The genetics of *Botrytis cinerea* resistance in tomato

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Dit onderzoek is uitgevoerd binnen de onderzoekschool ‘Experimental Plant Sciences’

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Proefschrift

Ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van Wageningen Universiteit,

Prof. dr. M.J. Kropff,

in het openbaar te verdedigen

op dinsdag 3 april 2007

des namiddags te vier uur in de Aula

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Thesis Wageningen University, The Netherlands, 2007
With references – with summaries in English and Dutch

ISBN 90-8504-628-9

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CHAPTER 1
General introduction

Introduction

Tomato (*Solanum lycopersicum*) is an economically important vegetable crop for both fresh market and processing industry. The Netherlands produced an estimated total of 645000 ton of tomatoes in 2005 (http://faostat.fao.org). Plants are continuously exposed to a wide range of pathogens. Of the average annual production losses worldwide, 38% is caused by diseases ($ 220 billion; Agrios 2005). One of the pathogens able to cause both pre- and post-harvest damage in tomato is the fungus *Botrytis cinerea*, also known as grey mould.

Botrytis cinerea

B. cinerea Pers.:Fr (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) is a necrotrophic pathogen with a wide host range (Elad et al. 2004; Jarvis 1977). Besides tomato, *B. cinerea* is able to infect other agronomically important crops such as grapevine, strawberry, cucumber, bulb flowers and ornamental plants. Conidia of *B. cinerea* are predominantly wind-dispersed but can also spread by insects (Holz et al. 2004). After conidia land on the host tissue, their germination and subsequent penetration of the host epidermis require moderate temperatures and a high relative humidity (>93% RH) or the presence of free surface water. The fungus can also produce sclerotinia, which are considered to be the most important structures involved in the survival of *Botrytis* species (Fig. 1; reviewed by Holz et al. 2004).

Resistance to *B. cinerea*

Two factors are of utmost importance for a successful *B. cinerea* infection: the capability to kill host cells and subsequent conversion of plant tissue into fungal biomass (Prins et al. 2000; van Kan 2006). The interaction of *B. cinerea* with its hosts can be compared to chemical warfare. *B. cinerea* can induce death of plant cells through production of secondary metabolites and proteins with a phytotoxic activity (van Kan 2006). Resistance to *B. cinerea* can affect entry into the host and further colonization of the surrounding tissue (van Baarlen et al. 2004a). Among mechanisms restricting entry are the development of structural barriers (papillae) and production of phenolic compounds at the entry points (van Baarlen et al. 2007). If the fungus succeeds in entering the host, growth can be restricted by secondary metabolites with an antimicrobial effect. The majority of these metabolites are effective against a broad spectrum of pathogens, but some pathogens are capable to detoxify these metabolites (Morrissey and Osbourn 1999; van Etten et al. 1989). Besides secondary metabolites, some plants are also capable to produce proteins which block the function of *B. cinerea* enzymes. The expression of fungal-genes and plant-genes resulting in production of proteins and metabolites at the right moment at the right place plays an important role in the outcome of the interaction (van Kan 2006).

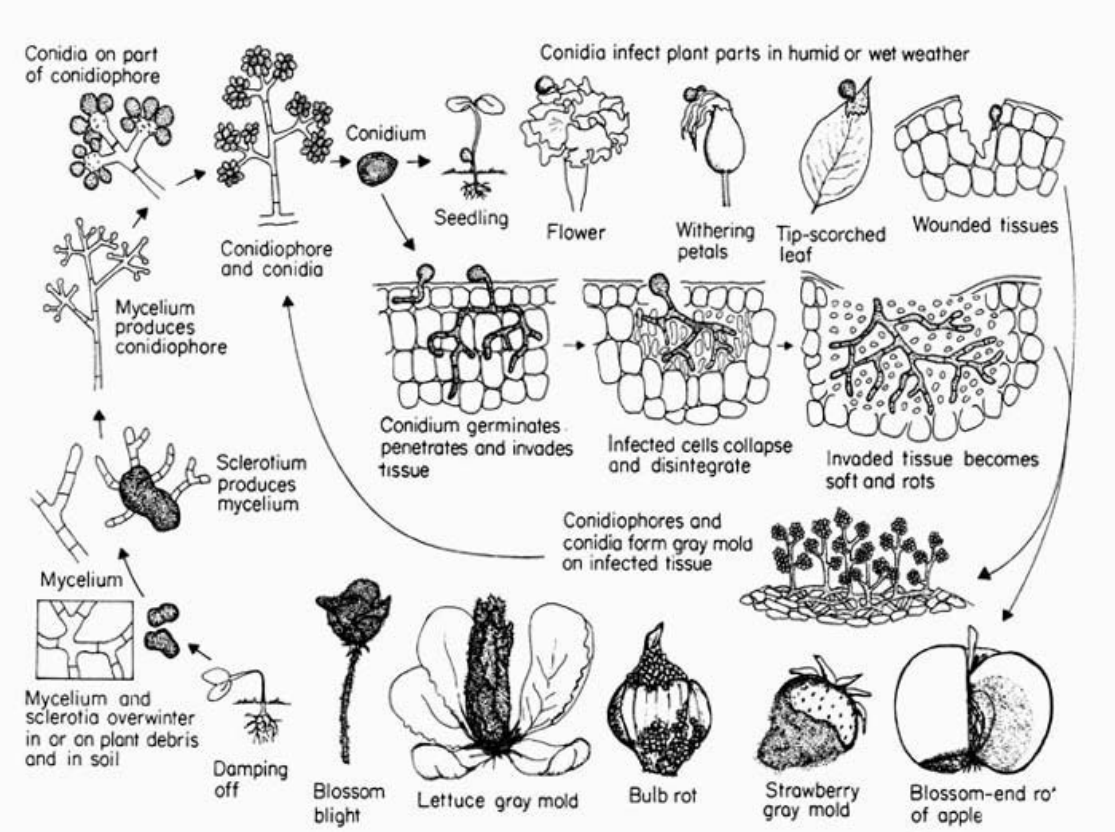

Fig. 1: Infection process, development and symptoms of diseases caused by *B. cinerea*. Reprinted from Plant Pathology, Agrios GN (2005), with permission from Elsevier.

Control of *B. cinerea* infection in greenhouses

In heated greenhouses, a *B. cinerea* infection in tomato is usually restricted to the stem (Dik and Wubben 2004; Shtienberg et al. 1998). Stem rot usually develops after pruning of lower leaves and side shoots (Verhoeff 1968) and results in a lower yield or premature plant death. Disease control frequently relies on fungicides (Leroux 2004) or on biocontrol (Elad and Steward 2004). These control strategies, however, are often ineffective as *B. cinerea* has acquired resistance against several of these agents. The effectiveness of control measures may also depend on environmental conditions (Elad and Steward 2004; Leroux 2004). Transgenic plants displaying increased resistance to *B. cinerea* under controlled laboratory conditions have been described, however, in a field trial the reduction of symptoms was less pronounced or not observed (Stotz et al. 2004). Implementation of transgenic *B. cinerea*-resistant crops in practice is unlikely to happen in the near future because of a lack of consumer acceptance in Europe. The development of non-transgenic cultivars by introgression of resistance genes from wild relatives into modern elite lines may provide an effective alternative.

Strategies for identifying quantitative loci conferring resistance to *B. cinerea*

High levels of resistance to *B. cinerea* have not been observed in cultivated tomato. Several wild *Solanum* species are crossable with tomato and many traits of economic importance were introgressed from wild *Solanum* species, including almost all the major disease resistances (Rick and Chetelat 1995; Rick and Yoder 1988). Resistance to *B. cinerea* is, however, not among the introduced traits. In general, resistance to pathogens can be qualitative resistance (i.e. based on genes with a major effect) or quantitative (i.e. resistance is based on genes with a minor effect). Resistance to *B. cinerea* is presumed to depend on multiple genes, each contributing to resistance in a quantitative manner, as no genes with major effects have been reported so far.

Geneticists have developed tools to map genes involved in quantitative traits (Quantitative trait locus, QTL). QTL mapping algorithms rely on a segregating population which is analyzed for molecular markers (=genotyping) and the trait(s) of interest (=phenotyping) followed by calculation of the genetic map and association of trait data with the marker data. The concepts and tools required for QTL mapping were extensively reviewed by Collard et al. (2005). Various types of mapping populations can be used, each having its specific characteristics with respect to the efficiency of mapping QTLs. The different types of populations which can be used are described below.

F_2 and BC_1

F_2 and BC_1 populations are commonly used for QTL mapping. An advantage of tomato is the relatively short time needed between identifying an accession with a positive trait and the analysis of a segregating population (approximately 9 months). F_2 and BC_1 populations were mainly used to detect QTLs for resistance to biotic (Bai et al. 2003; Chaerani et al. 2006; Foolad et al. 2002; Maliepaard et al. 1995; van Heusden et al. 1999) and abiotic stress (Foolad et al. 2003; Monforte et al. 1997; Saranga et al. 1993)

F_2 populations are useful to detect QTLs with additive and dominance effects. However, morphologic variation (due to the heterogeneous genetic background) of F_2 plants can make the evaluation of certain traits troublesome. This can be partially overcome by using a BC_1 population where each plant theoretically contains only 25% of the wild relative genome instead of the 50% present in a F_2 plant. However, it is not possible to discriminate dominance and additive effects of a QTL in a BC_1 population. Dominance will enlarge or reduce the explained variance of the QTL depending on the choice of recurrent parent.

An F_2 or BC_1 population is suitable to detect traits for which a few “major” loci are segregating, but “minor” QTLs will be overlooked. A simulation study showed that a population of at least 200 F_2/BC_1 plants is needed to pinpoint a QTL that explains 5% or more of the total variance, to a 40 cM interval (van Ooijen 1992). Development of near-isogenic lines (NILs) for each identified QTL is time consuming. Each F_2/BC_1 genotype is unique and mortal, therefore replicate analysis of a particular genotype in multiple locations is often not feasible. It is only possible to obtain replications using cuttings or after *in vitro* multiplication, which is either disease sensitive (cuttings) or very laborious (*in vitro*).

For tomato, a number of alternative strategies can be used to circumvent the difficulties described above, including advanced backcross (AB), inbred backcross lines (IBL), recombinant inbred lines (RIL), and introgression lines (IL).

Advanced backcross populations

In contrast to the use of BC_1 populations, Tanksley and Nelson (1996) proposed the advanced backcross (AB) methodology. The AB method aims at introgression of valuable QTL alleles from un-adapted donor lines (e.g. land races or wild species) into an established elite inbred line. QTL analysis is delayed until the second or third backcross generation. This theoretically reduces the percentage of wild genome in the elite genetic background of a BC_2 or BC_3 plant to 12.5 or 6.3% respectively. After identification of a QTL, a NIL can be readily developed, tested and utilized as a candidate for a new improved cultivar (Bernacchi et al. 1998a). During development of AB lines, marker assisted selection (MAS) is applied to reduce the frequency of unwanted donor alleles, such as, indeterminate growth in the case of field tomatoes. For tomato, the AB approach has been applied using accessions from most of the crossable wild *Solanum* species: *S. pimpinellifolium* LA722 (Tanksley et al. 1996); *S. arcanum* LA1708 (Fulton et al. 1997a); *S. habrochaites* LA1777 (Bernacchi et al. 1998b); *S. neorickii* LA2133 (Fulton et al. 2000) and *S. pennellii* LA1657 (Frary et al. 2004). Each AB population was screened to detect QTLs for agronomically favorable characteristics. The use of AB populations has a number of disadvantages: the method is unlikely to detect epistasis or recessive donor alleles. Furthermore, it is not possible to detect if a QTL is dominant or additive (Tanksley and Nelson 1996) and skewness may result in under- or overrepresentation of a locus (Fulton et al. 1997b). An AB population is not homozygous. Testing at multiple locations requires testing a larger set of plants, grown from seeds obtained by self pollination.

Recombinant inbred populations

The advantage of a recombinant inbred line (RIL) population is that it is inbred for many generations (e.g. F_9) through single seed descent. Each genotype is therefore almost homozygous and can be reproduced through selfing. Because each genotype contains approximately 50% of the parental genomes, epistatic interaction can readily be detected. As a consequence of the large proportion of each parental genome, the development of NILs is hampered and requires several rounds of backcrossing. RIL populations have been developed to identify loci for disease resistance (Diwan et al. 1999; Smith et al. 1999) and agronomic traits (Causse et al. 2001; Goldman et al. 1995; Saliba Colombani et al. 2001; Voorrips et al. 2000). Skewness of loci is not overcome using RIL populations, e.g. Paran et al. (1995) reported that 73% of the markers in a tomato RIL population significantly deviated from the expected 1:1 ratio, mainly towards overrepresentation of *S. lycopersicum* alleles.

Introgression line populations

Of all mapping populations mentioned, an IL population is the most time-consuming to develop. Alike to RIL populations, they take at least three years to develop. The development of an IL population requires repetitive MAS for selection of suitable genotypes in every (backcross) generation. Each IL ideally harbors a single defined chromosome segment that originates from the donor parent in an otherwise uniform genetic background and typically contains more than 95% of the recurrent cultivated parent genome (Zamir 2001). ILs facilitate the identification of QTLs because phenotypic variation between the IL and the recurrent parent is directly associated with the introgressed segment. Minimizing negative epistatic effects, caused by other regions of the wild genome, make identification of novel QTLs possible (Eshed and Zamir 1995), while, on the other hand QTLs relying on interactions are likely to remain undetected. It is not possible to discriminate between dominance and additive effects of QTLs. Each introgression line is homozygous; therefore such a population is immortal and repeated testing in multiple environments is feasible. Repetitive testing of an IL also allows identification of QTLs with a minor quantitative effect.

ILs in a tomato genetic background have been developed from *S. pennellii* LA716 (Eshed and Zamir 1994), *S. habrochaites* LA1777 (Monforte and Tanksley 2000a), *S. habrochaites* LA407 (Francis et al. 2001) and *S. lycopersicoides* LA2951 (Canady et al. 2005). Especially the *S. pennellii* IL population has been studied extensively for agronomic traits (Eshed and Zamir 1995; Rousseaux et al. 2005), as well as for resistance to *Fusarium* (Sela-Buurlage et al. 2001). ILs in tomato have been extremely helpful in fine mapping of QTLs (Eshed and Zamir 1996; Ku et al. 2001; Monforte et al. 2001; Monforte and Tanksley 2000b) and cloning of QTLs (Frary et al. 2000; Fridman et al. 2000; Liu et al. 2002).

Epistasis

Epistasis (the interaction between two or more genes to control a single phenotypic trait) can theoretically be detected in mapping studies, but there are only few reports of the detection of epistatic interactions in tomato. In an unbalanced F_2 population, only 4.9% of the tested epistatic interactions were significant (de Vicente and Tanksley 1993), leading the authors to conclude that epistasis was not a major factor. Using the *S. pennellii* IL population, Eshed and Zamir (1996) showed that epistasis did play a prominent role in yield-associated QTLs. Using a balanced, half diallel scheme, epistasis was detected in 28% of the interactions and it was generally less-than-additive (i.e. the effect of the two combined QTLs was lower than the sum of effects of each single QTL). Coaker and Francis (2004) studied the effect of epistasis between two QTLs conferring resistance to bacterial canker. In the F_2 generation of a cross between two inbred backcross lines (IBL, approximately 12% of wild genome), each containing one QTL, they selected 11 to 14 independent lines for each of the possible nine genotypic classes resulting in a population of 112 genotypes. Epistasis explained an additional 18% of the variation in this balanced set of genotypes.

These examples illustrate that the potential to accurately identify epistasis is much larger when deliberate crosses are made than when using a random population such as described by de Vicente and Tanksley (1993). A balanced experimental design (i.e. not skewed towards under- or over-representation of a locus) is an important prerequisite for the identification of epistasis.

QTLs and the environment (G x E interactions)

Several studies have demonstrated the occurrence of genotype x environment interactions. For example, experiments aimed at identifying QTLs for agronomic traits were performed using different AB backcross populations and each population was tested in at least three environments (Bernacchi et al. 1998b; Frary et al. 2004; Fulton et al. 1997a; Fulton et al. 2000). 35% of the identified QTLs could be detected in all three environments tested while on average over 60% of the QTLs were detected in at least two out of three environments. Similar results were observed in experiments using the *S. pennellii* LA716 IL population (Gur et al. 2004). We presume that reproducibility of experiments in greenhouses is higher due to a more controlled environment. However, due to the influence of the environment on QTL detection, replicate experiments are needed; preferentially performed using homozygous lines of RIL or IL mapping populations.

Power of QTL detection

The power to detect a QTL increases with larger population size and higher marker density but is adversely affected by skewness marker segregation. Skewness does not occur in IL populations because of the way they are constructed. Phenotyping problems are less pronounced in advanced populations which rely on backcrossing to a recurrent parent because such population contains a smaller portion of the wild species genome. Negative epistatic interactions between a QTL and the wild genetic background become less prominent in more advanced populations obtained through backcrosses. The possibility to develop immortal genotypes allows testing in multiple replicates and increases the probability to detect QTLs with smaller effects. Table 1 presents a ranking of population types for their power of QTL detection.

Table 1: Specific characteristics related to mapping populations used as tools for QTL mapping.

Population type	*No of generations*	*MAS needed*	*Effect of skewness*[a]	*Epistatic interactions detectable* [b]	*Replicates possible*	*Development of NILs* [c]	*Power of QTL detection* [d]
F_2	2	No	+	+	No[f]	-	-
BC_x	2-5	No	+	-	No[f]	±	-
AB	3-5	No[e]	±	-	No[f]	±	+
IBL	5-9	No	±	±	Yes	±	+
RIL	7-9	No	+	+	Yes	-	±
IL	3-7	Yes	-	-	Yes	++	++

[a] Skewness does (+) or does not (-) affect QTL analysis

[b] Epistatic interactions can be detected (+) or not (-)

[c] NILs can readily be developed (+) or will be time consuming to develop (-)

[d] Very powerful (++), powerful (+) or weak (-)

[e] MAS is used to select against unwanted phenotypes

[f] Replicated testing possible by testing a (large) set of offspring, via cuttings or via in vitro propagation

Outline of this thesis

The research described in this thesis, focuses on the identification of tomato QTLs conferring resistance to *B. cinerea*. From the study of ten Have et al. (2007), two partially resistant accessions: *S. habrochaites* LYC4 and *S. neorickii* G1.1601 were selected and segregating populations were developed. $F_{2\text{-}3}$ and IL strategies were used to identify loci conferring resistance, which were confirmed in backcross progenies.

Chapter 2 describes the identification of QTLs for resistance to *B. cinerea* originating from *S. habrochaites* LYC4 using an F_2 mapping approach. QTLs were confirmed using segregating BC_2S_1 progenies.

Chapter 3 describes the development of an IL population in which regions of the genome of the partial resistant parent *S. habrochaites* LYC4 were introgressed in the susceptible genetic background of *S. lycopersicum* cv. Moneymaker. This population of 30 genotypes was tested to identify QTLs conferring resistance to *B. cinerea*.

Chapter 4 describes the identification of QTLs for resistance to *B. cinerea* originating from *S. neorickii* G1.1601 in an F_3 population and their confirmation either in segregating BC_3S_1 populations or selected homozygous BC_3S_2 lines.

Chapter 5 describes agronomical and morphological characteristics of the *S. habrochaites* LYC4 IL population.

Chapter 6 provides a general discussion focusing on the strategies used to identify QTLs, the *Solanum* accessions used for introgressing resistance and the relationship between *S. habrochaites* LYC4 QTLs conferring resistance to *B. cinerea* and linked morphological characteristics.

CHAPTER 2

Three QTLs for *Botrytis cinerea* resistance in tomato

Published in Theor Appl Genet (2007)
http://dx.doi.org/10.1007/s00122-006-0458-0

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Tomato (*Solanum lycopersicum*) is susceptible to grey mold (*Botrytis cinerea*). Partial resistance to this fungus was identified in accessions of wild relatives of tomato such as *S. habrochaites* LYC4. In order to identify loci involved in quantitative resistance (QTLs) to *B. cinerea*, a population of 174 F_2 plants was made originating from a cross between *S. lycopersicum* cv. Moneymaker and *S. habrochaites* LYC4. The population was genotyped and tested for susceptibility to grey mold using a stem bioassay. *Rbcq*1, a QTL reducing lesion growth (LG) and *Rbcq*2, a QTL reducing disease incidence (DI) were identified. *Rbcq*1 is located on Chromosome 1 and explained 12% of the total phenotypic variation while *Rbcq*2 is located on Chromosome 2 and explained 15% of the total phenotypic variation. Both QTL effects were confirmed by assessing disease resistance in two BC_2S_1 progenies segregating for either of the two QTLs. One additional QTL, *Rbcq*4 on Chromosome 4 reducing DI, was identified in one of the BC_2S_1 progenies. F_2 individuals, homozygous for the *Rbcq*2 and *Rbcq*4 alleles of *S. habrochaites* showed a reduction of DI by 48%. QTLs from *S. habrochaites* LYC4 offer good perspectives for breeding *B. cinerea* resistant tomato cultivars.

Introduction

Grey mold: *Botrytis cinerea* Pers.:Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) is a necrotrophic pathogenic fungus with a wide host range (Elad et al. 2004; Jarvis 1977). Modern hybrid tomato (*Solanum lycopersicum*) cultivars are susceptible to *B. cinerea* although some cultivars show some quantitative resistance. During cultivation in heated greenhouses, leaves and fruit are rarely infected by *B. cinerea* but infections on stems are common (Shtienberg et al. 1998). Stem rot usually develops after pruning of lower leaves and side shoots (Verhoeff 1968) and results in lower yield or premature plant death.

Disease control in heated greenhouses currently relies on spraying systemic fungicides and/or the application of biocontrol agents. These control strategies may, however, often be ineffective as *B. cinerea* has become resistant against several of these agents (Elad et al. 2004). Transgenic plants displaying increased resistance to *B. cinerea* under laboratory conditions have been described (Elad et al. 2004) but their performance under commercial culturing conditions has rarely been tested. Implementation of transgenic *B. cinerea*-resistant crops in practice is unlikely to occur in the near future because of legal restriction and lack of consumer acceptance. Development of non-transgenic cultivars by introgression of resistance genes from wild relatives into modern elite lines may provide effective disease control.

Using a segregating population of a cross between a susceptible and a (partial) resistant parent combined with molecular marker analysis, genetic linkage maps can be constructed and quantitative trait loci (QTL) can be identified. Such a strategy has been successfully applied for identifying quantitative resistance against

several fungal and bacterial diseases in tomato (Bai et al. 2003; Foolad et al. 2002; van Heusden et al. 1999). Once identified, desirable QTLs can be introgressed rapidly into elite cultivars by marker-assisted selection (MAS) (Tanksley et al. 1998b). Quantitative resistance to *B. cinerea* has been identified in wild relatives of *S. lycopersicum* (Egashira et al. 2000; Nicot et al. 2002; Urbasch 1986). Although the resistance levels reported in these plants are high, the presumed polygenic inheritance and the lack of a standardized bioassay for quantitatively assessing resistance have limited the success of breeding for resistance. We used a stem bioassay suitable to quantify *B. cinerea* infection (ten Have et al. 2007) to assess susceptibility to *B. cinerea* in an interspecific segregating F_2 population, obtained after crossing the susceptible cultivar *S. lycopersicum* cv. Moneymaker with the *B. cinerea* resistant accession *S. habrochaites* LYC4 (ten Have et al. 2007; Urbasch 1986). The population was phenotyped using stem bioassays and genotyped using AFLP fingerprinting and PCR based co-dominant CAPS and SCAR markers. Two QTLs were detected in a F_2 population screen, while a third locus was identified in experiments aimed at confirmation of the two initial QTLs. This is the first report of a successful QTL study for resistance against *B. cinerea* in crop plants.

Materials and methods

Plant material

An interspecific cross between *S. lycopersicum* cv. Moneymaker (hereafter referred to as *SL*) and *S. habrochaites* LYC4 (hereafter referred to as *SH*) was made to produce F_1 seeds. Seeds of *SL* were in stock of Wageningen University and seeds of *SH* were obtained from the Institute for Plant Genetics and Crop Plant Research, Gatersleben, Germany. F_2 seeds, derived from a single F_1 plant, were sown to obtain an F_2 population of 174 individuals. The same F_1 was backcrossed to *SL* to obtain BC_1 seeds. 14 BC_1 plants were subsequently backcrossed to *SL* to obtain a BC_2 population of 59 individuals. Using marker-assisted selection, BC_2 genotypes were selected containing one of the two identified QTLs. These BC_2 plants were self pollinated to produce BC_2S_1 seeds. Two BC_2S_1 populations were grown: one population consisting of 60 BC_2S_1 individuals that segregated for the QTL for disease incidence and a second population consisting of 47 BC_2S_1 individuals that segregated for the QTL for lesion growth.

Stem assay and experimental design

Inoculum of *B. cinerea* strain B05.10 was prepared according to Benito et al. (1998). The stem assay was performed according to ten Have et al. (2007). In short, stems of 6-8 week-old plants were cut into six pieces of 5 cm length and inoculated with 5 µl inoculum, containing 10^6 conidia · ml^{-1}, applied to the top of each stem segment. Incubations were performed in plastic boxes (30 x 45 cm) at 15˚C in the dark at 100% relative humidity. In each box 22 genotypes were tested, including two *SL* controls. The infection progress was measured at day four and five after inoculation using a Vernier caliper. For each genotype, the percentage of successfully infected stem pieces was calculated (disease incidence, DI). The lesion growth rate (LG) was calculated as the increase in lesion size between day 4 and 5 (mm/day) only for the infected stem pieces.

To assess susceptibility in F_2 plants, cuttings were made for each F_2 genotype and tested independently in eight destructive disease assays. For each experiment, the cuttings and *SL* controls

were grown and tested using a completely randomized design. Nine boxes were required for each replicate experiment.

To confirm the identified QTLs, two BC_2S_1 families were grown. For each BC_2S_1 genotype, cuttings were grown and tested using a completely randomized design. Two independent experiments were performed in which susceptibility of each BC_2S_1 genotype was tested twice resulting in a total of four replicate observations. In each experiment, both *SL* and *SH* controls were included.

DNA isolation and marker analysis

Genomic DNA was isolated from two young (rolled up) leaves using a CTAB based protocol according to Steward and Via (1993), adjusted for high throughput DNA isolation using one ml micronic tubes (Micronic BV, Lelystad, The Netherlands). Leaves were ground using a Retsch MM 300 shaker at maximum speed (Retsch BV, Ochten, The Netherlands). The AFLP analysis of the F_2, BC_2 and BC_2S_1 populations was done according to Vos et al. (1995). AFLP fragments were resolved on a LI-COR 4200 DNA sequencer, essentially following the method of Myburg et al. (2001). The selective *Pst*I primer was labelled with an IRD700 or IRD800 fluorescent label. AFLP gel images were scored using the AFLP-Quantar Pro software package (Keygene, Wageningen, The Netherlands). Primer and adapter sequences have been described by Bai et al. (2003). The following ten primer combinations were used for genotyping: P14M48, P14M49, P14M50, P14M60, P14M61, P15M48, P18M50, P18M51, P22M50 and P22M51.

Sets of CAPS and SCAR primers were obtained from the “Solanaceae genomics website” (http://www.sgn.cornell.edu) or designed on sequences of genomic or cDNA clones available from the same source. Polymorphisms between *SL* and *SH* were determined using the CAPS digestion approach described by Bai et al. (2004). Marker sequences, PCR conditions and specific restriction endonucleases used for genotyping are presented in Appendix 1. CAPS markers were generally separated using a 2.5% agarose gel.

Data analysis

Marker data were analyzed and a genetic linkage map was calculated with Joinmap 3.0 (van Ooijen and Voorrips 2001). Skweness of a region was determined using a Chi-Square test. If all markers in a certain region were skewed at a probability of $P<0.005$, this region was considered as skewed. Phenotypic data were analyzed using the general linear model (GLM) approach as implemented in the software package SPSS 12.0 (SPSS Inc, Chicago, USA.). LG data were square root transformed to obtain a normal distribution. Mean estimates for each F_2 genotype were calculated using the following models: DI = constant + genotype + experiment and LG = constant + genotype + experiment + genotype x experiment. While analyzing BC_2S_1 data, information about experimental unit structures (box) were added and resulted in the following models to estimate mean values for each genotype: DI = constant + genotype + box + experiment and LG = constant + genotype + box + experiment + genotype x experiment. QTLs were calculated using the multiple QTL mapping procedure (MQM; Jansen and Stam 1994; F_2 dataset) or Kruskal-Wallis test (BC_2S_1 dataset) as embedded in MapQTL (van Ooijen 2003). Using the permutation test of MapQTL, a genome wide significance threshold ($P<0.05$) was calculated for each trait. Linkage maps and QTL plots were drawn using the MapChart software (Voorrips 2002). The correlation between traits was considered by interpreting Pearson correlation coefficients. Epistasis was analyzed using a two-way ANOVA and the gene action of each locus was tested using orthogonal contrast mean separation statistics (Cockerham and Zeng 1996; Table 4). Homogeneity of variance was tested using a Levene test and depending on the result, the contrast table was interpreted from the table which does assume equal variances or from the table which does not assume equal variances.

Results

Molecular marker analysis and genetic linkage map

An F_2 population (n=174) was grown of the cross between the susceptible cultivar *S. lycopersicum* cv. Moneymaker (hereafter referred to as *SL*) and the partially resistant accession *S. habrochaites* LYC4 (hereafter referred to as *SH*; ten Have et al. 2007; Urbasch 1986). This population was used to develop a genetic linkage map. Ten AFLP primer combinations resulted in 218 AFLP markers. Initially, all markers were scored dominantly, but 69 markers (32%) could be readily scored co-dominantly using Quantar-Pro software. To improve the linkage map, 51 co-dominant PCR based CAPS and SCAR markers were added (Appendix 1), mainly selected based on their positions in the *S. lycopersicum* LA925 x *S. pennellii* LA716 reference map (Tanksley et al. 1992). After identification of QTLs an additional set of CAPS or SCAR markers was developed that flank each of the QTLs.

CAPS markers known to be close to telomeres were often outside the region covered with AFLP markers. A representative example is provided by the linkage map of Chromosome 2 (Fig. 1), where the region below P22M50-93h could only be included after addition of CAPS markers TG537, TG91 and TG620. The resulting genetic linkage map consisted of 12 linkage groups, covering a total genetic length of 1087 cM. Ten linkage groups could be assigned to chromosomes using the CAPS and SCAR markers. The last two linkage groups were assigned using common *SL* markers, which have been mapped in previous crosses using the same *SL* cultivar (Bai et al. 2003; Haanstra et al. 1999).

Skewed segregation of markers was observed in regions of Chromosome 1, 5, 7 and 9. Skewness of most regions was in favor of *SH* alleles, with the exception of a region on Chromosome 1 where the occurrence of marker, TG224, which is linked to the *S* locus (Bernacchi and Tanksley 1997), was skewed towards *SL* while the more distal region was skewed in favor of *SH* alleles.

Botrytis cinerea ***resistance in the F_2 population***

To assess the quantitative level of susceptibility to *B. cinerea* for each F_2 genotype, eight independent disease assays were performed resulting in phenotypic data for 172 of the 174 F_2 individuals. Two traits were evaluated for each individual genotype: disease incidence (DI), expressed as the proportion of inoculated stem pieces that were successfully infected and lesion growth (LG), expressed as the mean growth (mm/day) of *B. cinerea* lesions of infected stem pieces.

Between experiments, the mean DI varied between 13 and 52%, while the mean LG varied from 6.2 to 8.4 mm/day (Table 1). Experiments with a low mean DI will bias the analysis of our data since relatively many genotypes will have a DI of 0%. In experiments 3 and 4, 61 and 49% of the genotypes, respectively showed a DI

of 0%. The data were analyzed using data of all experiments and excluding experiments 3 and 4. Including or excluding data from experiments 3 and 4 only influenced the significance of the QTLs, described in the next section, but did not lead to a different interpretation of the data.

Table 1 Mean disease incidence (DI) and mean lesion growth (LG) given as an estimation for the disease progress in each experiment.

Experiment	DI (%)	n[a]	LG (mm/day)	n[b]
1	32	170	6.2	115
2	34	153	7.7	114
3	13	148	8.2	53
4	18	109	6.5	57
5	47	53	7.2	44
6	36	135	7.6	109
7	52	152	8.4	129
8	21	142	6.5	80
Estimated mean[c]	37	172	7.4	172

[a] Number of individuals that were tested in this experiment

[b] Number of individuals that had at least one outgrowing lesion

[c] Estimated from data of experiments 1-2 and 5-8. Data from experiments 3 and 4 were excluded for reasons explained in the text

The mean DI of the susceptible control *SL* was 60% and the mean LG was 9.1 mm/day. Within the F_2 population the mean DI of individual genotypes varied between 5 and 86% and the mean LG varied between 2.6 and 12.3 mm/day. No correlation was observed between both traits (r=0.173, P>0.05).

We obtained an estimate of DI and LG for each genotype based on six determinations. The continuous distribution of DI and LG suggests that resistance to *B. cinerea* is quantitatively inherited and likely to be controlled by more than one gene.

QTL mapping in the F_2 population

Using a multiple QTL mapping approach (MQM), two QTLs were identified, for which the LOD score exceeded the genome-wide confidence threshold of 3.5 (P<0.05). The first QTL, located on Chromosome 1, reduced LG by 1.7 mm/day (LOD score of 4.3) and it explained 12% of the total phenotypic variation (Table 2, Fig. 1). This QTL was named *Rbcq*1 (Resistance to *B. cinerea* QTL on Chromosome 1). The second QTL, located on Chromosome 2, reduced DI by 22% (LOD score of 6.5) and it explained 15% of the total phenotypic variation. This QTL will be referred to as *Rbcq*2. In addition, the analyses suggested the presence of a third putative QTL, *Rbcq*4, on Chromosome 4 (LOD score 3.0) just below the genome-wide significance threshold. This QTL was confirmed in a later stage of this study and was included as a co-factor

in the MQM analysis. Alleles from *Rbcq*1 and *Rbcq*2 show additive effects and the increased resistance originated, as expected, from the *SH* alleles. QTL analyses were also done on data of independent experiments. Both QTLs *Rbcq*1 and *Rbcq*2 could be detected in data of experiments 1 and 2, separately, but the LOD peak did not reach the significance threshold of 3.5.

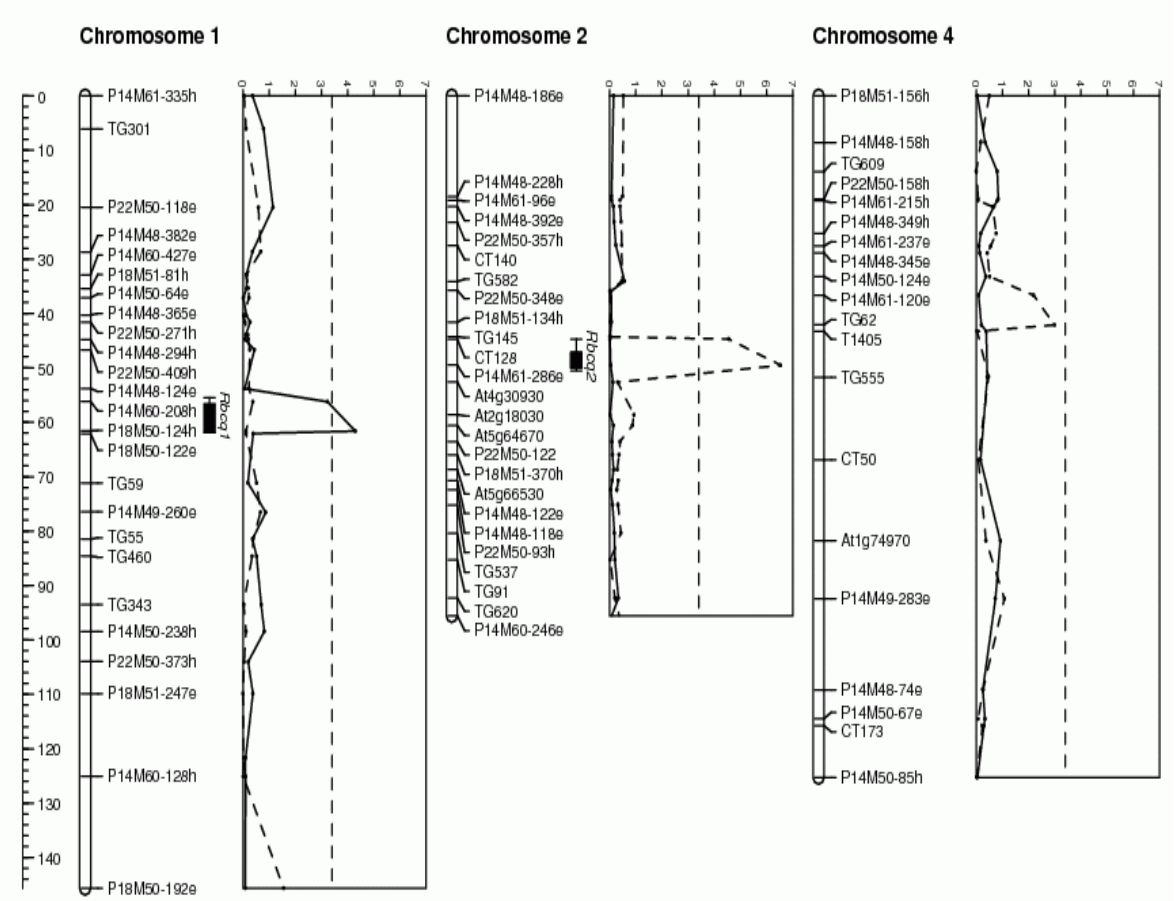

Fig. 1. The positions of QTLs for resistance to *B. cinerea* are shown on linkage maps of Chromosome 1, 2 and 4. Map positions are given in cM. QTL *Rbcq*1 on Chromosome 1 reduced lesion growth (solid line) while QTL *Rbcq*2 on Chromosome 2 reduced disease incidence (dashed line). The epistatic QTL *Rbcq*4, linked to TG62 on Chromosome 4, did not exceed the significance threshold in MQM mapping. Bars indicate the QTL intervals for which the inner bar shows a one-LOD support interval and the outer bar shows a two-LOD support interval. The LOD threshold value of 3.5 is shown as a dashed line.

Table 2 Summary of QTLs identified for DI and LG in multiple populations.

Locus	Population	Trait	n	*SL*[a]	n[b]	H	n	*SH*	n	Significance	% explained
*Rbcq*1	F_2	LG[c]	174	8.26	(13)	7.51	(73)	6.56	(76)	LOD 4.3	12%
	BC_2S_1a	LG	47	5.63	(6)	4.94	(12)	4.71	(29)	NS[d]	
	BC_2S_1b	LG	60							Not Present	
*Rbcq*2	F_2	DI[e]	174	54	(25)	39	(81)	32	(50)	LOD 6.5	15%
	BC_2S_1a	DI	47							Not Present	
	BC_2S_1b	DI	60	61	(12)	62	(25)	51	(14)	p<0.1	
*Rbcq*4	F_2	DI	174	49	(43)	39	(84)	37	(35)	LOD 3.0	7%
	BC_2S_1a	DI	47	66	(7)	71	(30)	50	(9)	p<0.01	
	BC_2S_1b	DI	60	61	(20)	56	(34)	63	(5)	NS	

[a] *SL* denotes homozygous for the *S. lycopersicum* allele, *SH* homozygous for the *S. habrochaites* LYC4 allele and H is used to describe heterozygous classes

[b] Number of observations for the linked co-dominant marker

[c] Expressed in mm/day

[d] Not significant

[e] Expressed in % infected pieces

Confirmation of QTLs

To confirm the QTLs *Rbcq*1 and *Rbcq*2, two BC_2 genotypes were selected which were heterozygous for *Rbcq*1 or *Rbcq*2. After selfing, one BC_2S_1 progeny segregated for *Rbcq*1 (BC_2S_1a, n=47) and the other segregated for *Rbcq*2 (BC_2S_1b, n=60). Both segregating populations were screened for susceptibility to *B. cinerea* in four replications. AFLP, CAPS and SCAR markers in the regions covering *Rbcq*1 and *Rbcq*2 were determined. Analyses of the two BC_2S_1 populations identified a significant variance between the ten boxes (P<0.001 for LG and P=0.007 for DI), therefore box was included in the GLM model. The mean DI during these tests was 58%, which is higher than compared to the DI of 37% observed during analysis of the F_2 population. This can be explained by assuming that additional resistance factors originating from *SH*, which were present in the F_2 population, are lacking in the BC_2S_1 genotypes. The mean DI of *SL* in these experiments was 68% while the mean LG was 6.1 mm/day. The resistant parent *SH* showed a mean DI of 37% and a mean LG of 2.2 mm/day. In population BC_2S_1a, homozygous presence of the resistance allele of *Rbcq*1 reduced LG by 0.92 mm/day (Table 2) which explains 24% of the parental variation. In the second population, BC_2S_1b, homozygous presence of the resistance allele of *Rbcq*2 reduced DI by 10% which explained 32% of the parental variation. According to a Kruskal-Wallis test, reduction of both LG and DI were not significantly different at the P<0.05 level. Nevertheless, the observed effect and magnitude of each effect confirmed the identified QTLs (Table 2).

Table 3 Mean values for disease incidence of the populations segregating for both *Rbcq*2 and *Rbcq*4.

		QTL on Chromosome 4 (Rbcq4)											
		F_2						BC_2S_1					
QTL on Chromosome 2 (*Rbcq2*)		*SL*[a]		H		*SH*		*SL*		H		*SH*	
	SL	68%	(3)[b]	42%	(9)	39%	(4)	67%	(4)	60%	(13)	56%	(2)
	H	48%	(16)	34%	(28)	37%	(6)	65%	(9)	57%	(15)	75%	(2)
	SH	29%	(8)	34%	(15)	20%	(12)	53%	(7)	49%	(6)	51%	(1)

[a] *SL* denotes homozygous for the alleles of *S. lycopersicum*, *SH* homozygous for the alleles of *S. habrochaites* LYC4 and H is used to describe heterozygous classes

[b] Number of individuals within each genotypic class

Identification of an additional locus for disease incidence

To examine whether additional loci could be identified that contribute to resistance to *B. cinerea*, each BC_2S_1 genotype was AFLP fingerprinted with the same 10 AFLP primer combinations as used for fingerprinting the F_2 population. A Kruskal-Wallis test showed a significant effect (P<0.01) on Chromosome 4 reducing DI in the population BC_2S_1a. Interestingly, this locus, *Rbcq*4, was already identified as a putative QTL in the F_2 population. *Rbcq*4 also segregated in the population BC_2S_1b but a contribution to a lower DI could not be observed in this population. Additional co-dominant CAPS and SCAR markers were determined in this region to allow a better calculation of the size of the effect of this locus (Table 2).

In order to determine if the combined effect of *Rbcq*2 and *Rbcq*4 would give a further decrease in susceptibility, mean values for the nine possible genotypic classes in both the F_2 and BC_2S_1b populations were calculated (Table 3) using the nearest co-dominant CAPS markers. No effect of *Rbcq*4 was detected in the population BC_2S_1b even though an effect of *Rbcq*2 and *Rbcq*4 was seen in the F_2 population. When both resistance alleles of *Rbcq*2 and *Rbcq*4 are absent, a mean DI of 68% was observed. The homozygous presence of either *Rbcq*2 or *Rbcq*4 resistance alleles reduced DI to 39 or 29% respectively while the homozygous combination of both *Rbcq*2 and *Rbcq*4 resistance alleles reduced DI to 20%. These results show that resistance alleles of both *Rbcq*2 and *Rbcq*4 reduce DI and that the effect of *Rbcq*2 is larger than the effect of *Rbcq*4. The combination of both *Rbcq*2 and *Rbcq*4 resistance alleles shows a less-than-additive epistatic interaction. The F_2 data showed that the gene action of *Rbcq*4 is partially dominant. Orthogonal contrast analysis testing for additive-by-additive, additive-by-dominant, dominant-by-additive and dominant-by-dominant epistatic interactions between *Rbcq*2 and *Rbcq*4 detected a significant additive (*Rbcq*2) by dominant (*Rbcq*4) epistatic interaction (P=0.047; Table 4).

Table 4 Orthogonal contrast codes used to determine gene action and type of epistasis of the DI QTLs *Rbcq*2 and *Rbcq*4 in the F_2 dataset.

Contrast[a]	SH_2SH_4[b]	SH_2H_4	SH_2SL_4	H_2SH_4	H_2H_4	H_2SL_4	SL_2SH_4	SL_2H_4	SL_2SL_4	p-*value*[c]
A *Rbcq*2	-1	-1	-1	0	0	0	1	1	1	0.003
D *Rbcq*2	1	1	1	-2	-2	-2	1	1	1	0.803
A *Rbcq*4	-1	0	1	-1	0	1	-1	0	1	0.015
D *Rbcq*4	1	-2	1	1	-2	1	1	-2	1	0.337
A_2 x A_4	1	0	-1	0	0	0	-1	0	1	0.193
A_2 x D_4	-1	2	-1	0	0	0	1	-2	1	0.047
D_2 x A_4	-1	0	1	2	0	-2	-1	0	1	0.541
D_2 x D_4	1	-2	1	-2	4	-2	1	-2	1	0.318

[a] A indicates additive gene action; D indicates dominant gene action; subscripts denote the QTL *Rbcq*2 (2) and *Rbcq*4 (4)
[b] *SL* denotes homozygous for the alleles of *S. lycopersicum*, *SH* homozygous for the alleles of *S. habrochaites* LYC4 and H is used to describe heterozygous classes; subscripts denote the QTL *Rbcq*2 (2) and *Rbcq*4 (4)
[c] Data, excluding experiment 3 and 4, was used for orthogonal contrast analysis and test significances are reported for the test which does not assume equal variances

Discussion

An F_2 population was created of the cross between *S. lycopersicum* x *S. habrochaites* LYC4 and a genetic linkage map was constructed spanning 1078 cM of the genome. This length is shorter than the 1200-1400 cM reported for other interspecific *S. lycopersicum* maps (Foolad et al. 2002; Haanstra et al. 1999; Tanksley et al. 1992). Although *Pst*I/*Mse*I derived AFLP markers have a better distribution over the genome compared to *Eco*RI/*Mse*I AFLP makers (Haanstra et al. 1999), the use of additional CAPS markers revealed that not all telomeric regions were covered by AFLP-markers. Nonetheless the map covers at least 85% of the genome. Skewness of loci was detected on several chromosomes and was often in favor of the presence of *SH* alleles. The most extreme skewness was observed on Chromosome 1 in the proximity of a region where a locus for *S*-RNAse based unilateral incompatibility has been described (Bernacchi and Tanksley 1997) and it was skewed towards homozygous *SL*. Another region on Chromosome 1 was skewed towards *SH*; QTLs have been described in this region affecting hybrid incompatibility in *S. habrochaites* LA1777 (Moyle and Graham 2005). Skewness has been described in multiple studies and is generally believed to be related to the presence of genes involved in reproductive behavior such as pollen, gamete and (or) zygote viability (Fulton et al. 1997a; Monforte and Tanksley 2000a; Paran et al. 1995). Skewness may result in failure to detect QTLs or misinterpretation of the effect of a QTL.

Quantitative resistance to *B. cinerea* was observed in the F_2 population between *SL* x *SH* and two loci were detected: *Rbcq*1 and *Rbcq*2. Both QTLs were validated in BC_2S_1 populations each segregating for *Rbcq*1 or *Rbcq*2 separately. In both populations, the expected decrease in resistance caused by *Rbcq*1 and *Rbcq*2

were observed (Table 2). The criteria for significance were not met due to the small population sizes (n=60 and 47) and skewness of segregation. Identification of the third locus *Rbcq*4 in the BC_2S_1 population aimed at confirming *Rbcq*1 suggested that certain QTLs were not detected in the original F_2 mapping population. The experimental variation in the bioassay as well as epistatic interactions, such as shown between *Rbcq*2 and *Rbcq*4 and genotype x environment (G x E) interactions allow only the identification of major QTLs. Transgressive segregation for DI and LG, beyond the level of the susceptible control *SL*, was observed in the F_2 population showing the presence of at least one unidentified QTL contributing to resistance in the susceptible parent.

The bioassay for measuring susceptibility of tomato to *B. cinerea* (ten Have et al. 2007) has proven to be a valuable tool although the test remains difficult to standardize. While the bioassay itself is performed under standardized lab conditions, the environment in which the plants are grown can vary (e.g. day length, hours of sun and temperature), affecting their physiological condition and presumably their susceptibility in a quantitative manner. Data analysis of the single F_2 experiments never revealed the presence of a significant QTL, mainly due to the limited levels of observation (only six stem pieces were inoculated per genotype per experiment). The identification of significant QTLs in all cases required combining data of multiple independent experiments.

The mechanisms underlying the increased resistance conferred by the QTLs remain to be resolved. *B. cinerea* is a necrotroph that relies primarily on three principles for subduing its host plants, i.e. the ability to kill host cells, the ability to decompose plant tissue and the ability to counteract plant defense responses (reviewed by van Kan 2006). In order to kill host cells, the fungus is able to produce multiple phytotoxic metabolites, such as botrydial and/or botcinolide (Reino et al. 2004), which may be important for virulence of certain *B. cinerea* strains (Siewers et al. 2005). Moreover, *B. cinerea* contains at least three genes encoding phytotoxic proteins (Chagué et al. 2006; Staats et al. 2006), which are expressed during infection (Chagué et al. 2006; Schouten and van Kan Unpublished). It could be envisaged that QTLs that reduce *B. cinerea* disease incidence confer a reduced sensitivity or detoxify either of these phytotoxic factors, but this remains to be determined. The second important virulence trait for the pathogen is its ability to decompose plant tissue by a spectrum of plant cell wall-degrading enzymes (Kars and van Kan 2004; ten Have et al. 2002). QTLs that reduce *B. cinerea* lesion growth rate might be related to slower plant tissue decomposition, possibly caused by altered cell wall composition or architecture. Thirdly, the pathogen must be able to counteract the action of antifungal plant metabolites. *S. lycopersicum* produces one major phytoanticipin named α-tomatin and a spectrum of sesquiterpene phytoalexins including rishitin (Grayer and Kokubun 2001; Hammerschmidt 1999; Kuć 1995). Enzymatic detoxification of α-tomatin was shown to be an important virulence trait

(Quidde et al. 1998). The QTLs identified in this study might be involved in conferring higher levels of antifungal plant metabolites, or in the production of slightly different metabolites which cannot efficiently be counteracted by *B. cinerea*. Future studies should identify the gene(s) and unravel the mechanisms that underlie the enhanced resistance levels conferred by *Rbcq*1, *Rbcq*2 and *Rbcq*4.

Regardless of the mechanisms involved, the QTLs identified in this study provide an excellent source of resistance that can be introgressed into commercial cultivars. Even though *Rbcq*1, *Rbcq*2 and *Rbcq*4 do not confer full resistance, it should be considered that the disease pressure in the bioassay was extremely favorable for the pathogen. Disease pressure in commercial greenhouses is lower and environmental conditions are less favorable for pathogen invasion. Thus the partial resistance conferred by the identified QTLs may in practice be sufficient to efficiently protect tomato against *B. cinerea*. Although *Rbcq*4 is not the QTL showing the largest reduction, its partial dominance, as observed in the F_2 dataset, makes it an interesting candidate for breeding resistance into F_1 hybrids, since introgression in only one parent is needed. We realize that our F_2 population is relatively small and that due to skewness in segregation, we have under-representation of several classes and therefore a balanced design will be needed to detect QTLs for resistance to *B. cinerea* to its full extent (de Vicente and Tanksley 1993). For identifying additional QTLs we have pursued to develop a complete introgression line (IL) population, offering multiple advantages. Firstly, the phenotypic variation between an IL and the control cultivar is directly associated with the introgressed segment. Secondly, each IL is homozygous allowing multiple testing. Thirdly, negative epistatic effects caused by other regions of the wild genome that may obscure a QTL are less likely to occur (Zamir 2001), allowing a more reliable assessment of quantitative disease resistance to *B. cinerea*. A complete IL population of *S. habrochaites* LYC4 in the genetic background of *SL* is under construction.

Acknowledgments

This project is sponsored by grants of the Dutch Technology Foundation (STW, grant no. WBI.4172) and De Ruiter Seeds. The authors would like to thank Paul Maris, Fien Meijer-Dekens, Marianne Sela and Danny Schipper for their contribution and valuable suggestions.

CHAPTER 3

The construction of a *Solanum habrochaites* LYC4 introgression line population and the identification of QTLs for resistance to *Botrytis cinerea*

Slightly modified from Theor Appl Genet (2007)
http://dx.doi.org/10.1007/s00122-006-0500-2

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Tomato (*Solanum lycopersicum*) is susceptible to grey mold (*Botrytis cinerea*). Partial resistance to this fungus has been identified in accessions of wild relatives of tomato such as *Solanum habrochaites* LYC4. In a previous F_2 mapping study, three QTLs conferring resistance to *B. cinerea* (*Rbcq*1, *Rbcq*2 and *Rbcq*4a) were identified. As it was probable that this study had not identified all QTLs involved in resistance, we developed an introgression line (IL) population (n=30), each containing a *S. habrochaites* introgression in the *S. lycopersicum* cv. Moneymaker genetic background. On average, each IL contained 5.2% of the *S. habrochaites* genome and together the lines provided an estimated coverage of 95%. The level of susceptibility to *B. cinerea* for each of the ILs was assessed in a greenhouse trial and compared to the susceptible parent *S. lycopersicum* cv. Moneymaker. The effect of the three previously identified loci could be confirmed and seven additional loci were detected. Some ILs contain multiple QTLs and the increased resistance to *B. cinerea* in these ILs is in line with a completely additive model. We conclude that this set of QTLs offers good perspectives for breeding *B. cinerea* resistant cultivars and that screening an IL population is more sensitive for detection of QTLs conferring resistance to *B. cinerea* than the analysis in an F_2 population.

Introduction

Cultivated tomato (*Solanum lycopersicum*) displays only a limited amount of genotypic and phenotypic variation. Wild relatives of tomato are a useful source of variation for improving tomato (Rick 1982). Several wild *Solanum* accessions are crossable with tomato but barriers like unilateral incompatibility, hybrid inviability and sterility sometimes have to be overcome to obtain viable progeny. After successful hybridization, the introgression of specific traits from wild *Solanum* is often laborious and time consuming. The practical feasibility of introgressing specific chromosomal regions can be hampered due to reduced recombination and/or linkage drag. Despite these difficulties, many favorable traits such as disease resistances (Bai et al. 2003; Haanstra et al. 2000; Kabelka et al. 2002), tolerance to abiotic stresses (Foolad et al. 2003) and agronomic traits (Bernacchi et al. 1998b; Eshed and Zamir 1995; Lecomte et al. 2004) have been successfully introduced into tomato.

The focus of our research was the identification of quantitative trait loci (QTL) for increased disease resistance to *Botrytis cinerea* Pers:Fr (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel). *B. cinerea* is a necrotrophic pathogenic fungus with a wide host range of at least 235 species (Elad et al. 2004; Jarvis 1977) and uses multiple strategies to subdue its host plant (van Kan 2006). Plants, on the other hand, possess multiple strategies to protect themselves against infection by *B. cinerea* (van Baarlen et al. 2004a). Different *B. cinerea* isolates vary in their capacity to deal with such defense mechanisms (Kliebenstein et al. 2005; Quidde et al. 1998), hampering the comparison of results between research groups using different fungal isolates

and host species. To date, mainly *Arabidopsis thaliana* is used as a model to study the interaction with *B. cinerea*. Glazebrook (2005) reviewed the complex interaction between *B. cinerea* and mutants of *A. thaliana*. Denby et al. (2004) performed QTL mapping to elucidate the genetics of the interaction between *B. cinerea* and *A. thaliana* and reported the presence of multiple, isolate-specific loci controlling resistance to *B. cinerea*.

Heirloom and modern hybrid tomato cultivars are susceptible to *B. cinerea* although a number of cultivars show quantitative resistance. A high level of resistance to *B. cinerea* has been identified in the wild accession *S. habrochaites* LYC4 (ten Have et al. 2007; Urbasch 1986). To study the genetics of this resistance, an F_2 mapping population (n=174) of the cross between *S. lycopersicum* cv. Moneymaker and *S. habrochaites* LYC4 has been developed (Chapter 2). Initially, two QTLs for resistance to *B. cinerea* were identified in this F_2 mapping study (denominated *Rbcq*1 and *Rbcq*2, located on Chromosomes 1 and 2 respectively). A third QTL (*Rbcq*4) that showed an interaction with *Rbcq*2 was detected in segregating BC_2S_1 progenies (Chapter 2).

A limitation of QTL-mapping in interspecific segregating F_2 populations is the wide variation in plant growth rate, morphology and physiology but also the presence of major QTLs that potentially disguise QTLs with minor effects. Furthermore, it is difficult to do repeated tests as each F_2 plant is a unique genotype. Alternatively, a genetic library consisting of a set of introgression lines (IL) can be used for mapping purposes. Each IL ideally harbors a single, defined chromosome segment that originates from the donor species in an otherwise uniform genetic background (Zamir 2001). Such lines facilitate the identification of QTLs because phenotypic variation between the line and the control cultivar is directly associated with the introgressed segment. Each line typically contains more than 95% of the recurrent cultivated parent genome and minor quantitative effects can easily be identified by comparison with the recurrent parent. Minimizing negative epistatic effects caused by other regions of the wild genome may lead to identification of novel QTLs (Eshed and Zamir 1995). Each line is homozygous and immortal and thus allows multiple testing (in multiple environments). Finally, sterility problems are less prominent due to the fact that the genetic constitution of each line is largely identical to the cultivated variety, which is usually very fit and fertile.

The first IL population has been developed in wheat and dates back to 1965 (Wehrhahn and Allard 1965), but the majority of IL populations have been developed during the last decade by means of marker assisted selection (MAS) using different marker systems and breeding approaches (Table 1). Besides tomato, IL populations have been developed for barley, cabbage, lettuce, melon, rice and wheat. Within tomato, ILs have been developed from *Solanum pennellii* LA716 (Eshed and Zamir 1994), *S. habrochaites* LA1777 (Monforte and Tanksley 2000a), *S. habrochaites* LA407 (Francis et al. 2001) and *S. lycopersicoides* LA2951 (Canady et al. 2005). ILs

in tomato have shown to be extremely helpful in the identification of QTLs (Eshed and Zamir 1995; Rousseaux et al. 2005), fine mapping of QTLs (Eshed and Zamir 1996; Ku et al. 2001; Monforte et al. 2001; Monforte and Tanksley 2000b) and cloning of QTLs (Frary et al. 2000; Fridman et al. 2000; Liu et al. 2002).

Table 1: Overview of strategies and marker systems used for the development of introgression line populations for different species.

Crop	Strategy	Marker system	Reference
Barley	BC_2DH	220 SSR markers	von Korff et al. 2004
Cabbage	$BC_{2\text{-}3}S_2$	138 RFLP markers	Ramsay et al. 1996
Lettuce	BC_4S_1	757 AFLP markers	Jeuken and Lindhout 2004
Melon	$BC_{3\text{-}4}S_1$	62 SSR markers	Eduardo et al. 2005
Rice	BC_1S_5	245 RFLP markers	Lin et al. 1998
Tomato	$BC_1S_6BC_3S_1$	350 RFLP markers	Eshed and Zamir 1994
	BC_2S_3	95 RFLP markers	Monforte and Tanksley 2000a
	BC_2S_5	58 RFLP markers	Francis et al. 2001
	BC_2S_6	116 RFLP markers	Canady et al. 2005
Wheat	BC_2S_2	65 SSR markers	Pestsova et al. 2001

In general, IL populations have been developed by MAS using co-dominant SSR and RFLP markers (Table 1). Screening a population with SSR or RFLP markers is labor intensive and results in a lower marker density (ranging from 62 to 350 markers) as compared to using AFLP markers (757 markers for lettuce). The advantage of SSR and RFLP markers, however, is that the positions of markers are usually known, allowing the selection of a core set of markers covering the complete genome. AFLP markers are high throughput markers but often with an unknown position and unequal coverage of the genome. Clustering of AFLP markers is a potential pitfall, as shown in *Solanaceae* for *Eco*RI/*Mse*I markers, which tend to cluster around the centromere whereas there was less clustering of *Pst*I/*Mse*I markers (Haanstra et al. 1999). In certain cases, however, AFLP markers may identify introgressions that remained undetected using RFLP markers (Bonnema et al. 2002).

Our research aimed at verification of the QTLs for resistance to *B. cinerea* that have previously been identified by QTL mapping in an interspecific F_2 population obtained from a cross of tomato with *S. habrochaites* LYC4 (Chapter 2) and the identification of additional QTLs. This paper describes the development of an IL population (BC_5S_2) of *S. habrochaites* LYC4 in the genetic background of an indeterminate growing tomato by using AFLP as the platform for MAS. Each of the developed ILs was screened for its level of susceptibility to *B. cinerea*.

Material and methods

Plant material and development of the ILs

The parents of the present study were the *B. cinerea* susceptible, indeterminate growing *S. lycopersicum* cv. Moneymaker (hereafter referred to as *SL*) and the resistant *S. habrochaites* LYC4 (hereafter referred to as *SH*, syn. *Lycopersicon hirsutum*; ten Have et al. 2007; Urbasch 1986). Seeds of *SL* were in stock of Wageningen University and seeds of *SH* were obtained from the Institute for Plant Genetics and Crop Plant Research, Gatersleben, Germany. One F_1 plant was self pollinated to obtain F_2 seeds and also backcrossed to *SL* to obtain BC_1 seeds. The F_2 seeds were sown to grow an F_2 population (n=174) which has previously been used for the construction of a genetic linkage map (Chapter 2). The BC_1 was used for development of the IL population (Fig. 1).

Marker analysis

Genomic DNA was isolated from two young (rolled up) leaves using a CTAB based protocol according to Steward and Via (1993), adjusted for high throughput DNA isolation using one ml micronic tubes (Micronic BV, Lelystad, The Netherlands) and grinded using a Retsch 300 mm shaker at maximum speed (Retsch BV, Ochten, The Netherlands).

AFLP™ analysis was done according to Vos et al. (1995). AFLP fragments were resolved on a LI-COR 4200 DNA sequencer, essentially following the method published by Myburg et al. (2001). The selective *Pst* primer was labeled with an IRD700 or IRD800 fluorescent label. AFLP gel images were scored using AFLP-Quantar™ Pro software (Keygene, Wageningen, The Netherlands). Primer and adapter sequences have been described by Bai et al. (2003).

CAPS primers were obtained from the “Solanaceae Genomics Website” (http://sgn.cornell.edu) or designed on sequences of genomic or cDNA clones available from the same source. Polymorphisms between *SL* and *SH* were determined using the CAPS digestion approach described by Bai et al. (2004). Marker sequences, PCR conditions, and specific restriction endonucleases used for genotyping are presented in Appendix 1. PCR products were separated using a 2.5% agarose gel.

Graphical genotyping

Graphical representation of the genotypes for each backcross population and the ILs were obtained using the software program GGT (van Berloo 1999). For the calculation of introgression size and genome percentages, the half-intervals flanking a marker locus were considered to be of the same introgression as implemented by the software. Missing marker data were estimated from the flanking markers; if these had identical genotypes than the missing marker was assumed to have the same genotype as the two flanking makers. If the two flanking makers had contrasting genotypes, the missing data were recorded as truly missing.

Disease evaluations

To assess susceptibility, 16 incomplete randomized blocks were used in which in total 11 replications for each IL were tested. Each block contained at least two *SL* plants and one plant of *S. lycopersicum* cv. Durinta, a commercial cultivar producing truss tomatoes with a fair level of resistance to *B. cinerea*. Six weeks after sowing, plants were transplanted into the soil of two greenhouse compartments with a set day/night regime of 19/15 ºC and a photo period of 16 hours light. After 11 weeks, two incisions of 15 mm were made into the stem of each plant at approximately 1.5 and 1.7 meters height using a knife. Each wound was inoculated with a one cm^2 agar plug containing a culture of *B. cinerea* B05.10 (Benito et al. 1998) and subsequently covered with tape. Two weeks later a third and fourth inoculation were performed on the same plants below the initial inoculation sites. Plants were watered

at the beginning of the evening to maintain a humid climate during the night. Disease parameters were measured nine and twelve days after inoculation. The parameters were: disease incidence as percentage of growing lesions (DI), lesion size (using a caliper) after 12 days minus the size of the inoculation wound (15 mm, LS), and lesion growth rate expressed as the difference in lesion size between nine and twelve days post inoculation expressed in mm/day (LG).

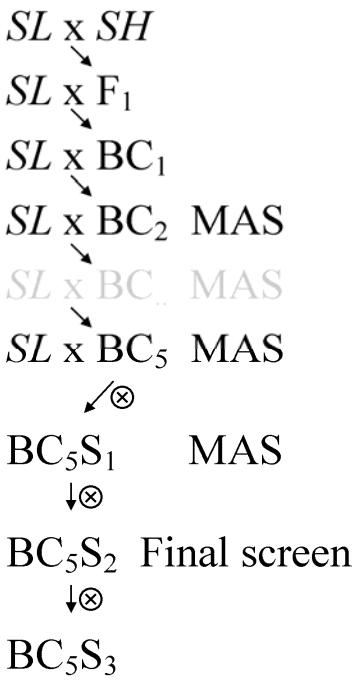

Fig. 1: Backcross and selection strategy used to obtain the *S. habrochaites* LYC4 IL population introgressed in the *S. lycopersicum* cv. Moneymaker genetic background. Starting from BC_2, marker assisted selection (MAS) was applied by using AFLP markers. The BC_5S_2 population was screened with a combination of AFLP and CAPS markers.

Statistical analysis

Statistical analysis was performed using SPSS 12.0 software (SPSS Inc, Chicago, USA). Using a general linearized model (GLM) procedure, means for each IL/trait were estimated. Mean values of the traits measured were compared to the susceptible control genotype *SL* using a Dunnett test (Dunnett 1955) and probabilities smaller than 0.05 were considered as significant. To analyze LS and LG, a square root transformation was applied to normalize the data of both traits. Correlations between traits were calculated using a Pearson correlation coefficient.

Results

IL population

An introgression line (IL) population of *S. habrochaites* LYC4 (*SH*) in the genetic background of *S. lycopersicum* cv. Moneymaker (*SL*) was generated according to the procedure illustrated in Fig. 1. One F_1 plant from the cross between *SL* and *SH* was backcrossed to *SL*. Subsequently a random set of 14 BC_1 plants was backcrossed to

SL to obtain a BC_2 progeny (n=59). All BC_2 plants were genotyped and a selected set was backcrossed to *SL*. This set was chosen in such a way that the combined introgressions covered as much of the *SH* genome as possible and that each alien chromosome was represented by three ILs. The backcrossing and selection procedure was repeated until BC_5 (Fig. 1). Eventually, 31 BC_5 plants were selected, mainly containing one or two heterozygous introgressions. These 31 plants were self pollinated. Up to 12 plants of each of the 31 BC_5S_1 families were screened with AFLP markers to obtain a selected BC_5S_2 progeny (n=44) homozygous for the (single) *SH* introgressions. The selected BC_5S_2 progeny was grown once more to allow a more detailed marker screen in which 457 AFLP markers (result of 10 AFLP enzyme/ primer combinations) and 34 CAPS markers were analyzed. The total of 457 AFLP markers reported here is a two-fold increase in marker density as compared with the F_2 population (Chapter 2). The marker screen resulted in the selection of a core set of 30 ILs with the highest possible coverage of the *SH* genome (Fig. 2) in as few lines as possible. Each selected line was self pollinated to obtain BC_5S_3 seeds.

The IL library consists of 15 lines with a single introgression, ten lines with two introgressions, four lines with three introgressions while one IL contained four introgressions. The proportion of *SH* genome ranged from 20 (1.7%) to 122 cM (10.6%) with an average of 60 cM (5.2 %). The IL library covers 95% of the F_2 AFLP linkage map (Chapter 2), which however, does not cover the entire *SH* genome. Additional CAPS analysis on Chromosomes 3 (top of the short arm), 4 (top of the short arm), 5 (long arm) and 9 (top of the short arm) provided markers that were nearer to the telomeres than the most distal AFLP marker. The sizes of the latter introgressions were estimated, using the high density RFLP map (Tanksley et al. 1992; http://www.sgn.cornell.edu) and were estimated to be 20, 50 and 32 cM for Chromosome 4, 5 and 9 respectively. The size of the introgression of the top arm of Chromosome 3 could not be estimated.

ILs homozygous *SH* for the top of Chromosome 3 (IL3-1) were not obtained. Plants homozygous *SH* for IL5-1 and 5-2 failed to set seeds and these lines could only be maintained as heterozygote for these introgressions. No ILs were obtained containing an introgression of Chromosome 1 harboring CAPS marker TG460, the distal end of the long arm of Chromosome 2 and the top of the short arm of Chromosome 8. Introgressions with the top of the short arm of Chromosome 7 and 9 were present in multiple ILs (Fig. 2). Selection against introgression on Chromosome 7 was hampered due to a strong skewness favoring the *SH* alleles. Introgressions for the top of Chromosome 9 could only be detected during the more detailed CAPS marker screen of the BC_5S_2 ILs, as mentioned above. Introgressions homozygous *SH* in this region were present in 30% of the ILs.

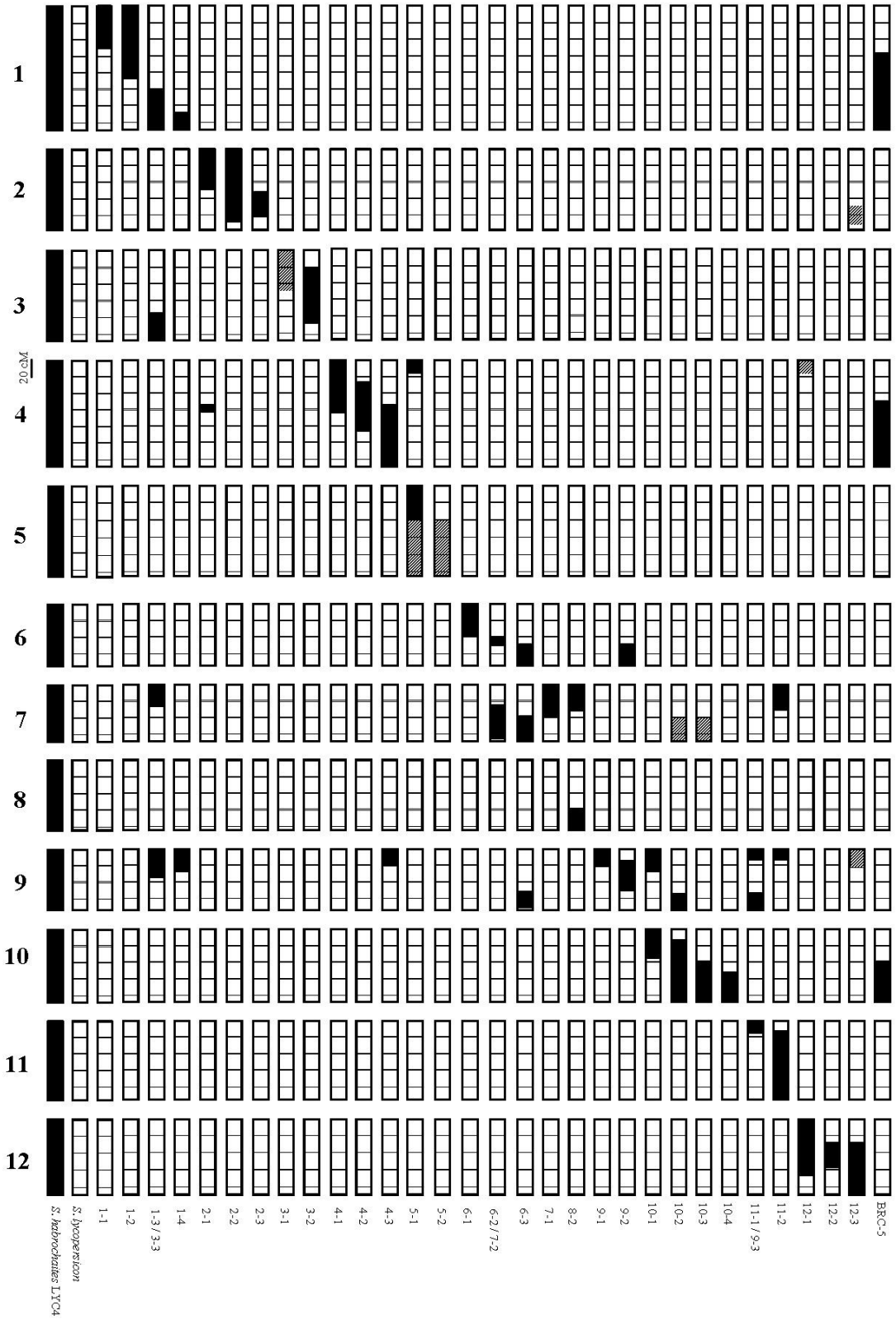

Fig. 2: Graphical representation of the genotypes of the *S. lycopersicum* cv. Moneymaker x *S. habrochaites* LYC4 introgression line population. All chromosomes are drawn to scale in 20 cM segments according to the F_2 genetic linkage map (Chapter 2) or estimated using the *S. lycopersicum* x *S. pennellii* linkage map (Tanksley et al. 1992; http://www.sgn.cornell.edu). Genetic length of the Chromosome 9 introgressions were estimated from recombination frequencies of BC_5S_2 marker data. Homozygous introgressions from *S. habrochaites* are in black and heterozygous introgressions in gray.

Table 2: Estimated mean disease incidence (DI), lesion size (LS) and lesion growth (LG) in the introgression lines (IL) and control lines. Means of each trait for each IL were compared to the mean of *S. lycopersicum* cv. Moneymaker (*SL*) using a Dunnett test and significant differences are marked with * (P<0.05) or ** (P<0.01).

IL	QTL	N[a]	Disease incidence (%)		Lesion size (mm)		Lesion growth rate (mm/day)	
1-1		41	65 ± 6.7		56		5.9	
1-2	*Rbcq*1?	40	47 ± 6.7		33		3.4	
1-3/3-3	*Rbcq*9b	29	45 ± 9.1	**	30	**	1.7	**
1-4	*Rbcq*9b	44	37 ± 6.4	**	34		2.4	*
2-1	*Rbcq*2	44	41 ± 6.4	**	30	*	3.0	
2-2	*Rbcq*2	44	37 ± 6.5	**	26	*	2.8	
2-3		44	58 ± 6.5		44		3.5	
3-1	*Rbcq*3	43	47 ± 6.6		41		2.8	*
3-2	*Rbcq*3	44	46 ± 6.5	*	35		4.2	
4-1	*Rbcq*4a	44	41 ± 6.4	**	26	**	2.5	*
4-2	*Rbcq*4a	42	45 ± 6.7	*	33		3.8	
4-3	*Rbcq*4b & *Rbcq*9a	20	51 ± 9.6		29		2.8	
5-1		44	61 ± 6.6		53		4.8	
5-2		43	69 ± 6.6		64		5.4	
6-1	*Rbcq*6	44	49 ± 6.5	*	44		3.6	
6-2/7-2		44	55 ± 6.3		39		3.7	
6-3		44	79 ± 6.5		49		4.6	
7-1		44	50 ± 6.4		35		3.1	
8-3		44	59 ± 6.5		43		3.9	
9-1	*Rbcq*9a	44	69 ± 6.5		34	*	3.0	*
9-2	*Rbcq*9b	44	49 ± 6.4	*	33		3.1	
10-1		43	60 ± 6.6		47		4.3	
10-2		44	62 ± 6.5		49		4.4	
10-3		44	70 ± 6.4		53		4.7	
10-4		44	76 ± 6.6		47		4.8	
11-1/9-3	*Rbcq*9b	44	48 ± 6.5	*	36		4.3	
11-2	*Rbcq*11	44	34 ± 6.4	**	33	*	3.2	
12-1	*Rbcq*12	44	51 ± 6.4		35		4.7	
12-2	*Rbcq*12	43	52 ± 6.4		37		4.0	
12-3	*Rbcq*9b & *Rbcq*12	24	24 ± 8.6	**	21	**	2.3	
SL		156	73 ± 4.0		46		4.6	
SH		44	3 ± 6.4	**	ND[b]			
BRC-5	*Rbcq*1 & *Rbcq*4b	39	15 ± 6.9	**	20	*	ND[c]	
Durinta		68	42 ± 5.5	**	29	**	2.3	**

[a] Number of inoculation sites

[b] Only one outgrowing lesion, observation excluded from the statistical analysis

[c] First observation of outgrowing lesions was after 12 days, therefore lesion growth could not be determined

Susceptibility of introgression lines to B. cinerea

The population of 30 ILs was grown in the greenhouse to the adult stage, inoculated with *B. cinerea* and evaluated for disease symptoms. On nine and twelve days after inoculation the susceptibility to *B. cinerea* was quantified by determining the parameters disease incidence (DI), lesion size (LS) and lesion growth rate (LG), the results of which are shown in Table 2. The resistant parent *SH* hardly showed symptoms while 73% of the inoculation sites on the susceptible control *SL* developed into an expanding lesion with an mean LS of 46 mm and a LG of 4.6 mm/day. In total 14 ILs showed reduced susceptibility as compared to *SL* and thus contain QTLs increasing resistance. Overall, 12 of these 14 ILs showed a significantly lower DI (24-49%), seven a significantly reduced LS (21-33 mm) and five a significantly lower LG (1.7-3.0 mm/day). Two lines, IL4-1 and IL1-3/3-3, showed a significant reduction of all three parameters (DI, LS and LG). The commercial cultivar *S. lycopersicum* cv. Durinta, with a fair level of resistance, also showed lower disease parameters as compared to the susceptible control, *SL*.

Pearson correlation coefficients were calculated to asses the correlations between DI, LS and LG. A significant correlation (P<0.01) was present between DI and LS (r=0.82); DI and LG (r=0.65) and LG and LS (r=0.82) and are in agreement with the presence of lines significantly reduced for more than one disease parameter. The high correlations show that most of the QTLs are involved in the reduction of all three parameters (DI, LS and LG). We designated the identified QTLs as Resistance to *B. cinerea* QTL (*Rbcq*, Table 2) followed by the number of the chromosome on which they are located, an analogous to the designation used for describing the QTLs in the F_2 population (Chapter 2).

Three QTLs, *Rbcq*2, *Rbcq*4a (previously named *Rbcq*4; Chapter 2) and *Rbcq*6, unambiguously conferred increased resistance. The lower levels of susceptibility in IL3-1 and IL3-2, containing overlapping introgressions, are most probably the effect of one QTL, i.e. *Rbcq*3.

Other ILs that are less susceptible to *B. cinerea* than the reference *SL* contain multiple introgressions, thus complicating the allocation of resistance loci to specific chromosomes. In some cases, the most likely location can be deduced. For instance IL9-2 contains introgressions of Chromosome 6 and Chromosome 9. The introgression on Chromosome 6 is also present in IL6-3, which is equally susceptible to *B. cinerea* as *SL*. Thus, the increased resistance of IL9-2 is likely caused by a QTL on Chromosome 9 and there is no contribution from Chromosome 6. Furthermore, IL9-1 is partly overlapping with IL9-2 and has a similar LS as IL9-2, but a DI similar to *SL*. Therefore we propose that two QTLs are present in IL9-2, *Rbcq*9a causing reduced LS in both IL9-1 and IL9-2 and *Rbcq*9b causing reduced DI only in IL9-2. By analogous deductions, the reduced disease symptoms in IL1-3/3-3, 1-4, 11-2 and 12-3 are likely to be caused by their Chromosome 9 introgressions which partly overlap

the introgressions of IL9-1 and IL9-2. However, IL12-3 is significantly more resistant than IL9-2 suggesting an additional effect on Chromosome 12 (*Rbcq*12). The reduced susceptibility in IL12-1 and IL12-2, although statistically not significant, suggest that *Rbcq*12 might be located in the region overlapping between IL12-1, 12-2 and 12-3. IL11-2 has a significantly lower DI than IL9-1 and the introgression on Chromosome 9 is smaller than in IL9-1. Therefore the lower DI is probably conferred by another QTL: *Rbcq*11. IL1-3/3-3 and 1-4 are not significantly less susceptible than IL9-2 therefore the resistance in these lines is probably conferred by the presence of *Rbcq*9b.

Due to the absence of *SH* alleles for the CAPS marker TG460, which is linked to *Rbcq*1, it is unclear whether this locus is represented within the IL population. However, from previous experiments (Chapter 2) a highly resistant BC_2S_2 genotype was selected, denominated BRC-5, containing three homozygous introgressions on Chromosomes 1 (including *Rbcq*1 and homozygous *SH* for TG460), 4 and 10, representing in total 18% of the *SH* genome (Fig. 2). BRC-5 was the least susceptible line showing a DI of only 15%. No decreased susceptibility has been identified in any of the four Chromosome 10 ILs even though these ILs cover the entire length of Chromosome 10. It is therefore unlikely that the Chromosome 10 introgression contributes to the resistance of BRC-5. The Chromosome 4 introgression of BRC-5 does not overlap with the previously mapped position of *Rbcq*4a. IL4-3 showed a decreased susceptibility, although statistically not significant, suggesting the presence of a second QTL *Rbcq*4b. The combined effect of *Rbcq*1 and *Rbcq*4b is likely responsible for the increased resistance of BRC-5, however this needs to be confirmed by experimental evidence.

In summary, ten QTLs were identified all increasing resistance to *B. cinerea*. The evidence for QTLs *Rbcq*4b, *Rbcq*11 and *Rbcq*12 is only circumstantial due to the presence of *Rbcq*9a/b, and their effects should be confirmed in the absence of the introgression on Chromosome 9.

Discussion

IL population

An IL population (n=30) was developed of *S. habrochaites* in the genetic background of the cultivated tomato *S. lycopersicum*. During generation of the IL population, 239 additional AFLP markers were used which were not identified during analysis of the initial F_2 population (Chapter 2) providing a nearly two-fold increase in marker density in spite of the use of the same AFLP primer combinations. A similar increase in marker density was observed during the development of a lettuce IL population (Jeuken and Lindhout 2004), mainly due to the reduced complexity of the AFLP-patterns of the ILs, thereby enabling the scoring of closely migrating or faint amplification products.

Although *Pst*I/*Mse*I markers are more equally distributed over the tomato chromosomes as compared to *Eco*RI/*Mse*I markers (Haanstra et al. 1999), ten randomly chosen primer combinations did not provide complete genome coverage. Compared to the high density RFLP reference map (Tanksley et al. 1992) about 32% of the genetic length of Chromosome 9 was not detected during the AFLP screenings. In an IL population of *S. pennellii* that was developed using 350 RFLP markers (Eshed and Zamir 1994), Chromosome 10 introgressions remained unnoticed using RFLP markers but could be detected using AFLP markers (Bonnema et al. 2002). A combination of AFLP with CAPS or SSR markers is a good strategy to develop introgression lines with a full genome coverage.

Reproductive behavior of the IL population

IL populations are generally believed to be less prone to reproductive problems, yet the development of some specific ILs has proven to be cumbersome. No ILs containing the top of Chromosome 8 were obtained. ILs generated with *S. pennellii* LA716 (Eshed and Zamir 1995) and *S. habrochaites* LA1777 (Monforte and Tanksley 2000a) as progenitors appeared to lack lines with introgressions for a small region on the short arm of Chromosome 8. Attempts to fixate this region of the wild relative remained unsuccessful, presumably due to the presence of a male sterility locus. Compared to both previously mentioned studies, a larger portion of Chromosome 8 is lacking in our population. Developing additional ILs covering at least part of this region should be feasible.

Plants that were homozygous *SH* for introgressions on the long arm of Chromosome 5 failed to set seeds. The *S. habrochaites* LA1777 IL population (Monforte and Tanksley 2000a) contains a QTL for reduced self seed set on Chromosome 5, possibly causing zygotic incompatibility resulting in early seed abortion (Moyle and Graham 2005). The occurrence of homozygous introgressions of Chromosome 7 and 9 was skewed towards homozygous *SH* resulting in the presence of these introgressions in multiple ILs (Fig. 2). Similar observations were made in other advanced interspecific tomato populations involving wild relatives such as *S. galapagense* (Paran et al. 1995), *L. peruvianum* (Fulton et al. 1997a) and *S. habrochaites* (Monforte and Tanksley 2000a).

One of the advantages of an IL population is that each line has a high resemblance to *SL* and therefore sterility problems are expected to be minimal. For the development of a full set of homozygous ILs, however, extra effort must be undertaken to select lines containing introgressions flanking deleterious genes (e.g. on Chromosomes 5 and 8) or select against overrepresentation of introgressions on which an advantageous gene is located (e.g. on Chromosomes 7 and 9).

Confirmation of previously identified loci for resistance to B. cinerea

Two different disease assays were applied to quantify resistance to *B. cinerea*. Initially, QTLs were detected in an F_2 population and confirmed in BC_2S_1 families (Chapter 2) using a bioassay on stem segments (ten Have et al. 2007) leading to the identification of three QTLs: *Rbcq*1 reducing LG, *Rbcq*2 and *Rbcq*4a both reducing DI (Chapter 2). Stem resistance in the IL population was quantified in whole adult plants; wound inoculated with a *B. cinerea* containing agar plug and disease progress was recorded during a longer period. In all tests, *Rbcq*2 and *Rbcq*4a conferred resistance to *B. cinerea* showing the robustness of each QTL. *Rbcq*1 could not be confirmed in the IL population, but it remains uncertain whether *Rbcq*1 was represented in the ILs tested. Marker analysis showed that the introgression in lines IL1-2 and IL1-3/3-3 do not overlap. A small *SH* introgression for Chromosome 1 is therefore not represented within the ILs (Fig. 2). *Rbcq*1 is however certainly present in BRC-5, the most resistant introgression line tested. Thus, all three previously identified loci for increased resistance to *B. cinerea* were detected using both bioassays.

Identification of additional resistance loci using an IL population

The main purpose of developing an IL population and assessing its resistance to *B. cinerea* was to identify additional QTLs to the ones previously identified in the F_2 population (Chapter 2). As resistance to *B. cinerea* is polygenic, multiple minor quantitative effects can easily be overlooked in an interspecific F_2 population. Seven additional QTLs were detected in the IL population: *Rbcq*3, *Rbcq*4b, *Rbcq*6, *Rbcq*9a, *Rbcq*9b, *Rbcq*11 and *Rbcq*12. No indications for any of the seven new QTLs were found in the analysis of the F_2 population, even when reexamining these data (data not shown). Most newly identified loci were less efficient in reducing symptoms compared to *Rbcq*2. These “minor loci” might have been identified in a larger F_2 population, but they were readily detected in an IL population. Also in an *Arabidopsis* RIL population, a larger number of QTLs for resistance to *B. cinerea* were reported, some of which were considered to be specific for distinct pathogen isolates (Denby et al. 2004). This IL population provides an excellent tool to study the possible existence of isolate-specific QTLs in tomato and study resistance levels in different environments.

Potential of pyramiding QTLs

None of the QTLs was sufficient to confer the full level of resistance of the *SH* parent. To reach such a level QTLs have to be combined. Pyramiding of ten QTLs is practically impossible and probably not necessary. An example of an additive effect is illustrated by *Rbcq9b* and *Rbcq*12. IL12-1 and IL12-2 with only *Rbcq*12 had on average a 22% lower DI (although not significant) while IL9-2 with *Rbcq*9b had a DI significantly decreased by 24%, as compared to *SL*. Assuming an additive model, the

combination of *Rbcq*9b and *Rbcq*12 would lead to a decrease in DI by 46%. The observation that IL12-3, combining both *Rbcq*9b and *Rbcq*12, had a DI that was 49% lower is in agreement with this additive model. These results show the potential of pyramiding multiple QTLs in order to get an elevated resistance to *B. cinerea* but redundancy of QTLs for resistance is expected. The IL population is an excellent tool to combine multiple QTLs and study their interaction in order to select the best set of QTLs for exploitation in a commercial breeding program. More detailed metabolome, proteome and transcriptome analysis of each of the ILs may provide insight in the resistance mechanism conferred by each QTL.

Acknowledgements

This project is sponsored by grants of the Dutch Technology Foundation (STW, grant no. WBI.4172) and De Ruiter Seeds BV, The Netherlands. The authors would like to thank Petra van den Berg, Pham Thi Thu Huong and Roeland Voorrips for their contribution and valuable suggestions.

CHAPTER 4

Quantitative resistance to *Botrytis cinerea* from *Solanum neorickii*

Submitted to Euphytica

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Tomato (*Solanum lycopersicum*) is susceptible to grey mold (*Botrytis cinerea*). Quantitative resistance to *B. cinerea* was previously identified in a wild relative, *S. neorickii* G1.1601. 122 F_3 families derived from a cross between the susceptible *S. lycopersicum* cv. Moneymaker and the partially resistant *S. neorickii* G1.1601 were tested for susceptibility to *B. cinerea* using a stem bioassay. Three quantitative trait loci (QTL) were detected: QTL3 and QTL9 reducing lesion growth (LG) and QTL4 reducing disease incidence (DI). For each QTL, a putative homologous locus was identified recently in another wild tomato relative, *S. habrochaites* LYC4. The effects of QTL3 and QTL4 were confirmed by assessing disease resistance in BC_3S_1 and BC_3S_2 progenies of *S. neorickii* G1.1601. The reduction in LG of QTL9 was not confirmed but rather, this locus conferred a reduced DI, similar to observations in the QTL study using *S. habrochaites*. The results are discussed in relation to other disease resistance loci identified in studies with other wild tomato relatives.

Introduction

Botrytis cinerea (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) is a necrotrophic fungus with a wide host range (Elad et al. 2004; Jarvis 1977). Modern hybrid tomato (*Solanum lycopersicum*) cultivars are susceptible to *B. cinerea* although some cultivars show a certain level of quantitative resistance (ten Have et al. 2007). The presumed polygenic inheritance has limited the success of breeding for resistance to *B. cinerea*.

Quantitative resistance to *B. cinerea* has been identified in several wild relatives of *S. lycopersicum* (Egashira et al. 2000; Nicot et al. 2002; ten Have et al. 2007; Urbasch 1986). A stem bioassay suitable to quantify susceptibility of tomato to *B. cinerea* was used to screen a collection of wild tomato accessions (ten Have et al. 2007), and two parameters were calculated: the proportion of outgrowing lesions or disease incidence (DI) and lesion growth rate expressed as the increase in lesion size in mm/day (LG). All four tested accessions of *S. habrochaites* showed quantitative resistance (ten Have et al. 2007). *S. habrochaites* LYC4 was used previously to study the genetic basis of this resistance (Chapters 2 and 3) and a total of ten quantitative trait loci (QTL) were identified illustrating the genetic complexity of resistance to *B. cinerea*. Also *S. neorickii* G1.1601 showed a certain level of resistance (ten Have et al. 2007). A F_2 mapping population of *S. lycopersicum* cv. Moneymaker x *S. neorickii* G1.1601, previously developed to identify QTLs for resistance to *Oidium neolycopersici* (Bai et al. 2003), was screened for susceptibility to *B. cinerea*. Since F_2 seeds were no longer available, F_3 families were used for the analysis. Segregating BC_3S_1 families and BC_3S_2 plants were generated in order to confirm the effects identified in the F_3 analysis. We report the identification of three QTLs, from *S. neorickii* G1.1601 involved in resistance to *B. cinerea*. Results of this

study were compared to previously identified QTLs for resistance to *B. cinerea* in *S. habrochaites* LYC4 (Chapters 2 and 3).

Material and methods

Plant material

Three tomato accessions were used in this study: *Solanum lycopersicum* cv. Moneymaker (hereafter referred to as *SL*), *S. neorickii* G1.1601 (hereafter referred to as *SN*) and *S. habrochaites* LYC4 (hereafter referred to as *SH*). A cross between *SL* and *SN* was made and an F_2 population (n=209) developed as described in detail by Bai et al. (2003). From this F_2 population, F_3 seeds of each genotype were collected, but only 122 F_2 plants produced enough F_3 seeds for further study. Marker data for 75 of the 122 F_2 plants used in this study were available.

For confirmation of the QTLs, three selected F_3 plants were backcrossed to *SL* to obtain BC_1 seeds. Two subsequent backcrosses to *SL* resulted in a BC_3 progeny of 53 plants and marker assisted selection (MAS) was used to select three plants containing either QTL. Three BC_3S_1 families (n=86 each) were genotyped to select plants homozygous *SL* or *SN* for the putative QTLs. A selected set of BC_3S_1 genotypes homozygous *SN* for the region of interest was grown to produce BC_3S_2 seeds.

Experimental setup and stem assay

For each of the 122 F_3 families, 5 seedlings were grown and their susceptibility to *B. cinerea* was evaluated. For logistic reasons the disease assays were divided (at random) into 13 experiments with equal numbers of plants (50 plants/week). Eight *SL* controls were included in each experiment.

For the BC_3S_1 families, six replicates were grown by taking cuttings of each genotype including a set of *SL*, *SN* and *SH* controls. To assess susceptibility to *B. cinerea* in the BC_3S_2 plants, two experiments were performed in which three replicates, grown from seeds, were tested in each experiment.

The stem assay was performed according to ten Have et al. (2007). In short, stems of 6-8 week-old plants were cut into six pieces of five cm length and the top of each segment was inoculated with a droplet of 5 µl inoculum, containing approximately 10^6 conidia $\cdot$ ml^{-1}. Inoculum of *B. cinerea* strain B05.10 was prepared according to Benito et al. (1998). Incubations were performed at 15 ˚C in the dark at 100% relative humidity. The infection progress was measured at day four and five after inoculation using a Vernier caliper. For each genotype, the percentage of successfully infected stem pieces was calculated (disease incidence, DI). The lesion growth rate (LG) was calculated as the increase in lesion size between day four and five (mm/day) for the infected stem pieces.

DNA isolation and marker analysis

12 plants of each F_3 family were grown and one leaf was harvested from each plant and pooled for DNA isolation in order to deduce the original F_2 genotype. The AFLP™ and CAPS analysis of the F_3, BC_3 and BC_3S_1 populations were performed as described previously (Chapters 2 and 3). The following ten AFLP primer combinations were used for genotyping: P14M48, P14M49, P14M50, P14M60, P14M61, P15M48, P18M50, P18M51, P22M50 and P22M51.

CAPS and SCAR primers were obtained from the “Solanaceae Genomics Website” (http://www.sgn.cornell.edu) or designed on sequences of genomic or cDNA clones available from the same source. Polymorphisms between *SL* and *SN* were determined using the CAPS digestion approach described by Bai et al. (2004). Markers, PCR conditions and restriction endonucleases used for genotyping are presented in Table 1.

Table 1: Primer sequences, annealing temperature, lengths of PCR products and enzymes revealing a polymorphism for CAPS/SCAR markers. Segregation of the CAPS/SCAR markers was determined in the F_3, BC_3S_1 or both populations.

Marker name	Chromosome	Primer sequence (5'-3')	PRC product length (bp)	Annealing Tm (°C)	Marker Type	Enzyme or allele size (bp)	Source[a]	F_3	BC_3S_1
TG40	3	GCGAGCTCGAATTCAATTCCAAC CGGGATTTTAGTTTTTCCGATCC	450	55	CAPS	*Alu*I	PBR	X	X
TG56	3	TTTGTACCATGATTGTCCGATC GGCATTCATCATTCAACATGC	380	55	CAPS	*Hpa*I	PBR		X
T1388	3	GCGATTTGGCTATCTGGGTA AACCGAAAGGCTTTTCCAAG	1000	55	CAPS	*Hpy*CH4V	SGN		X
TG599	3	GCATGCCTGCAGAGTGGTC ATTCGCTACCTTGAGGGCTG	350	65	CAPS	*Bsp*LI	PBR	X	X
TG42	3	TTCCTCACTGCTTGGACCAGC TAGAACTTGGCATCCCTTGAAG	560	55	CAPS	*Xba*I	PBR		X
TG549	3	ATGGAGAGAAGCTGGAACAC TTCTTAGAGCCCACCAGCAC	400	55	CAPS	*Mse*I	PBR	X	
T1143	3	GGAGAATGGGCATCTACAAA CCTTTAGGATGGATTCCG		55	SCAR	*SN*: 1580 +1050/ *SL*: 1000	PBR	X	X
T0707	4	TCGTGGATTATGGGCTTCTT GGTAAGGCTGCAACACATCA	560	55	CAPS	*Dde*I	PBR	X	X
TG339	4	GAAACCTTACCCCTCTA CGCTGTTTCTTGCCATTT	500	46	CAPS	*Hinf*I	PBR	X	X
TG272	4	GATTTTGCCCCCTCTACCA ACATCTTTTCCTTCCCTCTGC	352	55	CAPS	*Hinf*I	PBR	X	X
TG264	4	GGAACAGGTCAGGACAGCAT TGGCTAACTGACGAAGACGA	520	55	CAPS	*Mnl*I	PBR	X	X
T1405	4	CACCAACAACTAGCCCTTGA AAGCAATTCCTCCAGCTTCA	535	55	CAPS	*Bsa*JI	SGN	X	X
TG555	4	AATTCGGAGCTCACTGCTTC AGTACGGCATGCTTGCTATC	430	55	CAPS	*Hpy*CH4IV	PBR	X	X
CT50	4	GACGGCGTATTACGTTCAGA CTAGCACCCCAAAGGATGAG	390	55	CAPS	*Dde*I	PBR		X
TG498	4	CCTGCAGAGCATCAACGTAGT CACATCACAATGAGGAGAGCA		55	SCAR	*SN*: 500/ *SL*: 450	PBR		X
TG10	9	ATGATATCCACACCCCTGGA ATGCCTCGAAATTCAAATGC	587	55	CAPS	*Hae*III	PBR	X	X
*Tm*2a	9	AGCGTCACTCCATACTTGGAATAA AGCGTCACTCAAAATGTACCCAAA	1600	53	CAPS	*Acc*I	Sobir et al. 2000	X	X
TG551	9	CAACGAAAACCTTGGCACTC GAGATGAGCAGCATATGGAG	350	55	CAPS	*Hpy*CH4IV	PBR	X	X

[a] PBR: Developed at Wageningen University laboratory of Plant Breeding, mainly using data from SGN. SGN: primers published in the SGN database (http://www.sgn.cornell.edu) or published previously and references are given

Data analysis

Marker data were analyzed and a genetic linkage map was calculated with Joinmap® 3.0 (van Ooijen and Voorrips 2001). The susceptibility of the F_2 genotype was estimated by taking the means of the replicated disease assays of five F_3 plants. Phenotypic data of the BC_3S_1 and BC_3S_2 plants were analyzed using the general linear model (GLM) approach as implemented in SPSS 12.0 (SPSS Inc, Chicago, USA) using the following models: LG = constant + genotype + block + genotype * block and DI = constant + genotype + block. QTL analysis of the F_3 and BC_3S_1 populations were performed using the Kruskal-Wallis analysis as embedded in MapQTL® 5.0 (van Ooijen 2003). For analysis of the BC_3S_2 plants, the parameter genotype was replaced by the parameter CAPS in the GLM model which contained information about the presence or absence of the identified QTL regions. Data of the grouped BC_3S_2 plants were analyzed by comparing mean observations of each group to the mean observation of *SL* using a Dunnett test (Dunnett 1955). Linkage maps were drawn using MapChart (Voorrips 2002). The correlation between traits was examined by interpreting Pearson correlation coefficients.

Results

Analysis of the F_3 lines

F_3 seeds of the cross between the susceptible cultivar *S. lycopersicum* cv. Moneymaker (*SL*) and *S. neorickii* G1.1601 (*SN*) were available (Bai et al. 2003) in sufficient quantity for 122 of the original 209 F_2 plants. For 75 of these 122 F_2 individuals, marker data were available; therefore we decided to (re)genotype all 122 F_3 families. Ten AFLP primer combinations resulted in a total of 234 markers: 120 *SL* specific and 114 *SN* specific. 192 AFLP markers were placed on the paternal and maternal linkage maps (data not shown).

A quantitative *B. cinerea* disease assay on stem segments (ten Have et al. 2007) yielded data on disease incidence (DI) and lesion growth (LG). The frequency distributions of both traits suggested normal, quantitative trait characteristics (data not shown). The susceptible control, *SL,* showed a DI and LG comparable to previous experiments (Table 2; Chapter 2). Kruskal-Wallis (KW) analysis identified three linkage groups putatively containing a QTL for decreased susceptibility to *B. cinerea*. Based on the map of Bai et al. (2003) these linkage groups could be assigned to Chromosomes 3, 4 and 9 and the putative QTLs will be referred to as QTL3, QTL4 and QTL9, accordingly. To integrate the maternal and paternal linkage groups of Chromosome 3, 4 and 9, thirteen co-dominant CAPS markers were developed (Table 1). Using the integrated linkage maps, the effect of each putative QTL was recalculated using KW analysis (Table 3). All three QTL regions showed a skewed segregation resulting in a deficit of plants homozygous *SL* for QTL3 and QTL4 and a deficit of plants homozygous *SN* for QTL9 (Table 3). In spite of the correlation between DI and LG (Pearson: r=0.258, $P<0.01$), all QTLs were associated with a single trait: QTL3 and QTL9 conferred a reduced LG while QTL4 conferred a reduced DI.

Table 2: Mean disease incidence (DI) and lesion growth (LG) of the controls.

Population	*SL*			*SN*			*SH*		
	n[a]	DI	LG	n	DI	LG	n	DI	LG
F_3	104	70 ± 7	6.9 ± 0.3						
BC_3S_1	14	70 ± 7	6.6 ± 0.3	7	71 ± 9	5.1 ± 0.3	7	45 ± 9	4.8 ± 0.4
BC_3S_2	57	55 ± 3	5.3 ± 0.2	11	36 ± 7	5.1 ± 0.4	4	16 ± 12	1.8 ± 0.9

[a] Number of plants tested

Confirmation of the QTLs using BC_3S_1 and BC_3S_2 plants

53 BC_3 plants were genotyped using AFLP and three BC_3 plants were selected heterozygous for the alleles of either QTL3, QTL4 or QTL9 in a genetic background

as similar as possible to the recurrent parent *SL* (Table 4). Only one BC_3 plant was identified containing the *SN* allele of QTL9. However, this plant was also heterozygous for the QTL3 and QTL4 alleles. Three BC_3S_1 families were grown and marker assisted selection (MAS) was used to obtain a more balanced test design (Table 4). In 3 of the 20 plants homozygous for the *SN* allele of QTL9, *SN* alleles of QTL3 and QTL4 were absent. As a result, experiments aimed at confirming QTL9 were only performed using BC_3S_2 lines.

Table 3: Effect of the QTLs identified in the F_3 population. Mean values for disease incidence (DI) and lesion growth (LG) are presented, along with their significance as determined using a Kruskal-Wallis test.

		DI (%)				LG (mm/day)			
Marker	QTL	*SL*[a]	H	*SN*	sign[b]	*SL*	H	*SN*	sign
TG599	QTL3-LG	51	50	45		5.6	5.4	5.0	x
		(11)	(49)	(33)		(11)	(49)	(33)	
TG339	QTL4-DI	59	47	47	x	5.7	5.0	5.3	
		(13)	(31)	(47)		(13)	(31)	(47)	
TG551	QTL9-LG	47	50	49		5.6	5.2	5.0	
		(31)	(53)	(19)		(31)	(53)	(19)	

[a] *SL* denotes homozygous for the alleles of *S. lycopersicum*, *SN* homozygous for the alleles of *S. neorickii*, and H describes the heterozygous class. The number of observations within each class is indicated between parentheses
[b] Significance: (x; $P<0.1$)

Table 4: Description of the three BC_3S_1 families. Each QTL and the additional introgressions for each family are described. Initially, 86 BC_3S_1 plants were grown for each family. After marker assisted selection (MAS), 50 plants were selected to be evaluated for susceptibility to *B. cinerea*.

QTL	Trait	Additional in BC_3 plants	Segregation of the BC_3S_1 families [a]	Number of BC_3S_1 plants evaluated [a,b]
QTL3	LG	Parts C2 & C6 [c]	21:33:16	17:17:16
QTL4	DI	Parts C1 and C12	21:40:25	17:17:15
QTL9	LG	QTL3 & QTL4 & part C12	25:41:20	20:10:20

[a] Segregation ratio: homozygous *SL* : heterozygous : homozygous *SN*
[b] Plants, homozygous *SN* were grown to produce BC_3S_2 seeds
[c] C = Chromosome

While assessing susceptibility to *B. cinerea* in the BC_3S_1 families, only the first experiment showed a sufficient level of infection (overall mean DI of 63%; observed four days post inoculation). Replicate experiments in subsequent weeks showed a low level of infection (overall mean DI < 20%) and were therefore discarded. In the first assay, the control *SL* showed the expected level of susceptibility (Table 2; Chapter 2). However, the susceptibility of the controls *SN* and *S. habrochaites* LYC4

(*SH*) was higher than previously reported (Chapter 2; ten Have et al. 2007) suggesting an overall high disease pressure in this experiment.

Susceptibility to *B. cinerea* in BC_3S_2 lines was assessed in two independent experiments. The mean DI of the two experiments was 29 and 62% respectively, and yielded, on average, five independent observations for each BC_3S_2 line. The susceptible control *SL* and the partial resistant control *SH* showed the expected level of susceptibility (Table 2) but *SN* was more susceptible than previously reported (ten Have et al. 2007).

Experiments aimed at confirming QTL3, in the BC_3S_1 population, did not lead to identification of a significant reduction of LG, yet the presence of the homozygous *SN* resistance allele resulted in a reduced LG (Table 5). A significant reduction was observed while testing the BC_3S_2 lines ($P<0.01$, group baa, Table 6). QTL4, reducing DI, could not significantly be confirmed using either BC_3S_1 (Table 5) or BC_3S_2 lines (group aba, Table 6). However, in both BC_3S_1 population and BC_3S_2 lines, a reduction in DI was observed in the presence of the *SN* resistance allele. The LG reducing effect of QTL9 was not confirmed in the BC_3S_2 lines (group aab, Table 6). Instead, a reduction in DI was observed for lines homozygous for the *SN* allele of QTL9 (-9 %).

Table 5: Effect of QTL3 or QTL4 in its respective BC_3S_1 family. Mean values for disease incidence (DI) and lesion growth (LG) are presented.

		DI (%)			LG (mm/day)		
Marker	QTL	*SL* [a]	H	*SN*	*SL*	H	*SN*
TG599	QTL3-LG	69	67	72	5.0	5.2	4.3
		(16)	(17)	(16)	(16)	(17)	(16)
TG339	QTL4-DI	68	52	58	6.1	6.2	6.4
		(17)	(17)	(15)	(17)	(17)	(15)

[a] *SL* denotes homozygous for the alleles of *S. lycopersicum*, *SN* homozygous for the alleles of *S. neorickii*, and H describes the heterozygous class. The number of observations within each class is indicated between parentheses. None of the effects were significant

In addition, BC_3S_2 lines were tested in which a combination of loci were present (Table 6). Two plants were homozygous for the *SN* alleles of QTL4 and QTL9 (group abb) and showed a lower DI than plants containing either *SN* alleles of QTL4 or QTL9. Plants containing a combination of the *SN* alleles of QTL3 (higher DI and lower LG compared to *SL*) and QTL9 (lower DI and similar LG compared to *SL*; group bab) were as susceptible as *SL*. However, the observed mean was not deviating from the mean estimated from lines containing each QTL separately.

Table 6: Estimated mean values for disease incidence (DI) and lesion growth (LG) of the BC_3S_2 lines. Means of each line/trait were compared to the mean of *S. lycopersicum* cv. Moneymaker (*SL*) using a Dunnett test.

QTL Genotype [a]	n[b]	Mean DI (%)[c]		Std error	Mean LG (mm/day)		Std error
aab	3	46		7	5.8		0.28
aba	8	48		5	4.9	x	0.19
abb	2	33	x	8	5.2		0.38
baa	13	62		4	4.5	**	0.16
bab	1	61		11	5.0		0.38
SN	11	36	x	7	5.1		0.35
SH	4	16	*	12	1.8	**	0.91
SL	57	55		3	5.3		0.19

[a] aab = QTL genotype at Chromosome 3, Chromosome 4 and Chromosome 9, a = homozygous *SL* and b= homozygous *SN*

[b] number of lines BC_3S_2 genotype tested

[c] Significant differences are marked with x (P<0.1), * (P<0.05) or ** (P<0.01)

Discussion

While performing the *B. cinerea* stem assays, susceptibility of the controls varied between experiments (Table 2). In experiments with a harsher infection, quantitative resistance of *SN* is less robust than the quantitative resistance of *SH.* The higher resistance of *SH* suggests the presence of a larger number of QTLs, or more effective QTLs. Stem morphology and vascular development of *SH* stems might also play a role in resistance (Coaker et al. 2002). Variation in the bioassays and environment influences the confirmation of QTLs for resistance to *B. cinerea* (Chapter 2). External influences can be minimized by growing plants in climate rooms but this is logistically not feasible for experiments on this scale.

Initially, three QTLs were identified while analyzing the F_3 families. The resistance allele from each QTL was derived from *SN*. The LG reducing effect of the QTL3 resistance allele could be confirmed using BC_3S_2 lines. The LG reducing effect of the QTL9 resistance allele was not confirmed, but this locus instead conferred a decrease in DI. No confirmation was obtained for the DI reducing effect of the QTL4 resistance allele. However, the presence of the QTL4 resistance allele reduced DI in all three populations. Criteria for significances were not met due to the lack of replications (BC_3S_1 and BC_3S_2) or the lack of the number of lines tested per group (BC_3S_2).

The positions of each *SN* QTL were compared to previously mapped QTLs, conferring resistance to *B. cinerea*, from *SH* (Chapters 2 and 3). Both QTL4 and QTL9 from *SN* are located at positions homologous to the *SH* QTLs *Rbcq*4a and *Rbcq*9b (Fig. 1). CAPS analysis of the *SH* ILs containing *Rbcq*3 (Chapter 3) does not exclude the possibility that *SN* QTL3 is at a homologous position. We postulate that the three *SN* QTLs identified in this study have QTLs at homologous positions in *SH*.

Experiments aimed at confirming the LG reducing effect of QTL9 unexpectedly resulted in the identification of a QTL reducing DI. Previous analysis of the *SH* IL population, resulted in identification of two QTLs on Chromosome 9: *Rbcq*9a reducing LG and *Rbcq*9b reducing DI (Chapter 3). The region homologous to *Rbcq*9a is homozygous *SL* in this set of BC_3S_2 plants and may explain why only a reduced DI was observed. Future experiments aimed at confirming the reduction in LG should focus on testing lines containing *SN* alleles in the region homologous to *Rbcq*9a.

The *SN* QTL9 did not confer a reduction in DI in the *SL* x *SN* F_3 population (Table 3), as was also observed for the *SH Rbcq*9b in the *SL* x *SH* F_2 population (Chapter 2). Segregation of multiple DI-reducing loci combined with the under-representation of *SN* alleles for Chromosome 9 might have obscured identification of this locus. Several BC_3S_2 lines contained multiple QTLs. Two plants contained both QTL4 and QTL9 (group abb) and the additional reduction in DI of these lines suggests a fully additive model, showing the potential of pyramiding these two QTLs.

Figure 1 shows a comparison between QTL positions identified in this study and previously mapped QTLs conferring resistance to *B. cinerea* (Chapters 2 and 3), mapped positions of resistance genes (R-genes), resistance gene analogs mapped by Zhang et al. (2002) and QTLs conferring resistance to other diseases (Tables 7 & 8). The positions of *B. cinerea* QTLs may be homologous with the positions of R-genes or QTLs conferring resistance to *Phytophtora infestans* and *Xanthomonas campestris* (Chromosome 3); *Ralstonia solanacearum*, *Phytophtora infestans* and *O. neolycopersici* (Chromosome 4); *Alternaria solani*, *Fusarium oxysporum* and TMV (Chromosome 9). It remains to be determined whether loci at homologous positions involve the same genes triggering a general defense mechanism such as papillae formation or phytoalexin production or whether the observed homologous positions are coincidental. The recessive gene *ol-2*, identified in *S. lycopersicum* var. *cerasiforme* and mapped at a position homologous to QTL4, is involved in papillae formation (Bai et al. 2005). The observation that QTL4 acts dominant implies that resistance to *B. cinerea* and to *O. lycopersici* cannot be conferred by the same gene. However, it cannot be excluded that each species contains a similar ancestral gene, which has diverged into alleles conferring specificity to different pathogens. The isolation of *B. cinerea* resistance genes, followed by complementation and subsequent testing of these plants for resistance to multiple pathogens, might resolve such questions.

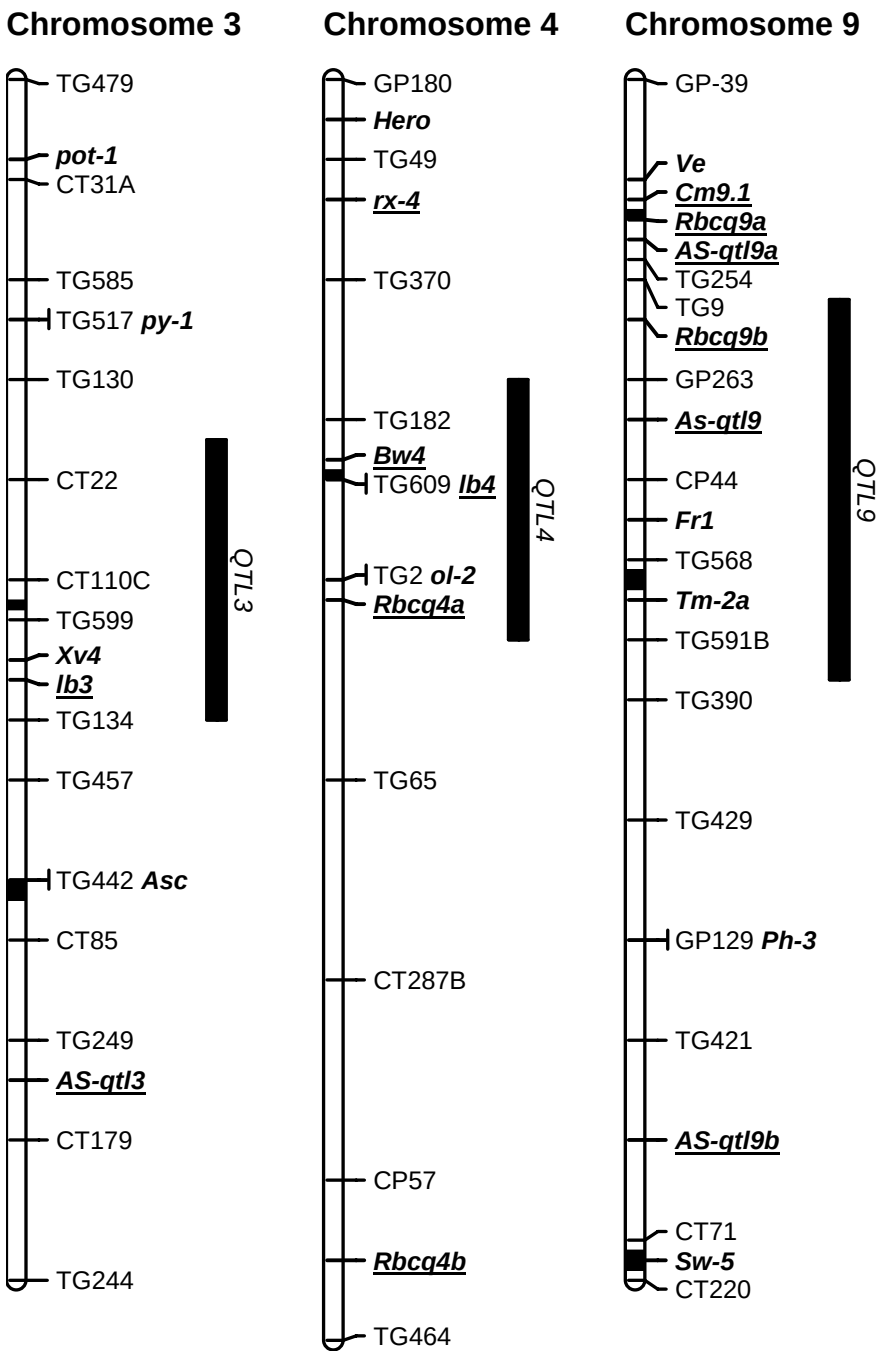

Fig. 1: Overview of resistance loci mapped on tomato Chromosomes 3, 4 and 9: markers are from the core RFLP map (Tanksley et al. 1992). Closed blocks within the bars show the approximate locations of mapped resistance gene analogs (Zhang et al. 2002). The approximate locations of monogenic resistance genes (R genes) and quantitative trait loci (QTL, underlined) for disease-resistance are shown in bold. For clarity, some loci were renamed for ease of display. Explanation of abbreviations of the R genes or QTLs is presented in Tables 7 and 8 respectively. The approximate locations of QTLs identified in this study are presented as bars on the right hand side of each chromosome.

Table 7: Qualitative resistance genes mapped on the tomato Chromosomes 3, 4 and 9.

Chromosome	Locus	pathogen	Common name	Resistance originating from [a]	Reference
3	*Asc*	*Alternaria alternata*	Black mold	*S. chilense* G1.1701, *S. chmielewskii* CPRO731089, *S. neorickii* G1.1604, *S. pennellii* LA716 and G1.1611, *L. peruvianum* G1.1860 and *S. pimpinellifolium* G1.1704	van der Biezen et al. 1995
9	*Fr1*	*Fusarium oxysporum*	Fusarium vascular wilt	*L. peruvianum*	Vakalounakis et al. 1997
4	*Hero*	*Globodera rostochiensis*		*S. pimpinellifolium* LA1792	Ganal et al. 1995
4	*ol-2*	*Oidium neolycopersici*	Powdery mildew	*S. lycopersicum* var. *cerasiforme*	Ciccarese et al. 1998; de Giovanni et al. 2004
9	*Ph-3*	*Phytophthora infestans*	Late blight	*S. pimpinellifolium* L3708	Chunwongse et al. 2002
3	*pot-1*	Potato virus Y	PVY	*S. habrochaites* PI247087	Legnani et al. 1995; Parrella et al. 2002
3	*Py-1*	*Pyrenochaeta lycopersici*	Corky root rot	*L. peruvianum*	Doganlar et al. 1998
3	*pot-1*	Tobacco etch virus	TEV	*S. habrochaites* PI247087	Parrella et al. 2002
9	*Tm-2a*	Tomato mosaic virus	TMV	*L. peruvianum*	Young et al. 1988
9	*Sw-5*	Tomato spotted wilt virus	TSWV	*L. peruvianum*	Stevens et al. 1995; Stevens et al. 1991
9	*Ve*	*Verticillium dahliae*	*Verticillium* wilt	*L. peruvianum*	Diwan et al. 1999; Zamir et al. 1993
3	*Xv4*	*Xanthomonas campestris*	Bacterial spot	*S. pennellii* LA716	Astua-Monge et al. 2000

[a] according to the new nomenclature *L. peruvianum* is divided into 4 *Solanum* species (Peralta et al. 2005). This division is unknown for most accessions mentioned in this table.

Table 8: Quantitative resistance loci with at least one locus mapped on the tomato Chromosomes 3, 4 or 9.

Chromosome	Locus	pathogen	Common name	Resistance originating from	Reference
9	*As-qtl9*	*Alternaria solani*	Early blight	*S. arcanum* LA2157	Chaerani et al. 2007
3 and 9	*AS-qtl3* and *AS-qtl9a&b*	*Alternaria solani*	Early blight	*S. habrochaites* PI126445	Foolad et al. 2002; Zhang et al. 2003
3, 4 and 9	*Rbcq3, Rbcq4a&b* and *Rbcq9a&b*	*Botrytis cinerea*	Gray mold	*S. habrochaites* LYC4	Chapters 2 and 3
3, 4 and 9	*QTL3, QTL4* and *QTL9*	*Botrytis cinerea*	Gray mold	*S. neorickii* G1.1601	This chapter
9	*Cm9.1*	*Clavibacter michiganensis*	Bacterial canker	*S. arcanum* LA2157	van Heusden et al. 1999
3 and 4	*lb3* and *lb4*	*Phytophtora infestans*	Late blight	*S. habrochaites* LA2099	Brouwer et al. 2004; Brouwer and St. Clair 2004
4	*Bw4*	*Ralstonia solanacearum*	Bacterial wilt	*S. lycopersicum* Hawaii 7996	Thoquet et al. 1996a; Thoquet et al. 1996b
4	*rx-4*	*Xanthomonas campestris*	Bacterial spot	*S. lycopersicum* Hawaii 7998	Yang et al. 2005

The resistance alleles of QTL4 and *Rbcq*4a act dominantly in reducing DI (Chapter 2). Over-dominance of QTL4 was observed in the BC_3S_1 population, but an independent confirmation is needed. Dominant QTLs for resistance to *B. cinerea* are advantageous in commercial F_1 hybrid cultivar development. Disease tests, using the stem assay, resulted in identification of QTLs either reducing DI or reducing LG

(Chapter 2). QTLs generally contributed to both a lower DI and LG in a greenhouse assay on mature plants (Chapter 3). QTL3 and QTL4 might therefore be effective in reducing both DI and LG when used in commercial breeding programs. Besides *S. habrochaites* LYC4, *S. neorickii* G1.1601 is an alternative source for QTLs conferring resistance to *B. cinerea*.

Acknowledgements

This project was sponsored by the Dutch Technology Foundation (STW, project WBI.4172) and De Ruiter Seeds BV, The Netherlands. The authors would like to thank Pham Thi Thu Huong and Yaxin Zhang for their contributions and the staff at Unifarm for taking care of the plants.

CHAPTER 5

Agronomic and morphological characteristics of a *Solanum habrochaites* LYC4 introgression line population

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Tomato (*Solanum lycopersicum*) has been the subject of extensive quantitative trait loci (QTLs) mapping studies. Most of this work has been conducted on transient populations (e.g. F_2 or backcross). We developed an introgression line (IL) population in which the *S. habrochaites* LYC4 genome was introgressed into the genetic background of *S. lycopersicum* cv. Moneymaker. This IL population was developed to identify loci conferring resistance to the necrotrophic fungus *Botrytis cinerea*. Since an IL population is homozygous and permanent, it is an excellent source for further exploitation. In this study, a total of 31 agronomic and morphological characteristics related to plant development and quality were determined.

Introduction

Tomato (*Solanum lycopersicum*) is a major vegetable and a high-value export crop. As with many crop plants, cultivated tomatoes carry only a small fraction of the genetic variation that is available in landraces and related wild species (Tanksley and McCouch 1997). Because of this, plant breeders screen landraces and wild genetic resources for valuable traits that can be introduced into modern varieties (Zamir 2001).

S. habrochaites, a wild relative of the cultivated tomato, is native to Peru and the southern parts of Ecuador and has very small, green, hairy fruit. *S. habrochaites* is often found at higher elevations in river valleys (between 1800 and 3300m) and is mainly an out-breeder with long exerted stigma. Most accessions of *S. habrochaites* are self-incompatible, but accessions with successful self-pollination produce weak progeny suffering from inbreeding depression. The subspecies *S. habrochaites* f. *glabratum* can readily be crossed with tomato. Leaves and stems of *S. habrochaites* f. *glabratum* are nearly free of hairs and the slender stems are darkly pigmented with anthocyanin (http://tgrc.ucdavis.edu/key.html; revised from Rick et al. 1990). *S. habrochaites* f. *glabratum* is predominantly found at lower latitudes (0-6 degrees south) in the south-western part of Ecuador.

S. habrochaites and *S. habrochaites* f. *glabratum* accessions harbor genes for many favorable traits such as resistance genes for pests and diseases (Brouwer et al. 2004; Foolad et al. 2002; Huang et al. 2000; Kabelka et al. 2002; Legnani et al. 1995; Maliepaard et al. 1995; Moreira et al. 1999; Parrella et al. 2000; Vidavsky and Czosnek 1998), fruit quality (Bernacchi et al. 1998a; Bernacchi et al. 1998b; Kabelka et al. 2004; Monforte and Tanksley 2000b), sesquiterpene biosynthesis (Frelichowski and Juvik 2005; van der Hoeven et al. 2000), trichomes (Antonious et al. 2005; Maliepaard et al. 1995), hybrid incompatibility (Bernacchi and Tanksley 1997; Moyle and Graham 2005) and chilling tolerance (Goodstal et al. 2005; Venema et al. 2005).

In previous research, we have identified resistance to *Botrytis cinerea* in *S. habrochaites* LYC4 (ten Have et al. 2007). To elucidate the genetics of resistance to *B. cinerea*, we used a QTL mapping approach in an F_2 population (Chapter 2).

However, as we suspected, we did not identify all loci and developed a *S. habrochaites* LYC4 introgression line (IL) population (Chapter 3). The main advantage of an IL population is that such a population is homozygous and can be propagated indefinitely (Zamir 2001). An IL population can easily be screened for a large number of traits in different experiments and can readily be grown in a number of replications in more than one environment. The latter is important for the analysis of quantitative traits. In this paper, we describe the analysis of a set of characteristics related to plant, fruit and flower morphology in the *S. habrochaites* LYC4 IL population.

Material and Methods

Population and glasshouse trial

The introgression line (IL) population consists of 30 lines derived from the cross between *S. lycopersicum* cv. Moneymaker (hereafter referred to as *SL*) and the wild accession *S. habrochaites* LYC4 (*SH*) followed by repeated back crosses, as described in Chapter 3. The developed IL population is depicted in Figure 1. The ILs and *SL* controls were grown in a heated greenhouse from July till December 2005 in two experimental blocks using a fully randomized design. Each plot consisted of a single row of three plants. The majority of observations were done on the middle plant of each plot. Observations were separated into four categories: plant characteristics, flower characteristics, fruit characteristics and yield.

The plant characteristics that were examined included: the mean increase in plant length measured at weekly intervals expressed in cm/day (PL), the mean number of branches until the first fruit truss (BR), the mean stem diameter, measured in the middle of the segment above the second fruit truss (SD), the mean number of fruit trusses below 3 meters (TR), and the stem length between the second and sixth fruit truss, expressed as mean distance in cm between trusses (TD).

The flower characteristics that were examined included: functional sterility (FST), sepal length (SPL), petal length (PTL), anther length (AL), style length (SL), anther sterile length (ASL) and anther fertile length (AFL). All measurements were performed as the mean length of five flowers at anthesis of the two outer plants of each plot, according to Georgiady et al (2002; Fig. 2). Opening of the third flower on the second truss was evaluated on all plants and is presented as the mean number of days after sowing (FL) according to the descriptors for tomato (International plant genetic resources institute 1996). Ovule development is presented as the mean number of days required between opening of the first flower and a visual swelling of the ovule (OV).

The fruit characteristics included: fruit weight (FW), fruit diameter (FD), fruit height (FH), peduncle length (PE), pericarp thickness (PCP), green shoulder (GS), fruit color (FC: red, orange or yellow), internal fruit color (IFC: red, orange, yellow or green) and self seed set calculated as all seeds harvested from the first four trusses (SS: SS < 500 seeds (-); 1000>SS>500 (±) and SS>1000 (+)). Data were collected from the first four trusses of the middle plant of each plot. On these plants, trusses were pruned to seven fruit per truss. Soluble-solids content (BRX), titratable acids (TA, expressed as % citric acid) and pH were measured on two representative fruit from each plot. Mean fruit shape (FS) was obtained by dividing FD and FH and by classifying the fruit according to the UPOV standards (FSU, Fig. 3).

Yield (YIELD) of each IL was obtained by multiplying the total fruit weight of the first four trusses (4FW) with the total number of trusses (TR). Multiplication of YIELD with BRX resulted in the determination of B*Y and an index was calculated in which the B*Y index of *SL* was set as 1. All abbreviations of the traits are summarized in Table 1.

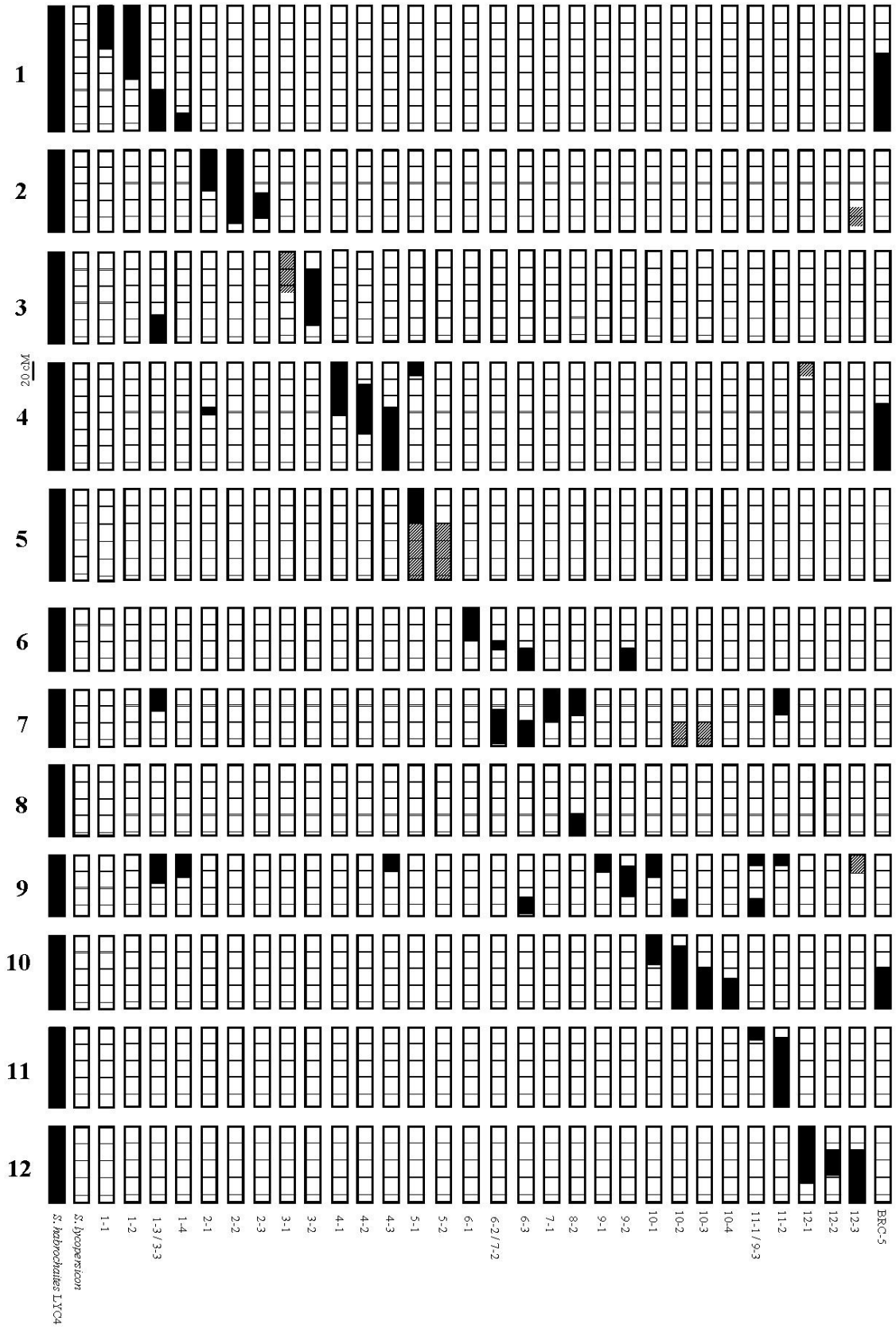

Fig. 1: Graphical representation of the genotypes of the *S. lycopersicum* cv. Moneymaker x *S. habrochaites* LYC4 introgression line population. Homozygous introgressions from *S. habrochaites* are drawn in black and heterozygous introgressions in gray in the white *S. lycopersicum* cv. Moneymaker genetic background.

Statistical Analysis

Estimated means of each trait/IL were calculated using the linear model fitting the formula: Trait = constant + IL + BLOCK. The mean of each IL was compared to the mean of *SL* using a Dunnett test (Dunnett 1955). Significant deviations from *SL* are denoted with * ($P<0.05$) or ** ($P<0.01$). Dunnett tests were performed using the mvtnorm library of the statistical software environment R, version 2.3.1 (R Development Core Team 2006). When necessary, a data transformation was applied to normalize residuals. Correlations among traits were calculated using a Pearson correlation coefficient.

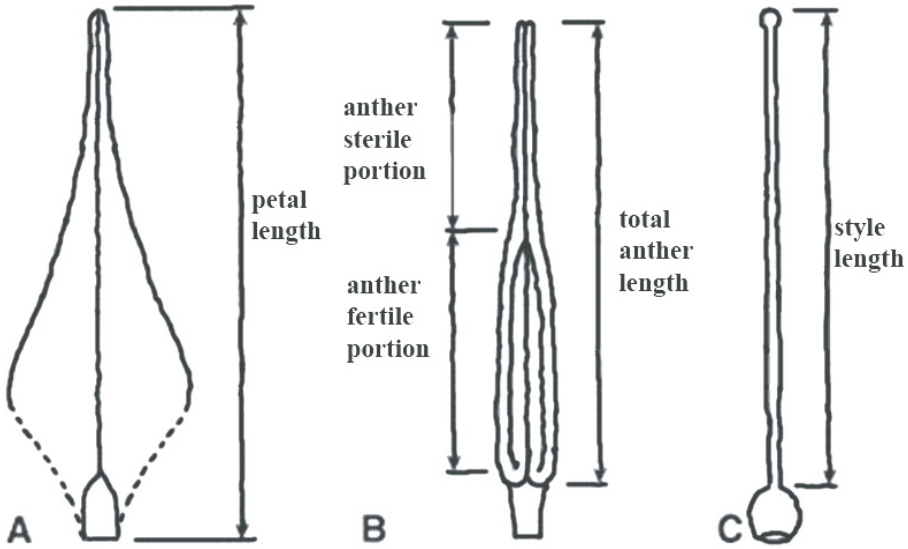

Fig. 2: Schematic view of the morphological flower characteristics measured. (A) Petal length was measured as the length of the petal from its apex to its junction with the receptacle. (B) Anther length was measured as the entire length of the anther. Anther fertile length was measured as the length of the stomia, from their basal end of their junction at the midline of the anther. Anther sterile length was measured as the distance from the junction of the stomia to the apical end of the anther. (C) Style length was measured from the junction of the style and ovary to the tip of the stigma. Figure taken from Georgiady et al. (2002).

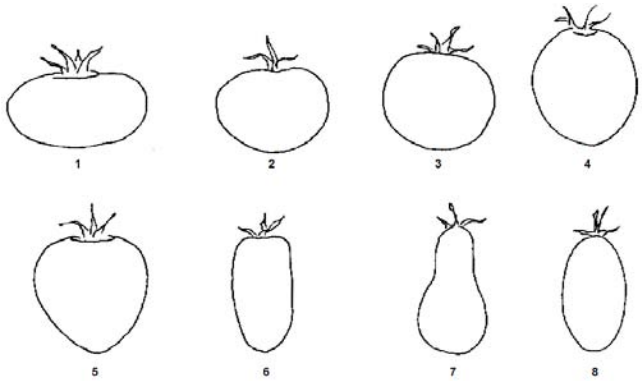

Fig. 3: Predominant fruit shapes in tomato (recorded after the fruits turn color). Figure is taken from descriptors for tomato (*Lycopersicon* spp.; International plant genetic resources institute 1996). The fruit shapes are: 1 flattened (oblate), 2 slightly flattened, 3 rounded, 4 high rounded, 5 heart-shaped, 6 cylindrical, 7 pyriform, and 8 Ellipsoid (plum-shaped).

Table 1: Used abbreviations and units

Abbreviation	Trait
4FW	Fruit weight of the first four trusses (gram)
AFL	Anther fertile length (mm)
AL	Anther length (mm)
ASL	Anther sterile length (mm)
B*Y	Brix * Yield
B*Y Index	B*Y, index *SL* = 1
BR	Number of branches until the first inflorescence
BRX	Brix
FC	Fruit color
FD	Fruit diameter (mm)
FH	Fruit height (mm)
FL	Opening of the third flower on the second truss (days)
FS	Fruit shape index
FST	Functional sterility
FSU	Fruit shape according to UPOV
FW	Fruit weight (gram)
GS	Green shoulder
IFC	Internal fruit color
OV	First visual swelling of an ovule (days)
PCP	Pericarp thickness (mm)
PE	Peduncle length (mm)
pH	pH of the apoplastic fluids
PL	Increase in plant length (cm/day)
PTL	Petal length (mm)
SD	Stem diameter (mm)
SL	Style length (mm)
SPL	Sepal length (mm)
SS	Seed set
TA	Titratable acids (% citric acid)
TD	Length between the second and sixth fruit trusses (cm/truss)
TR	Fruit trusses until the wire
YIELD	Yield (Kg)

Results & Discussion

Phenotypes for 31 traits related to plant development and quality were determined in the *S. habrochaites* LYC4 introgression line (IL) population. Estimated mean observations for each trait are summarized in Table 2 (plant characteristics), Table 3 (flower characteristics) and Table 4 (fruit characteristics).

Correlation between traits

Table 5 shows the correlation coefficients between most of the traits measured in this study. Eight traits (4FW, B*Y index, FC, FST, FSU, IFC, GS and SS) were not included in the correlation matrix, either because traits are related (e.g. B*Y and B*Y index, and 4FW and FW), or a trait was not measured on a metric scale. Significant (P<0.05) correlations were observed between many traits.

In general, all observed correlations were expected because either the traits were related or correlations were published previously, such as, the correlations between the flower characteristics: SPL, PTL, AL, SL, ASL, and AFL (Georgiady et al. 2002), and the correlations between the fruit characteristics: FW, HD, FD, PCP (Doganlar et al. 2002). The number of branches till the first inflorescence (BR) is negatively correlated with the total number of fruit trusses till 3 meter (TR; r=-0.53), yield (YIELD; r=-0.39), and fruit weight (FW; r=-0.35), but positively correlated with the time till opening of the third flower on the second truss (FL, r=0.78). Additional branches till the first inflorescence (BR) not only negatively affected maturity type, but these plants also produced smaller fruit. This correlation was also reported previously by Banerjee and Kallo (1989) and the authors speculate that the observed association is due to pleiotropic effects.

The positive correlation of yield (YIELD) and fruit weight (FW; r=0.79) and the negative correlation of yield and brix (BRX; r=-0.52) were also known (Bernacchi et al. 1998b; Fulton et al. 1997a; Fulton et al. 2000; Stevens 1986; Stevens and Rudich 1978). The negative relationship between yield and brix represents the major factor limiting simultaneous improvement of these two traits (Grandillo et al. 1999b; Stevens and Rudich 1978).

Association of loci with introgressions of Chromosome 12

In this study the mean observation of an IL was compared to the mean of the control cultivar *SL* for each trait separately using a Dunett test (Table 2, 3 and 4). If a significant deviation from the mean of *SL* was identified in an IL, the introgression and the size of the introgression in which the locus that has an effect on a trait is known. If introgressions of different ILs are overlapping, this information can be used to further delimit the interval in which the gene must be located.

For example, there are three ILs with partly overlapping introgressions for Chromosome 12 (IL12-1, IL12-2 and IL12-3; Fig. 1 and 4). Compared to *SL*; IL12-1,

IL12-2 and IL12-3 all showed a slower plant growth (PL), additional branches till the first inflorescence (BR) and delayed flowering (FL). Therefore, loci controlling these traits are predicted to be located in the overlapping region of the introgressions of IL12-1, IL12-2 and IL12-3 (Fig. 4). However, FL in IL12-2, IL12-3 and IL12-1 was respectively 6.2, 10.6 and 15.0 days. The additional delay in FL for IL12-3 and IL12-1 suggests either the presence of an additional locus in the overlapping part of the introgression of IL12-3 and IL-12-1, an additional locus in the unique introgressions of IL12-1, interaction between loci, or the presence of an unidentified *SH* introgression on other chromosome locations.

Compared to *SL*, negative effects on fruit weight (FW), fruit height (FH), fruit diameter (FD), pericarp thickness (PCP), peduncle length (PE) and ovule development (OV) were observed in IL12-3. Therefore, loci controlling these traits should be located on the region unique for the introgression in IL12-3. Both IL12-1 and IL12-3 showed a poorer seed set than *SL* and the locus controlling this trait is located on the distinctive *SH* introgression of these two ILs.

A direct association between a phenotype and an introgression could not always be made as, for example, for internal fruit color (IFC). IFC of IL12-2 is green and the introgression of IL12-2 completely overlaps with the introgressions in the two other ILs (Fig.4; IL12-1 with orange and IL12-3 with red IFC). To explain green IFC, an interaction between the introgression and the *SL* genetic background might be required or an undetected *SH* introgression can be present in IL12-2. Further analysis is required to understand the genetics of IFC.

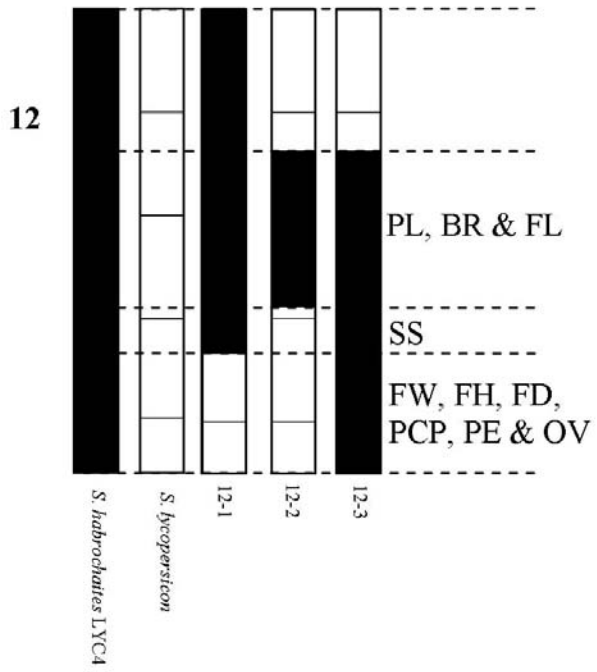

Fig. 4: Graphical genotype of the Chromosome 12 ILs. The traits affected by the presence of the homozygous *SH* introgressions are presented next to the associated segment.

Other associations between traits and ILs

Using similar reasoning, the locus responsible for the green shoulder disorder (GS) was assigned to an introgression of Chromosome 10. The *SH* allele must act dominantly as GS was observed during each backcross. Yellow fruit were only observed in IL3-2, but not during any backcross. The allele responsible for this trait must therefore be recessive.

Functional sterility (FST) was observed in IL2-2 and IL5-1. A major QTL, *se2.1* is responsible for a large portion of the phenotypic variation for this stigma exertion in *S. pennellii* (Bernacchi and Tanksley 1997; Fulton et al. 1997b). High resolution mapping of *se2.1* indicated the presence of at least five tightly linked genes: one controlling style length, three controlling stamen length, and the other affecting anther dehiscence (Chen and Tanksley 2004). The *S. pennellii* QTL *se2.1* is in a region homologous to the *SH* introgression present in IL2-2. In IL2-2, style length (SL) is increased and anther dehiscence is affected but not stamen length (AL). In agreement with the study of Chen and Tanksley (2004) the long style allele acts also dominantly in the *SH* introgression line IL2-2, because style exertion was observed during each backcross.

Maturity type can be defined by four different components and each of these components has shown to be a unique and heritable trait (reviewed by Doganlar et al. 2000). Two of these components; FL and OV were measured in the IL population. In agreement with Doganlar et al. (2000), we identify different ILs in which the mean FL (IL1-2, IL12-1, IL12-2 and IL12-3) or mean OV (IL3-2 and IL12-3) significantly deviated from the mean of *SL*. However, presence of these loci never resulted in an earlier maturity. FL is correlated with number of branches till the first inflorescence (BR). Loci affecting the number of branches until the first inflorescence (BR) are located on Chromosome 2, 4, 5, 7, 8, 9 and 12 (Table 2). BR is influenced by the environment in which the plant is growing (Dieleman and Heuvelink 1992). Because the environment in our experiment is constant, we show that there is also genetic variation present for this trait in the IL population. Unfortunately, all loci increased the number of branches till the first inflorescence and therefore a later maturity.

Fruit weight (FW) is influenced by several loci on different chromosomes. Introgressions on Chromosome 1, 2, 3, 4, 8, 10, and 12 significantly reduced FW (Table 4). This result is in line with studies reviewed by Grandillo et al. (1999a) where he describes that the major QTLs for FW were identified on Chromosomes 1, 2, 3, 4, 8 and 9. Fine mapping of FW QTLs in the *S. pennellii* LA716 IL population suggests that there are at least three FW QTLs on Chromosome 2 (Eshed and Zamir 1995). Further analysis, using ILs with a smaller introgression, is needed to identify these FW QTLs in the *S. habrochaites* IL population. IL5-1 showed the largest increase in YIELD and B*Y. However, the introgressed region is to a large extent heterozygous and the observed YIELD and B*Y may have been caused by hybrid vigor. ILs homozygous for the *SH* allele need to be developed in order to test whether the

increase in YIELD is also present in the homozygous IL. FW and BRX are in general negatively correlated (Stevens and Rudich 1978), however, IL1-4 showed a significantly increased BRX but a FW similar to *SL* (Tables 2 and 4). Analysis of *S. pennellii* ILs, containing an introgression in the corresponding region, resulted in similar observations (Baxter et al. 2005; Eshed and Zamir 1995). This locus is likely useful for improvement of Brix in tomatoes.

Not every IL, for example IL9-1, showed a significant deviation from *SL* for the traits measured in this study. On the other hand, the introgression in IL9-2 resulted in a significant deviation for the majority of the traits measured in this study. Both ILs, however, do contain a locus conferring resistance to *B. cinerea* (Chapter 3). The difference in number of associations between IL9-1 and IL9-2 is biased because of the correlations between traits (Table 5).

Conclusion

The current study has shown some of the potential of the *S. habrochaites* LYC4 IL population: a resourceful tool which easily allows fine mapping of loci, identification of loci and screening for any trait of interest.

Acknowledgements

The authors would like to thank Dr. ir. Ep Heuvelink, of the Horticultural Production Chains Group, Wageningen University and Dr.ir. Roeland Voorrips, Plant Research International, for their valuable suggestions.

Table 2: Plant characteristics and total yield [a]

IL	PL		BR		SD		TR	TD	4FW[b]	YIELD[c]	B*Y[c]	B*Y index[c]
1-1	2.35	*	8.7		12.5		9.0	19.1	1000	9.0	39.6	0.8
1-2	1.85	**	9		13.4		ND	ND	344	NA	NA	NA
1-3/3-3	1.45	**	8.9		9.4	**	NA	11.0	143	NA	NA	NA
1-4	2.35	*	9		15.5		12.0	18.1	901	10.8	60.5	1.2
2-1	2.85		8.3		13.3		9.5	25.6	1780	16.9	74.4	1.5
2-2	3.35		10.2	**	16.0		11.0	18.3	554	6.1	33.5	0.7
2-3	3.65	**	9.9	*	18.9	**	6.0	26.8	1996	12.0	71.9	1.5
3-1	2.55		8.9		12.3		9.0	25.1	1001	9.0	37.8	0.8
3-2	2.85		7.6		12.2		14.0	16.8	586	8.2	40.2	0.8
4-1	2.05	**	9.5	*	15.1		11.0	18.9	965	10.6	43.5	0.9
4-2	2.15	**	8.7		13.6		ND	15.1	885	NA	NA	NA
4-3	2.75		8.9		13.9		8.0	32.5	366	2.9	19.0	0.4
5-1	2.55		9.1		15.5		11.5	15.0	2085	23.9	110.3	2.2
5-2	2.75		10.4	**	16.5		10.5	19.0	1203	12.6	60.6	1.2
6-1	2.55		7.8		13.1		9.5	20.8	1383	13.1	63.1	1.3
6-2/7-2	2.55		8		14.3		12.0	23.4	852	10.2	51.1	1.0
6-3	2.75		8.8		15.2		11.0	19.0	1362	14.9	58.4	1.2
7-1	2.65		9.7	*	14.2		9.5	22.1	636	6.0	31.4	0.6
8-2	2.15	**	11.2	**	15.2		9.0	20.7	312	2.8	14.0	0.3
9-1	2.45		9		14.2		9.5	23.0	957	9.1	45.5	0.9
9-2	2.55		10.7	**	17.4	*	8.0	30.0	580	4.6	27.4	0.6
10-1	2.25	**	8.5		14.6		13.0	17.8	381	5.0	30.7	0.6
10-2	3.55	**	8.8		14.8		8.5	24.1	1281	10.9	59.9	1.2
10-3	2.55		8		15.2		8.0	30.8	1444	11.6	45.1	0.9
10-4	2.45		8.8		14.4		9.0	20.3	1935	17.4	85.3	1.7
11-1/9-3	2.85		9		15.0		9.5	21.3	675	6.4	34.6	0.7
11-2	2.95		7.5		17.0		10.0	20.3	667	6.7	34.7	0.7
12-1	2.35	*	13	**	12.5		6.5	30.1	868	5.6	29.3	0.6
12-2	1.95	**	11.5	**	12.5		8.0	26.5	1148	9.1	37.7	0.8
12-3	2.45		11.9	**	14.4		6.0	32.3	455	2.7	14.7	0.3
BRC-5	4.15	**	10.2	**	15.1		7.0	33.5	581	4.1	23.6	0.5
SL	2.85		8.0		14.2		8.5	22.3	1352	11.5	49.4	1.0

[a] significant deviations from *SL*, as determined using a Dunnett test, are presented next to each mean. Probability of $P<0.05$ (*) or $P<0.01$ (**) is indicated. Abbreviations are summarized in Table 1

[b] The Dunnett test is only performed for fruit weight (FW, Table 4)

[c] The Dunnett test was not performed for derived traits

Table 3: Flower characteristics [a]

IL	SPL		PTL		AL		SL	ASL	AFL		FST	FL		OV	
1-1	13.6		7.83		6.54		3.26	1.78	4.76		-	51.9		5.5	
1-2	15.8		8.97		7.99		3.85	2.16	5.83		-	59.1	**	ND	
1-3/3-3	7.7	**	6.99		6.11		4.81	1.69	4.42		-	52.2		8.8	
1-4	12.7		7.59		5.49		3.43	1.56	3.93		-	49.7		4.7	
2-1	12.8		7.18		5.67		4.19	1.49	4.18		-	51.3		4.7	
2-2	18.3	**	8.27		5.49		6.42	1.28	4.21		+	53.8		7.8	
2-3	ND		ND		ND		ND	ND	ND		ND	50.8		6.1	
3-1	14.9		7.83		6.06		4.95	1.44	4.63		-	52.4		5.7	
3-2	14.8		7.68		5.93		4.31	1.53	4.40		-	51.5		16.8	**
4-1	14.3		7.81		6.71		4.51	1.79	4.92		-	52.2		4.0	
4-2	13.1		7.67		5.74		4.39	1.68	4.07		-	51.2		3.8	
4-3	14.9		9.02		6.53		3.32	1.99	4.54		-	56.5		6.9	
5-1	15.8		7.48		5.61		6.20	1.62	3.99		+	51.7		7.7	
5-2	13.3		6.11		5.11		3.87	1.40	3.71		-	53.4		5.5	
6-1	8.9	*	6.04		4.62		2.91	1.40	3.22		-	49.6		6.3	
6-2/7-2	12.0		8.50		6.90		6.58	1.90	5.00		-	51.6		9.3	
6-3	13.5		7.20		6.08		4.13	1.54	4.54		-	49.9		4.7	
7-1	10.5		6.81		5.03		4.49	2.04	3.39		-	52.9		4.2	
8-2	9.0	*	7.19		6.10		5.66	1.24	4.45		-	53.6		4.5	
9-1	12.4		6.89		6.03		4.28	1.52	4.51		-	51.4		5.2	
9-2	20.2	**	10.96	**	8.24	*	7.73	1.85	6.40	*	-	52.9		6.0	
10-1	12.2		7.11		5.31		3.74	1.55	3.77		-	50.7		10.5	
10-2	11.2		7.08		6.18		3.90	1.72	4.46		-	50.9		4.3	
10-3	10.0		6.87		6.04		3.93	1.69	4.35		-	49.7		4.8	
10-4	9.5		6.84		6.00		3.91	1.69	4.31		-	49.7		4.2	
11-1/9-3	11.8		7.72		6,31		4.97	1.67	4.64		-	51.7		6.5	
11-2	12.2		7.97		6.78		5.76	1.88	4.90		-	49.1		6.3	
12-1	12.0		7.09		5.58		4.30	1.38	4.21		-	65.9	**	7.7	
12-2	10.2		7.41		6.20		4.79	1.72	4.48		-	57.1	**	6.7	
12-3	10.9		5.97		4.12		3.17	1.21	2.91		-	61.5	**	16.4	**
BRC-5	ND		ND		ND		ND	ND	ND		ND	55.2		11.8	
SL	12.9		7.42		6.08		5.14	1.66	4.14		-	50.9		5.0	

[a] significant deviations from *SL*, as determined using a Dunnett test, are presented next to each mean. Probability of $P<0.05$ (*) or $P<0.01$ (**) is indicated. Abbreviations are summarized in Table 1

Table 4: Fruit Characteristics [a]

IL	*FW*	*FH*	*FD*	*PCP*	*FS*	*FSU*	*PE*	*pH*	*TA*	*BRX*	*GS*	*FC*	*IFC*	SS
1-1	36.8	42.27	58.60 *	5.69	0.92	2	12.73	3.81	0.56	4.4	-	red	red	+
1-2	24.3	35.11	32.03	4.36	1.07	2	8.41	3.91	0.61	3.4	-	orange	ND	-
1-3 / 3-3	19.3 **	30.47 **	32.95 **	4.47 **	0.91	3	7.83 **	ND	ND	ND	-	orange	green	-
1-4	67.2	41.92	48.97	6.07	0.86	2	11.27	3.90	0.90	5.6 *	-	red	red	+
2-1	70.4	41.08	51.33	5.75	0.79 *	2	16.94 **	3.91	0.63	4.4	-	red	orange	+
2-2	28.3 **	34.85 *	36.48 **	5.03 *	0.92	6	8.56 *	3.99	0.76	5.5	-	red	red	-
2-3	41.0 **	38.52	42.05	5.60	0.87	5	9.56	3.87	0.95	6.0 *	-	red	red	+
3-1	76.0	42.41	53.65	6.32	0.75 **	2	11.36	3.83	0.67	4.2	-	red	orange	+
3-2	31.8 **	33.30 **	39.47 *	5.32	0.83	2	8.93	3.63	0.58	4.9	-	yellow	yellow	-
4-1	49.8	36.89	46.60	5.19	0.81	1	9.6	3.95	0.48	4.1	-	red	orange	+
4-2	62.4	41.92	49.06	6.57	0.86	2	10.8	3.83	0.54	4.4	-	red	orange	+
4-3	29.2 *	33.49 **	37.48 **	5.05	0.86	3	9.44	3.90	0.83	6.5 **	-	red	red	-
5-1	76.3	44.29	51.91	6.04	0.84	2	8.8	3.74	0.62	4.6	-	red	red	+
5-2	65.3	41.13	49.31	6.32	0.82	2	10.73	3.72	0.59	4.8	-	red	red	+
6-1	49.2	41.20	43.36	5.65	0.87	2	11.04	3.80	0.55	4.8	-	red	green	+
6-2 / 7-2	45.9	38.30	42.23	5.56	0.88	3	8.66	4.14	0.54	5.0	-	red	orange	+
6-3	77.9	46.15	50.91	5.99	0.94	3	13.95 *	3.76	0.72	3.9	-	orange	orange	+
7-1	41.8	37.07	41.80	6.04	0.88	2	9.15	3.92	0.59	5.2	-	orange	green	+
8-2	8.2 **	21.85 **	22.71 **	2.42 **	0.83	3	5.67 **	3.81	0.55	5.0	-	orange	red	+
9-1	60.8	41.31	47.24	6.57	0.87	2	11.41	3.80	0.76	5.0	-	red	red	+
9-2	28.6 **	32.42	35.76 *	4.29 *	0.89	2	14.51 **	3.71	1.04	5.9 **	-	orange	orange	-
10-1	29.5 *	33.50	35.86 *	4.06 **	0.91	3	11.71	3.93	0.98	6.2 **	+	red	red	-
10-2	63.2	43.29	48.29	5.78	0.94	3	6.93 **	4.03	0.59	5.5	+	red	green	+
10-3	81.1	49.69	53.26	6.44	0.87	2	9.85	4.02	0.52	3.9	-	red	green	+
10-4	83.7	48.34	54.59	7.52	0.86	2	11.71	3.89	0.63	4.9	-	red	orange	+
11-1 / 9-3	46.0	41.95	42.44	5.88	0.90	3	12.4	3.76	0.70	5.4	-	red	orange	±
11-2	36.4	34.64	39.90	5.29	0.85	2	9.22	3.93	0.47	5.2	-	red	orange	±
12-1	47.6	38.11	44.65	6.43	0.84	2	9.32	3.87	0.57	5.2	-	red	orange	-
12-2	40.3	37.36	42.52	5.90	0.86	2	9.07	3.81	0.64	4.1	-	red	green	+
12-3	10.8 **	21.39 **	26.95 **	3.58 **	0.82	2	6.67 **	4.07	0.93	5.4	-	red	red	-
Brc-5	33.8 **	36.28	39.73 *	5.23	0.86	3	9.82	4.21	0.83	5.8	-	orange	green	-
SL	58.3	42.27	47.42	6.18	0.88	2	10.70	3.84	0.66	4.3	-	red	red	+

[a] significant deviations from *SL*, as determined using a Dunnett test, are presented next to each mean. Probability of $P<0.05$ (*) or $P<0.01$ (**) is indicated. Abbreviations are summarized in Table 1

Table 5: Correlation between traits [a]

[a] Only significant correlations (P<0.05) are presented. Correlations with a P<0.01 are presented in gray. Abbreviations are summarized in Table 1

CHAPTER 6
General discussion

The research described in this thesis focuses on the identification of quantitative trait loci conferring resistance to *Botrytis cinerea* from two partial resistant wild *Solanum* accessions: *S. habrochaites* LYC4 and *S. neorickii* G1.1601. Aspects of the research described in Chapters 2, 3, 4 and 5 will be discussed. Furthermore, ideas will be presented on how the developed introgression lines might contribute to increase our knowledge on the interaction between *B. cinerea* and tomato.

Introduction

Over half of the 42 pathogen resistance traits identified in tomato's wild relatives have been introduced into cultivated tomato (Peralta and Spooner 2001; Rick and Chetelat 1995). At the onset of this research, resistance to the necrotroph *Botrytis cinerea* was not among the introduced resistances. Partial resistance to *B. cinerea* was identified in wild *Solanum* accessions (Nicot et al. 2002; Rick 1987; Rick and Chetelat 1995; ten Have et al. 2007; Urbasch 1986). The research described in this thesis focused on identification of quantitative trait loci (QTL) conferring resistance to *B. cinerea* from two partial resistant wild *Solanum* accessions: *S. habrochaites* LYC4 and *S. neorickii* G1.1601 (Chapters 2, 3 and 4).

Power of QTL detection

Partial resistance to *B. cinerea* is controlled by quantitative trait loci (QTL, Chapters 2, 3 and 4). In Chapter 1, we proposed that an introgression line population is the most powerful population to detect QTLs. As a practical example, we identified QTLs conferring resistance to *B. cinerea* from *S. habrochaites* LYC4 using both an F_2 mapping approach and an introgression line (IL) approach (Chapter 2 and 3). Results from both F_2 and IL study will be discussed in relation to the criteria used in the general introduction to rank the power of different population types (Chapter 1).

Initially, two QTLs conferring resistance to *B. cinerea* were detected in the *S. habrochaites* LYC4 F_2 population (n=174; *Rbcq*1 and *Rbcq*2; Chapter 2). The level of resistance to *B. cinerea* was assessed using a stem segment assay (ten Have et al. 2007). During the confirmation of these QTLs in segregating BC_2S_1 families, a third QTL (*Rbcq*4a) was discovered from which the effect could only be detected in absence of *Rbcq*2. Using orthogonal contrast analysis, *Rbcq*4a could be detected in data of the F_2 population (Chapter 2). The main reasons that this locus was initially overlooked was because of its less-than-additive epistatic interaction with *Rbcq*2 (Chapter 2).

Analysis of an F_2 population might not identify all genes involved in resistance. Therefore, we developed the *S. habrochaites* LYC4 introgression line population (n=30). The level of resistance to *B. cinerea* of each of the ILs was assessed in a greenhouse trial. All three QTLs detected in the F_2 population (Chapter 2) could be confirmed in the IL population (Chapter 3) and seven additional loci were identified in

the IL population. None of these seven new QTLs could be detected in the F_2 population, even when re-examining the original data (data not shown). In essence, the power of IL populations in identifying additional loci was reviewed by Zamir (2001). The genome of each IL typically contains more than 95% of the genome of the recurrent parent *Solanum lycopersicum* cv. Moneymaker (*SL*). QTLs can easily be identified by comparison of the mean phenotype of an IL with the mean phenotype of *SL*. *Rbcq*2 and *Rbcq*4a were among the loci showing the largest decrease in infection in the ILs, suggesting that only QTLs with large quantitative effects conferring resistance to *B. cinerea* can be identified in an F_2 population.

The F_2 study already showed that confirmation of a *B. cinerea* resistance phenotype requires multiple testing of a line (Chapter 2). Each IL is genetically uniform and allows testing of multiple plants per IL, thereby reducing the standard error of the mean and allowing identification of QTLs with a minor effect. Negative epistatic interactions with other regions of the wild genome or less-than-epistatic interactions may obscure identification of QTLs as was shown with the less-than-additive epistatic interaction between *Rbcq*2 and *Rbcq*4a. This interaction was only detected after the identification of *Rbcq*4a in a BC_2S_1 progeny (Chapter 2). Skewness can obscure a locus segregating in a F_2 population. For example, segregation of the region encompassing *Rbcq*9a and *Rbcq*9b was skewed in the F_2 population. *Rbcq*9a and *Rbcq*9b, however, could be readily detected in the IL population.

In addition to the reasons reviewed by Zamir (2001), functionally redundant QTLs are difficult to detect in segregating F_2 populations, but can easily be detected in an IL population. The regulation of two ethylene receptors, *NR* and *LeETR4*, provides such an example of functionally redundant genes (Tieman et al. 2000). Transgenic plants with reduced *NR* mRNA levels exhibit normal ethylene sensitivity and elevated levels of *LeETR4* mRNA. Over-expression of *NR* in lines with lowered *LeETR4* gene expression eliminates the ethylene-sensitive phenotype. Functionally redundancy might also be regarded as an epistatic interaction. Among the seven newly identified *B. cinerea* resistance loci, some loci might be functionally redundant.

Power of QTL detection – A breeders point of view

The introgression of a minimum number of QTLs, yet obtaining the largest increase in resistance is important from a breeder's point of view. Among the additional advantages of an IL population is that directed crosses of individual *B. cinerea* resistance QTL-containing ILs can be made. Using marker assisted selection (MAS), skewness can be eliminated in the F_2 progeny of such a cross by selecting an equal number of individuals for each of the nine possible genotypic classes. A balanced population is much more powerful to determine the combined effect of the QTLs than in an unbalanced population (Chapter 1). Interactions between QTLs can readily be identified and dominance of each QTL can also be determined. This is important since dominance of a QTL is advantageous for development of F_1 hybrid cultivars.

BRC-5 is the most resistant line in our tests with a disease incidence of only 15% (Chapter 3). We expect that the resistance of this line is caused by two or perhaps three interacting loci (Chapter 3). The inoculation pressure in our experiments was higher than in a greenhouse. Therefore, we anticipate that introgressing two to three QTLs might be enough to reach an absolute level of *B. cinerea* resistance in a commercial tomato production greenhouse.

Confirmation of QTLs identified in the IL population

One aspect of QTL analysis is that their detection depends on the environment (Chapter 1). In our study, susceptibility to *B. cinerea* in the IL population was only assessed once (Chapter 3). Since the IL population is homozygous, resistance to *B. cinerea* could be assessed in an independent experiment (Chapter 1; Zamir 2001). Analogous to the greenhouse disease assay described in Chapter 3, an independent *B. cinerea* disease assay was performed on the *S. habrochaites* LYC4 IL population (De Ruiter Seeds, Bergschenhoek). Nine of the ten QTLs could be confirmed in this disease assay (data not shown) demonstrating the robustness of each QTL but also confirming the advantage of detection of QTLs in an IL population.

Quantitative *Botrytis cinerea* disease assay

Both a quantitative stem segment assay (ten Have et al. 2007) and a quantitative greenhouse assay on mature plants (Chapter 3) have proven to be valuable tools to assess susceptibility to *B. cinerea* (Chapter 2, 3 and 4). Determination of susceptibility to *B. cinerea* in transient populations was performed using the stem segment assay (ten Have et al. 2007) while for the IL population the greenhouse assay was used (Chapter 3). Both assays can be divided into two steps: plants were grown in the greenhouse and followed by assessing susceptibility to *B. cinerea* under controlled lab conditions on cut stem pieces (stem assay) or on growing plants (greenhouse assay). In the next section we will discuss the quantitative variation observed within the stem segment bioassays performed under laboratory conditions, as well as environmental factors that may quantitatively influence *B. cinerea* infections while the plants are growing in the greenhouse.

While assessing susceptibility using the stem assay, 22 genotypes could be tested in one box (Fig. 1). Eight to ten boxes were usually needed for each experiment. Analysis of each experiment showed variation between boxes within one experiment for disease incidence (Fig. 2a or 2b) and lesion growth (Fig. 2c or 2d). Susceptibility was assessed in two independent disease assays showing the variation between experiments for disease incidence (Fig. 2a and 2b) and lesion growth (Fig. 2c and 2d). Within one experiment, the time between cutting and inoculating the stem pieces differs up to six hours. However, no relationship between time of cutting and disease incidence (Fig. 2a and 2b) or lesion growth (Fig. 2c and 2d) was observed. These results are in agreement with the results of O'Neill et al.

(1997) in which disease incidence was similar in stem pieces inoculated 0 and 9 hours after cutting. Relative humidity or phytohormone concentration inside a box might lead to differences in disease incidence between boxes (Diaz et al. 2002; Holz et al. 2004; Sharon et al. 2004). Condensation of water could be observed on the lid of each box, therefore the relative humidity was presumably high enough for outgrowth of *B. cinerea* infections. Ten Have et al. (2007) have shown that ethylene biosynthetic genes are induced by *B. cinerea* infection in all wild *Solanum* accessions tested in this study. The gaseous phytohormone ethylene plays a role in induced resistance and can induce certain types of pathogenesis-related proteins or phytoalexins (van Loon et al. 2006). Through stimulation of the phenylpropanoid pathway, it can reinforce cell walls in various plant species (van Loon et al. 2006). It is, however, unclear whether in the experimental setup for the inoculations ethylene could accumulate to a concentration high enough to affect resistance in the stem pieces in a quantitative manner.

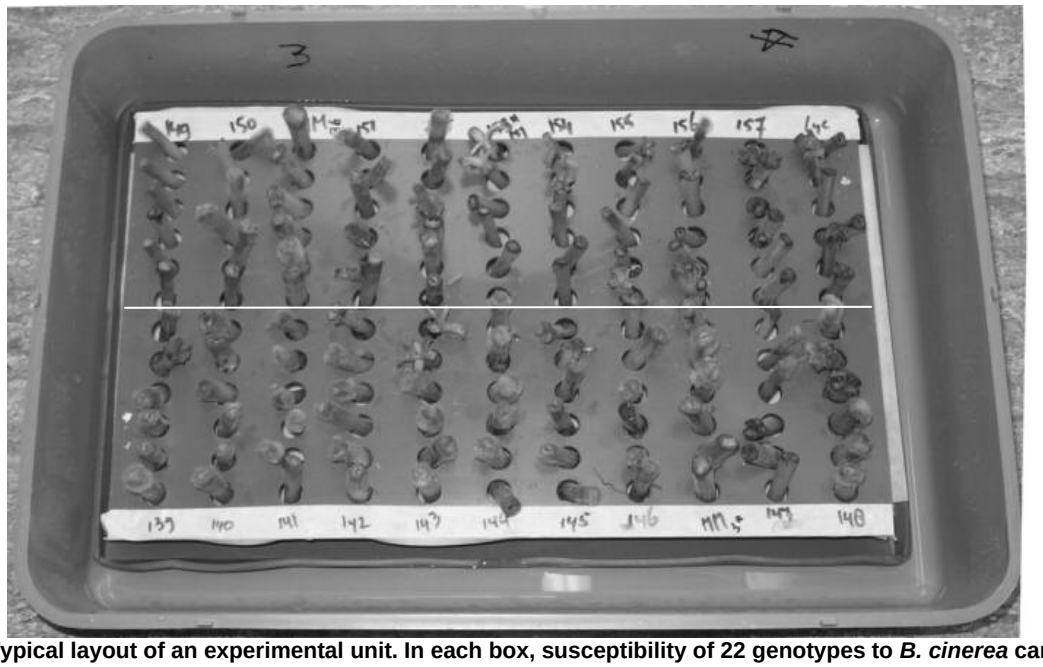

Fig. 1: Typical layout of an experimental unit. In each box, susceptibility of 22 genotypes to *B. cinerea* can be assessed. Each genotype is represented by six stem pieces (ten Have et al. 2007).

Variation was not only observed within one experiment, but also between experiments (Fig 2a and 2b). In several cases, data from a complete experiment were discarded because the mean disease incidence in the experiment was below 20% (Chapter 2, Chapter 4). The quality of inoculum was verified in all experiments by determining the germination efficiency of conidia. It was established that low disease incidence was not caused by poor germination. This suggests that as yet

unidentified physiological differences between sets of plants, possibly related to their cultivation history, plays a role in the success of an experiment.

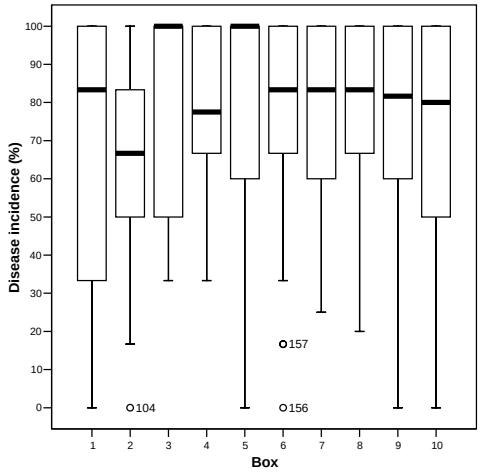

Fig 2a

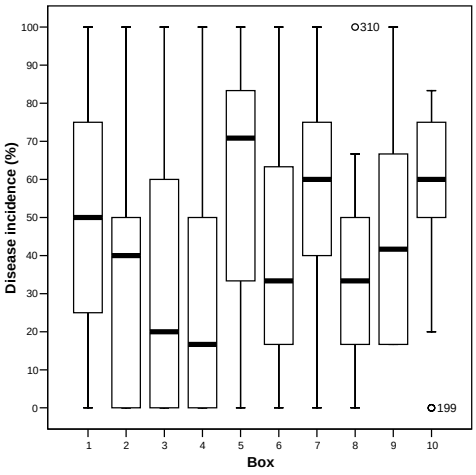

Fig 2b

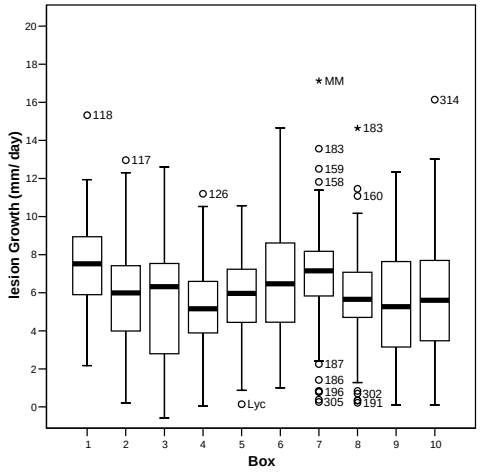

Fig 2c

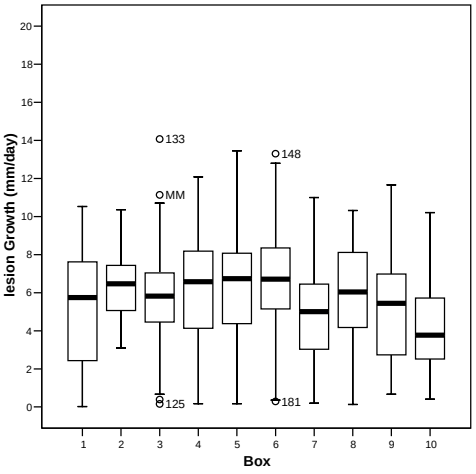

Fig 2d

Fig. 2: Box-plots for disease incidence (Fig. 2a and 2b) and lesion growth (Fig. 2c and 2d) of two independent disease assays. Both assays were performed on BC_2S_1 plants to confirm the *S. habrochaites* QTLs: *Rbcq*1, *Rbcq*2, and *Rbcq*4a (Chapter 2). The median (presented as a line within each box), 50% and 95% quartiles and extremes (dots) are presented for each box. Box 1 contains plants cut in the morning while box 10 contains plants cut in the afternoon. The box number can therefore be considered as a time scale.

For both the stem assay and the greenhouse assay, the environment plays a role in the development of disease caused by *B. cinerea*. Factors which influence *B. cinerea* infections in the greenhouse include greenhouse climate, light, CO_2 levels, cultivar, plant spacing, fertilizer, and irrigation regime (Dik et al. 1999; Dik and Wubben 2004; Islam et al. 1998a; O'Neill et al. 1997; Yermiyahu et al. 2006). Elad and Steward (2004) reviewed strategies for biocontrol of *Botrytis*-incited diseases with (dead cells of) filamentous fungi, bacteria and yeasts which might promote

induced systemic resistance (ISR). Islam et al. (1998b) showed that pre-treatment of *Vicia faba* leaflets with red light (600-700) reduced the area of necrosis significantly as compared to leaflets kept under near-UV light (320-400 nm), blue light (400-520 nm) or without light. The composition of the nutrient solution can also affect the symptoms observed on the plant after infection with *B. cinerea* (Dik and Wubben 2004; Yermiyahu et al. 2006). These studies show that various factors influence *B. cinerea* disease assays. Therefore, controlled growth is preferred to exclude undesirable variability in the environment, but the scale of our experiments prevents complete control of all parameters.

Due to variation in the disease assays, as described in previous paragraphs, replications of experiments are necessary. While analyzing the data of the *S. habrochaites* LYC4 F_2 population, the three QTLs could only be identified after combining the data of six replications, while none was detected in the independent experiments (Chapter 2). Each of these three loci could be confirmed using independent disease assays on BC_2S_1 plants (Chapter 2) and in the *S. habrochaites* LYC4 IL population (Chapter 3), showing that replicate analysis of a genotype always provided consistent conclusions, even when variation occurred between assays.

Cost of *B. cinerea* resistance

Resistance of a plant is determined by many physiological factors besides the possession of resistance genes (Albar et al. 1998; Bergelson and Purrington 1996; Brown and Simmonds 2006; Coaker et al. 2002). The *S. habrochaites* LYC4 IL population was evaluated for *B. cinerea* resistance (Chapter 3) and for agronomic and morphological traits (Chapter 5). These data (summarized in Table 1) were used to investigate the correlation between resistance to *B. cinerea* and either plant growth rate (PL), stem diameter (SD) or yield and resistance. This analysis indicates that yield was significantly correlated both with disease incidence (DI; Pearson: r=0.46; $P<0.05$), and with lesion size (LS; Pearson: r=0.52; $P<0.01$).

Overall, resistance to *B. cinerea* was correlated with a lower yield of the ILs, which might be regarded as a cost of resistance, as described previously for other pathosystems (Bergelson and Purrington 1996; Brown 2002; Brown 2003; Tanksley et al. 1998a). For example, a yield penalty of 16% has been reported for tobacco mosaic virus (*TM-2*^{a}) resistance in tomato (Tanksley et al. 1998a) and a 5.4% lower grain weight has been reported for powdery mildew (*mlo*) resistance in barley (Kjaer et al. 1990). Bergelson et al. (1996) postulated that several cases of cost of resistance appeared to be due to linkage drag rather than to pleiotropic effects. This could be also the case in tomato since many loci affecting fruit weight have been identified in crosses with wild tomato (Grandillo et al. 1999a). Brown (2002; 2003), however, proposed that the cost of *mlo* resistance is pleiotropic. It remains to be determined whether the correlation between *B. cinerea* resistance and yield in the *S. habrochaites* LYC4 IL population is due to linkage drag or to pleiotropic effects.

Table 1: Mean phenotypic observations for *B. cinerea* resistance (disease incidence, DI; Lesion size, LS and lesion growth, LG) and plant growth (plant growth PL and stem diameter, SD) and yield (Brix * Yield index) related traits.[a]

IL	DI (%)[b]		LS (mm)		LG (mm/day)		PL (cm/day)[c]		SD (mm)		Yield (Kg)[d]
1-1	65		56		5.9		2.35	*	12.5		9.0
1-2	47		33		3.4		1.85	**	13.4		NA
1-3/3-3	45	**	30	**	1.7	**	1.45	**	9.4	**	NA
1-4	37	**	34		2.4	*	2.35	*	15.5		10.8
2-1	41	**	30	*	3.0		2.85		13.3		16.9
2-2	37	**	26	*	2.8		3.35		16.0		6.1
2-3	58		44		3.5		3.65	**	18.9	**	12.0
3-1	47		41		2.8	*	2.55		12.3		9.0
3-2	46	*	35		4.2		2.85		12.2		8.2
4-1	41	**	26	**	2.5	*	2.05	**	15.1		10.6
4-2	45	*	33		3.8		2.15	**	13.6		NA
4-3	51		29		2.8		2.75		13.9		2.9
5-1	61		53		4.8		2.55		15.5		23.9
5-2	69		64		5.4		2.75		16.5		12.6
6-1	49	*	44		3.6		2.55		13.1		13.1
6-2/7-2	55		39		3.7		2.55		14.3		10.2
6-3	79		49		4.6		2.75		15.2		14.9
7-1	50		35		3.1		2.65		14.2		6.0
8-2	59		43		3.9		2.15	**	15.2		2.8
9-1	69		34	*	3.0	*	2.45		14.2		9.1
9-2	49	*	33		3.1		2.55		17.4	*	4.6
10-1	60		47		4.3		2.25	**	14.6		5.0
10-2	62		49		4.4		3.55	**	14.8		10.9
10-3	70		53		4.7		2.55		15.2		11.6
10-4	76		47		4.8		2.45		14.4		17.4
11-1/9-3	48	*	36		4.3		2.85		15.0		6.4
11-2	34	**	33	*	3.2		2.95		17.0		6.7
12-1	51		35		4.7		2.35	*	12.5		5.6
12-2	52		37		4.0		1.95	**	12.5		9.1
12-3	24	**	21	**	2.3		2.45		14.4		2.7
BRC-5	15	**	20	*	ND		4.15	**	15.1		4.1
SL	73		46		4.6		2.85		14.2		11.5

[a] significant deviations from *SL*, were tested using a Dunnett test, and are presented next to each mean. Probabilities of $P<0.05$ (*) or $P<0.01$ (**) are indicated

[b] Data originally presented in Table 1 in Chapter 3

[c] Data originally presented in Table 2 in Chapter 5

[d] Yield is a estimated from FW of the first 4 clusters multiplied with the number of cluster until 3 meters. Deviation from *SL* was not tested

QTLs from *S. habrochaites* and *S. neorickii*

In our research, we identified QTLs conferring resistance to *B. cinerea* in *S. habrochaites* LYC4 and *S. neorickii* G1.1601. *S. habrochaites* is native in various forest types, from pre-montane to dry forests on the western slopes of the Andes from Central Ecuador to Central Peru. *S. neorickii* is found from South Peru (Department of Apurimac) to South Ecuador (Department of Azuay) in dry inter-

Andean valleys at elevations between 1950-2600 m and is often found trailing over rocky banks and roadsides (http://www.sgn.cornell.edu/about/solanum_nomenclature.pl). *S. habrochaites* LYC4 is more resistant to *B. cinerea* than *S. neorickii* G1.1601 (ten Have et al. 2007; Chapter 4). This might be related to the more humid in which *S. habrochaites* is growing; such conditions are advantageous for *B. cinerea* infections (Holz et al. 2004). *S. habrochaites* LYC4 might therefore have additional or more effective QTLs.

QTLs conferring resistance to *B. cinerea* were identified at homologous positions in *S. habrochaites* LYC4 and *S. neorickii* G1.1601 (Chapter 4). These loci might have evolved from a common ancestor and may be involved in general defense responses (Chapter 4). If costs are indeed associated with *B. cinerea* resistance, it is likely that only species which grow in a habitat with increased disease pressure have adapted and carry additional resistance loci. To test the hypothesis that *S. neorickii* G1.1601 carries fewer *B. cinerea* resistance loci than *S. habrochaites* LYC4, it would be required to develop a *S. neorickii* G1.1601 IL population.

The relation between stem, leaf, flowers and fruit resistance

Besides stems, *B. cinerea* is able to infect leaves, flowers and fruit of tomato. In several studies, both stem and leaf resistance in a set of wild tomato accessions was reported (Egashira et al. 2000; Nicot et al. 2002; ten Have et al. 2007), but there was a poor correlation between the resistance level of stems and leaves on most accessions. This suggests that different loci may be involved in conferring resistance to *B. cinerea* in stem segments or leaves. Only a few accessions, such as *S. habrochaites* LYC4, showed quantitative resistance in both stem and leaves (ten Have et al. 2007; Urbasch 1986). Using the IL population, it can be determined whether the same or distinctive loci from *S. habrochaites* LYC4 confer stem and leaf resistance to *B. cinerea*. Additionally, flower and fruit resistance can be tested in the *S. habrochaites* LYC4 IL population.

Conclusion

In spite of the many years of breeding for disease resistant tomato cultivars, *B. cinerea* was not among the introgressed resistances (Chapter 1). The work described in this thesis shows the potential of developing of *B. cinerea*-resistant tomato cultivars. Major QTLs conferring resistance to *B. cinerea* were detected in transient mapping populations (Chapter 2 and 4). Despite the longer development time of an introgression line population, we showed that an IL population was necessary to unravel the complex genetics of resistance to *B. cinerea* (Chapter 3 and 6). The availability of this IL population, in addition, offered an opportunity to screen for other traits, such as agronomical and morphological characteristics (Chapter 5). The availability of ILs with QTLs conferring resistance to *B. cinerea* will assist

breeders in further fine mapping of each QTL (Chapter 3) and determine whether the possible cost of resistance is caused by linkage drag or pleiotropic effects (Chapter 6). Crosses between two ILs carrying separate QTLs will be useful to determined which loci should be combined to obtain the maximal level of resistance while introgressing the least number of QTLs into elite breeding material (Chapter 3 and 6).

Future research

The mechanisms underlying the increased resistance conferred by the identified QTLs remain to be resolved. *B. cinerea* relies primarily on three principles for subduing its host plants: the ability to kill host cells, the ability to decompose plant tissue and the ability to counteract plant defense responses (reviewed by van Kan 2006). The *S. habrochaites* LYC4 IL population is an ideal tool to further investigate the mechanisms underlying *B. cinerea* resistance.

Differences in the level of resistance might be due to cellular defense responses that can be visualized using histological techniques. The formation of papillae, the presence of lysosome-like vesicles and intracellular accumulation of H_2O_2 and nitric oxide upon infection with *B. cinerea* can be visualized (van Baarlen et al. 2007). The defense response of each IL can be studied using such histological techniques.

Some of the *S. habrochaites* LYC4 QTLs identified might be effective only against certain *B. cinerea* isolates but fail to be effective against other isolates, as was reported in Arabidopsis (Denby et al. 2004). The IL population can readily be used to determine whether the resistance QTLs in tomato act in an isolate-specific manner.

Nowadays, a diverse set of tools is available allowing identification of changes in metabolites (e.g. phytoalexins and phytoanticipins), proteins (e.g. cell wall degrading enzymes or enzymes degrading plant metabolites with an antifungal effect) or gene expression profiles. The use of these innovative techniques should provide new leads to further understanding of the mechanism involved in resistance to *B. cinerea* and to develop innovative disease control strategies.

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Appendix

Table A1: Primer sequences, lengths of PCR products and annealing temperature for the CAPS/SCAR markers developed for *Solanum habrochaites* LYC4 x *S. lycopersicum* cv. Moneymaker populations and which marker is used to screen the F_2 (Chapter 2), BC_2S_1 (Chapter 2) and/or IL (Chapter 3) populations. The enzyme(s) revealing a polymorphism for each CAPS maker are also presented.

Marker name	Chromosome	Primer sequence (5'-3')	Observed PCR product length (bp)	Annealing Tm (°C)	Marker Type	Enzyme	Source[a]	F_2	BC_2S_1	IL
TG301	1	AGAGCTCAGGATGAACAGAACACGAA	800	55	CAPS	*Hpy*CH4IV	Bonnema et al. 1997	X		
		TTCTGCCAATTTCTTTGACCAACC								
TG224	1	GTATACTCAACAGAGCTTCAGAC	500	55	CAPS	*Hae*III	PBR	X		X
		GTGTAGATACCTGCACTCTCAG								
TG59	1	AACTCTACGCTGCACTGCTG	300	55	CAPS	*Hpa*II	PBR	X	X	X
		CTGAAGCTCACCTTGAGGTG								
TG460	1	CGAAATGTGACTATTCCAGAG	400	55	CAPS	*Alu*I	PBR	X		X
		GTAATTTGCACTTCTTGCCT								
TG343	1	AAGTTGCCAATAGCCAAGTC	500	55	CAPS	*Hpy*188I	PBR	X		
		GAAACTTATATTTGTGCATCCAG								
CT140	2	TCACTCGGGCTAAAATCTTC	550	55	CAPS	*Dde*I	PBR	X		
		GGGCTCAAAAGTTAACTCCA								
T1706	2	ATCTTGAACCAGGAGCCTTC	200	55	CAPS	*Alu*I	PBR	X		
		AGTATCTAGAGTAGTTGTCTCAC								
TG582	2	GGATATGTTTCATTGGCAAGC	1650	55	CAPS	*Dde*I	PBR	X		
		ACGGACTAGCAGAAGCAGCTG								
TG453	2	ATCACCATGAGGAAGACTGC	650	55	CAPS	*Dde*I	PBR	X		
		GGTCTGCAAGGTGGTCAGC								
TG145	2	TTCATTTGCTGGACAGGCAGTC	850	55	CAPS	*Hpy*CH4IV	PBR	X	X	X
		TGTCATCAAACGACCAGAGAC								
CT128	2	GTAAAGGCCAATTTGCTGCAC	500	55	CAPS	*Hpa*II	PBR	X		
		AGCTCCCCATCAACACAACTTG								
C2_At4g30930	2	ATCATACCTTCTCTCTCCAAACCC	700	55	CAPS	*Bse*DI	SGN	X	X	X
		TCGCCATTGCTCACTTTAAACTG								
C2_At5g64670	2	TGATAAATGCTGGGAAGATTGACTC	220	55	SCAR		SGN	X		X
		ATCAACCTCGCTCCCATCTTCTATTTG								
C2_At5g66530	2	TTCAGCAATGGCATTTGCAAGTGTG	900	55	CAPS	*Hinf*I	SGN	X	X	
		ACCATTGAATACAGCATCTGGTCGAAC								
TG337	2	TCCACAGTTATTGCTTCTTGTTTC	450	57	CAPS	*Mnl*I	Coaker and Francis 2004			X
		GGGTGTGTCTGTTTGACAGC								
TG537	2	TACCCGAGGCTCAGAAACAC	446	57	CAPS	*Hinf*I	Coaker and Francis 2004	X	X	
		CATCAACAGGAGATCGGTTTT								
TG91	2	TGCAGAGCTGTAATATTTAGAC	400	55	CAPS	*Dra*I	Alpert and Tanksley 1996	X		X
		CGGTCTCAGTTGCAACTCAA								
TG620	2	CTCTGTGCCAGAGCTCGAA	503	57	CAPS	*Eco*RI	Coaker and Francis 2004	X		
		TTTTACCTGGCGGAGAACTG								

Marker name	Chromosome	Primer sequence (5'-3')	Observed PCR product length (bp)	Annealing Tm (°C)	Marker Type	Enzyme	Source[a]	F_2	BC_2S_1	IL
TG40	3	GCGAGCTCGAATTCAATTCCAAC CGGGATTTTAGTTTTTCCGATCC	450	55	CAPS	*Hpy*CH4IV	PBR			X
TG56	3	TTTGTACCATGATTGTCCGATC GGCATTCATCATTCAACATGC	380	55	CAPS	*Hpa*III	PBR			X
TG585	3	TGGAAAGCCAGACACACAGA CAGGGGTATCAGTAGGCAGTG	580	55	CAPS	*Hinf*I	SGN			X
T1388	3	GCGATTTGGCTATCTGGGTA AACCGAAAGGCTTTTCCAAG	1000	55	CAPS	*Hin*1II	SGN			X
T0753	3	TGGTGCAACAAATCCCGAGC AGTTATCATAATGGCTAGCTTG	1500	55	SCAR		PBR			X
C2_At5g60160	3	ACACAATGCTAATCAACGTTATGC TCATCCACCGCCCACATTTC	500	55	CAPS	*Hin*1II	SGN			X
TG599	3	GCATGCCTGCAGAGTGGTC ATTCGCTACCTTGAGGGCTG	350	65	CAPS	*Dde*I	PBR			X
C2_At5g49970	3	AATTGGCAGGCTTGAGTGTTGC TCCCACCATTGTTACCAGGACCAC	850	55	CAPS	*Hpy*CH4IV	SGN			X
TG42	3	TTCCTCACTGCTTGGACCAGC TAGAACTTGGCATCCCTTGAAG	560	55	CAPS	*Mnl*I	PBR	X		X
TG134	3	CTACACAATTGTCACAGAAGTG GAGATCATTGGTATACAGCTG	500	55	SCAR		PBR	X		X
C2_At1g61620	3	ATGCATTCTAGAATGCCTTTTGTC TCCCTGGCTTTCTGCAGCATC	1350	55	CAPS	*Hae*III	SGN			X
TG549	3	ATGGAGAGAAGCTGGAACAC TTCTTAGAGCCCACCAGCAC	400	55	CAPS	*Mse*I	PBR			X
CT229	4	ATGGGCTGGGATCGTAGTAAA AAGCTTGCGATTCCCATAACA	336	55	CAPS	*Mwo*I	PBR			X
T1068	4	CAAAGCAATGGGCAATGGT ACACAGCAGTTTCAGTAGGAC	304	55	CAPS	*Hinc*II	PBR			X
TG609	4	TTGCCTACTTATAACCCTGTGGA ATATGACTAGGAGGCAATGACTGA	380	55	CAPS	*Alu*I	PBR	X	X	
TG652	4	CGGGGATCTGATAAGGAAGG ATAAGGGATCGTCAAGGAGTAACA	550	55	SCAR		PBR	X		
TG272	4	GATTTTGCCCCCTCTACCA ACATCTTTTCCTTCCCTCTGC	352	55	CAPS	*Mnl*I	PBR		X	X
TG264	4	GGAACAGGTCAGGACAGCAT TGGCTAACTGACGAAGACGA	520	55	CAPS	*Hae*II	PBR			X

Marker name	Chromosome	Primer sequence (5'-3')	Observed PCR product length (bp)	Annealing Tm (°C)	Marker Type	Enzyme	Source[a]	F_2	BC_2S_1	IL
TG62	4	CATGCCTAGTTGCAGTGTCC TTCAGCAGCAAGCAAAGATG	410	63	CAPS	*Dde*I	PBR	X	X	X
T1405	4	CACCAACAACTAGCCCTTGA AAGCAATTCCTCCAGCTTCA	535	55	CAPS	*Bsa*JI	SGN	X		X
TG574	4	CCATTCATCATCAGCTGGAA GTGCATGCTGGGTCAAGTAG	200, 220	55	SCAR		PBR	X	X	
TG555	4	AATTCGGAGCTCACTGCTTC AGTACGGCATGCTTGCTATC	430	55	CAPS	*Hpy*CH4IV	PBR	X	X	
CT50	4	GACGGCGTATTACGTTCAGA CTAGCACCCCAAAGGATGAG	390	55	CAPS	*Dde*I	PBR	X	X	X
C2_At1g74970	4	TCATCATCAACTATCGTGATGCTAAG ACGCTTGCGAGCCTTCTTGAGAC	1000	55	CAPS	*Hinf*I	SGN	X		
CT173	4	TGAGCACGGTATTACGCAG TTAGTAGTATGTGTGGCAGTCAC	1700	55	CAPS	*Hpy*CH4IV	PBR	X	X	
TG441	5	TGTCAGCATAGGCTTTTCCA CGGTCGGGAAAAATGACA	550	55	CAPS	*Rsa*I	Coaker and Francis 2004	X		X
CD31	5	ATCTCGGGATCATGGTTGAC ATFFCCAFAGAAATTCCAAA	501	55	CAPS	*Hinf*I	Coaker and Francis 2004	X		X
TG318	5	CAAGCCATAGAAATTGCCGTA TGCTCTCTCTGTGATGGAAGC	450	55	SCAR		Coaker and Francis 2004	X		X
TG358	5	CAACTTTTCCAGGTTCATTTTCTC ACACCTACATGCTACTAAGGGGTC	700	55	CAPS	*Dde*I	Brouwer and St. Clair 2004			X
TG60	5	TTGGCTGAAGTGAAGAAAAGTA AAGGGCATTGTAATATCTGTCC	400	55	CAPS	*Hpy*CH4IV	PBR			X
CT138	5	ACCAGCCCCGGAAGATTTTA GCGGTCAACTTCAGCAACTAT	364	55	CAPS	*Rsa*I	PBR	X		X
TG183	7	CTACTTGTCTGCCAAGGATTAC CGTGCCGTTCAAGAAGAGTG	1200	55	SCAR		Hemming et al. 2004			X
TG266	7	TGCAGAGCCAGTGTCACTTT CCATCCTTTGATTCCAAAGC	1500, 1550	55	SCAR		PBR	X		X
CT114	7	ATTGAAGAATGGCGGTGAAG ATGCCAACTTCTTGGCAAAC	1125	55	CAPS	*Dde*I	PBR	X		X
TG176	8	TTGAGATGTAGATTCTACAG CGCATAATCATGTGAGCAG	1000	55	CAPS	*Mnl*I	PBR	X		
CT92	8	CTCACTATCCAACTTACTGAGC TGTAGTTGTATTACTAACACAGC	700	55	CAPS	*Sau*96	PBR	X		

Marker name	Chromosome	Primer sequence (5'-3')	Observed PCR product length (bp)	Annealing Tm (°C)	Marker Type	Enzyme	Source[a]	F_2	BC_2S_1	IL
TG302	8	CTCTCCGGGTGGCTATTACA	400	55	CAPS	*Alu*I	SGN	X		
		TCTTGGGACTCCTCCTTTTCT								
CT64	8	GAGGAGAGATTCTTGGAC	300	55	CAPS	*Hae*III	PBR	X		
		TGAGGTTGATAGTGGGTG								
T1359	8	TTTGAGAGGCATGATGGTCA	850	55	CAPS	*Hpy*CH4IV	PBR	X		
		TCCCACCGGTTAAACTCATC								
TG294	8	ATTGGCTGCAATGATGGATT	900	50	CAPS	*Mnl*I	PBR	X		
		CTAAGCAGGACGGCCATCTA								
TG254	9	TTGGGAATATAGTGTAGGAAG	380	55	CAPS	*Fok*I	Bai et al. 2004			X
		CTGGAAAGGGGAAAGAC								
TG223	9	CAAGAAAATATTGTGTAGTGTTCTCCA	700	55	CAPS	*Mnl*I	PBR			X
		TCCCCCTCTTCATCAAATTC								
TG10	9	ATGATATCCACACCCCTGGA	587	55	CAPS	*Alu*I	PBR			X
		ATGCCTCGAAATTCAAATGC								
*Tm2*a	9	AGCGTCACTCCATACTTGGAATAA	1600	53	CAPS	*Acc*I	Sobir et al. 2000			X
		AGCGTCACTCAAAATGTACCCAAA								
U38666	9	AGCTGCCGTGTCCTGTATCA	400	55	CAPS	*Hin*fI	Bai et al. 2004			X
		ACTCATGTTCACGCCACTTTCTTA								
CT203	10	TAGAATATGGGAAGCGAAATG	400	55	CAPS	*Hae*III	Bai et al. 2004			X
		GAGAGGAAGCGTAATAGG								
CT240	10	ATCCCAAGTACCCTCGCATTAGT	850	55	CAPS	*Hae*III	Bai et al. 2004	X		X
		AGCCTTCTTTGTCCCATCAG								
CT113	11	ACAACGGGCAACAGACGCAACC	850, 350	55	SCAR		Bai et al. 2004	X		
		AGCTCGAGGATGGCCGCACTTT								
TG497	11	CGGAGAGTGAAGATGCATTG	850	55	CAPS	*Dde*I	SGN	X		
		AAGTTCCAGAGGGAGCACAA								
TG47	11	CCTCATTGGGCGGTCATT	420	55	CAPS	*Dde*I	Bai et al. 2004	X		
		TCCCACTCCCAAGCTATACTAACAA								
TG393	11	TGGATTTGATTAGCCGAAGG	750, 650	55	SCAR		SGN	X		
		CCAAGAATCCCAGAAGGAGA								
CT19	12	GAATCAGCAATCCGTAAT	250	55	CAPS	*Nla*III	Bai et al. 2004	X		
		CATGCAAGCAAGGTCCACAAC								
TG68	12	TTTGATTACACCTGCCTTTACATA	420	55	CAPS	*Dde*I	Bai et al. 2004	X		
		TTTTGAATCCCCTTTTACCAT								
TG565	12	CTGCAAAACAAGAATCACACT	380	55	CAPS	*Mnl*I	Bai et al. 2004	X		
		TCTGCGAGGTAGGGGTAAG								
TG296	12	TGTTCTGTCGGCATAAAGT	373	55	CAPS	*Hpy*CH4IV	Bai et al. 2004	X		X
		TGCTAAAACGGACCTACAA								

[a] PBR: developed at Plant Breeding, mainly using data from SGN. SGN: primers available at SGN. When published, references are given.

Summary/Samenvatting

Summary

Botrytis cinerea Pers:Fr (teleomorph: *Botryotina fuckeliana* (de Bary) Whetzel) is a necrotrophic pathogenic fungus with a wide host range of at least 235 species (Elad et al. 2004; Jarvis 1977). Modern hybrid tomato (*Solanum lycopersicum*) cultivars are susceptible to *B. cinerea* although some cultivars show a certain level of quantitative resistance. In heated greenhouses, *B. cinerea* infection in tomato is usually restricted to the stem (Dik and Wubben 2004; Shtienberg et al. 1998). Stem rot usually develops after pruning of lower leaves or side shoots (Verhoeff 1968) and results in a lower yield or premature plant death. Disease control frequently relies on fungicides (Leroux 2004) or biocontrol (Elad and Steward 2004). However, *B. cinerea* has developed resistance against several of these control measures (Elad and Steward 2004; Leroux 2004). The development of *B. cinerea* resistant cultivars by introgressing resistance alleles from wild tomato relatives may provide an effective alternative.

Previously, quantitative resistance to *B. cinerea* has been identified in wild relatives of tomato (Egashira et al. 2000; Nicot et al. 2002; ten Have et al 2007; Urbasch 1986). The scope of this thesis was to identify quantitative trait loci (QTL) conferring resistance to *B. cinerea* originating from *S. habrochaites* LYC4 and *S. neorickii* G1.1601. Susceptibility to *B. cinerea* was assessed using a quantitative stem assay (ten Have et al. 2007). Three QTLs originating from *S. habrochaites* LYC4 (Chapter 2) and three QTLs originating from *S. neorickii* G1.1601 (Chapter 4) conferring resistance to *B. cinerea* were identified using transient (i.e. $F_{2\text{-}3}$) mapping populations. We presumed that not all QTLs conferring resistance to *B. cinerea* were identified in these two populations and proposed that an introgression line population would be more powerful for detection of QTLs (Chapter 1). For that reason, we developed a *S. habrochaites* LYC4 introgression line (IL) population (n=30; Chapter 3). Most ILs contained one *S. habrochaites* introgression in the *S. lycopersicum* cv. Moneymaker genetic background. Susceptibility of each IL to *B. cinerea* was assessed in a greenhouse trial (Chapter 3). In addition to the three QTLs identified in the F_2 population, seven new QTLs were identified using this introgression line population (Chapter 3).

None of the QTLs separately conferred a level of resistance comparable to the resistant parent *S. habrochaites* LYC4 (Chapters 3 and 4). In order to obtain *B. cinerea* resistant tomato, QTLs need to be combined. In these studies, we have observed examples of combined QTLs acting in an additive manner (Chapter 3) and of combined QTLs showing less-than-additive epistatic interactions (Chapter 2). Redundancy of certain QTLs is also likely (Chapters 3 and 6). Using the *S. habrochaites* LYC4 IL population, controlled crosses can readily be made to test which combinations of QTLs will result in the highest level of resistance while introgressing the minimum number of QTLs.

We propose that introgressing three QTLs will be sufficient to obtain *B. cinerea* resistant plants. One resistant BC_2S_1 genotype, identified while confirming the QTLs identified in the F_2 population (Chapter 2), was selected and bred in. The level of resistance of this inbred (BRC-5) was comparable to the wild parent *S. habrochaites* LYC4 (Chapter 3). The high level of resistance of BRC-5 is likely caused by the (inter)action of two, possibly three QTLs (Chapters 3 and 6).

Each of the three *S. neorickii* G1.1601 QTLs was located at position homologous to the *S. habrochaites* LYC4 QTLs (Chapter 4). These loci might have evolved from a common ancestor and may be involved in general defense responses (Chapter 4).

Variation was observed within the disease assays (Chapters 2, 4 and 6), yet replicate analyses of a genotype led to consistent conclusions. An IL population is an ideal tool for replicate analysis (Chapter 3). The *S. habrochaites* LYC4 IL population can also easily be screened for additional traits, such as agronomic and morphological characteristics (Chapter 5). Genetic variation was observed for most traits. This data not only showed the additional potential use of this population for further breeding research, but also provided indications for a correlation between a lower yield and *B. cinerea* resistance (Chapter 6).

This study identified loci conferring resistance to *B. cinerea*, which will be used to develop tomato cultivars that are (partially) resistant to *B. cinerea*. The ILs that were developed will provide useful tools for further studies to unravel the resistance mechanisms governed by individual QTLs (Chapter 6).

Samenvatting

Botrytis cinerea Pers:Fr (telomorph: *Botryotina fuckeliana* (de Bary) Whetzel) is een necrotrofe pathogene schimmel die infecties kan veroorzaken in tenminste 235 plantensoorten (Elad et al. 2004; Jarvis 1977). Moderne hybride tomaten (*Solanum lycopersicum*) cultivars zijn vatbaar voor infecties door *B. cinerea*. Toch zijn er cultivars die een kwantitatief niveau van resistentie vertonen. Normaal gesproken beperken infecties van *B. cinerea* in tomaat zich tot de stengel (Dik en Wubben 2004; Shtienberg et al. 1998). Stengelrot ontwikkelt zich meestal na het verwijderen van de onderste bladeren of van de dieven (Verhoeff 1968) wat resulteert in lagere opbrengst of het voortijdig afsterven van de plant. Bespuiten met fungiciden (Leroux 2004) of het toepassen van biologische bestrijding zijn veelgebruikte methodes om *B. cinerea* infecties te voorkomen (Elad en Steward 2004). *B. cinerea* heeft echter resistentie ontwikkeld tegen veel van deze beheersmethodieken (Elad en Steward 2004; Leroux 2004). Het ontwikkelen van *B. cinerea* resistente cultivars, doormiddel van het inkruisen van resistentie allelen uit wilde verwanten van tomaat, kan een effectief alternatief zijn.

Resistentie tegen *B. cinerea* is gevonden in wilde verwanten van tomaat (Egashira et al. 2000; Nicot et al. 2002; ten Have et al 2007; Urbasch 1986) en deze resistentie was meestal kwantitatief. Het doel van dit promotieonderzoek was om kwantitatieve loci (QTLs) te identificeren die bijdragen aan resistentie tegen *B. cinerea* uit de wilde tomaten accessies: *S. habrochaites* LYC4 en *S. neorickii* G1.1601. Vatbaarheid voor *B. cinerea* is bepaald met een kwantitatieve stengeltoets (ten Have et al. 2007). Drie QTLs afkomstig uit *S. habrochaites* LYC4 (Hoofdstuk 2) en drie QTLs afkomstig uit *S. neorickii* G1.1601 (Hoofdstuk 4) zijn gevonden na analyse van resistentie in tijdelijke (i.e. $F_{2\text{-}3}$) karteringspopulaties. Het was waarschijnlijk dat we in deze tijdelijke karteringspopulaties nog niet alle loci hadden geïdentificeerd, die bijdragen aan *B. cinerea* resistentie. Een populatie van introgressielijnen (IL) is krachtiger voor de detectie van QTLs dan tijdelijke kartering populaties (Hoofdstuk 1). Daarom hebben we een *S. habrochaites* LYC4 IL populatie ontwikkeld (n=30; Hoofdstuk 3). In de meeste lijnen is er één introgressie-fragment uit *S. habrochaites* LYC4 ingekruist in de genetische achtergrond van *S. lycopersicum* cv. Moneymaker. Vatbaarheid voor *B.* cinerea in de IL populatie is bepaald in een kasproef (Hoofdstuk 3). Naast de drie eerder geïdentificeerde QTLs werden zeven extra QTLs voor *B. cinerea* resistentie geïdentificeerd in deze populatie (Hoofdstuk 3).

Geen van de afzonderlijke QTLs resulteerde in een niveau van resistentie, dat overeen kwam met de resistente ouder *S. habrochaites* LYC4 (Hoofdstuk 3 en 4). Dit betekent dat QTLs gecombineerd zullen moeten worden om *B. cinerea* resistente tomaten te verkrijgen. In onze experimenten hebben we voorbeelden gezien van QTL combinaties die additief werken (Hoofdstuk 3) maar ook van QTL combinaties

die een interactie vertonen waardoor het effect van de QTL combinatie minder was dan wat verwacht werd op basis van het effect van elk afzonderlijk QTL (Hoofdstuk 2). Sommige QTLs zijn waarschijnlijk redundant (Hoofdstuk 3 en 6). De *S. habrochaites* LYC4 IL populatie is geschikt voor het maken van gecontroleerde kruisingen tussen QTL bevattende ILs. Nakomelingen van deze kruising, geselecteerd op de aanwezigheid van de gewenste QTLs, kunnen getest worden op resistentie tegen *B. cinerea* om te bepalen welke en hoeveel QTLs minimaal ingekruist moeten worden om een redelijk tot hoog niveau van resistentie te verkrijgen.

De verwachting is dat teneinde een acceptabel niveau van resistentie te verkrijgen het voldoende is om drie QTLs in te kruisen. Eén resistente BC_2S_1 lijn, die verkregen werd tijdens experimenten om de F_2 QTLs te bevestigen (Hoofdstuk 2), is ingeteeld. Het resistentie niveau van deze inteeltlijn (BRC-5) was vergelijkbaar met dat van *S. habrochaites* LYC4 tijdens de kastoetsen (Hoofdstuk 3). Het hoge niveau van resistentie in BRC-5 wordt waarschijnlijk veroorzaakt door de (inter)actie van twee, wellicht drie, QTLs (Hoofdstuk 3 en 6).

De positie van elk van de drie QTLs uit *S. neorickii* G1.1601 kwam overeen met een QTL uit *S. habrochaites* LYC4 (Hoofdstuk 4). Homologe loci kunnen geëvolueerd zijn uit een gemeenschappelijke voorouder en kunnen betrokken zijn bij algemene resistentie reacties (Hoofdstuk 4).

Ondanks de variatie in de ziektetoetsen (Hoofdstuk 2, 4 en 6) resulteerde herhaald toetsen van een genotype altijd in een consistente conclusie. Een IL populatie is een ideale populatie voor het uitvoeren van herhalingen (Hoofdstuk 3). De *S. habrochaites* LYC4 IL populatie kon dan ook eenvoudig geanalyseerd worden voor aanvullende eigenschappen, zoals agronomische en morfologische kenmerken (Hoofdstuk 5). Voor de meeste van de geanalyseerde eigenschappen werd genetische variatie geïdentificeerd. Doordat ook opbrengst is bepaald van elke introgressielijn hebben we gevonden dat een hoger niveau van resistentie tegen *B. cinerea* gecorreleerd is met een lagere opbrengst (Hoofdstuk 6).

De in deze studie geïdentificeerde loci voor resistentie tegen *B. cinerea* zullen gebruikt worden voor de ontwikkeling van (partieel) resistente tomatencultivars. De ontwikkelde IL populatie is een krachtig gereedschap voor verdere studies waarin het resistentie mechanisme van de afzonderlijke QTLs ontrafeld kan worden (Hoofdstuk 6).

Van/over de auteur

Nawoord

De klus is geklaard, het spreekwoordelijke nietje zit erdoor en ik kan dus nu met een voldaan gevoel deze laatste woorden typen. Dit werk is echter niet tot stand gekomen zonder de inzet van vele andere mensen die ik daarvoor zal proberen te danken. Ik ben echter bang dat ik ook vast wel iemand vergeet. Dus voor diegene de erepositie om als eerste bedankt te worden: BEDANKT!

In het speciaal wil ik Sjaak van Heusden bedanken voor alle deuren die hij heeft weten te openen. Ik heb je inzet altijd kunnen waarderen hoewel ik dat op mijn gebruikelijke wijze niet altijd heb laten merken. Als copromotor is waarschijnlijk het laatste woord aan jou tijdens de promotie, maar ik hoop van harte dat we nadien nog lang woorden met elkaar kunnen wisselen.

Ook zonder de hulp van mijn tweede copromotor Jan van Kan, promotor Richard Visser en tomatoloog Pim Lindhout zou dit boekje niet zijn zoals U het op dit moment leest. Hun interesse, relativerende blik en/of inhoudelijke discussies zijn erg belangrijk geweest tijdens verschillende, soms frustrerende, stadia van dit onderzoek.

Ook wil ik graag de Technologiestichting STW en De Ruiter Seeds bedanken voor hun bijdrage aan dit onderzoek en de discussies tijdens de halfjaarlijkse gebruikersbijeenkomsten. Paul, de samenwerking met jou heb ik in het bijzonder gewaardeerd.

This thesis could also not have been completed without the help of my students Pham, Yaxin, Nasos, Nasos, Martijn and Manja. Their help was invaluable. I also would like to thank my various roommates during this period. This period would have been less pleasant without you. I would also like to thank the members of the resistance group. They all contributed in a positive way to completion of this thesis.

Op een aantal momenten, toen het erg belangrijk was, heb ik ook assistentie gekregen van Fien en Petra. Jullie resultaten hebben een belangrijke bijdrage geleverd aan het tot stand komen van dit proefschrift. Dat geldt ook voor het werk uitgevoerd door Arjen en Ralph. Door wat jullie al hadden gedaan kon ik een vliegende start maken. Dank hiervoor! Zonder de zorg van het Unifarm team bestaande uit Alex, André, Maarten, Maarten & Teus zou geen er geen enkele tomaat geoogst zijn.

Christel, Dianka, Danny, Erik, Gerard, Hossein, Huub, Koen, Linda, Remmelt, Marina & Ton. Op de één of andere manier hebben jullie (vaak ongemerkt) een positieve bijdrage kunnen leveren tot wat nu mijn proefschrift te noemen is.

Marleen, Olga, Roeland, Sjaak & Yuling. Jullie hebben meer voor mij betekend dan alleen een goede collega. Naast onze interacties in het werk hoop ik ook nog lang van interacties met jullie te kunnen genieten buiten de werkvloer. Zonder de steun van mijn ouders, familie en in het speciaal Ania was dit proefschrift nooit geworden zoals het nu is. Dank hiervoor.

Curriculum vitae

Richard Finkers werd geboren op 24 februari 1972 te Slagharen. In 1988 behaalde hij het MAVO diploma aan de Rijksscholen Gemeenschap te Coevorden. Hierna heeft hij het MLO diploma gehaald aan het Technisch college Drenthe en aansluitend een studie HLO gevolgd aan de Hanzehogeschool Groningen, welke in 1996 is afgesloten met een diploma met als specialisatie biotechnologie. Tijdens deze studie heeft hij aan een stage voltooid bij het toenmalige ID-DLO (Dr. Hilde Smith) met als onderwerp: Identificatie van virulentiefactoren van de pathogeen *Streptococcus suis* die hersenvliesontsteking veroorzaakt bij varkens.

In 1996 is hij als analist begonnen bij het toenmalige ATO-DLO (Dr. Miranda van der Rhee en Dr. Gerard Rouwendal) aan kwaliteitsaspecten van potplanten. Sinds 1999 werkt hij bij Plant Research International en is betrokken geweest bij diverse projecten in o.a. appel, goudsbloem, paprika, tomaat en ui gericht op karakterisatie van kwaliteit en/of resistentie gebruik makend van moleculaire merkers. Sinds 2003 heeft hij in samenwerking met de vakgroep Plantenveredeling van Wageningen Universiteit de mogelijkheid gekregen om promotieonderzoek te verrichten.

List of Publications

Articles

Finkers R, van den Berg P, Meijer Dekens F, Bai Y, van Berloo R, ten Have A, van Kan JAL, Lindhout P, van Heusden AW (2007) Quantitative resistance to *Botrytis cinerea* from *Solanum neorickii*. Euphytica: Submitted

Finkers R, van den Berg P, van Berloo R, ten Have A, van Heusden AW, van Kan JAL, Lindhout P (2007) Three QTLs for *Botrytis cinerea* resistance in tomato. Theor Appl Genet: http://dx.doi.org/10.1007/s00122-006-0458-0

Finkers R, van Heusden AW, Meijer-Dekens F, van Kan JAL, Maris P, Lindhout P (2007) The construction of a *Solanum habrochaites* LYC4 introgression line population and the identification of QTLs for resistance to *Botrytis cinerea* Theor Appl Genet: http://dx/doi.org/10.1007/s00122-006-0500-2

Voorrips RE, Finkers R, Sanjaya L, Groenwold R (2004) QTL mapping of anthracnose (*Colletotrichum* spp.) resistance in a cross between *Capsicum annuum* and *C. chinense*. Theor Appl Genet 109: 1275-1282

Book chapter

Broude NE, van Heusden AW, Finkers R (2004) High multiplexity PCR based on PCR suppression. In: Demidov VV, Broude NE (eds) DNA amplification. Horizon Bioscience, Boston, pp 61-76

Congress proceedings

Finkers R, van Heusden AW, ten Have A, van Kan JAL, Lindhout P (2005) Identification of QTLs for *Botrytis cinerea* resistance in *S. habrochaites* LYC4. In: Mol Plant-Microbe Interact. International society for Molecular Plant-Microbe interactions, Merida, Mexico vol 5, pp 625-629

Sreenath HL, Finkers R, van Heusden AW (2002) Isolation and analysis of resistance gene analogues (RGAs) in coffee. In: Proceedings of Placrosym XV, Mysore, India

van de Weg WE, Voorrips RE, Finkers R, Kodde LP, Jansen J, Bink MCAM (2004) Pedigree genotyping: a new pedigree-based approach of QTL identification and allele mining. Acta Hortic 663: 45-50

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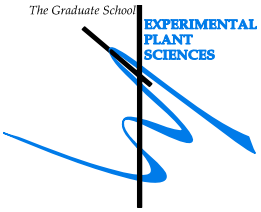
Voorrips RE, Verkerke W, Finkers R, Jongerius R, Kanne J (2000) Inheritance of taste components in tomato. Acta Physiol Plant 22: 259-261

Patent

van Kan JAL, ten Have A, Lindhout P, Finkers R, van Berloo R, van Heusden AW (2006) *Botrytis*-resistant tomato plants. In: Office EP (ed) EP1652930, pp 45

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: **Richard Finkers**
Date: **3 April 2007**
Group: **Plant Breeding, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
New approaches for disease control of *Botrytis cinerea*	Apr 11, 2003
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
High Multiplexity of PCR Based on PCR Suppression	2004
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
Nijmegen	Jun 2, 2005
Wageningen	Sep 19, 2006
► **EPS theme symposia**	
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Leiden University	Jun 23, 2005
► **NWO Lunteren days and other National Platforms**	
NWO Lunteren meetings (2-days)	2001-2006
► **Seminars (series), workshops and symposia**	
Flying Seminars	2001-2006
Weekly Seminars Plant Breeding	2003-2006
► **Seminar plus**	
► **International symposia and congresses**	
Plant, Animal & Microbe Genomes X Conference, San Diego, California (USA)	Jan 12-16, 2002
Plant & Animal Genome XII, San Diego, California (USA)	Jan 10-14, 2004
XII International Congress on Molecular Plant Microbe Interactions, Merida (Mexico)	Dec 14-19, 2005
VI International Solanaceae Conference, Madison, Wisconsin (USA)	Jul 23-27, 2006
Plant Resistance Symposium, Budapest (Hungary)	Aug 31-Sep 2, 2006
► **Presentations**	
AFLP Markers in Plant Systematics and Breeding	May 16-18, 2001
Plant, Animal & Microbe Genomes X - Poster	Jan 12-16, 2002
XII international congress on Molecular Plant microbe interactions - Oral presentation	Dec 14-19, 2005
VI International Solanaceae Conference - Poster	Jul 23-27, 2006
Plant Resistance Symposium - Oral Presentation	Aug 31-Sep 2, 2006
► **IAB interview**	Jun 3, 2005
► **Excursions**	
Subtotal Scientific Exposure	*18.2 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
AFLP Markers in Plant Systematics and Breeding	May 16-18, 2001
Mass Spectrometry in Biology	Sep 3-7, 2001
Springschool Bioinformatica	Mar 31-Apr 2, 2004
Principes of ~Omics Data Analysis	Nov 7-10, 2005
Multivariate Analysis	Apr 19-27, 2005
► **Journal club**	
Literature discussions Plant Breeding	2001-2006
► **Individual research training**	
Subtotal In-Depth Studies	*9.0 credits**

4) Personal development	*date*
► **Skill training courses**	
Guide to Digital Scientific Artwork	Dec 6-7, 2001
Presentation Skills	Apr 24-May15, 2006
Scientific Writing	Sep 26-Nov 14, 2006
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**38.3**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

The work described in this thesis was performed at the Laboratory of Plant breeding of Wageningen University. Main financial support of the research project was provided as STW project “New approaches for disease control of *Botrytis cinerea*: sources of partial resistance in *Lycopersicon* accessions and the identification of new pathogenicity factors as potential targets for inhibitors” (WBI4712). The project was funded for two additional years by De Ruiter Seeds B.V., Bergschenhoek, The Netherlands and for an additional six months with strategic funding from the Laboratory of Plant breeding of Wageningen University.

Printed at Gildeprint Drukkerijen, Enschede.

Front cover:
Natural *Botrytis cinerea* infection developed after pruning of the side shoot.

Back cover:
Botrytis cinerea infected introgression lines (Greenhouse assay; top row).
Details from the *Botrytis cinerea* laboratory disease test (bottom row).