for alignment of linkage groups was a diploid reference map (Chapter 2), using the map position of common multi-allelic SSR markers. The resulting linkage maps were drawn using MapChart software (Voorrips 2001).

Marker-trait association analysis

Various marker-trait association analyses were performed using the phenotypic means from resistance tests with the isolates 2 and F1 (Chapter 5). Traits analyzed were the disease scores at 11 dpi (DS), latency period (LP) and rate of symptom development (RSD) observed for each of the two isolates. Simplex markers were subjected to marker-trait association analysis. The analyses consisted of two steps. The first step comprised a single-marker ANOVA using GenStat 6.0 (Payne et al. 2002) to pre-select markers significantly ($P<0.05$) associated with resistance. The second step was a subset selection of the pre-selected markers by using stepwise multiple regression analysis to fit the following equation with a maximum contribution to resistance:

$$Y_j = \mu + \Sigma \beta_k m_{jk}$$

where, Y_j is the phenotypic value of genotype j; μ population mean; β regression coefficient for marker k; m_{jk} the presence of marker k in genotype j. The best subsets of predictor variable (markers) in regression were selected by using the Rselect procedure from the GenStat Precedure Libraries (Payne et al. 2002). This procedure evaluates all possible subsets of predictor variables and selects a small number of best subsets of markers by using t-statistic criterion ($P<0.005$) for measuring goodness of fit (Payne et al. 2002).

Results

Segregation patterns for molecular markers

A total of 26 AFLP primer pairs, i.e.16 E-M and 10 P-M, were used for genotyping the tetraploid K5 population, resulting in 237 easily scorable polymorphic markers. The number of polymorphic markers per primer pair varied from two to 24, with an average of 9.1 markers per pair (data not shown). The classification of the uni- and bi-parental markers depending on segregation ratio is presented in Table 1. Less than half (99 out of 237) of the markers showed a uni-parental simplex and about one quarter (66 out of 237) of the markers a bi-parental simplex segregation (Table 1).

A selection of 20 SSR primer pairs from the subset of the primers that gave markers mapped on the diploid reference map (Chapter 2) was used to genotype the present population. This yielded 56 polymorphic allelic markers (Table 1). Among the markers, more than half (29 out of 56) of the

markers showed uni-parental simplex segregation pattern and about one fifth (11 out of 56) fitted the ratio expected for bi-parental simplex markers. For both types of AFLP and SSR markers, differences in allele dosage were found (Table 1). In addition to simplex markers, 29 duplex, 24 triplex and 6 quadruplex markers were detected.

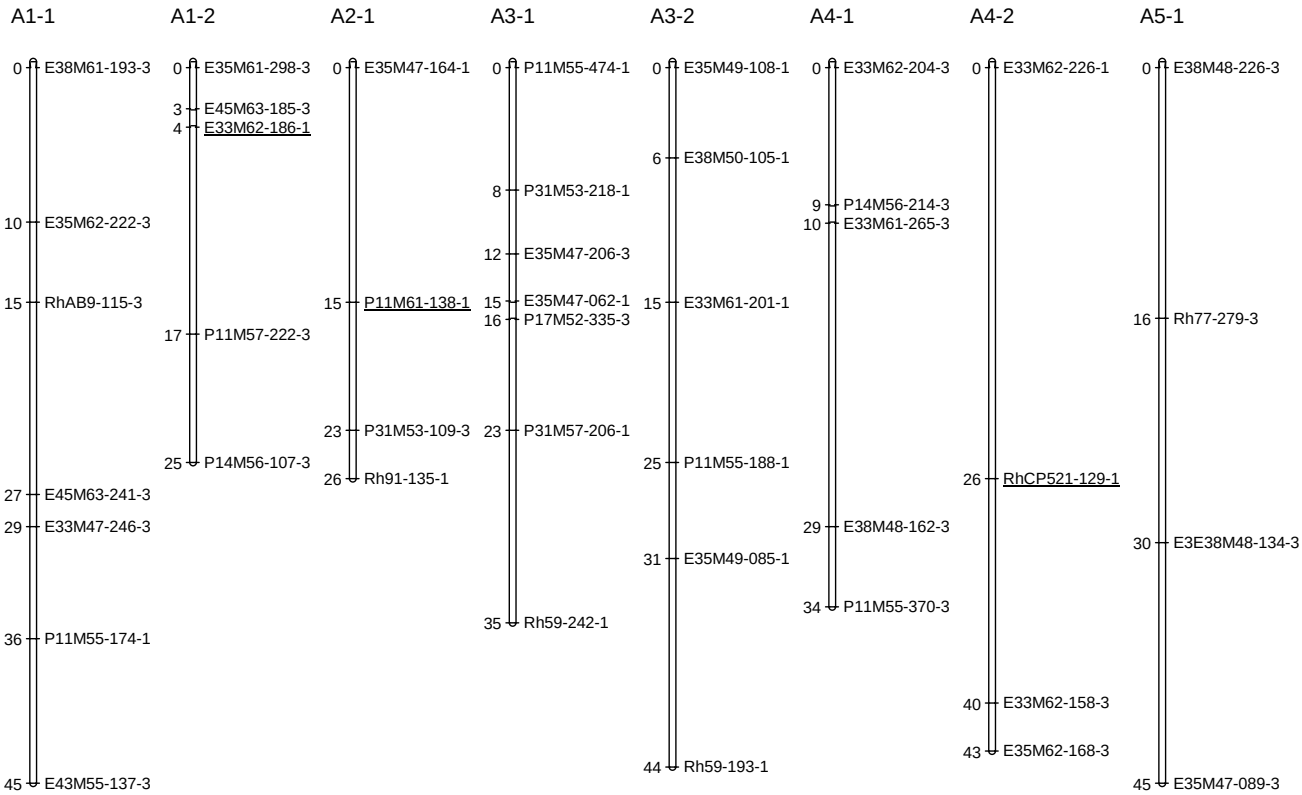
Genetic map

Parental linkage maps were separately constructed with JoinMap® 3.0 (Van Ooijen and Voorrips 2001) using only the uni- and bi-parental simplex markers. For parent P540, a set of 134 markers, comprising 57 uni-parental and 77 bi-parental markers, was used to construct the maternal map; 102 of these markers could satisfactorily be mapped. In all, 24 linkage groups each including three or more markers were obtained (Figure 1). The total length of those linkage groups was 695 cM. Among the 24 linkage groups, the mapping of the multi-allelic SSR markers enabled assignment of 15 groups to one of the seven chromosomes using the diploid map (Chapter 2) as reference for alignment.

For parent P867, a set of 148 markers, comprising 71 uni-parental and 77 bi-parental markers (Table 1), was available for map construction. The analysis resulted in a map of 17 linkage groups with 110 markers. The total length of this map was 697 cM. Among the 17 linkage groups, 14 groups were assigned to one of the seven chromosomes of the reference map. The linkage groups are depicted in Figure 1. Due to the lack of anchor markers a few linkage groups containing markers from P540 or P867 could not be assigned to one of the chromosomes of the reference map.

Table 1 Classification of segregation of uni-parental and bi-parental markers based on the segregation ratios observed. The markers with origin code P_1 refer to markers originating from P540 and P_2 from P867.

Marker type	Marker segregation type	Marker origin	Number of markers fitting expected segregation ratio						Total
			Uni-parental		Bi-parental			Others	
			1:1	5:1	3:1	11:1	35:1		
AFLP									
	1	P_1	48	14				4	66
	2	P_2	51	9				12	72
	3	P_1 & P_2			66	22	6	5	99
SSR									
	1	P_1	9	2				1	12
	2	P_2	20	4				4	28
	3	P_1 & P_2			11	2		3	16
Total			128	29	77	24	6	29	293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10 E35M62-222-3
15 RhAB9-115-3
27 E45M63-241-3
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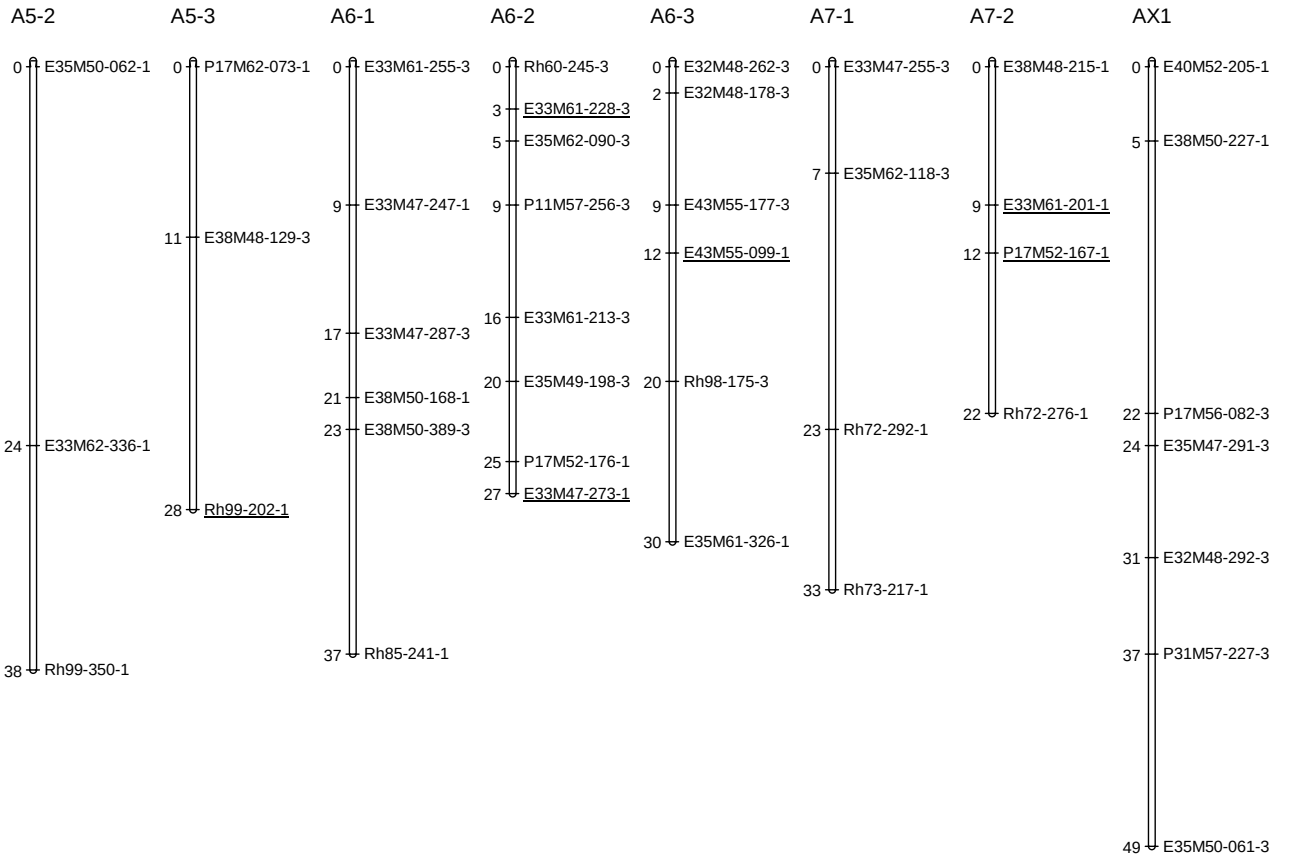
36 P11M55-174-1
45 E43M55-137-3
A1-2
0 E35M61-298-3
3 E45M63-185-3
4 E33M62-186-1
17 P11M57-222-3
25 P14M56-107-3
A2-1
0 E35M47-164-1
15 P11M61-138-1
23 P31M53-109-3
26 Rh91-135-1
A3-1
0 P11M55-474-1
8 P31M53-218-1
12 E35M47-206-3
15 E35M47-062-1
16 P17M52-335-3
23 P31M57-206-1
35 Rh59-242-1
A3-2
0 E35M49-108-1
6 E38M50-105-1
15 E33M61-201-1
25 P11M55-188-1
31 E35M49-085-1
44 Rh59-193-1
A4-1
0 E33M62-204-3
9 P14M56-214-3
10 E33M61-265-3
29 E38M48-162-3
34 P11M55-370-3
A4-2
0 E33M62-226-1
26 RhCP521-129-1
40 E33M62-158-3
43 E35M62-168-3
A5-1
0 E38M48-226-3
16 Rh77-279-3
30 E3E38M48-134-3
45 E35M47-089-3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24 E33M62-336-1
38 Rh99-350-1
A5-3
0 P17M62-073-1
11 E38M48-129-3
28 Rh99-202-1
A6-1
0 E33M61-255-3
9 E33M47-247-1
17 E33M47-287-3
21 E38M50-168-1
23 E38M50-389-3
37 Rh85-241-1
A6-2
0 Rh60-245-3
3 E33M61-228-3
5 E35M62-090-3
9 P11M57-256-3
16 E33M61-213-3
20 E35M49-198-3
25 P17M52-176-1
27 E33M47-273-1
A6-3
0 E32M48-262-3
2 E32M48-178-3
9 E43M55-177-3
12 E43M55-099-1
20 Rh98-175-3
30 E35M61-326-1
A7-1
0 E33M47-255-3
7 E35M62-118-3
23 Rh72-292-1
33 Rh73-217-1
A7-2
0 E38M48-215-1
9 E33M61-201-1
12 P17M52-167-1
22 Rh72-276-1
AX1
0 E40M52-205-1
5 E38M50-227-1
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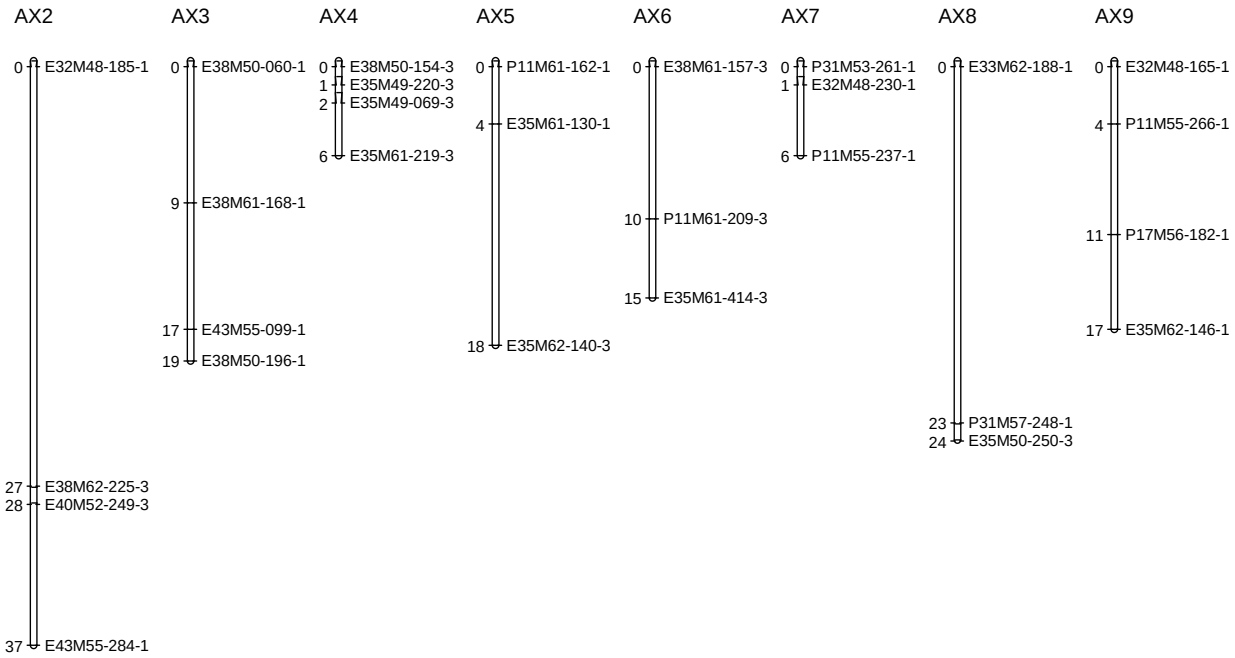
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9 E38M61-168-1
17 E43M55-099-1
19 E38M50-196-1
AX4
0 E38M50-154-3
1 E35M49-220-3
2 E35M49-069-3
6 E35M61-219-3
AX5
0 P11M61-162-1
4 E35M61-130-1
18 E35M62-140-3
AX6
0 E38M61-157-3
10 P11M61-209-3
15 E35M61-414-3
AX7
0 P31M53-261-1
1 E32M48-230-1
6 P11M55-237-1
AX8
0 E33M62-188-1
23 P31M57-248-1
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4 P11M55-266-1
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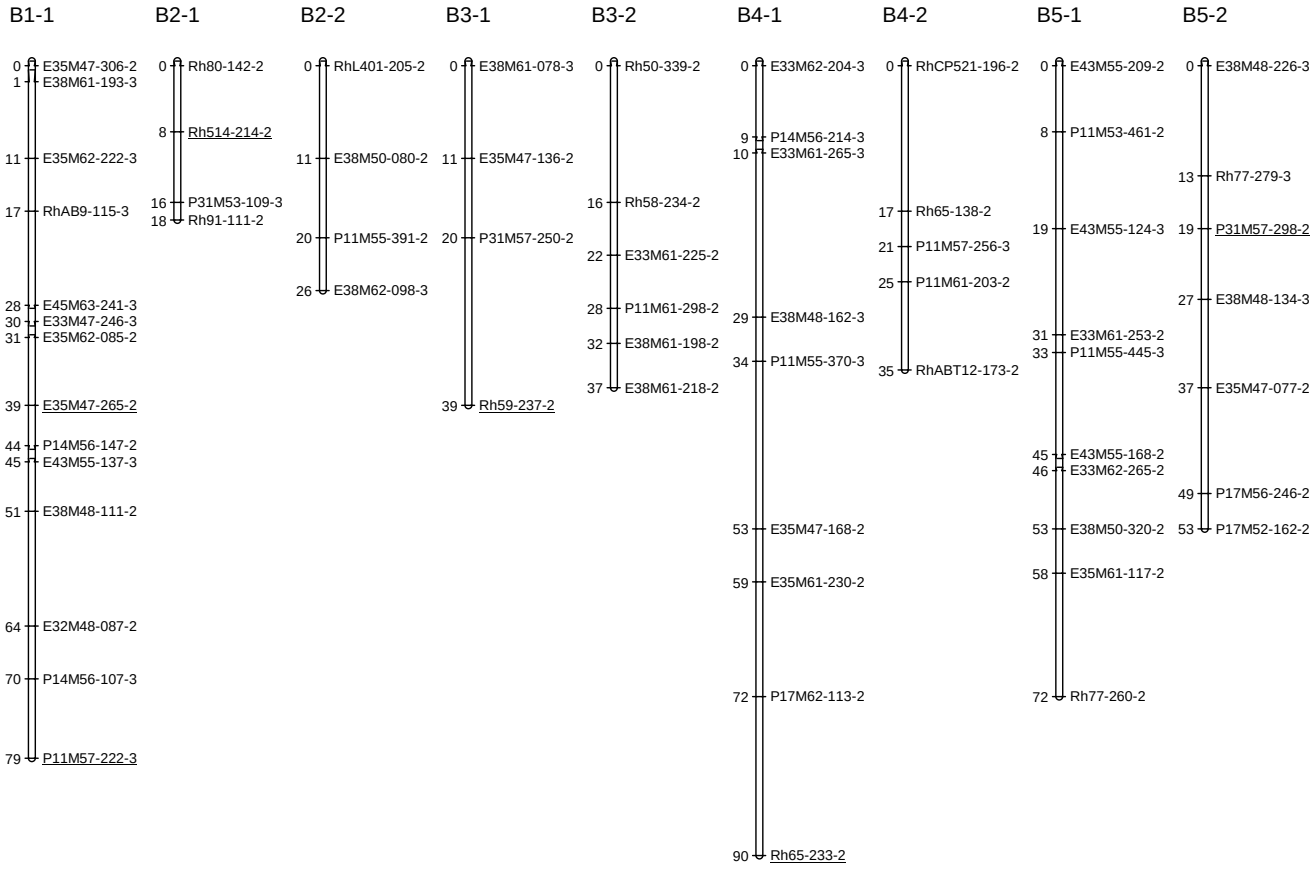
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1 E38M61-193-3
11 E35M62-222-3
17 RhAB9-115-3
28 E45M63-241-3
30 E33M47-246-3
31 E35M62-085-2
39 E35M47-265-2
44 P14M56-147-2
45 E43M55-137-3
51 E38M48-111-2
64 E32M48-087-2
70 P14M56-107-3
79 P11M57-222-3
B2-1
0 Rh80-142-2
8 Rh514-214-2
16 P31M53-109-3
18 Rh91-111-2
B2-2
0 RhL401-205-2
11 E38M50-080-2
20 P11M55-391-2
26 E38M62-098-3
B3-1
0 E38M61-078-3
11 E35M47-136-2
20 P31M57-250-2
39 Rh59-237-2
B3-2
0 Rh50-339-2
16 Rh58-234-2
22 E33M61-225-2
28 P11M61-298-2
32 E38M61-198-2
37 E38M61-218-2
B4-1
0 E33M62-204-3
9 P14M56-214-3
10 E33M61-265-3
29 E38M48-162-3
34 P11M55-370-3
53 E35M47-168-2
59 E35M61-230-2
72 P17M62-113-2
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46 E33M62-265-2
53 E38M50-320-2
58 E35M61-117-2
72 Rh77-260-2
B5-2
0 E38M48-226-3
13 Rh77-279-3
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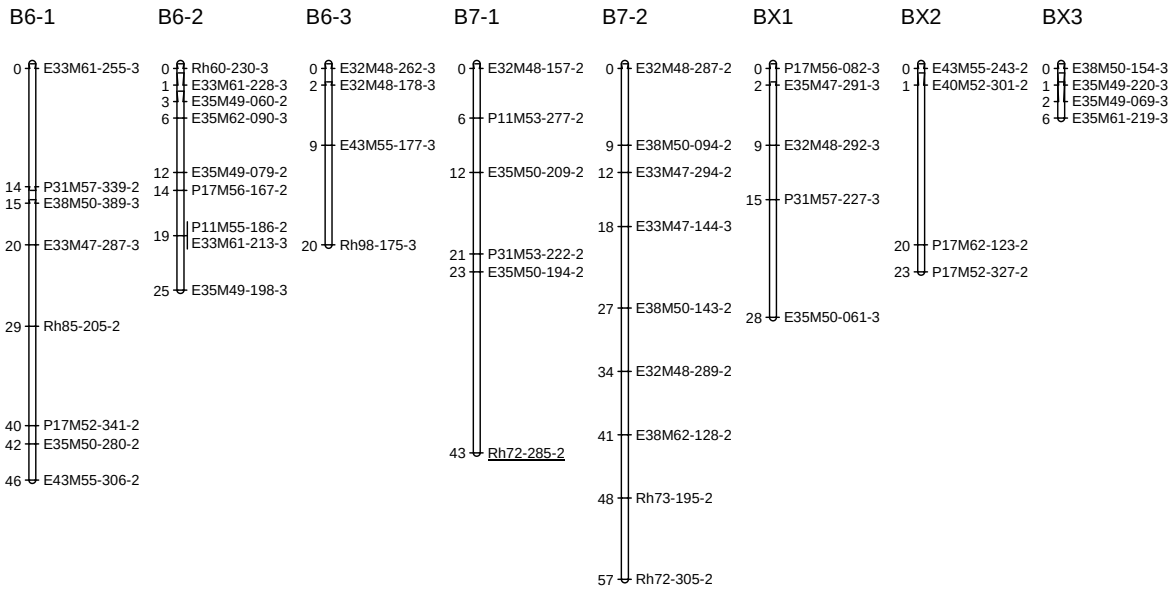

Figure 1 Parental P540 (A) and P867 (B) maps with marker loci putatively associated with resistance. Chromosome codesA1 to A7 and B1 to B7 are similar to those of the diploid maps (Chapter 2). Linkage group codes were extended with a serial number for each of the homologous linkage groups found for a chromosome. Linkage groups without alignment to one of the chromosomes of the diploid reference map are marked with "X". Map distances are given in cM at the left of each linkage group. Marker loci associated with powdery mildew resistance are underlined.

Table 2 Selection of marker loci putatively associated with three components of powdery mildew resistance, being the disease scores at 11 days post inoculation (DS), latency period (LP) and the rate of symptom development (RSD). The effect of a marker to the resistance (regression coefficient β) is indicated.

Isolate	Marker	DS			LP			RSD		
	code	Marker locus	β	Site	Marker locus	β	Site	Marker locus	β	Site
Isolate 2	m_1	E33M62-186-1	0.40	A1-2	E33M62-186-1	0.40	A1-2	E33M47-273-1	0.08	A6-2
	m_2	E33M47-273-1	0.39	A6-2	Rh65-233-2	0.35	B4-1	RhCP521-129-1	0.07	A4-2
	m_3	Rh514-214-2	-0.58	B2-1	E38M48-220-3	-0.58		Rh99-202-1	-0.10	A5-3
	m_4	E43M55-161-3	-0.40		E35M47-265-2	-0.39	B1-1	E31M57-298-2	-0.07	B5-2
Isolate F1	m_1	E33M61-201-1	2.52	A7-2	E33M61-201-1	3.60	A7-2	E33M61-201-1	0.19	A7-2
	m_2	P11M61-138-1	0.39	A2-1	P17M52-167-1	2.50	A7-2	E38M61-050-2	0.11	
	m_3	E38M50-104-2	0.37		Rh72-285-2	0.68	B7-1	Rh59-237-2	0.10	B3-1
	m_4	P17M52-167-1	-1.48	A7-2	E33M61-228-3	0.64	A6-2	E43M55-099-1	0.10	A6-3
	m_5	E33M62-343-2	-0.59					P11M57-222-3	-0.09	B1-1

Marker-trait association

To find candidate marker loci associated with genes for powdery mildew resistance to the two isolates used in the resistance tests, separate marker-trait association analyses were performed for each individual component of resistance. The analysis was preceded by ANOVA for each marker to select only those markers showing a significant difference between the two marker classes, i.e. presence vs. absence of the markers, or with and without specific marker alleles, for further analysis. The numbers of "promising" markers for each of the three resistance traits varied from 16 to 28. Multiple regression analysis was subsequently performed using the sets of markers having a significant effect on the corresponding resistance trait as predictor variables. This resulted in subsets of markers accounting for a moderate proportion of phenotypic variation for the resistance traits under study. For each combination of resistance trait and isolate test, four or five most significant (P<0.005) markers associated with resistance were selected. Adding more markers to the subsets did not result in significant improvement of R^2. Four markers were identified for components of resistance to isolate 2, four or five markers for resistance to isolate F1 (Table 2). The whole set of specific regression equations are presented in Table 3. Phenotypic variation of resistance explained by the marker loci ranged from 10.4 to 22.3 % for the traits (Table 3). Some marker loci, for example, E33M62-186-1, E33M47-273-1, E33M61-201-1 and P17M52-167-1 associated with different resistance traits. None of the markers were detected to be associated with resistance to both isolates.

Map position of markers associated with resistance

The map position of the markers putatively associated with genes for resistance is indicated in Table 2 and Figure 1. Obviously, these markers were distributed over several chromosomes. P540 has apparently genes accounting for resistance located on at least six different chromosomes, i.e.

Table 3 Multiple regression equations describing the variation for three components of powdery mildew resistance, i.e. the disease scores at 11 days post inoculation (DS), latency period (LP) and the rate of symptom development (RSD), contributed by a set of selected marker loci (m_1 to m_5, Table 2), which were used as explanatory variables. The regression equation is indicated in Materials and Methods. R^2 is a measure for the proportion of the total variation of the trait contributed by the marker loci.

Isolate	Regression equation	R^2
Isolate 2	$DS = 4.53 + 0.40m_1 + 0.39m_2 - 0.58m_3 - 0.40m_4$	12.9
	$LP = 6.23 + 0.40m_1 + 0.35m_2 - 0.58m_3 - 0.39m_4$	10.4
	$RSD = 0.74 + 0.08m_1 + 0.07m_2 - 0.10m_3 - 0.07m_4$	12.8
Isolate F1	$DS = 2.58 + 2.52m_1 + 0.390m_2 + 0.37m_3 - 1.48m_4 - 0.59m_5$	21.7
	$LP = 7.01 + 3.60m_1 + 2.50m_2 + 0.68m_3 + 0.64m_4 + 1.08$	22.3
	$RSD = 0.54 + 0.19m_1 + 0.11m_2 + 0.10m_3 + 0.10m_4 - 0.09m_5$	17.0

chromosomes 1, 2, 4, 5, 6 and 7; while P867 has putative genes on six chromosomes, i.e. chromosomes 1, 2, 3, 4, 5 and 7; which contributed to variation for powdery mildew resistance.

Discussion

The AFLP and SSR primer pairs used to generate markers were chosen on the basis of our experience with the construction of a diploid reference map (Chapter 2). The aim was to generate as many informative markers as possible and to create allelic bridges between linkage groups of the tetraploid maps to facilitate QTL analysis. A total of 237 AFLP markers and 56 allelic SSR markers were generated. The most likely segregation ratio for each marker was determined based on χ^2 tests for deviation from the expected segregation ratio. Ninety percent of the markers showed simplex or duplex segregation ratios.

By using a strategy of first mapping uni-parental simplex markers and then adding bi-parental simplex markers, two parental maps were constructed with a total length of 695 cM for the P540 map and 697 cM for the P867 map. The allelic SSR markers are informative anchor markers for assignment of the linkage groups to one of the seven designated rose chromosomes of the reference map (Chapter 2). They are also useful for assessment and alignment of homologous chromosomes from the set of tetraploid parental maps. At present, however, the number of linkage groups is far from complete and a considerable number of markers could not be assigned to one of the linkage groups of the parental maps. The occupancy of linkage groups with markers is not dense and some linkage groups could not be assigned to specific chromosomes due to a lack of anchor markers. Obviously, more markers are required to get a complete coverage of the tetraploid map.

The present study has shown that the parents of the population K5, from a genetic point of view, can be autotetraploids. The segregation ratios observed for multi-allelic markers indicated that random chromosome pairing of homologous chromosomes is common. The parents of the population were shown to have only a small proportion of polymorphic markers with double dosage of marker alleles. The segregation ratios indicated that 10 % of the markers correspond to a parental combination of nulliplex and duplex, 8 % to a combination of simplex and duplex, and 2 % to duplex and duplex. This phenomenon agrees with that found in autotetraploids like potato (Meyer et al. 1998) and alfalfa (Skinner et al. 2000). The genetic mapping of the population with simplex markers also ended up with the identification of some homologous chromosomes. Future confirmation of the genome constitution of the parents can be made by the observation of typical meiotic chromosome association frequencies (Lata 1982; Ma et al. 2000) or with codominant molecular markers (Byrne and Crane 2003) when a sufficient number of such markers is available.

Marker-trait association analysis was employed to detect marker loci for powdery mildew resistance in the present study by using both uni- and bi-parental markers. Although uni- and bi-

parenal simplex markers are efficient for mapping QTLs in tetraploids (Xie and Xu 2000), we applied regression of traits on markers, because of the insufficient genome coverage of the parental maps. Marker loci associated with genes for different resistance components have been found on different chromosomes and different chromosome regions, indicating a polygenetic nature of powdery mildew resistance in the present tetraploid population. Both of the parents were a partially resistant and the population showed transgression for resistance to both isolates (Chapter 5), indicating that each parent contributed alleles to the resistance. The proportion of the total variation of the traits contributed by the best sub-set of marker loci is in general not high. However, a considerable part of the genetic variation of the traits probably has been detected considering the fact that the traits had intermediate heritabilities (Chapter 5). Nevertheless, no marker locus associated with major genes for resistance were found. This could either mean that many genes with small effects are involved in powdery mildew resistance or that, due to the limited number of markers available in the present study, major genes could not be detected. The later may also, to some extent, explain why no common markers were found for resistance to both isolates in the present study. Different sets of QTLs may also exist separately for quantitative resistance to different isolates in roses as found by Qi (1998) for barley leaf rust resistance.

Both qualitative and quantitative resistances to powdery mildew have been found in other plant species like in barley (Jorgensen 1994; Williams 2003), wheat (Mingeot et al. 2002), tomato (Bai 2004) and *Arabidopsis thaliana* (Schiff et al. 2001). This study suggests that both qualitative and quantitative resistances to powdery mildew also occur in roses. A major dominant gene (*Rpp1*) for race-specific resistance to powdery mildew has been identified on chromosome 3 of diploid rose (Linde and Debener 2003; Linde et al. 2004). The current findings of multiple marker loci for resistance to different isolates point towards quantitative resistance to powdery mildew in rose. Quantitative resistance controlled by multiple genes, each having minor effect, is considered durable. Since the minor genes each may have a different function in plant defense, the loss of only one or few resistance genes will not lead to a dramatic infection of the plant (Qi 1998).

Additional genotyping of the present population may unmask major genes affecting powdery mildew resistance. Rose breeders would greatly benefit from the identification and mapping of the markers linking genes for polygenic resistance to powdery mildew. Such markers will allow them to introgress this resistance, possibly from several sources, into their elite breeding materials. In the future, the markers found in this study can be used for pyramiding resistance genes into rose genotypes through marker-assisted selection.

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Chapter 7

General Discussion

Screening methodology

Energy in greenhouses is mainly needed to control temperature and air humidity and to give light for plant growth. Energy efficiency of greenhouse cultivation is the ratio of energy fixed in biomass and the amount of energy needed for plant production. This can be improved through a better of light interception, cultivation methods, crop varieties and CO_2 enrichment (Van der Velden 1992). Therefore, breeding for rose cultivars with vigorous growth and excellent adaptation to low energy levels was started in the early 1980s, following an initiative of the Dutch Government to lower the energy use in greenhouse industries (De Vries et al. 1980).

Proper evaluation methods for assessment of phenotypic traits relevant to the energy efficiency of crops are needed for genetic improvement. They should be simple, fast and easily applicable. Good methods also are one of the most important prerequisites for a successful analysis of genetic variation. Strategies for the evaluation of growth vigour and powdery mildew resistance have been worked out in the current study. They proved to be suitable and useful in genetic studies that can improve breeding efficiency in roses.

Growth vigour

Measurements of growth vigour in rose are difficult, time-consuming and costly. This holds certainly true if large numbers of genotypes have to be tested. Therefore, work has been done to design and test a fast and well-standardized screening procedure for vigour, based on only one single shoot per cutting. Various characteristics of these shoots measured before flowering were used as indicators of growth vigour. The pilot study gave clear clues for the experimental design and the number of cuttings per clone needed to get an effective test for growth vigour (Chapter 3). It was also proven that collection of genotypic data was possible in a rather short period of time for each of the vigour-related traits.

A second aspect of testing for vigour addressed in the pilot study concerned the temperature conditions for testing. Temperature is one of the climatic factors to be considered to optimize selection of rose cultivars to be used in energy saving greenhouses, together with factors as light and relative air humidity (Berninger and Philouze 1988). In the pilot study (Chapter 3) some genotypes from the diploid mapping population as well as its parents were tested at two suboptimal

temperatures (16 and 20 ^{o}C). Most genotypes responded in a similar way to lowering the temperature by 4 ^{o}C for most of the vigour-related traits. In other words, genotype x environment interaction was of minor importance. The outcome of the diploid study was in agreement with the studies of De Vries and co-workers in the 1980s. De Vries et al. (1982) conducted an experiment with 15 Hybrid tea rose F1 populations in nine growth conditions, i.e. combinations of three light levels of 8, 16, 24 Wm^{-2} (visible) irradiation and three temperatures of 16, 20 and 24 ^{o}C, and concluded that the effects of temperature on shoot growth were basically the same under high and lower light intensity; temperature and light were independent factors, which suggested that genotypes adapted to both low light and low temperature might be selected under the test regimes. In an earlier study, De Vries et al. (1980) evaluated the seedlings from 30 Hybrid tea rose F1 populations in greenhouse at six constant temperatures (10, 14, 17, 20, 23 and 26 ^{o}C) under natural light conditions. The results of these studies suggested that temperatures about 3 ^{o}C lower than normal temperature (~ 23 ^{o}C) used for rose cultivation in greenhouses are the best for selection of energy-efficient genotypes. One single temperature of 20 ^{o}C was finally chosen for the large-scale testing needed for the vigour evaluation of the diploid mapping population, using temperature-controlled greenhouses. This temperature is suboptimal for rose cultivation and therefore suitable for improvement of crop adaptation to energy-saving conditions.

Growth vigour is a complex trait. Dissection of this trait into components, each of which might be under control of different sets of genes, can be useful since selection on these components may be more efficient to improve growth vigour. To this end we evaluated the diploid mapping population in two large greenhouse experiments at 20 ^{o}C for ten vigour-related traits, using the screening procedure developed. The analyses showed that we were able to reduce satisfactorily the experimental error in the assessment of growth vigour. The evaluation on second shoots of vigour-related traits has proven to be a simple and fast procedure for large-scale screening of genotypes for growth vigour. The results showed that the variation observed in the mapping population for vigour-related traits was quantitative and highly heritable; total shoot dry weight and leaf area are good criteria for early selection of rose genotypes with vigorous growth under suboptimal growth conditions (Chapter 3).

Resistance to powdery mildew

In the evaluation of genotypes for powdery mildew resistance, both an effective inoculation assay and a proper quantification method for the disease development are essential. In comparison to other inoculation methods like dusting of dry spores of powdery mildew on plants, wet spore inoculation was shown to be quite effective and to have some clear advantages as well (Chapter 5). Firstly, wet inoculation allows relatively easy control over the quantity of inoculum and gives uniform distribution of spores, which are the critical factors for a good resistance test (Linde and Debener, 2003). Secondly, wet inoculation reduces the chances of infection of the test plants by

other fungi like *Botrytis*, which is not easy to be controlled in *in vitro* tests. Thirdly, with wet inoculation it is easy to handle in a repeatable and reliable way with large numbers of plants under greenhouse conditions, which is particularly useful in a rose breeding setting.

Three measures for disease resistance were used to describe genotypic differences within the tetraploid population with respect to the disease development upon inoculation with two isolates, i.e. disease score at 11 days post inoculation (DS), latency period (LP) and rate of symptom development (RSD) (Chapter 5). The analysis of the relationship between components of resistance indicated that a close relationship between DS and LP (r=-0.83 to isolate 2 and r=-0.88 to isolate F1), an intermediate relationship between DS and RSD (r=0.56 to isolate 2 and r=0.37 to isolate F1), and a weak relationship between LP and RSD (r=-0.24 to isolate 2 and r=-0.14 to isolate F1) exist. QTL analyses of the variation for each of the components provided evidence for a partly common genetic basis (Chapter 6), for example, marker E33M62-186-1 is associated with DS and LP, E33M47-273-1 with DS and RSD for isolate 2; E33M61-201-1 with DS, LP and RSD, P17M56-167-2 with DS and LP for isolate F1. Statistical analyses showed that disease score at 11 dpi and/or latency period are suitable criteria for selection and identification of genetic materials with quantitative resistance.

Molecular maps

Diploid population and its maps

The mapping population that we used to study the genetics of growth vigour, derived from *Rosa multiflora*, one of the ancestral parents of modern roses, has a large genetic variation for growth vigour. The two parents showed significant differences for most of the measured vigour-related traits and the population displayed transgressive segregation (Chapter 3). A diploid population was chosen on purpose, although pot and cut roses are tetraploid. The reason is that the inheritance of traits is relatively simple at the diploid level (disomic versus tetrasomic). This strategy has often been employed in genetic studies in the polyploid species like potato, alfalfa and sugarcane. The intensive molecular studies conducted at the diploid level have resulted in knowledge on the inheritance of several traits of interest in these species and provided information useful for breeding of related polyploid crops (Bonierbale et al. 1988; Jacobs et al. 1995; Meyer et al. 1998; Haynes and Christ 1999; Bryan et al. 2002). The current mapping study represents the first step of investigation into the inheritance of vigour, which will enable marker-assisted selection in cultivated rose breeding.

Genotyping of the diploid population with different types of molecular markers has ended up with the most advanced rose maps with respect to genome coverage and marker density. The maps have helped QTL mapping for vigour (Chapter 4) and will presumably be used to map genes for

other traits of interest. The SSR markers, randomly distributed over the maps, offer good opportunities for alignment with other maps.

Tetraploid population and its maps

The tetraploid K5 population was made specifically for the present study through crossing of two rose cultivars, each with partial resistance to powdery mildew, aiming at unveiling the inheritance of mildew resistance, in particular partial resistance. The population showed continuous variation of resistance to the two tested monospore isolates (Chapter 5).

The genotyping of the population with AFLPs and SSRs has resulted in parental maps with many linkage groups (Chapter 6). Homologous linkage groups were identified with allelic SSR markers. The construction of linkage maps in tetraploid rose has proven not to be easy. It is time consuming and expensive to generate a sufficient number of reliable markers for genetic mapping, especially in a population with a large size like the present population with 181 offspring. The number of markers for efficient use in tetraploid mapping is limited as compared to diploid mapping. A large proportion of the markers with higher allele dose (duplex or higher) cannot be effectively used in tetraploid mapping analysis. Even for bi-parental simplex markers, without prior linkage information as in the present study, the estimation of recombination rate of linked markers is troublesome because these markers may have different linkage phases in both parents. For that reason only uni-parental simplex markers can be reliably used for genetic mapping regardless of disomic or tetrasomic inheritance (Rajapakse et al. 2001). For an ideal linkage analysis in a tetraploid population, an extremely large number of markers, preferably codominant ones, have to be generated and mapped, with a sufficient number of markers and anchor points on each linkage group. The latter markers are essential for identifying the homologous groups.

Anchor markers

The comparison of the diploid (Chapter 2) and tetraploid populations (Chapter 6) with respect to the generation of molecular markers was possible since the same AFLP primer combinations were used. Both types of population showed similar numbers of polymorphic markers, i.e. on average 10 markers per primer combination in the diploid population and about 9 in the tetraploid population. For SSR, twenty SSR primer combinations used in the two populations resulted in a wide variety of SSR alleles (Table 1). Theoretically, the maximum number of distinct alleles per primer combination is four in two diploid parents and eight in two tetraploid parents. In the diploid population some primer combinations gave rise to the maximum number of alleles. The actual numbers of alleles generated by a specific primer combination was, however, lower. Some primer combinations, for example, Rh72, Rh77 and Rh98, yielded relatively high numbers of alleles in both populations (Table 1). Some primer combinations detected possibly identical alleles in both populations, i.e. those having the same or similar in size. In general, the number of polymorphic

markers generated by AFLP and SSR primer combinations in diploid and tetraploid populations did not differ much. However, the number of markers useful for mapping in the tetraploid roses is lower.

The map positions of allelic SSR markers on the diploid and tetraploid maps are anchor points for the alignment of the two maps. This enabled the assignment of most of the linkage groups of the tratraploid maps to one of the seven linkage groups of the diploid reference map (Chapter 6).

Genetic analysis

Vigour study

Various statistics describing the variation of vigour-related traits (Chapter 3) as well as the corresponding QTL analyses (Chapter 4) indicated that the variation for these traits is of a quantitative genetic nature. QTLs clustered in small regions on some chromosomes like the cluster on linkage group 2 which included major QTLs for all the ten vigour-related traits. The QTLs for highly correlated traits co-localized in several chromosome regions, indicating that correlation is due to pleiotropy or linkage.

Table 1 A comparison of the number and size of the alleles generated in the diploid 94/1 population and the tetraploid K5 population.

SSR primer combination	Allele size (bp)	
	Diploid 94/1 population	Tetraploid K5 population
Rh50	336, 339, 343	335, 339
Rh58	248, 254, 269	234, 260, 295
Rh59	197, 200, 216	193, 203, 211, 237, 242
Rh60	234, 240, 252	137, 230, 245
Rh65	128, 130, 132	130, 132, 138, 168, 233
Rh72	115, 276, 283, 285	260, 268, 276, 285, 292, 305
Rh73	160, 162, 172	195, 210, 217
Rh76	156, 196	156
Rh77	232, 249, 258, 264	248, 255, 260, 279, 298, 323
Rh80	134, 148	133, 142, 146, 182
Rh85	207, 217, 221	205, 215, 241
Rh91	111, 119, 135	111, 135
Rh98	153, 170, 175, 221	153, 156, 165, 172, 175, 223
Rh99	179	179, 202, 260, 283, 350
RhAB38	149, 173, 190	126, 150, 163, 171
RhAB9-2	101, 108, 124	105, 115, 118, 122
RhABT12	167, 173, 181	173
RhL401	210	205
Rh514	197, 201, 205	187, 205
RhCP521	128, 140, 147	129, 196, 216, 249

It is difficult to estimate at this moment the value of the different vigour QTLs segregating in the diploid population for breeding of tetraploid roses. Genes affecting vigour might act differently in other genetic background (Groover et al. 1994). Nevertheless, the markers linked to the QTLs for vigour in the present study are potential handle for marker-assisted selection in rose breeding.

Powdery mildew resistance

In the marker-trait association analysis of powdery mildew resistance we found markers with a medium effect on resistance. They are located in different linkage groups, providing evidence for the segregation of minor genes for quantitative resistance in the population. The finding of the present study agrees with the observations that both qualitative (Linde and Debener, 2003; Linde et al., 2004) and quantitative resistance exist in roses as well as in other crops (Jorgensen, 1994; Schiff et al. 2001; Williams 2003; Mingeot et al. 2002; Bai 2004).

Prospects and future research

Improvement of genetic maps

To improve the current diploid and tetraploid maps, more anchor points such as genomic SSRs and EST-SSRs, should be developed. Rose EST data that are becoming more and more available in public domains will be helpful for this purpose. Other interesting gene-based anchor points are polymorphic functional genes. To this end, expression profiling using cDNA-microarray analysis or cDNA-AFLP can be performed to discover relationships between DNA sequences and gene function in rose. The following step is the development of allele-specific markers for the genes controlling target traits, in particular single nucleotide polymorphisms (SNPs).

A consensus map for rose, combining the genetic maps generated from different mapping populations with different molecular markers in different laboratories is under construction. Comparative mapping of rose with plant species within the Rosaceae family like peach and apple, as well as with plant species outside the Rosaceae family like *Arabidopsis thaliana* and rice, will provide novel opportunities for searching genes of interest like the ones for resistance to diseases (Rajapakse 2003).

Fine mapping of QTLs

The resolution of QTL mapping obtained from this initial study could be improved by adding more genetic markers, which would allow the identification of markers more closely linked to the mapped QTLs for vigour and powdery mildew resistance. Different populations can be used to allow an examination of more recombinant types and to verify the QTLs detected in the present studies. Common QTLs identified in different populations and environments are ideal targets for

marker-assisted selection and for basic research towards cloning and characterization of genes affecting quantitative traits. This valuable information could be used in any available population to monitor the inheritance of a specific chromosomal segment or to evaluate the variation available in a specific gene pool at a particular locus. Molecular markers closely linked with the QTLs could then be transformed into a PCR-based marker assay to simplify the detection of specific QTL alleles among selected genotypes.

Linkage disequilibrium (LD) or association mapping, based on natural populations to identify the association of a marker locus with genes for a target trait, has provided an alternative way for genetic studies and is currently being used in plants (Hansen et al. 2001; Reming et al. 2001; Rafalski 2002; Simko et al. 2004). It is a potential way for direct testing of candidate genes and alleles in complex tetraploid species (Simko et al. 2004).

Marker-assisted selection

The efficient use of the genetic variation in vigour from unadapted rose germplasm or wild relatives of modern cultivars is essential for continuous genetic improvement. Transfer of desirable characters from the diploid related species into modern cultivars without affecting other traits will be a challenge for rose breeders. Factors affecting genetic exchange between parental genomes in tetraploid hybrids could be addressed by marker analysis in segregating populations or from patterns of introgression (Herrera et al. 2002). The improved knowledge of the target traits in the present study will accelerate the application of marker-assisted selection on target genes and alleles in rose breeding.

Further studies on powdery mildew resistance

Precise genetic mapping, high-resolution chromosome haplotyping and extensive phenotyping of genotypes for powdery mildew resistance by the present methods as well as by LD mapping will provide more information on molecular resistance mechanisms. Cytological and histological studies of the interaction of rose plant and powdery mildew pathogen will reveal the cellular resistance mechanisms. This information will help phenotyping of test plants and will be a prerequisite for the development of sophisticated breeding strategies in rose.

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Summary

Rose (*Rosa*) is one of the important ornamental crops worldwide. Greenhouse production of cut rose and pot rose requires much fossil fuel for optimal plant growth and is adversely affected by infection with powdery mildew (*Podosphaera pannosa*). The heavy energy input and frequent application of fungicides to control the disease contributes considerably to the product cost and environmental pollution. Genetic improvement of cultivars will be facilitated by a better understanding of the genetic variation and the inheritance architecture of the traits determining vigour and mildew resistance. To this end procedures were developed to screen for plant vigour and mildew resistance. These methods were subsequently used with success to study the genetic variation for the two traits present in a diploid and a tetraploid population, respectively. The populations were also molecularly characterized to enable genetic dissection of the variation of the traits. The achievements of the studies are presented in different chapters of this thesis.

A high-density genetic map with a number of anchor markers was created for vigour study, using the diploid rose population (Chapter 2). Linkage maps were constructed using a total of 520 molecular markers including AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers. Seven linkage groups, each putatively corresponding to one of the seven rose chromosomes, were identified for female and male linkage groups spanning 487 cM and 490 cM, respectively. The linkage groups likely cover more than 90 % of the rose genome. The corresponding female and male linkage groups were subsequently integrated. The present linkage maps with robust types of markers are currently the most advanced ones in rose with regard to marker density and genome coverage. The mapped SSR and RFLP markers provide good anchor points for future map alignment studies.

Phenotypic evaluation of the diploid population for vigour and its related traits was separately conducted in greenhouses in Denmark (DK) and The Netherlands (NL) under suboptimal growth conditions. A screening procedure for large-scale evaluation of rose genotypes for vigour was developed (Chapter 3). The population showed a continuous quantitative variation as well as a considerable transgression for all the traits. Genetic differences among the tested entries were highly significant and tended to be large for most of the traits in comparison to the effects of genotype by environment interaction. The estimates for heritability were high (68 to 92 %) and the relationships among most of the traits were also high (r = 0.65 to 0.95). Total shoot dry weight and leaf area are suggested to be good criteria for early selection of rose genotypes with vigorous growth.

QTL analyses for each of ten vigour-related traits identified ten chromosomal regions, scattered over the seven linkage groups, containing QTLs for one or more traits (Chapter 4). Considering each trait separately, a total of 42 QTLs was found. Among these QTLs, 24 were common in both

DK and NL experiments, whereas eight were only detected in the NL experiment and ten in the DK experiment. The number of QTLs for individual traits varied from three to five, each determining 12 % to 35 % phenotypic variation. QTLs for highly correlated traits were frequently colocalized, indicating a common genetic basis. Clustering of QTLs for different traits was noted in some chromosome regions, suggesting co-localization of several separate genes or/and pleiotropy.

The tetraploid population and its parents were tested for powdery mildew resistance under greenhouse conditions with two well-defined monospore isolates (2 and F1) using artificial inoculation with spore suspensions (Chapter 5). Disease score at 11 days post inoculation, latency period and rate of symptom development were used to describe the variation in resistance within the population. The tests for both isolates exhibited a wide and significant variation among genotypes for resistance. The distribution of the genotypic means of the disease scores was continuous and showed a considerable transgression. Analysis of the data indicated that the two isolates differed in pathogenicity. It is the first time that in rose an inoculation assay with spore suspensions has been successfully used. It is a reliable and effective way to screen large numbers of genotypes under greenhouse conditions for mildew evaluation in rose genetic and breeding studies.

The tetraploid population for powdery mildew resistance was further molecularly characterized to allow genetic map construction and marker-trait association analysis (Chapter 6). Uni-parental as well as bi-parental simplex AFLP and SSR markers were used to construct female and male maps. These analyses resulted in 23 and 17 separate linkage groups for the maps. The length of the respective maps was 695 and 697 cM. Multi-allelic SSR markers were successfully used to assign most of the linkage groups to one of the seven rose chromosomes. Marker-trait association analyses identified a number of marker loci presumably linked to genes for mildew resistance. These marker loci determined only a moderate part of the heritable variation. Some isolate-specific markers were found from both parents and distributed on different chromosomes, indicating polygenic resistance of the population.

The findings of the present study provide knowledge of the inheritance of the target traits at molecular level, which paves the road towards marker-assisted selection for breeding of new rose cultivars with vigorous growth for more efficient energy use and durable resistance to powdery mildew. This will finally result in energy efficient in the cultivation of roses in greenhouse.

Samenvatting

Roos (*Rosa*) is wereldwijd een van de belangrijkste siergewassen. Kasteelt van snijrozen en potrozen vereist veel fossiele brandstof voor een optimale plantengroei en –ontwikkeling en deze wordt vaak gehinderd door een infectie met echte meeldauw (*Podosphaera pannosa*). De zware energiebelasting en frequente bespuiting met fungiciden om ziekte te controleren draagt aanzienlijk bij aan de productiekosten en milieuverontreiniging. Kennis over de erfelijkheid van eigenschappen als groeikracht en meeldauwresistentie zijn nodig om tot genetische verbetering van cultivars te komen. In dit proefschrift werden procedures ontworpen om groeikracht en meeldauwresistentie te onderzoeken. Deze procedures werden vervolgens gebruikt om de genetische variatie voor twee eigenschappen te bestuderen in een diploïde en tetraploïde populatie. De populaties werden daartoe moleculair gekarakteriseerd om genetische ontleding van de variatie van eigenschappen mogelijk te maken.

Een genetische merkerkaart met hoge dichtheid aan moleculaire merkers en een aantal ijkmerkers werd geconstrueerd voor de analyse van groeikracht bij een diploïde populatie en is beschreven in Hoofdstuk 2. Koppelingsgroepen werden geconstrueerd met gebruikmaking van 520 moleculaire merkers, waaronder AFLP, SSR, PK, RGA, RFLP, SCAR en morfologische merkers. Zeven koppelingsgroepen, waarvan elke koppelingsgroep waarschijnlijk correspondeert met een van de zeven chromosomen, werden geïdentificeerd voor de moederlijke en vaderlijke koppelingsgroepen (resp. 487 en 490 cM). Het is zeer waarschijnlijk dat de koppelingsgroepen meer dan 90 % van het roosgenoom omspannen. Vervolgens werden de moederlijke en vaderlijke koppelingsgroepen geïntegreerd. De in dit onderzoek gecreëerde merkerkaart met robuuste merkers is een van de meest geavanceerde in roos met betrekking tot merkerdichtheid en genoomdekking. De in kaart gebrachte SSR en RFLP merkers verschaffen goede referentiepunten om vergelijking met toekomstige merkerstudies mogelijk te maken.

Fenotypische evaluatie van de diploïde populatie voor groeikracht en de daarbij behorende eigenschappen werden afzonderlijk uitgevoerd in Denemarken en Nederland onder suboptimale groeicondities. Een screeningsprocedure voor grootschalige evaluatie van groeikracht van roosgenotypen werd ontwikkeld en is beschreven in Hoofdstuk 3. De populatie liet een continue kwalitatieve variatie en een aanzienlijke transgressie zien is voor alle eigenschappen. Genetische verschillen onder de geteste nummers waren erg significant en waren vrij hoog voor de meeste eigenschappen in vergelijking tot de genotype x milieu interactie. De schattingen voor erfelijkheidsgraad waren hoog (68 – 92 %), evenals de correlatie tussen de meeste eigenschappen (r= 0,65 – 0,95). Scheutdrooggewicht en bladoppervlak bleken goede criteria voor vroege selectie van rozengenotypen op verhoogde groeikracht.

QTL analyse voor elk van de tien groeikrachtgerelateerde eigenschappen identificeerde tien

chromosomale gebieden, verdeeld over zeven koppelingsgroepen welke QTL's bevatten voor een of meer eigenschappen. Dit is beschreven in Hoofdstuk 4. Wanneer elke eigenschap afzonderlijk wordt bekeken, zijn 42 QTL's gevonden. Onder deze QTL's waren er 24 die zowel de proeven in Denemarken als in Nederland voorkwamen. Acht werden alleen gevonden in Nederland en tien alleen in Denemarken. Het aantal QTL's voor individuele eigenschappen varieerde van drie tot vijf, waarbij elke eigenschap een fenotypische variatie van 12 – 35 % verklaarde. QTL's voor eigenschappen met een hoge correlatie vielen vaak samen, wat duidt op een gemeenschappelijke genetische basis. Clustering van QTL's voor verschillende eigenschappen werd waargenomen in sommige chromosomale gebieden. Dit suggereert co-localisatie van verschillende aparte genen en/of pleiotropie.

De tetraploïde populatie en haar ouders werden getest op resistentie voor echte meeldauw onder teeltcondities met twee goedgedefinieerde monospore-isolaten en is beschreven in Hoofdstuk 5. Ziektescores op elf dagen na inoculatie, latentieperiode en snelheid van ziekte-ontwikkeling werden gebruikt om de variatie in resistentie te beschrijven. Testen met beide isolaten afzonderlijk toonden een grote en significante variatie in resistentie tussen de genotypen. De verdeling van de genotypische gemiddelden voor elk van de ziektescores was continue en liet een aanzienlijke transgressie zien. Analyse van de data gaf aan dat de twee isolaten verschilden in pathogeniciteit. Het is voor het eerst dat een natte inoculatie succesvol is toegepast bij roos. De toets bleek een betrouwbare en efficiënte manier om grote aantallen genotypen onder teeltcondities te testen op meeldauwresistentie.

De tetraploïde populatie welke werd gebruikt voor het onderzoek naar echte meeldauw resistentie werd verder moleculair gekarakteriseerd om een genetische map te construeren en merker-eigenschap associatiestudies te doen. Dit is beschreven in Hoofdstuk 6. Simplex AFLP en SSR merkers werden gebruikt voor de constructie van merkerkaarten voor elk van de ouders. Deze analyse resulteerde in een kaart voor de moeder met 23 koppelingsgroepen en een voor de vader met 17. De kaartlengte besloeg respectievelijk 695 en 697 cM. Multi-allele SSR merkers werden met succes gebruikt om de meeste koppelingsgroepen toe te kennen verbinden aan een van de zeven chromosomen. Merker-eigenschap associatie studies identificeerde een aantal merkerloci welke waarschijnlijk gekoppeld liggen met resistentiegenen voor echte meeldauw. Deze merkerloci verklaarden echter maar een beperkt deel van de erfelijke variatie. Sommige isolaat-specifieke merkers waren afkomstig van verschillen de ouders en waren gekarteerd op verschillende chromosomen, wat duidt op de aanwezigheid van polygene resistentie in de populatie.

De uitkomsten van de huidige studie voorzien in kennis van de erfelijkheid van de doeleigenschappen op een moleculair niveau. Dit maakt de weg vrij voor merkergestuurde selectie in de veredeling van nieuwe rozencultivars met verbeterde groeikracht bij suboptimale temperatuur en duurzame resistentie tegen echte meeldauw. Uiteindelijk zal dit resulteren in efficiënt gebruik van energie en verlaagd gebruik van fungiciden bij de teelt van kasrozen.

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Zifu Yan
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October, 2005

Curriculum Vitae

Zifu Yan was born on July 15, 1962, in Zhengyang, Henan Province, China. He obtained his BSc degree in Agronomy in 1982 from Henan Agricultural University and then worked there as a teacher and wheat breeding researcher. In 1999, he participated in the MSc Program in Plant Breeding at Wageningen University and got his MSc degree in 2001. He continued a four year AIO (PhD) research project at Plant Research International and the Laboratory of Plant Breeding, Wageningen University, The Netherlands, which ended up with the completion of this thesis.

This research is part of a larger research program "Rassen onder Glas met Minder Gas" aimed at studying the perspectives of improving the energy efficiency of Dutch greenhouse crops by breeding. The present study was sponsored by the Dutch Product Board for Horticulture (PT), The Netherlands Agency for Energy and the Environment (NOVEM) and the companies Plant Research International B.V., Terra Nigra B.V., Agriom B.V. and Poulsen Roser Aps.

PE&RC PhD Education Statement Form

With the educational activities listed below the PhD candidate, Zifu Yan, has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 22 credits (= 32 ECTS = 22 weeks of activities)

Review of literature (3 credits)

- Presentation: Study of growth vigour and powdery mildew resistant in rose (2002)
- Presentation: Genetic research on rose using molecular markers (2003)
- Presentation: progress of the project, Plant Research International (2004)

Post-Graduate Courses (4.2 credits)

- AFLP markers in plant systematics and breeding (2001)
- Guide to digital scientific art work (2001)
- Disease resistance in plants (2002)
- Techniques for writing and presenting a scientific paper (2004)

Deficiency, Refresh, Brush-up and General Courses (4 credits)

- Written English (2001)
- Advanced phytopathology (2001)
- Plant-pathogen interaction (2001)

PhD Discussion Groups (5 credits)

- Plant resistance group (2001-2004)
- Energy-efficiency program (2001-2005)
- Genetic resources and diversity in production ecology (2001-2004)

PE&RC Annual Meetings, Seminars and Introduction Days (3 credits)

- PE&RC annual meetings (2001-2005)
- EPS days (2001-2005)

International Symposia, Workshops and Conferences (5 credits)

- Dutch national symposium plant science (2002)
- Presentation: Genetic mapping towards QTL analysis in rose, 21st International Symposium on Classical versus Molecular Breeding of Ornamentals (2003)
- Presentation: Genetic dissertation of growth vigor with molecular markers in rose, Plant and Animal Genome XII Conference (2004)

Laboratory Training and Working Visits (2 credits)

- Mildew *in vitro* maintain and inoculation techniques. Institute for Ornamental Plant Breeding, Ahresburg, Germany (2001)
- Working visit “consensue rose map”, INRA, Genetics and Horticulture, France (2003)