How do plant species defend themselves against *Oidium lycopersici*?

Mapping of monogenic and polygenic resistance in *Lycopersicon* species

Cai-Cheng Huang

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Stellingen (Propositions)

1. With DNA markers and QTL mapping, complex forms of disease resistance and their underlying genes are now far more accessible. Someday soon, the distinction between manipulating qualitative and quantitative disease resistance may finally disappear (Young, 1996, Annu Rev Phytopathol 34:479-501).

2. The successful cloning of *Brix9-2-5* and *fw2.2*, QTLs controlling respectively sugar content and fruit weight of tomato, indicates that a QTL may indeed correspond to a gene (Fray *et al.*, 2000, Science 289:85-88; Fridman *et al.*, 2000, PNAS 97:4718-4723).

3. The movement of the nucleus of cowpea cells to the penetration site of an invading rust fungus (*Uromyces vignae*) is diagnostic for the resistance reaction (Heath *et al.*, 1997, New Phytol 135:689-700).

4. A plant species can be considered as a host of *Oidium lycopersici* if this fungus can successfully and repeatedly reproduce on this plant species (this thesis).

5. It is still unclear whether *Oidium lycopersici* (formerly *Oidium lycopersicum*), a fungus responsible for the recent outbreaks of powdery mildew on tomato, is identical to the one described in 1888 in Australia, or originated from another powdery mildew fungus which has extended its host range to include tomato (this thesis).

6. Cultures can be different, but no culture is superior to any other.

7. The "chicken and egg" problem is an issue not only for nature scientists but also for sociologists.

8. The Dutch are the Chinese of Europe. For example, the Dutch hope to hit two running hares by throwing one stone while the Chinese want to target two flying eagles by shooting one arrow.

Stellingen behorende bij het proefschrift: How do plant species defend themselves against *Oidium lycopersici?* - Mapping monogenic and polygenic resistance in *Lycopersicon* species, door Caicheng Huang, in het openbaar te verdedigen op 25 april 2001, te Wageningen.

谨以此书敬献给

我敬爱的父母
我亲爱的太太
我可爱的儿子
我挚爱的朋友

To my parents
For Li-E, Hai-Yun and He-Yun

Contents

Chapter 1

General introduction

Tomato

The cultivated tomato belongs to the species *Lycopersicon esculentum* Miller. *Lycopersicon* is a relatively small genus within the very large and diverse family Solanaceae, which consists of about 1500 species in circa 90 genera. The centre of origin of the family is Central America. All species in the genus *Lycopersicon* show remarkable cytogenetic uniformity having a chromosome base number of $x = 12$ (Taylor, 1986). They are divided into the esculentum-complex and the peruvianum-complex based on crossabilities (Rick, 1976). Other members of the esculentum-complex are: *L. pimpinellifolium, L. cheesmanii, L. parviflorum, L. chmielewskii, L. hirsutum* and *L. pennellii*. Members of the peruvianum-complex are *L. peruvianum* and *L. chilense*. The two complexes are isolated from each other by strong hybridization barriers. Though some accessions in the esculentum-complex (*viz. L. hirsutum* and *L. pennellii*) are strictly self-incompatible, most members are completely self-compatible, and have a strong tendency to inbreeding. In contrast; both species in the peruvianum-complex, *viz. L. peruvianum* and *L. chilense*, are self-incompatible and, consequently, are outbreeders (Taylor, 1986).

The tomato was introduced into the Old World in the 16th century, and since about 1800 it has been cultivated in most parts of the world (Boswell, 1949). The name tomato probably derived from 'tomatl' in the Nahua tongue of Mexico (Kalloo, 1991). The cultivated tomato genetically is very uniform. There is less than 3% of DNA polymorphisms within the group of old tomato cultivars compared to 24.5% within *L. esculentum* var *cerasiforme* (Williams and Clair, 1992). The low overall genetic diversity of cultivated tomato may be due to continuous breeding (single-seed descent or pedigree selection). Also, presumably, the entire population of tomato cultivars in Europe and US derives from a very limited amount of tomato seeds/plants (and accessions) introduced in Europe in the 16th century. Worldwide, the tomato crop covers 3500 million ha, producing $9.5 \cdot 10^{16}$ kg fruits per year (FAO, 2000).

Tomato is one of the best-studied crop plants, due to its easy crossability, clear genetics and economic importance (*e.g.* Rick, 1975). More recently, the small genome content (Arumuganathan and Earle, 1991), the high density molecular marker maps (Tanksley *et al.*, 1992; Haanstra *et al.*, 1999), the successful isolation of genes and the well-developed

transformation protocols have rendered tomato very suitable as a model organism for genetic and genomic studies.

Biotic stress of tomato

Pests: More than a hundred different pest species have been recorded on tomato crops (see review by Berlinger, 1986). The most important ones are nematodes, thrips, aphids, moths, whiteflies and beetles. They cause damage to all parts of tomato plants by sucking (often transmitting virus), chewing and rasping. Since the late 1960's, a lot of efforts have been made to breed tomato varieties resistant to these pests (Berlinger, 1986). For example, the resistance to nematodes has been widely characterized and one of the corresponding resistance genes *Mi* (also conferring resistance to aphid) has been mapped by using molecular markers and eventually has been cloned (Kaloshian *et al.*, 1998; Rossi *et al.*, 1998; Williamson *et al.*, 1994; Yaghoobi *et al.*, 1995) (Table 1), which has facilitated breeding tomato cultivars resistant to nematodes (and aphid).

Diseases: Over 200 diseases have been reported to affect tomato plants (Watterson, 1986). Before the introduction of resistant cultivars, *Fusarium* wilt was perhaps the most destructive disease to tomato. In some areas of the western US the processing tomato industry was virtually destroyed by beet curly top virus. In other temperate regions, diseases such as late blight, *Septoria* leaf spot, bacterial canker and bacterial speck have built up to epidemic proportion, completely ruining crops. While bacterial wilt and bacterial spot have devastated plantings in the warm humid tropics, protected crops have also been plagued by damaging diseases. *Fusarium* crown rot, corky root, *Didymella* stem rot, black dot, leaf mould and tomato mosaic have all caused serious problems for glasshouse tomato growers (Watterson, 1986). Thanks to disease resistance breeding, the damage resulting from tomato diseases has been substantially reduced and in some cases eliminated. For example, *Fusarium* wilt is readily controlled by using resistant cultivars containing the *I*-genes (Table 1). In tomato, in total, more than 15 resistance genes (R-genes) have been introduced into modern cultivars. Most known R-genes have been mapped by using molecular markers (Table 1). As a result, some of the introgressions of R-genes have been facilitated marker-assisted selection (MAS). Meanwhile, quantitative trait loci (QTLs) for resistance to a number of diseases (and pests) have been identified (Table 1).

Among the diseases that have been recorded, there are three types of powdery mildews in tomato, caused by *Leveilula taurica*, *Erysiphe orontii* and *Oidium lycopersici*. As in this thesis we focus on one of the powdery mildew diseases, *O. lycopersici*, a more detailed introduction to the powdery mildew diseases is given below.

Table 1. List of mapped resistance genes/QTLs in tomato

R-gene	Pathogen	Origin of the R-gene	Chromosome	Reference
Asc	*Alternaria alternata* f.sp. *lycopersici*	*L. pennellii*	3 (cloned)	Van der Biezen *et al.* (1995); Brandweracht (pers. comm.)
Cf-1	*Cladosporium fulvum*	*Lycopersicon*	1	Dickinson *et al.* (1993)
Cf-2	*C. fulvum*	*L. pimpinellifolium*	6 (cloned)	Dickinson *et al.* (1993); Dixon *et al.* (1996)
Cf-4	*C. fulvum*	*L. hirsutum*	1 (cloned)	Jones *et al.* (1993); Balint-Kurti *et al.* (1994); Thomas *et al.*, (1997)
Cf-4A	*C. fulvum*	*L. hirsutum*	1 (cloned)	Takken *et al.* (1998)
Cf-5	*C. fulvum*	*L. esculentum* var. *cerasiforme*	6 (cloned)	Balint-Kurti *et al.* (1994); Dixon *et al.* (1998)
Cf-9	*C. fulvum*	*L. pimpinellifolium*	1 (cloned)	Balint-Kurti *et al.* (1994); Jones *et al.* (1994)
Frl	*F. oxysporum* f.sp. *radicis-lycopersici*	(not reported)	9	Vakalounakis *et al.* (1997)
Hero	*Globodera rostochiensis*	*L. pimpinellifolium*	4	Ganal *et al.* (1995)
I-1	*Fusarium oxysporum* race 1	*L. pimpinellifolium*	7	Sarfatti *et al.* (1989, 1991)
I-2	*F. oxysporum* race 2	*L. pimpinellifolium*	11 (cloned)	Sarfatti *et al.* (1989); Simons *et al.*, (1998)
I-3	*F. oxysporum* race 3	*L. pennellii*	7	Bournival *et al.* (1990); McGrath *et al.* (1987); Tanksley and Costello (1991)
Lv	*Leveillula taurica*	(not reported)	12	Chunwongse *et al.* (1997)
Mi	*Meloidogyne* spp., *Macrosiphum euphorbiae*	*L. peruvianum*	6 (cloned)	Williamson *et al.* (1994); Rossi *et al.* (1998)
Mi-3	*M. incognita, M. javanica*	*L. peruvianum*	12	Yaghoobi *et al.* (1995)
Ol-1	*Oidium lycopersici*	*L. hirsutum* G1.1560	6	Van der Beek *et al.* (1994); Huang *et al.* (2000a)
Ol-3	*O. lycopersici*	*L. hirsutum* G1.1290	6	Huang *et al.* (2000b)
OPG12H	*Liriomyza trifolii*	*L. hirsutum* f. *glabralum*	2	Moreira *et al.* (1999)
Ph-1	*Phytophthora infestans*	*L. pimpinellifolium*	7	Pierce (1971)
Ph-2	*P. infestans*	*L. pimpinellifolium*	10	Moreau *et al.* (1998)
Ph-3	*P. infestans*	*L. pimpinellifolium*	9	Chunwongse *et al.* (1998)
Prf	*Pseudomonase syringae*	(not reported)	5 (cloned)	Salmeron *et al.* (1996)
Pto	*Pseudomonase syringae*	(not reported)	5 (cloned)	Martin *et al.* (1991); Martin *et al.*, 1993
py-1	*Pyrenochaeta lycopersici*	*L. peruvianum*	3	Doganlar *et al.* (1998)
QTLs (3)	*Clavibacter michiganensis* ssp. *michiganensis*)	*L. peruvianum* LA2157	5, 7 & 9	Van Heusden *et al.*, 1999
QTLs (3-6)	*Pseudomonase syringae*	(not reported)		Danesh and Young 1994; Thoquet *et al.*, 1996

QTLs (3)	Oidium lycopersici	L. parviflorum G1.1601	one on 12, two unassigned	(present study)
Ra (RAPD)	TYLCV	L. pimpinellifolium	6	Chague et al. (1997)
Rb (RAPD)	TYLCV	L. pimpinellifolium	7	Chague et al. (1997)
Rc (RAPD)	TYLCV	L. pimpinellifolium	8	Chague et al. (1997)
Rd (RAPD)	TYLCV	L. pimpinellifolium	9	Chague et al. (1997)
rx-1, rx-2, rx-3	Xanthomonas campestris pv. vesicatoria	L. esculentum	1	Yu et al. (1995)
Sm	Stemphilium	L. pimpinellifolium	11	Behare et al. (1991)
Sw-5	TSWV	L. peruvianum	9 (cloned)	Stevens et al. (1995, 1996); Brommenschenkel and Tanksley (1997)
Tm-1	tobacco mosaic virus	L. hirsutum	2	Levesque et al. (1990)
Tm-2a	tobacco mosaic virus	L. peruvianum	9	Young et al. (1988)
Ty-1	TYLCV	L. chilense, L. hirsutum, L. pimpinellifolium, L. cheesmanii	6	Zamir et al. (1994); Chague et al. (1997)
Ty-2	TYLCV	L. hirsutum	11	Hanson et al. (2000)
Ve	Verticilium race 1	L. esculentum	7	Juvick et al. (1991); Zamir et al. (1995); Diwan et al. (1999)

Powdery mildews

Powdery mildews are plant fungi with white hyphae growing mostly on the surface of the aerial parts of living plants and with haustoria growing in the epidermal cells of their hosts. They produce large one-celled conidia on isolated aerial unbranched conidiophores (Yarwood, 1978). They are typical biotrophic fungi, that need living tissue to grow and sporulate. The possible origins and the meanings of the term "powdery mildew" have been extensively discussed (Yarwood, 1978). Braun (1987, 1995) has reviewed the development of the genus name of powdery mildew, *Erysiphe*, and pointed out that, though many extensive observations on Erysiphaceae were conducted, only de Bary (cited by Braun, 1987, 1995) realized the true relationship between the spherical dark ascocarps, the white mycelial patches and the conidiophores, and between the fungus and the host (Braun, 1995). In the 20th century, important monographs on powdery mildews have been written by Salmon (cited by Blumer, 1967), Blumer (1967), Amano (1986, with Hirata's [1966] great contribution) and Braun (1987, 1995).

Of many powdery mildews only the asexual stage is known (Anamorph). *Oidium* is the generic name of the anamorphic (imperfect) states of the Erysiphaceae (Anonymous, 1975), which includes *Euoidium* and *Pseudoidium*, *i.e. Oidium* with catenate and solitary conidia respectively. Due to the lack of a sexual stage and the variability of their morphology, classification of powdery mildews is sometimes ambiguous. Actually, most species names of Erysiphaceae refer to a host genus on which they are first found (Yarwood, 1978). Of the 90 or more named species of *Oidium* (Hirata, 1966), 84 were named with reference to the host genus (Yarwood, 1978). This designation of Erysiphaceae reflects that each isolate (pathogenically distinct culture) is usually restricted to a single host genus or closely related genera; some are even restricted to only one plant species. Therefore, the most obvious character to recognize a powdery mildew is the host on which it is found. Yet, each morphologic species of Erysiphaceae usually has several host genera and species (Yarwood, 1978).

The host range of powdery mildews (Erysiphaceae) covers nearly 10,000 plant species in over 1,600 genera, 169 families and 44 orders. Most hosts belong to the dicotyledons (161 families, over 9,000 species). Only eight families and 662 host species belong to the monocotyledons, and most of them are Gramineae (Amano, 1986). The host species include both cultivated and wild species. Some genera of the Erysiphaceae can be classified as typical herb parasites, *e. g. Erysiphe, Leveillula, Sphaerotheca*, and some as tree parasites, *e. g. Podosphaera, Microsphaera, Uncinula, Phyllactinia, Pleochaeta*, though there are exceptions in both groups (Hirata, 1957, 1976). There are numerous hosts that are simultaneously affected by more than one powdery mildew genus, *e. g. Erysiphe* and *Sphaerotheca* on cucumber, *Erysiphe, Sphaerotheca* and *Leveillula* on tomato (Hirata, 1976). Some powdery mildew species can grow on more than 1000 host plant species. For example, *Erysiphe cichoracearum* has a host range of at least 1753 plant species (Amano, 1986). Such a host range does not necessarily imply that the host range of individual isolates is equally wide. A forma specialis or an isolate of a powdery mildew fungus may be confined to one plant species, such as the isolates of *E. cichoracearum* on tomato (Abiko, 1983) and tobacco (Reddy *et al.*, 1979), and *Sphaerotheca fuliginea* on eggplant (Abiko, 1978, 1982). Other formae speciales or isolates may have a wider host range. For instance, an isolate of an *Erysiphe* sp. on eggplant may also infect tomato, tobacco and, to some extent, cucumber (Whipps and Helyer, 1994). In addition, cucumber, melon and courgette are as susceptible to an isolate of *S. fuliginea* f. sp. *lycopersicum* as tomato (Angelov *et al.*, 1993). Host range studies are laborious and contaminations may easily occur. In addition, too low number of genotypes per plant species tested and climate conditions in greenhouse, *etc.* may also obstruct the determination of host range (Niks, 1987). Hence, accurate information on host range is often lacking.

In tomato (*Lycopersicon esculentum*), powdery mildews have frequently been reported to occur in all climatological regions of the world (Hirata, 1966). They belong to three species. Firstly, the polyphagous species *Erysiphe orontii* Cast. (also known as *E. cichoracearum* DC. *pro parte* and as *E. polyphaga* Hammarl., see Braun, 1987), is widely distributed over tropical and temperate regions (Wicks and Clare, 1981; Price, 1981). It is ectophytic and grows on both sides of leaves and on stems. It is characterized by the formation of catenary conidia. Its anamorph state "*Oidium*" produces conidia in chains. Secondly, *Leveillula taurica* (Lév.) G. Arn. is more prevalent in the tropics and subtropics (Price, 1981). It is characterized by the development of endophytic mycelia, growing in and between the mesophyll cells of tomato leaves. Its conidiophores, producing solitary pear-shaped conidial spores, arise from the internal mycelia (through stomata) and appear as a white mould at the underside of the leaves (Correll *et al.*, 1987). Very likely, a third powdery mildew species has been frequently reported in the Northern hemisphere. It belongs to the order of Erysiphales but, as no perfect stage has been described yet, it is preferably designated as *Oidium lycopersicum* Cook & Mass., a powdery mildew that was described in 1888 (*e.g.* Noordeloos and Loerakker, 1989; Whipps *et al.*, 1998). The fungus is entirely ectophytic and grows only on the upper side of the leaves and, on severely infected plants, also on the stems. Its conidiophores arise from the external mycelia, and produce solitary conidia,. This fungus differs from *E. orontii* mainly because 1) it produces solitary conidia while *E. orontii* produces catenary conidia and, 2) the length/width ratio of *O. lycopersicum* conidia is larger than two, while that of *E. orontii* is less than two. Still, several authors have designated the causal agent as *E. polyphaga* or *E. cichoracearum* (Corbaz, 1993; Bélanger amd Javis, 1994; Boiteux, 1994), or *E. orontii* Castagne (Cook *et al.*, 1997). However, in none of these studies the perfect stage has been described and consequently, these designations are erroneous. This has certainly led to conflicting data and misinterpretations. As long as no convincing evidence about a perfect stage is presented we used the species name *O. lycopersicum* in our earlier publications but later we refer to this pathogen as *O. lycopersici* as has been recommended by The International Code of Botanical Nomenclature (Mieslerová and Lebeda, 1999). As in this thesis we focus on *O. lycopersici*, this species is described in more detail below.

O. lycopersici

- Occurrence

O. lycopersicum Cook & Mass. (= *O. lycopersici*) on tomato has already been described in Australia in 1888, but later reports on its occurrence are scarce (Blumer, 1967). Surprisingly, only since late in the 1980's, outbreaks of tomato powdery mildew in greenhouses and fields

have been frequently reported around the world (e.g. Mieslerová and Lebeda 1999). The first occurrence was reported in the Netherlands (Simonse, 1987; Paternotte, 1988), then in France (Blancard, 1988), UK (Fletcher *et al.*, 1988) and Germany (Gabler *et al.*, 1990). In a short period this powdery mildew spread to most of the European countries: Sweden (Forsberg, 1989), Czech Republic (Lebeda and Rod, 1990), Italy (Aloi and Garibaldi, 1990; Stravato, 1993), Switzerland (Corbaz, 1993), Greece (Vakalounakis and Papadakis, 1992), Bulgaria (Neshev, 1993), Poland (Kozik, 1993; Sobolewski and Robak, 1994), Romania (Puscasu and Cristu, 1994), Slovak Republic (Paulech, 1995, cited by Mieslerová and Lebeda, 1999), Hungary (Kiss, 1996; Milotay and Dormanns-Simon, 1997) and Russia (Ignatova *et al.*, 1997). At the same time it appeared in Canada in 1993 (Bélanger and Jarvis, 1994), which is considered as the first report in North America, and in different areas of the USA (Arredondo *et al.*, 1996; Karasevicz and Zitter, 1996; Smith *et al.*, 1997; White *et al.*, 1997; Pernezny, 1998). Meanwhile, its appearances were also reported in Asia (LL Black, pers. comm.; XQ Zheng, pers. comm.; Kumar *et al.*, 1995) and in South America (Mendoza-Zamora and Meza, 1990; Boiteux, 1994). Globally, it has become a common disease not only in the protected cultivation in greenhouses, but also in the open production in the field. The cultivated tomato is very susceptible to the disease. When conditions are favourable large areas can become infected in only several weeks. Though the fungus can routinely be controlled by fungicides, this is not desirable for a safe and healthy production. Therefore, development of resistant cultivars is required.

- Origin

After its first description in Australia in 1888, *O. lycopersici* had hardly been documented in Europe (Blumer, 1967) before the new outbreak in 1986. It is an intriguing question how the fungus could remain nearly unnoticed over a century and suddenly spread over the world. Was the fungus always present at a low density, but did a more aggressive mutant arise? Did the spread and increase of the tomato production on a global scale generate more favourable environments for the fungus? Alternatively, the pathogen may originate from the centre of origin of tomato in South America, and be imported inadvertently to the areas of tomato cultivation. But this seems not likely because it has not been reported in Mexico and the Andes region. Another possibility is that a powdery mildew "jumped" from its host species to tomato by the acquisition of pathogenicity to the latter species, as has been documented for other pathogens like *Monilinia* (Sclerotiniaceae) (Holst-Jensen *et al.*, 1997), pitch canker (*Fusarium subglutinans* f. sp. *pini*) (Storer *et al.*, 1994) and rust (Uredinales) (Savile, 1971; Baum and Savile, 1985). In order to find out whether there is/are such "jump(s)", host range study is required to search for the relationship between *O. lycopersici* and other powdery mildew species and thus to provide clues to the possible "jump(s)".

- Host range

In their host range study, Whipps *et al.* (1998) considered any accession or species that supports sporulation to any extent as evidence for an alternative host of tomato powdery mildew. As a consequence, many plant species are listed as hosts of *O. lycopersici* (Whipps *et al.*, 1998). In general, the reported host ranges of tomato powdery mildew are still inconsistent. For instance, it includes, in some reports, *Solanaceae* species (Fletcher *et al.*, 1988) but in other reports cucumber and melon (Ignatova *et al.*, 1997; Corbaz, 1993) (see also Chapter 3). These differences are likely due to inconsistencies in the bioassays like differences in genotypes of tested species, or in environmental conditions or even contamination by other powdery mildew species, or in the definition of "susceptibility". Because powdery mildews are air-borne, any disease test experiment on host range study should be carried out in an isolated way, preferably under spore-proof conditions (see also Chapter 3). Some authors consider any plant species, on which the fungus can grow to some extent, as host of that fungus. **We only consider a plant species as a host of *O. lycopersici* if the fungus can repeatedly successfully reproduce on this plant species.**

- Maintenance of *O. lycopersici*

Several methods have been described in literature about storing spores of biotrophic fungi under extreme cold conditions (e.g. Dahmen et al., 1983). But there is no protocol available for storing powdery mildew fungi like *O. lycopersici*. It has to be maintained and propagated on tomato plants. As this is time and space consuming, and always implies the risk of contamination and shift in pathogen population identity, a protocol for storage of *O. lycopersici* still needs to be developed.

Resistance of tomato to *O. lycopersici*

The cultivated tomato is susceptible to *O. lycopersici* (Kozik, 1993; Neshev, 1993; Teubner and Neuhaus, 1993). In wild *Lycopersicon* species many resistant accessions have been identified (Kozik, 1993; Laterrot and Moretti, 1993; Neshev, 1993; Lindhout *et al.*, 1994). The resistance to *O. lycopersici* in *L. hirsutum* G1.1560 is monogenic and incompletely dominant. The resistance gene from G1.1560 has been named *Ol-1*, and mapped on chromosome 6 (Van der Beek *et al.*, 1994). Resistance in *L. esculentum* var. *cerasiforme* accession LA1230 (plant LC-95) is controlled by a recessive gene, *ol-2* (Ciccarresse *et al.*, 1998). The inheritance of resistance in most other accessions is still unknown.

Resistance mechanisms to powdery mildews (*Erysiphaceae*) can be roughly classified as pre- and post-haustorial. Pre-haustorial resistance is based on prevention or reduction of haustorium formation and is not associated with plant cell necrosis. This type of resistance has been reported in quantitative race-non-specific types of resistance (Heath, 1981 & 1982; Carver and Carr, 1977). Post-haustorial resistance is based on defence mechanisms that are elicited after a haustorium (initial) is formed, and is usually associated with plant cell necrosis (hypersensitive response, HR). This type of resistance usually inherits qualitatively and typically is race-specific (Heath, 1981 & 1982). In general, quantitative race-non-specific resistance is supposed to be more durable than the qualitative race-specific one. In order to facilitate breeding programmes, it is necessary to analyze the inheritance of resistance and to map the corresponding resistance genes in some of the resistant accessions, and to investigate the resistance mechanism to predict the durability of resistance.

Scopes of this thesis

The aims of this study were i) to investigate the resistance mechanism in (wild) tomato and other horticultural crop species, and to determine the host range of *O. lycopersici*; ii) to assess the genetic variation of the field isolates of the causal agent(s) collected world-wide; iii) to unravel the inheritance of resistance in two or three resistant accessions and to map the resistance genes by using molecular markers. All experiments are presented in this thesis, Chapters 2-8, and are summarized as follows.

Chapter 2 describes the development of *O. lycopersici* on susceptible cv Moneymaker and characterizes the defence response to *O. lycopersici* in three wild tomato species (*L. hirsutum, L. parviflorum* and *L. peruvianum*), and in several resistant advanced breeding lines (ABLs) carrying *Ol*-genes.

In Chapter 3, the susceptibility of 56 accessions from 25 plant species to a Dutch *O. lyco-persici* isolate is evaluated to see whether there is/are alternative host(s) of this pathogen. Moreover, in order to understand better the interaction between *O. lycopersici* and plant species outside the genus *Lycopersicon*, the infection process of the fungus on, and the responses of, these plant species is investigated histologically.

In Chapter 4, genetic variation of tomato powdery mildew isolates from Canada, Czech Republic, France, Hungary, the Netherlands and USA is assessed by using AFLP analysis. Also, the AFLP fingerprints and morphological data (size of spores, spore arrangement) of tomato powdery mildew were compared with those of 12 other powdery mildew species, in order to find out the possible origin of tomato powdery mildew.

In Chapter 5, fine-mapping of *Ol-1* is done by using a newly generated F_2 population (N=150) of Moneymaker × *L. hirsutum* G1.1560 and 15 additional RFLP markers which co-segregate with the resistance gene *Ol-1*. Bulked segregant analysis (Michelmore *et al.*, 1991) with RAPDs was applied as a bridge to develop SCARs (Paran and Michelmore, 1993) that would serve as convenient and diagnostic PCR markers in commercial breeding programmes. The obtained SCARs will provide a key tool in rapidly detecting the resistance locus in practical breeding and future research.

In Chapter 6, *Ol-1* and *Ol-3* are compared. This chapter describes i) inheritance of resistance to *O. lycopersicum* in *L. hirsutum* G1.1290, one of the resistant *L. hirsutum* accessions, and mapping of the resistance gene, designated *Ol-3*, ii) fine mapping of *Ol-1* which originated from *L. hirsutum* G1.1560, another resistant accession of *L. hirsutum*, and iii) tests of allelism for resistance in G1.1290 and G1.1560.

In Chapter 7, the inheritance of resistance to *O. lycopersici* in *L. parviflorum* G1.1601 is investigated by using an F_2 population. Then a genetic linkage map based on AFLP markers was constructed. Furthermore, QTL analysis for the resistance is performed by using the disease evaluation data and the obtained AFLP map. Finally, the level of resistance of plants containing different doses of resistant alleles/QTLs was compared.

Chapter 8 describes the inheritance of resistance of *L. peruvianum* LA2172 to *O. lycopersici* based on the segregation ratio of an LA2172 derived "F_2", determined by a disease test.

The last chapter discusses the origin(s) of *O. lycopersici*, the possible gene-for-gene interaction of tomato-*O. lycopersici* system, and the organization of the *Ol*-genes.

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Chapter 2

The resistance to powdery mildew (*Oidium lycopersicum*) in *Lycopersicon* species is mainly associated with hypersensitive response[1]

Huang CC, Groot T, Meijer-Dekens F, Niks RE, Lindhout P and Stam P

Chapter 2

The resistance to powdery mildew (*Oidium lycopersicum*) in *Lycopersicon* species is mainly associated with hypersensitive response

Huang CC[1], Groot T, Meijer-Dekens F, Niks RE, Lindhout P

Department of Plant Breeding, The Graduate School of Experimental Plant Sciences, Wageningen Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands. Fax: +31-317-483457.
[1]To whom correspondence should be addressed.

Abstract

The cultivated tomato (*Lycopersicon esculentum*) is susceptible to powdery mildew (*Oidium lycopersicum*). Six accessions of three related *Lycopersicon* species show high levels of resistance (Lindhout *et al.*, 1994b). The present research aimed at describing the development of *O. lycopersicum* on susceptible cv Moneymaker and characterizing the defence response to *O. lycopersicum* in *Lycopersicon* accessions by histological analysis. Spore germination and (primary) haustorium formation in resistant accessions were as frequent as in the susceptible *L. esculentum* cv Moneymaker. A high frequency of necrosis of epidermal cells in which a haustorium was formed appeared to be the major defence response, indicating that resistance to *O. lycopersicum* in the *Lycopersicon* genus was predominantly based on the hypersensitive reaction. However, the resistance in *L. parviflorum* was less associated with hypersensitivity than in other resistant accessions, suggesting the existence of a different but still unknown resistance mechanism. In addition, evidence is provided that the level of resistance could depend on the genetic background and the plant age.

Key words: *Lycopersicon*, powdery mildew, *Oidium lycopersicum*, resistance, hypersensitive response

Introduction

Since 1986 the occurrence of powdery mildew caused by *Oidium lycopersicum* Cooke &
Massee (Noordeloos & Loerakker, 1989) has been frequently reported in greenhouse tomato
crops in Western Europe (Lindhout *et al.*, 1994b). The disease has also spread rapidly to Eastern
Europe: in 1989 in Bulgaria (Neshev, 1993) and soon afterwards in Poland (Kozik, 1993).
Screening of a large collection of cultivated and wild *Lycopersicon* accessions has shown that
the cultivated tomato was susceptible to *O. lycopersicum* (Lindhout *et al.*, 1994b; Kozik, 1993;
Teubner *et al.*, 1993; Neshev, 1993 and Burgerjon *et al.*, pers. comm.[1990]). High levels of
resistance were found in *L. hirsutum* (PI247087) (Laterrot and Moretti, 1993), (G1.1257,
G1.1290, G1.1560 and G1.1606=CPRO742208), in *L. parviflorum* (G1.1601=CPRO731089)
and in *L. peruvianum* (LA2172) (Lindhout *et al.*, 1994b). *L. hirsutum* LA1775 and *L. pennellii*
LA716 were completely and moderately resistant, respectively (Kozik, 1993). Moreover, after
natural infection, immunity was observed in three accessions of *L. hirsutum* (var. *glabratum*, f.
typicum and one unknown accession), and in one accession each of *L. peruvianum* (var.
glandulosum), *L. chmielewskii* and *L. minutum* (Neshev, 1993).

The resistance of *L. hirsutum* G1.1560 to *O. lycopersicum* is controlled by an
incompletely dominant gene, *Ol-1*, on chromosome 6 near the RFLP markers TG25 and GP79
(Van der Beek *et al.*, 1994). The resistance in *L. parviflorum* G1.1601 may be controlled by a
recessive gene, provisionally designated *ol-2* (Lindhout *et al.*, 1994a), but more recent research
suggested a polygenic inheritance of the resistance (unpublished). The inheritance of resistance
in other accessions such as in *L. hirsutum* G1.1290 and *L. peruvianum* LA2172 is under
investigation.

Oligogenic and incomplete resistance to *O. lycopersicum* derived from an interspecific
hybrid of tomato x *L. hirsutum* PI247087 has been integrated into different varieties (Laterrot
and Moretti, 1995). In addition, a commercial hybrid (DRW 4061) with resistance to *O.
lycopersicum* has recently been released (Nunnink, 1996).

Various resistance mechanisms to powdery mildews (*Erysiphaceae*) and rusts have been
reported, and can be roughly classified as pre- and post-haustorial. Post-haustorial resistance is
usually associated with plant cell necrosis (hypersensitive reaction, HR). This is the mechanism
typical of the major genic race-specific resistance, e.g. in cereals to powdery mildews (Koga *et
al.*, 1990; Tosa and Shishiyama, 1984a) and to rust fungi (Heath, 1981 & 1982; Niks and
Dekens, 1991). With prehaustorial resistance formation of haustoria is prevented or reduced by
papillae and is not associated with plant cell necrosis. This type of mechanism has been reported
in quantitative race-non-specific types of resistance (Heath, 1981 & 1982; Carver and Carr,
1977), and also in plants inoculated with non-pathogenic ("inappropriate") powdery mildew

fungi (e.g. Carver and Carr, 1977; Tosa and Shishiyama, 1984b). Though most studies focus on one type of resistance, in nature several resistance mechanisms may exist in the same plant-pathosystem (e.g. Tosa and Shishiyama, 1984b; Koga *et al.*, 1990). Research on the histology of the interactions of powdery mildews with their dicotyledonous host plants is scarce. Neger showed in 1923 that resistance to *Erysiphe cichoracearum* was brought about by enclosure of the haustoria of the fungus in a gummy substance which prevented further fungal development (cited by Lupton, 1956). Hypersensitivity seemed to be the prevailing mechanism of resistance in clover varieties resistant to *E. polygoni* (Smith, 1938). However, resistance in pea to *E. pisi* (*Pisum sativum*) is of the prehaustorial type, similar to that in some non-host and partial resistance interactions (Stumpf and Gay, 1989), while resistance in apple to *Podosphaera leucotricha*, another member of *Erysiphaceae*, appeared to be based on inhibition of spore germination, probably by leaf cuticles (Korban and Riemer, 1990).

Macroscopically, resistance to *O. lycopersicum* in wild tomato species is characterized by a very low infection frequency and a strongly restricted mycelium growth and lack of sporulation (Lindhout *et al.*, 1994b). However, the infection process of the fungus and the nature of the defence reaction in the host plants are still unknown. The present paper describes the development of *O. lycopersicum* on susceptible cv Moneymaker and characterizes the histological reactions of three wild tomato species and several resistant advanced breeding lines (ABLs) with *O. lycopersicum*. We report that the resistance in tomato to *O. lycopersicum* is predominantly associated with the hypersensitive response.

Materials and methods

Plant and fungal materials

Five highly resistant accessions *L. peruvianum* LA2172, *L. hirsutum* G1.1257, G1.1290, G1.1560 and G1.1606 as well as one completely resistant accession *L. parviflorum* G1.1601 (Lindhout *et al.*, 1994b) were obtained from the Centre of Genetic Resources, Wageningen, The Netherlands. Seven indeterminate advanced breeding lines (ABLs), originating from *L. hirsutum* G1.1560 (ABL1560.1, ABL1560.2, ABL1560.3), or *L. hirsutum* G1.1290 (ABL1290.1, ABL1290.2, ABL1290.3, ABL1290.4), were obtained from breeding programmes for resistance to *O. lycopersicum*. Moneymaker, as susceptible control, was maintained at the authors' Department.

The stock of *O. lycopersicum* originated from infected commercial tomato plants (Lindhout *et al.*, 1994a), and was maintained on cv Moneymaker plants in a growth chamber at 20±2 °C with 70±5% RH and a 16 hour photoperiod.

Disease tests

Disease tests consisted of three experiments. In Experiment 1, six resistant accessions of wild species were investigated in October 1995. In Experiment 2, the seven ABLs were investigated in November 1995. In Experiment 3, six wild accessions and some ABLs were tested again in July 1996. The experiments were set up according to complete randomized block designs with four (in Experiment 2) or six blocks (in Experiments 1 & 3). Each block contained one plant of every genotype as an experimental unit. Each experiment was divided into two sets, each consisting of two blocks in Experiment 2 and three in Experiments 1 and 3 respectively.

Two inoculation methods were applied. Tomato seedlings at the four true leaf stage or cutting-derived plants with 4 to 5 leaves (designated "older plants") in one set, were inoculated by print-inoculation of three leaflets each of two fully expanded leaves per plant. For this *printing* method a direct contact of the sporulating leaves with the healthy ones was established by gently pressing these leaves together. Plants in another set were inoculated by using a second inoculation method, i.e. *spraying* with a spore suspension (3×10^4 or 12×10^4 conidia.ml^{-1}). The inoculum was prepared by washing conidial spores from freshly sporulating leaves of heavily infected Moneymaker plants in tap water and used immediately. The inoculated plants were grown at 20 ± 3 °C with $70\pm10\%$ RH under natural light supplemented with artificial light to provide a photoperiod of 16 hours per day.

Sampling and staining

For description of the infection process on susceptible tomato plants, leaf segments of each Moneymaker plant were harvested from the print-inoculated leaves at 17, 24, 41, 65, 89, 137 and 185 hours post inoculation (HPI). For investigation of the mechanisms of resistance, leaf segments of each plant were sampled at 41, 65, 96 and 137 HPI. The sampled leaf segments, 1×3 cm in size, were fixed in acetic acid-ethanol (1:3, v:v), and stained with 0.03% trypan blue in lactophenol-ethanol (1:2, v:v) as described by Hering and Nicholson (1964).

Micro- and macroscopic evaluations

Conidia were considered to have germinated when they had formed a germ tube with a primary appressorium or a germ tube of at least half the length of the spore. The percentage of germination was microscopically assessed on a sample of 100 conidia per leaf segment. A germinated spore which produced a primary appressorium or a primary haustorium, was considered as an infection-unit (IU). Thirty infection-units per leaf segment were observed. The presence of a primary haustorium (which was formed by the primary appressorium), the number of hyphae (which were at least as long as the spore), secondary appressoria (formed on hyphae) and secondary haustoria (developed from secondary appressoria) per IU were recorded as well

as the number of secondary appressoria per hypha. The number of hyphae per IU, and of secondary appressoria per IU and per hypha were considered as growth components of *O. lycopersicum*. Also, the presence of cell necrosis and papilla formation was recorded as components of the resistance mechanism. Sporulation on the print-inoculated plants (leaf segments) was also recorded microscopically, according to scales "-" to "++++". Here "-" meant no sporulation, and "±" to "++++" indicated the severity of sporulation from very faint to abundant.

Sporulation on the spray-inoculated plants was evaluated macroscopically at 1, 2 and 3 weeks post inoculation (WPI), according to the same scales as for microscopic evaluations.

Data analysis
The numeric data were statistically analyzed by using the computer software *SPSS*5.0. Duncan's multiple range test (DMRT) was used to compare the means for all possible pairs of genotypes.

Results

Inoculation method
The spray-inoculation with spore suspensions containing either 3×10^4 or 12×10^4 conidia.ml^{-1}, resulted in an even distribution of only a few spores per square centimetre of leaf segment. However, the density of spores was far too low for a reliable microscopic evaluation of the infection process. The print-inoculation resulted in a sufficiently high spore density but a very heterogeneous spore distribution. Even though many spores were clustered and could not be scored, this inoculation method allowed thousands of spores to be scored on each leaf segment. Mock-inoculation by *printing* with healthy leaves did not cause visible damage. Hence, the *printing* method was applied in further experiments.

Infection process of O. lycopersicum on susceptible cv. Moneymaker
Typically, a spore produced a short germ tube, ending in a primary appressorium, from which a primary haustorium was formed. From the primary appressorium or from another pole of the spore, a first hyphae (primary hyphae) arose, that formed small opposite or spread, lobed-shaped (secondary) appressoria from which secondary haustoria arose. Later, the primary hyphae branched to secondary hyphae. We refer to all haustoria and hypha of higher order than primary as secondary. The progress of the infection process of *O. lycopersicum* on the susceptible cv. Moneymaker is presented in Fig. 1. Spore germination and primary appressorium formation started before 17 HPI, but the percentage of germinated spores

Infection process
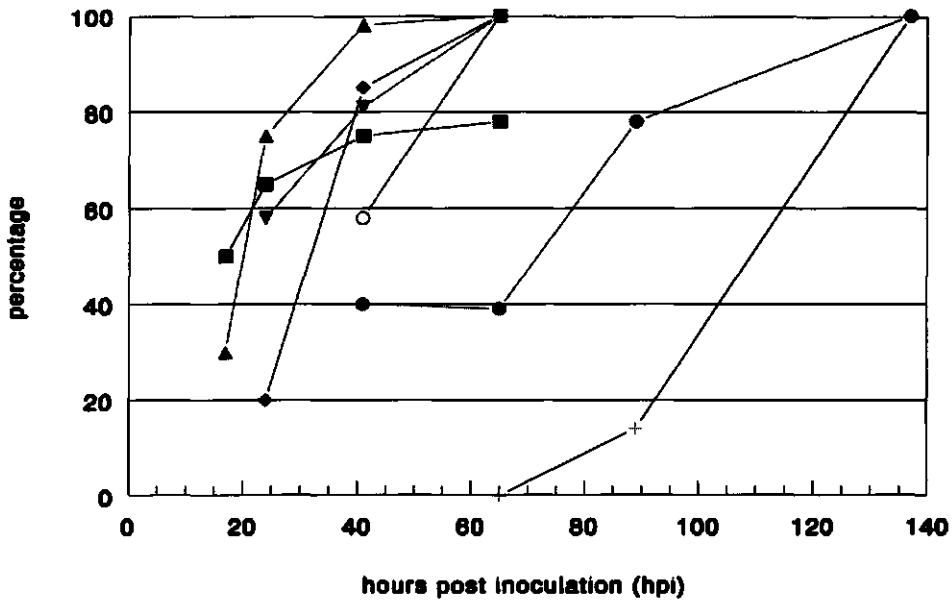
Fig.1 Infection process of *Oidium lycopersicum* on susceptible *L. esculentum* cv Moneymaker

• percentage germinated spores (17-65 HPI)

▲ percentage germinated spores with primary haustoria (17-65 HPI)

▼ percentage germinated spores with primary hyphae (24-65 HPI)

◆ percentage infection-units with secondary hyphae (24-65 HPI)

○ percentage infection-units with secondary appressoria (41-65 HPI)

• percentage infection-units with secondary haustoria (41-137 HPI)

+ percentage infection-units with conidiophores (89-137 HPI)

continued to increase to about 80% at 65 HPI. Per time point there was (great) variation in the percentage of germinated spores between blocks. For example, it varied between blocks from 20% to 75% at 41 HPI in Experiment 1 and from 26% to 81% at 65 HPI in Experiment 3. Within 41 HPI, nearly all primary appressoria had formed a primary haustorium. Primary and secondary hyphae were observed from 24 HPI. On these hyphae appressoria and haustoria were formed first between 24 and 41 HPI. At 65 HPI, branching of secondary hyphae were observed. At 89 HPI, each infection-unit on average had produced 9.8 hyphae which frequently interlaced each other. At that time also the first conidiophores were observed. At 137 HPI, mycelia interlaced extensively, and fully developed conidiophores were observed as erect mycelial structures. At 185 HPI, conidiophores matured and the top cell (the new generation of spores) became swollen like a normal spore and thus might be ready to be released. One vegetative generation cycle of the pathogen was then completed. No further sampling of leaves was carried out.

The compatible infection process elicited plant cell reactions to only a limited extent. At 41 and 65 HPI, 2% to 12% of the (primary or secondary) haustorium-invaded cells became necrotic. Up to 137 HPI, one percent of the appressoria (including primary appressoria) had induced papilla formation. However, even if papillae had been induced, haustorium formation succeeded in 50% of the cells with a papilla.

Infection process of O. lycopersicum *in resistant accessions*
Based on the development of *O. lycopersicum* on Moneymaker, further microscopic observations were focused on the samples harvested at 41 or 65 HPI.

Germination The percentage of spore germination in some accessions varied considerably between blocks. For example, it varied from 10% to 80% at 41 HPI in *L. peruvianum* LA2172, and from 16% to 81% at 65 HPI in *L. hirsutum* G1.1560. However, the percentage of germination was not significantly different between resistant accessions and Moneymaker, and among resistant accessions (Tables 1 & 2). Thus, spore germination was not affected on resistant accessions, indicating that resistance became effective only after spore germination.

Primary haustorium formation At 41 and 65 HPI, at least 70% of the infection-units had formed a primary haustorium. There was no significant difference in the frequency of primary haustorium formation between resistant accessions and Moneymaker (Tables 1 & 2), indicating that resistance to fungal infection did not take place before primary haustorium formation.

Table 1. Development of *O. lycopersicum* on wild *Lycopersicon* accessions, expressed as percentage of germination, formation of primary and secondary haustorium, induction of necrosis by primary and secondary haustorium, number of hyphae per infection-unit (IU) and number of appressorium per IU and per hypha (at 65 HPI unless otherwise indicated)¹

Accession	Germination (%)	Primary haustorium (%)	Secondary haustorium (%)		Necrosis first haustorium (%)		Necrosis second haustorium (%)	No. of hyphae per IU	No. of appressoria per hypha	No. of appressoria per IU	Sporulation²	
			41 HPI	65 HPI	41 HPI	65 HPI					Print	Spray
L.esculentum cv. MM	15a³	94a	45b	82b	12a	3a	3a	3.2c	1.7b	5.5b	+++++	++++
L.peruvianum LA2172	23ab	83a	20ab	39a	45bc	62d	32b	2.1ab	0.9a	1.9a	+	±
L.parviflorum G1.1601	43b	90a	18ab	66ab	24ab	27ab	4a	2.7bc	1.3ab	3.5ab	+++	+
L.hirsutum G1.1257	17a	91a	22ab	58ab	31(28)⁴ab	53(29)cd	20(37)ab	2.5abc	1.4ab	3.2ab	±	-
L.hirsutum G1.1290 ±	25ab	96a	35b	64ab	31(29)ab	50(42)bcd	30(40)b	2.6bc	1.3ab	3.4ab	±	-
L.hirsutum G1.560	13a	92a	9a	37a	65c	66d	32b	1.9a	0.8a	1.5a	+	-
L.hirsutum G1.1606	17ab	97a	42b	62ab	24ab	33abc	19ab	2.5abc	1.1ab	2.9a	++	+

1) Means over three blocks, *Experiment 1*.

2) Sporulation was evaluated microscopically for the print-inoculated plants (leaf segments) and macroscopically (2 WPI) for the spray-inoculated ones. -: no sporulation, ± to ++++ indicated the severity of sporulation from very faint to abundant.

3) Means followed by a different letter in each column are significantly different at the 5% level, determined by *DMRT* after *arcsine* (for percentages, if necessary) or *square root* (for whole numbers) *transformation*.

4) The figures in parenthesis indicate percentages of spreading necrosis over single cell necrosis.

Table 2. Development of *O. lycopersici* on wild *Lycopersicon* accessions and in advanced breeding lines, expressed as percentage of germination, formation of primary and secondary haustorium, induction of necrosis by primary and secondary haustorium, number of hyphae per infection-unit (IU) and number of appressorium per IU and per hypha at 65 HPI[1]

Accession	Germination (%)	Primary haustorium (%)	Secondary haustorium (%)	Necrosis primary haustoria (%)	Necrosis secondary haustoria (%)[3]	No. of hyphae per IU	No. of appressoria per hypha	No. of appressoria per IU	Sporulation[2]
L.esculentum cv.MM	61c	99a	39a	1a	4	4.0d	0.7a	2.90b	++++[4]
L.peruvianum LA2172	44abc	91a	15a	39(8)cde	88	2.0a	0.7a	1.40a	±
L.parviflorum G1.1601	40abc	76a	4a	32(21)bcd	33	3.0bcd	0.7a	2.07ab	-
L.hirsutum G1.1257	64c	97a	18a	58(17)e	72	3.3bcd	0.7a	2.50ab	-
L.hirsutum G1.1290	52abc	93a	18a	39(7)cde	59	3.7cd	0.6a	2.13ab	-
L.hirsutum G1.1560	51abc	99a	18a	47(14)de	58	3.2bcd	0.8a	2.57ab	-
L.hirsutum G1.1606	51abc	94a	17a	21(9)bc	27	3.8d	0.7a	2.87b	-
ABL1560.1 (s)	54abc	91a	12a	13b	36	3.2bcd	0.7a	2.10ab	+
ABL1560.1 (o)[5]	23a	97a	14a	55(22)de	84(12)	2.4abc	0.7a	1.73ab	±
ABL1560.2 (s)	47abc	92a	8a	19(2)bc	50	3.4bcd	0.7a	2.47ab	+
ABL1560.2 (o)	25ab	98a	13a	27(12)bcd	64	3.2bcd	0.7a	2.27ab	±
ABL1560.2 (o)	39abc	88a	22a	36(12)bcde	54	2.8abc	0.7a	1.77ab	-
ABL1560.3 (o)	39abc	99a	4a	47(17)de	67	1.9a	0.8a	1.53a	±
ABL1290.4 (o)	45abc	88a	35a	48(28)de	91	2.4abc	0.7a	1.70ab	+

1) Means over 3 blocks, *Experiment 3*; other notes are identical to those in Table 1.

2) Sporulation was evaluated macroscopically at 2 WPI by spraying.

3) The data were not statistically analyzed, because secondary haustoria were often not present, causing many missing values.

4) No difference in sporulation was observed between seedlings and older plants of Moneymaker.

5) Older plants (o) derived by cutting from the corresponding ABL seedlings (s).

Hypha and appressorium formation Compared to susceptible cv Moneymaker, significant reductions in the fungal growth components (i.e. number of hyphae per IU, and of secondary appressoria per IU and per hypha) were observed in *L. peruvianum* LA2172. The reductions in *L. hirsutum* G1.1560 were also great but not always significant (Table 1 & 2). This indicated a strong reduction in the growth and development of the fungus after primary haustorium formation in these two resistant accessions. No significant difference in appressorium formation between the other four accessions and Moneymaker was observed. This illustrated that resistance in these accessions did not significantly influence the early development of the fungus. Furthermore, similar hypha production on the resistant accessions and Moneymaker revealed that the resistance acted at a later stage of fungal development.

Cell necrosis In all resistant accessions, many epidermal cells in which a primary haustorium was formed became necrotic, indicating a hypersensitive response (HR). Compared to Moneymaker, the percentage of haustoria which induced (single cell) necrosis was significantly higher in *L. peruvianum* LA2172 and in *L. hirsutum* G1.1560 at 41 HPI, and in most resistant accessions except for *L. parviflorum* G1.1601 and *L. hirsutum* G1.1606 at 65 HPI (Table 1). In another experiment (Table 2), HR was observed much more frequently in all resistant accessions than in Moneymaker, though the level of hypersensitivity differed among resistant accessions. In both experiments, the frequency of HR was much lower in *L. parviflorum* G1.1601 and in *L. hirsutum* G1.1606 than in the other four resistant accessions at any stage after haustorium formation. In G1.1257 and G1.1290, necrosis was also observed in the cells adjacent to the haustorium-invaded cells (spreading necrosis). However, this variation of spreading necrosis between resistant accessions was not repeatable (Table 1 & 2).

Secondary haustorium formation The frequency of secondary haustorium formation varied among resistant accessions. For example, the number of secondary haustoria formed in *L. peruvianum* LA2172 and in *L. hirsutum* G1.1560 at 65 HPI in one experiment, was significantly lower than that in other resistant accessions and in Moneymaker (Table 1). But no significant difference in secondary haustorium formation between Moneymaker and any resistant accession was observed in another experiment (Table 2), indicating that the fungal growth and development was significantly retarded by resistance mainly after secondary haustorium formation.

Necrosis was also induced by secondary haustoria. As with primary haustoria, the frequency of cells which became necrotic was much higher in resistant accessions than in Moneymaker. The percentage of secondary haustoria which induced necrosis was, again, much lower in G1.1601 and G1.1606 than in the other four accessions (Tables 1 & 2).

Papilla formation Papillae beneath some appressoria were observed at very low frequencies in all accessions including the susceptible Moneymaker. On average, only 0-9% of the appressoria induced papillae. Haustoria were present in at least 50% of the cells where a papilla was induced. Therefore, papilla formation seemed not to be an effective nor a common mechanism of resistance to *O. lycopersicum*.

Sporulation The development of infection-units was not always stopped when epidermal cells, in which primary haustoria were formed, had become necrotic. One or more new hyphae were usually formed on the other side of the germinating spore, when the growth of the primary hypha was blocked in association with necrosis. The secondary hyphae produced new appressoria and subsequently new haustoria. Eventually, all haustoria could be associated with cell necrosis, and the infection-units may have been arrested completely. Therefore, sporulation on print-inoculated plants was considerably poorer in the resistant accessions than in susceptible cv Moneymaker. Only slight sporulation was sometimes observed, micro- and macroscopically, in *L. parviflorum* G1.1601 and in *L. hirsutum* G1.1606. Sporulation on spray-inoculated plants was almost absent in all resistant accessions (Tables 1 & 2).

Infection process of O. lycopersicum *in ABLs*
During disease tests, the inoculated leaves of the resistant accessions died 1-2 weeks after inoculation, probably due to lack of adaptation of these wild species to greenhouse conditions. In order to minimize the effect of leaf senescence on the accuracy of disease evaluation, and to study the resistance response in an *L. esculentum* genetic background, ABLs derived from *L. hirsutum* G1.1560 and 1290 were evaluated in two experiments. The results of Experiments 2 & 3 were similar and only those of Experiment 3 are presented (Table 2). In this experiment the levels of resistance were also evaluated in seedlings and in older plants of some ABLs to study the effect of plant age.

Compared to Moneymaker, no significant reduction was observed in spore germination on the ABL seedlings and on older plants. As in the wild accessions, appressorium formation in ABLs was almost as good as in Moneymaker, irrespective of plant age except for the older plant of ABL1560.3. Primary and secondary haustorium formation during the first 65 hours post inoculation was not reduced in ABLs, regardless of plant age. Less hypha formation was only observed in most of the older plants (Table 2). These observations indicated that the resistance in ABLs also did not significantly affect the early fungal development.

Necrosis of epidermal cells in which primary or secondary haustoria had been formed, was also commonly observed in ABLs. So was spreading necrosis except in ABL1560.1

seedlings (Table 2). Papilla formation was as low as in wild accessions, and again not an important component of resistance. Undoubtedly, the resistance to *O. lycopersicum*, introgressed into cultivated tomato, was mainly associated with HR. Eventually, almost no sporulation was visible on the ABLs.

The levels of resistance in older plants and seedlings were also compared (Table 2). The percentage of germination tended to be lower in the older plants than in the seedlings. The frequency of primary (and secondary) haustoria inducing necrosis was much higher in the older plants than in seedlings. Also, the print-inoculated leaves of all older plants (and ABL1560.2 seedlings) became seriously necrotic and died even one week after inoculation, demonstrating a possible effect of plant age on the level of resistance. Moreover, the frequency of cell necrosis was significantly higher in *L. hirsutum* G1.1560 than in its deriving ABL at the seedling stage. This demonstrated that the levels of resistance might also depend on genetic background.

Discussion

The resistance to *O. lycopersicum* in tomato is clearly not based on the inhibition of spore germination. This is in accordance with reports that the rate of spore germination of *Erysiphe polygoni* (Cirulli, 1976) and *E. pisi* (Singh and Singh, 1983) on resistant pea cultivars was the same as on susceptible ones, but in contrast to the observation that the germination of *E. pisi* conidia was inhibited on resistant pea plants (Reeser *et al.*, 1983). As in pea to *E. pisi* (Singh and Singh, 1983) and in barley to *E. graminis* f.sp. *hordei* (Andersen and Torp 1986), the resistance to *O. lycopersicum* also does not rely on the inhibition of appressorium formation. Also, papilla formation was rare and ineffective and hence not an important defence mechanism to *O. lycopersicum* infection in tomato. This is in contrast to the occurrence and effectiveness of papilla formation in barley to *E. graminis* f.sp. *hordei* (Koga *et al.*, 1990; Clark *et al.*, 1995) and to an inappropriate *forma specialis E. graminis* f.sp. *tritici* (Tosa and Shishiyama, 1984b), in oat (*Avena* sp.) to *E. graminis* f.sp. *avenae* (Carver and Carr, 1977) and in pea to *E. pisi* (Stumpf and Gay, 1989). Apparently, the importance of papilla formation to the resistance mechanism is pathosystem dependant.

As in many other pathosystem (e.g. Greenberg, 1997; Moerschbacher and Reisener, 1997), the resistance to *O. lycopersicum* in the wild resistant accessions and in the advanced breeding lines is posthaustorial. This resistance is clearly associated with a hypersensitive response. This HR is often not confined to the cells in which the haustorium is formed, but may also spread to the adjacent cells. This spreading necrosis was also observed in the resistance of clover to *E. polygoni* (Smith, 1938), barley to powdery mildews (e.g. Toyoda *et al.*, 1978; Koga *et al.*, 1990; Aist and Bushnell, 1991) and several crop species to rust (Heath, 1981).

Posthaustorial resistance associated with hypersensitivity usually indicates a race specific major genic resistance (Heath 1981 & 1982). The posthaustorial resistance to *O. lycopersicum* in tomato might be race-specific, like in many other pathosystems. The resistance originating from *L. hirsutum* G1.1560 has been proven to be monogenic (Van der Beek *et al.*, 1994), and may also be race specific and based on a gene-for-gene interaction. However, this remains to be demonstrated. Till now, there is no evidence that *O. lycopersicum* consists of races that differ in their ability to infect tomato lines with the various *Ol* genes.

There is evidence that the level of resistance to *O. lycopersicum* is affected by the genetic background and by plant development stage. For instance, the frequency of necrosis of epidermal cells, in which the haustorium was formed, tended to be lower in *L. esculentum* background (ABLs) than in their wild species, and in seedlings than in older plants. This is in agreement with the observation that the level of resistance to *E. graminis* f.sp. *avenae*, originating from resistant wild species, was reduced in an oat cultivar (Carver and Carr, 1977). The observation that cell necrosis was less frequent in ABL seedlings compared to older plants may indicate an influence of plant development stage on the level of resistance to *O. lycopersicum*. This is in agreement with the observation that resistance to *E. graminis* f.sp. *avenae* in oat was expressed more strongly in the fifth than in the first formed leaves (Carver and Carr, 1977). In addition, increase in resistance with age has also been reported in soybean against *Phytophthora megasperma* var. *sojae* (Paxton and Chamberlain, 1969; Ward *et al.*, 1981), *Ph. megasperma* f. sp. *glycinea* (Bhattacharyya and Ward, 1986) and soybean rust *Phakopsora pachyrhizi* (Melching *et al.*, 1988), and in North American cultivars of cowpea (*Vigna unguiculata*) to the cowpea rust fungus *Uromyces vignae* (Heath, 1994). Genes for complete resistance in cowpea to cowpea rust (Heath, 1994) and QTLs for partial resistance in barley to leaf rust *Puccinia hordei* (Qi *et al.*, 1998) at different plant development stages have been identified. Whether the different levels of resistance observed between young and older tomato plants in the present study is due to activation of different genes still needs to be verified.

Quantitative differences in the level of resistance observed between the wild *Lycopersicon* accessions may be due to the functioning of different genes. For example, the resistance in *L. parviflorum* G1.1601 on which the rate of HR was low, seems to be different from that in other wild accessions, because microscopically, the growth of the fungus during the first 65 h.p.i. was similar to that on Moneymaker, but macroscopically, the resistance was almost complete (also Lindhout *et al.*, 1994b). This resistance which may be polygenic or quantitatively inherited, is supposed to be more durable. In case the resistance of the various origins are governed by different genes, pyramiding these genes in one tomato cultivar may increase the durability of the resistance in this pathosystem.

Acknowledgement

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Chapter 3

Host range of *Oidium lycopersici* occurring in The Netherlands[1]

Huang CC, Biesheuvel J, Lindhout P, Niks RE

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Host range of *Oidium lycopersici* occurring in The Netherlands

Huang CC[1], Biesheuvel J, Lindhout P, Niks RE

Laboratory of Plant Breeding, Graduate School Experimental Plant Sciences, Wageningen University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands.
Fax: +31-317-483457
[1] To whom correspondence should be addressed.

Abstract

Nine accessions of three cucurbit species, 10 of eight legume species, three of lettuce (*Lactuca sativa*) and 34 of 14 Solanaceae species were inoculated with a Dutch isolate of the tomato powdery mildew fungus (*Oidium lycopersici*) to determine its host range. Macroscopically, no fungal growth was visible on sweet pepper (*Capsicum annuum*), lettuce, petunia (*Petunia* spp.) and most legume species (*Lupinus albus, L. luteus, L. mutabilis, Phaseolus vulgaris, Vicia faba, Vigna radiata, V. unguiculata*). Trace infection was occasionally observed on melon (*Cucumis melo*), cucumber (*Cucumis sativus*), courgette (*Cucurbita pepo*), pea (*Pisum sativum*) and *Solanum dulcamara*. Eggplant (*Solanum melongena*), the cultivated potato (*Solanum tuberosum*) and three wild potato species (*Solanum albicans, S. acaule* and *S. mochiquense*) were more heavily infected in comparison with melon, cucumber, courgette, pea and *S. dulcamara*, but the fungus could not be maintained on these hosts. All seven tobacco (*Nicotiana tabacum*) accessions were as susceptible to *O. lycopersici* as tomato (*Lycopersicon esculentum* cv Moneymaker), suggesting that tobacco is an alternative host. This host range of the tomato powdery mildew differs from that reported in some other countries, which also varied among each other, suggesting that the causal agent of tomato powdery mildew in The Netherlands differ from that in those countries. Histological observations on thirty-six accessions showed that the defense to *O. lycopersici* was associated with posthaustorial hypersensitive response.

Key words: Tomato, powdery mildew, *Oidium lycopersici*, host range, host resistance

Introduction

Many species of powdery mildew can grow on more than 100 host plant species. For example, *Erysiphe cichoracearum* has a host range of at least 1753 plant species (Amano, 1986). Such a host range of a powdery mildew species does not necessarily imply that the host range of individual isolates is equally wide. A forma specialis or an isolate of a powdery mildew fungus may be confined to one plant species, such as the isolates of *E. cichoracearum* on tomato (Abiko, 1983) and tobacco (Reddy *et al.*, 1979), and *Sphaerotheca fuliginea* on eggplant (Abiko, 1978; Abiko, 1982). However, there are examples of wide host ranges of other formae speciales or isolates. For instance, an isolate of an *Erysiphe* sp. on eggplant could also infect tomato, tobacco and, to some extent, cucumber (Whipps and Helyer, 1994). In addition, cucumber, melon and courgette were as susceptible as tomato to an isolate of *S. fuliginea* f. sp. *lycopersicum* (Angelov *et al.*, 1993).

On tomato several species of powdery mildew have been reported to occur. One of these species, *Leveillula taurica*, is characterized by the development of endophytic mycelium (Palti, 1988). Another species, *Erysiphe orontii* (also known as *E. cichoracearum* and *E. polyphaga*, see Braun, 1987), is exclusively ectophytic, and characterized by the formation of conidia in long chains. Since 1986, outbreaks have been reported of another, morphologically distinct, species of tomato powdery mildew, both in greenhouses and fields around the world (Mieslerová and Lebeda, 1999). This powdery mildew fungus is ectophytic and differs morphologically from *Erysiphe orontii* and *Leveillula taurica* on tomato (Noordeloos and Loerakker, 1989). The causal agent has been identified as *O. lycopersicum* in some countries (e.g. Noordeloos and Loerakker, 1989; Whipps *et al.*, 1998), but was provisionally designated *Erysiphe* sp. in many other countries. In the present paper we will refer to this species as *O. lycopersici* as has been recommended by The International Code of Botanical Nomenclature (Mieslerová and Lebeda, 1999). The formation of conidia singly is a key character in distinguishing *O. lycopersici* from *E. cichoracearum* and *Sphaerotheca* species, although pseudo-chains of 3 to 8 conidia were sometimes observed in humid conditions (Noordeloos and Loerakker, 1989). Since in all reports, due to the lack of cleistothecia, no complete description of the morphology of the tomato powdery mildew is presented, it remains an open question whether the powdery mildews referred to as "*Erysiphe* sp" (Table 1) may belong to *O. lycopersici*. For example, the tomato powdery mildew in UK, that was reported as *E. orontii* Castagne (Cook *et al.*, 1997), was designated as *O. lycopersicum* by Whipps *et al.* (1998). In our studies (e.g. Lindhout *et al.*, 1994; Huang *et al.*, 1998), the Dutch isolates of the recently occurring tomato powdery mildew, which produce conidiospores singly, were very similar to the *O. lycopersici* as described by Noordeloos and Loerakker (1989). We therefore consider this species as the causal agent of the relatively novel powdery mildew on tomato.

Table 1. Conidiospore arrangement and host range of the novel tomato powdery mildew

Causal agent[1]	Origin	Conidia borne in chain	Susceptibility					Reference
			Tobacco (*N. tabacum*)	Cucumber (*C. sativus*)	Potato (*S. tuberosum*)	Eggplant (*S. melongena*)	Sweet pepper (*C. annuum*)	
Oidium sp	USA-California	yes	+	-	nd	nd	-	Arredondo et al., 1996
Erysiphe sp	USA-Connecticut	no	+	nd	nd	+	nd	Smith et al., 1997
O. lycopersici	Czech Rep.	yes/no*	-	+	nd	nd	nd	Mieslerová and Lebeda, 1999
O. lycopersicum	Russia	nd	+	+	+	nd	-	Ignatova et al., 1997
Erysiphe sp	Canada	nd**	+	nd	nd	nd	nd	Cerkauskas 1997 (pers. comm.)
Erysiphe sp	Hungary	yes	-	-	nd	nd	nd	Kiss, 1996
Erysiphe sp	UK	yes/no	+	-	+	+	-	Fletcher et al., 1988
Erysiphe sp	UK	yes/no	+?	+	nd	+	-?	Whipps and Helyer, 1994
O. lycopersicum	UK	no	(+)	+	-/+	+	-	Whipps et al., 1998
O. lycopersici	NL	no	+	-	±	±	-	present study

Notes:

+ : susceptible, - : resistant; ?: uncertain, ±: not consistently susceptible; (): uncertain, because the pathogen originated from eggplant, and was pathogenic on tomato; nd: not determined; other *Nicotiana* species; ±: not consistently susceptible in all replications; -/+ four out of 44 tested cultivars were susceptible.

* : sometimes in chains but sometimes singly.

**: mixture of conidiospores in chains and singly, observed by the present authors.

[1] Designation as used by the respective authors

The origin of the organism(s) causing the recent outbreaks of tomato powdery mildew in the world is unknown. The pathogen may originate from the center of origin of tomato in South America, and be imported inadvertently to the areas of tomato cultivation. Another possibility is that a pathogen "jumped" from its host species to tomato by the acquisition of pathogenicity to the latter species, as has been documented for other pathogens like *Monilinia* (Sclerotiniaceae) (Holst-Jensen *et al.*, 1997), pitch canker (*Fusarium subglutinans* f. sp. *pini*) (Storer *et al.*, 1994) and rust (Uredinales) (Savile, 1971; Baum and Savile, 1985). Such a jump to tomato may have occurred in one or different powdery mildew species or forms, so that this novel "tomato powdery mildew" may be of one or various origins.

All authors agree that all tomato cultivars are susceptible to this newly occurring powdery mildew. Reports differ on the host range of the pathogen (Table 1). In some locations the host range includes Solanaceae species (Fletcher *et al.*, 1988), and in other locations cucumber (Ignatova *et al.*, 1997) (Table 1) and melon (Corbaz, 1993). These differences might be due to plant genotypes, environmental conditions or the definition of susceptibility. But these results might also indicate existence of genetic variation of the pathogen(s) responsible for the recent outbreaks. Therefore, host range studies may also provide clues to the possible origin(s) of the pathogen(s).

The most extensive studies on host range (and morphology) of the recently occurring tomato powdery mildew were conducted by Whipps *et al.* (1998). In their study, based on morphology, the causal agent of tomato powdery mildew was designated *Oidium lycopersicum* (Table 1, hereafter we refer to it as British *O. lycopersici* isolate). They mainly focused on the early stages of sporulation (two weeks after inoculation) and the morphology of the causal agent on tomato and some alternative hosts. They considered any accession or species that supported sporulation to any extent as an alternative host of tomato powdery mildew. In the present study, we evaluated the susceptibility of 25 plant species to a Dutch *O. lycopersici* isolate to see whether there is/are alternative host(s) of this pathogen. We consider those plant species as alternative hosts of *O. lycopersici* only if they allow the fungus to successfully reproduce for more than one generation. In order to better understand the interaction between *O. lycopersici* and plant species outside the genus *Lycopersicon*, we investigated histologically the infection process of the fungus on, and the responses of, these plant species.

Materials and methods

Plant and fungal material

Fifty-six accessions of 25 plant species were used in this study (Table 2). Eggplant (*Solanum melongena*), sweet pepper (*Capsicum annuum*), cucumber (*Cucumis sativus*), melon (*Cucumis melo*) and courgette (*Cucurbita pepo*) plants were raised in greenhouses at 24±2 °C, lettuce (*Lactuca sativa*) at 18±2 °C, tomato and other *Solanaceae* accessions at 20±2 °C, and legumes at 20±1 °C in a growth chamber with a 16-h day length. The light intensity was 10-40 Watt.m^{-2} in the greenhouses depending on the weather, and at least 20 Watt.m^{-2} in the growth chamber.

Three field isolates of *O. lycopersici* were collected from infected commercial tomato plants at three locations of The Netherlands (Lindhout *et al.*, 1994). The stocks of these isolates were maintained on tomato cv Moneymaker in separate growth chambers at 20±1 °C with 70±3% RH and 16-h day length with the same light intensity as described above.

Inoculation tests

Two inoculation tests (IT) were conducted, according to a complete randomized block design with four blocks for IT1 and six blocks for IT2, to evaluate the susceptibility of these accessions. Each block contained one plant per genotype as an experimental unit. *L. esculentum* cv Moneymaker served as susceptible control. In each test, one to three additional plant(s) of each accession were mock-inoculated with tap water, and added randomly to the blocks of inoculated plants. In these experiments, all plants were inoculated at the four true leaf stage. Plants in two blocks of IT1 and in three blocks of IT2 were inoculated by spraying with a conidiospore suspension (3-4x10^4 conidia.ml^{-1}). The inoculum was prepared by washing heavily infected tomato leaves in tap water and used immediately. Because of their smooth and waxy leaf surface on which inoculum drops easily fell off, all the legume plants were inoculated by shaking the sporulating tomato leaves above them. To ensure a high density of conidiospores on leaf segments for histological studies, at least three leaves per plant (except for legumes) in other blocks of each test were print-inoculated by gently pressing *Oidium*-infected tomato leaves onto the healthy leaves (Huang *et al.*, 1998). The inoculated plants were grown in a well-isolated greenhouse at 20±2 °C with 70±10% RH under natural light supplemented with artificial light to 16 hours per day. The light density was 10-40 Watt.m^{-2} depending on the weather.

Sampling and staining

For microscopical study on the infection process of *O. lycopersici*, leaf samples of 1×3 cm^2 were cut at 41 and 65 hours after inoculation (hai) from the print-inoculated leaves of all accessions or some representatives of each species, excluding legumes. They were fixed in acetic acid/ethanol (1:3, v/v), stained in 0.03% trypan blue in lactophenol/ethanol (1:2, v/v), and cleared in a nearly saturated aqueous solution of chloral hydrate (Huang *et al.*, 1998).

Table 2. Degree of susceptibility of different plant species/accessions tested against a Dutch isolate of *Oidium lycopersici*

Immune[1]		Slightly susceptible		Moderately susceptible		Susceptible	
Capsicum annuum (DR)[2]	PI123469	C. melo (DR)	PI125956	S. melongena	PI286107	Nicotiana	904750309
	PI183922		PI136223	(CPRO, De Ruiter &	PI175917	tabacum (BGUN)	904750310
Cucumis melo (DR)	PI187331	Cucumis sativus	PI222782	PGRCU,	PI358232		904750318
Lactuca sativa (CPRO)	PI179895	(DR)	PI206953	Bruinsma)	169328		904750304
	CGN14653		PI204692		169329		904750305
	CGN05237	Cucurbita pepo	Sardanz		169331		934750160
	CGN04884	(CPRO)	Albina	(Rijk Zwaan)	10-73		934750092
Petunia hybrida (BGUN)	804750083		Marha		10-90		Moneymaker
	91475O153		Finale		10-15		
P. nyctaginiflora (BGUN)	95475O063	Pisum sativum (LPB)	Gastro	(Enza Zaden)		Lycopersicon	
	95475O067		Paloma			esculentum (LPB)	
Solanum nigrum (BGUN)	944750095	Solanum dulcamara	914750008	S. tuberosum (LPB)[3]	breeding line		Bonica
Lupinus albus (DA)	00TPA0079	(BGUN)	914750046	S. albicans (BGUN, LT)	PI365376		breeding line
L. luteus (DA)	00TPA0077		924750023				Moneymaker
L. mutabilis (DA)	84TPE0649	S. villosum ssp.	814750090	S. acaule ssp. punae	BGRC7958		
Phaseolus vulgaris (DA)	00TPA0097	puniceum (BGUN)		(BGUN, LT)			
				S. mochiquense	BGRC32672		
Vicia faba (DA)	00TPA0096			(BGUN, LT)			
Vigna radiata (DA)	71TPE0042			S. mammosum	924750111		
V. unguiculata (DA)	72TPE0532			(BGUN)			

1) Immune: no infection; Slightly susceptible: infection only occasionally observed; Moderately susceptible: early sporulation similar to that on Moneymaker but nearly disappearing within two to three weeks; Susceptible: sporulation consistently similar to that on Moneymaker, even several weeks after inoculation.

2) Letters in bracket after each plant species indicate the donor(s) of the accession(s). BGUN-Botanical Garden of the University of Nijmegen, Nijmegen, The Netherlands. CPRO-Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands. LA, LPB & LT-Laboratory of Agronomy, Laboratory of Plant Breeding & Laboratory of Taxonomy, Wageningen University, Wageningen, The Netherlands. PGRCU-Plant Genetic Resources Conservation Unit, University of Georgia, 1109 Experiment Street, Griffin, GA 30223-1797, USA. Bruinsma, Enza Zaden, De Ruiter and Rijk Zwaan are Dutch seed companies.

3) Potato cultivars and breeding lines tested in another project also consistently showed moderate susceptibility.

Macro- and microscopic observations

To determine the susceptibility of each accession (Table 2), sporulation and plant tissue necrosis
were evaluated macroscopically at 7, 10, 14, 21 and 28 days after inoculation (dai). Leaf
samples were analyzed using a phase-contrast light microscope. Fungal growth parameters were
recorded, including percentage of conidiospore germination, percentage of infection units (IUs)
which formed secondary hyphae, number of hyphae per IU, percentage of IUs which produced
secondary haustoria and number of secondary haustoria per IU (Table 3) as described previously
(Huang *et al.*, 1998). Thirty IUs per leaf sample were observed. An infection unit refers to a
germinated conidiospore that produced at least one primary appressorium.

Reproduction of O. lycopersici

To check conidium production of *O. lycopersici* on different plant species, three infected plants
of eggplant and tobacco were separately transferred from the greenhouse to two growth
chambers. Infected leaves of eggplant and tobacco plants were used as inoculum sources to
inoculate tomato cv Moneymaker plants. Transfers of tomato-tobacco-tomato were cycled over
a five-month period. Conidiospore production on tomato, eggplant and tobacco was measured
by applying a drop of 15 µl of 0.5% Tween solution to a sporulating leaf area of about 0.2 cm^2.
This drop of solution was re-collected, and the conidiospore concentration in this drop was
measured by using a haemocytometer. Conidiospore shape and size as well as conidiospore
arrangement (i.e. singly or in chains) were also observed microscopically.

Statistical analysis

All data were statistically processed by ONEWAY model using a computer software SSPS5.0.
Duncan's Multiple Range Test (DMRT) was applied to compare means.

Results

Macroscopical evaluations of susceptibility

In total, 25 plant species were evaluated in two inoculation tests for their susceptibility to a
Dutch *O. lycopersici* isolate. The choice of plant species was mainly based on the earlier studies
listed in Table 1. During ITs, special care such as using over-pressure growth chambers or
greenhouse compartments with spore proof ventilation system, was taken to avoid inadvertent
cross contamination. Since the mock-inoculated plants never showed any sign of infection, the

observed infections were due to the inoculum applied. In general, there was no variation in susceptibility within species, except for melon (*Cucumis melo*). However, a large variation occurred between species. Based on the susceptibility, compared with Moneymaker, the 25 plant species could be grouped into four classes (Table 2). 1) Immune: no infection observed. 2) Slightly susceptible: infection only occasionally observed. 3) Moderately susceptible: early sporulation similar to that on Moneymaker but nearly disappearing within two to three weeks. Within this class, sporulation on eggplant was most abundant compared to that on other species. 4) Susceptible: sporulation consistently similar to that on Moneymaker, even several weeks after inoculation.

Infection process of O. lycopersici

The infection process of *O. lycopersici* on different accessions was mainly investigated from the print-inoculated leaf samples collected at 65 hai, unless indicated otherwise. Because the trend of variation for the fungal growth parameters between the two ITs was similar and there were only two replicates in IT1, we only present data obtained in IT2.

Germination of conidia Significant variation in the percentage of conidiospore germination within plant species was only observed in lettuce and *S. dulcamara* (Table 3). Except for one accession each of melon, sweet pepper, lettuce, two of *S. dulcamara* and all the three of courgette, conidiospores germinated equally well on the remaining plant species or accessions as on the susceptible control Moneymaker. Thus, conidiospore germination was not affected on most of the nonhost plants.

Fungal growth Typically, each conidiospore produced a short germ tube, ending in a primary appressorium. This structure is referred to as an infection unit (IU), from which a primary haustorium was formed. From the primary appressorium or from the other pole of the conidiospore, a first hypha (primary hypha) arose, that formed small opposite, lobe-shaped secondary appressoria from which secondary haustoria arose. Later, the primary hyphae branched to secondary hyphae. We refer to all haustoria and hypha of higher order than primary as secondary (Huang *et al.*, 1998). At 41 hai, 62-100% of IUs had formed a primary haustorium. No significant difference was found in haustorium formation within or between plant species at that time. This indicates that there was no effective prehaustorial resistance. At 65 hai, in general, the highest values of other growth parameters were obtained from tomato (cv Moneymaker), tobacco, eggplant, four wild potato species (*Solanum albicans, S. acaule, S. mochiquense* and *S. mammosum*), melon and cucumber. The development of the fungus on lettuce, sweet pepper and petunia was very poor (Table 3). These observations corresponded well with the macroscopic evaluations (Table 2).

Table 3. Development of *O. lycopersici* on different plant species and accessions, expressed as percentage of germination, induction of necrosis by primary haustorium, percentage of infection-units (IU) forming secondary hyphae, number of hyphae per IU, formation of secondary haustorium and number of haustoria per IU at 65 hai (means over 30 IUs)

Susceptibility class[1]	Plant species and accessions		Germination (%)	Sec. hyphae (%)	#Hyphae	Sec. haustoria (%)	#haustoria	Necrosis (%)[2]
1	*Capsicum annuum*	PI123469	63*bc*[3]	52*efghi*	1.60*defghi*	68*fghijk*	1.8*efghi*	58*hijkl*
		PI183922	47*ab*	3*abc*	0.40*abc*	10*abc*	0.1*abc*	31*efghij*
		PI187331	64*bc*	29*defg*	1.13*de*	36*cdef*	0.6*bcde*	30*efghij*
1	*Cucumis melo*	PI179895	64*bc*	71*hijklm*	2.40*ghijk*	82*ijkl*	3.0*ijk*	30*efghij*
1	*Lactuca sativa*	CGN14653	70*bc*	2*ab*	0.13*a*	6*ab*	0.1*abc*	74*kl*
		CGN05237	57*abc*	1*ab*	0.10*a*	0*a*	0.0*a*	58*ijkl*
		CGN04884	33*a*	0*a*	0.20*ab*	4*ab*	0.1*ab*	64*jkl*
1	*Petunia hybrida*	914750153	87*c*	8*abcd*	0.37*abc*	11*bcd*	0.2*abc*	28*defghij*
1	*P. nyctaginiflora*	954750063	87*c*	23*cdef*	0.70*bcd*	27*cde*	0.4*bcd*	23*cdefgh*
		954750067	88*c*	32*defg*	1.07*def*	37*defg*	0.6*cdef*	21*cdefg*
2	*C. melo*	PI125956	72*bc*	74*ijklm*	2.37*ghij*	88*jklm*	2.7*hij*	10*abcde*
		PI136223	56*ab*	71*hijklm*	2.63*hijkl*	72*ijk*	3.4*ijkl*	18*cdefg*
2	*Cucumis sativus*	PI222782	68*bc*	50*efgh*	1.43*defg*	66*hijk*	2.0*ghi*	40*fghijk*
		PI206953	64*bc*	81*hijklmn*	2.40*ghijk*	91*klm*	3.2*ijkl*	28*defghij*
		PI204692	75*bc*	59*ghijkl*	1.80*efghi*	86*ijkl*	2.5*hij*	7*abcd*
2	*Cucurbita pepo*	Sardanz	55*ab*	52*fghij*	1.77*efghi*	57*efghi*	1.9*ghi*	23*cdefg*
		Albina	56*ab*	35*defg*	1.07*cde*	38*defg*	0.7*cdef*	4*abcd*
		Marba	53*ab*	50*efgh*	1.60*fgh*	61*fghij*	1.4*defgh*	32*efghij*
2	*Solanum dulcamara*	914750008	71*bc*	54*fghijk*	2.23*fghi*	67*ijk*	2.1*ghi*	46*ghijkl*
		914750046	33*a*	34*defg*	1.13*def*	38*cdefgh*	1.3*defg*	22*cdefgh*
		924750023	29*a*	31*defg*	0.90*cde*	32*cdef*	0.6*cdef*	18*bcdef*
2	*S. villosum* ssp. *puniceum*	814750090	63*bc*	15*bcde*	1.05*cdef*	37*cdefgh*	0.5*bcde*	78*l*
3	*S. melongena*	PI286107	88*c*	83*ijklmn*	3.60*jklmn*	89*jklm*	5.4*lm*	24*cdefgh*
		PI175917	89*c*	84*iklmn*	3.60*jklmn*	86*ijkl*	4.8*klm*	17*cdefg*
		PI358232	89*c*	88*lmn*	3.77*klmn*	91*klm*	5.4*lm*	13*bcdef*
3	*S. albicans*	PI365376	88*c*	93*mn*	3.93*lmn*	93*klm*	4.5*jklm*	14*bcdef*
3	*S. mochiquense*	BGRC32672	84*bc*	77*hijklmn*	2.95*ijklm*	82*ijkl*	2.9*hijk*	23*cdefghi*
3	*S. mammosum*	924750111	81*bc*	82*hijklmn*	2.73*hijkl*	81*ijkl*	2.3*ghi*	24*cdefgh*
4	*Nicotiana tabacum*	904750309	81*bc*	96*mn*	3.90*lmn*	92*klm*	3.5*ijkl*	0*a*
		904750310	72*bc*	97*n*	4.27*mn*	94*lm*	4.3*jklm*	4*abc*
		904750318	82*bc*	98*n*	5.20*mn*	97*lm*	5.9*m*	0*a*
4	*Lycopersicon esculentum*	Moneymaker	85*c*	98*n*	4.57*n*	100*m*	5.9*m*	1*a*

1) Class 1: Immune; class 2: Slightly susceptible; class 3: Moderately susceptible; class 4: Susceptible. (see Table 2).

2) Except for tobacco and tomato, almost all infected leaf areas of other plant species became necrotic after 14 dai.

Means followed by a different letter in each column are significantly different at 5% level, determined by *Duncan's multiple range test* after *arcsine* (for percentages) and *square root* (for whole numbers) transformation.

Defense mechanism Papilla formation induced by primary appressoria was only observed on *Petunia hybrida* 804750083 in IT1, which was not tested in IT2 because of lack of seeds. This indicated that papilla formation might be important in the resistance in this petunia accession. In the other resistant accessions, cell necrosis was the predominant response of the epidermal cells to the fungal infection. The percentage of primary haustoria which induced cell necrosis (hypersensitive reaction, HR), varied considerably between accessions (Table 3). Except for tobacco, and one accession each of cucumber and courgette, the percentage of cell necrosis was significantly higher than in tomato. The highest percentage of cell necrosis were recorded in *S. villosum* ssp. *puniceum*, lettuce, sweet pepper, and one accession each of cucumber and *S. dulcamara*. This indicated that the resistance in these accessions was associated with HR. However, since the percentage of cell necrosis in courgette cv Albina, cucumber PI204692 and other petunia accessions was relatively low, non-host resistance of these accessions to *O. lycopersici* may be based on a non-hypersensitive type of resistance.

Asexual reproduction of O. lycopersici

Considering the early abundant sporulation on eggplant and tobacco, cross-inoculation was carried out to confirm the possible adaptation of *O. lycopersici* from tomato to these species. Conidiospores from eggplant and tobacco were used to inoculate tomato plants by print-inoculation. Sporulation on tomato, inoculated with conidiospores from tobacco, occurred as rapidly as from tomato to tomato, enabling a continuous host switch between tomato and tobacco over a five-month period of experiments. In cross-inoculation from eggplant to tomato, sporulation was retarded seven days as compared with that from tomato to tomato. Conidiospores were still produced singly on eggplant and tobacco; the shape and size of conidiospores produced on eggplant and tobacco were similar to that on tomato.

The number of conidiospores produced on tomato, tobacco and eggplant at two to three weeks after inoculation, was 6.0×10^4, 4.5×10^4 and 1.1×10^4 conidiospores.cm^{-2} respectively, when these plant species were inoculated with conidiospores produced on tomato. The number of conidiospores produced on eggplant was significantly lower than on tomato and tobacco, but the difference between tomato and tobacco was not significant. This suggests that tobacco is as good as tomato in supporting *O. lycopersici* reproduction.

Discussion

In the present study, tobacco may be the only species besides tomato that can be considered as host of *O. lycopersici* because the fungus could successfully maintain a polycyclic infection. Though eggplant and some other *Solanaceae* species also supported early infection to some degree, they could not be considered hosts because the fungus could not be maintained on these species. The Dutch isolate was not pathogenic to sweet pepper nor to cucurbits. This was in accordance with the host range reported for a British (Fletcher *et al.*, 1988) and a Californian isolate (Arredondo *et al.*, 1996; Table 1). Like the Californian isolate (Arredondo *et al.*, 1996), the Dutch isolate was not pathogenic to cowpea (*Vigna unguiculata*). The other studies on host ranges either included fewer plant species or gave different results, such as pathogenicity to cucurbits and/or eggplant (Table 1). The differences in host range may be due to different genotypes, environmental conditions and particularly due to the definition of susceptibility (e.g. Whipps *et al.*, 1998 and references herein). In the present study, initially, pea appeared to be susceptible to *O. lycopersici*. Even though most of the inoculation tests were conducted in isolated growth chambers or greenhouse compartments with spore-proof filters, detailed cross-infection experiments showed that this result was due to contamination with pea powdery mildew. This stresses the need for demonstrating host status of a plant species by repeated cross-infection cycles of the fungus between its original host and the other plant species. In our study only tobacco met these criteria and is considered as an alternative host for *O. lycopersici*.

The size and shape of conidiospores of the Dutch *O. lycopersici* isolate formed on tobacco and eggplant were identical to that on tomato. This is in agreement with the constant size of a British *O. lycopersici* isolate on alternative hosts (Whipps *et al.*, 1998). In addition, conidiospores of the Dutch isolate were still produced singly on tobacco and eggplant. This is in contrast to other studies in which conidiospore arrangement varied on different hosts (Fletcher *et al.*, 1988; Whipps and Helyer 1994). Such a morphological change and the lack of the sexual stage make the present taxonomic identification of this powdery mildew fungus ambiguous. Indirect approaches such as host range tests may be helpful to determine the relationship of the tomato powdery mildew with other powdery mildew species (also Cook *et al.*, 1997).

The defense response to *O. lycopersici* in most plant species and accessions tested in this study seems not to be based on the inhibition of conidiospore germination. This is in agreement with the resistance in pea cultivars to pea powdery mildew (Cirulli, 1976; Singh and Singh, 1983) and in barley to its nonpathogenic powdery mildew *E. cichoracearum*, but differs from the resistance mechanism in cucumber to *E. graminis* (Staub *et al.*, 1974). Apparently, leaf properties of the plants are unlikely to be critical to the germination process of *O. lycopersici* and, therefore, are probably not an important factor in determining the host range. The resistance to *O. lycopersici* in the nonhosts is also not based on the inhibition of the formation of a primary haustorium. This resembles the resistance in cucumber to *E. graminis*, but differs from that in

barley to *E. cichoracearum* (Staub *et al.*, 1974). The defense response is associated with a posthaustorial hypersensitive response, and maybe other, non-hypersensitive, defense mechanism(s). This is in agreement with the resistance in cucumber to *E. graminis* (Staub *et al.*, 1974), but in contrast to the common observation that the growth and development of rust and powdery mildew fungi ceases in nonhost plant species before or during formation of the first haustorium (Johnson *et al.*, 1982; Elmhirst and Heath, 1989).

O. lycopersici has been only occasionally mentioned in the literature since its first description in Australia one century ago (Blumer 1967). Apparently, it did not cause heavy damage in tomato until 1986. It is an intriguing question whether the *O. lycopersici* causing the recent outbreaks Europe belongs to the same species as the one found in Australia, or whether it originates from powdery mildews of other plant species. Currently, we are investigating the genetic differences in DNA composition among powdery mildew isolates including tobacco powdery mildew by applying molecular markers. Such DNA fingerprints may provide further indication about the origin of the causal agent(s) of the recently occurring tomato powdery mildew.

Acknowledgements

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Chapter 4

Assessment of genetic variation in tomato powdery mildew by AFLP fingerprinting[1]

Huang CC, Niks RE and Lindhout P

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[1] Submitted.

Chapter 4

Assessment of genetic variation in tomato powdery mildew by AFLP fingerprinting

Huang CC[1], Niks RE and Lindhout P

Laboratory of Plant Breeding, The Graduate School of Experimental Plant Sciences, Wageningen University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands. [1]Current address: Novartis Seeds BV, P.O. Box 2, 1600 AA Enkhuizen, The Netherlands.

Corresponding author: Niks, R. E. e-mail: rients.niks@pv.dpw.wau-ur.nl

Abstract

Tomato powdery mildew has become an important disease in tomato production around the world. Though in some countries such as the Netherlands and the UK the causal agent has been morphologically identified as *Oidium lycopersici*, its identity still remains unclear in many other countries. The origin of the pathogen is unknown. The aim of the present study was to assess the genetic variation of 11 field isolates of tomato powdery mildew from Canada, Czech Republic, France, Hungary, the Netherlands and USA, and to look for a possible origin of tomato powdery mildew by comparing the AFLP fingerprints of tomato powdery mildew with that of 12 other powdery mildew species. We also recorded morphological features of the powdery mildew isolates i.e. the size and the arrangement of conidial spores and compared these data with DNA polymorphisms for taxonomic purposes. We did not find any correlation between AFLP fingerprints and size or arrangement (i.e. solitary or in chains) of conidial spores. Our results demonstrated that 1) *O. lycopersici* isolates have many AFLP bands in common, and thus are considered to be genetically very similar; 2) *O. lycopersici* has hardly AFLP markers in common with other powdery mildew species. We conclude that there was only one anamorph of powdery mildew responsible for the recent outbreaks on tomato.

Keywords: Tomato, powdery mildew, *Oidium lycopersici*, genetic variation, AFLP fingerprinting, similarity analysis

Introduction

Since 1986, tomato powdery mildew (*O. lycopersici*) has become an important disease in
tomato production around the world (Whipps *et al.*, 1998; Huang *et al.*, 2000). Most modern
tomato cultivars are susceptible. Both *Pseudoidium* (*Oidium* with solitary conidia) and
Euoidium (*Oidium* with catenary conidia) anamorphs were repeatedly reported as causal
agents of these recently emerging epidemics. However, published data on the morphology of
tomato powdery mildew were often contradictory which hampers their exact identification
(Kiss *et al.*, 1999). Based on the morphology of the asexual stage, the causal agent in the
Netherlands and the UK has been identified as *Oidium lycopersicum* (Noordeloos and
Loerakker, 1989; Whipps *et al.*, 1998; recently renamed *Oidium lycopersici* by Mieslerová and
Lebeda, 1999[Mieslerová and Lebeda, 1999]), which was described in Australia for the first
time in 1888 and has been only occasionally mentioned in the literature till 1986 (e.g. Blumer,
1967). The origin(s) of the organism(s) causing the recent outbreaks of tomato powdery mildew
in the world is unknown. It is an intriguing question whether the *O. lycopersici* causing the
recent outbreaks at least in the Netherlands belongs to the same species as the one found in
Australia, or whether it originates from powdery mildews of other plant species which extended
their host range to include tomato. Conflicting reports on morphology and host range (e.g.
Whipps *et al.*, 1998; Huang *et al.*, 2000) suggest that more than one powdery mildew species,
form or race are responsible for the recent outbreaks. However, since the morphology of the
fungus is highly variable and its host ranges reported so far vary, it is still doubtful whether
such differences reflect genetic diversity of the pathogen(s) reported worldwide. Therefore,
DNA analysis is a prerequisite to determine the genetic variation among tomato powdery
mildew isolates collected from different geographical regions of the world, and to trace the
possible relationship of *O. lycopersici* with other powdery mildew species. This knowledge may
enable us to speculate which related species is the progenitor of *O. lycopersici*. Because the
number of anamorph(s) of the causal agent will strongly influence breeding strategies,
knowledge of genetic differences of tomato powdery mildew(s) will be of great importance in
tomato breeding programmes for powdery mildew resistance.

AFLP™ is a powerful PCR-based technique for detecting differences between
organisms (Vos *et al.*, 1995). It is advantageous over other molecular techniques like RFLP,
RAPD and rDNA sequence analyses in terms of time consumption, amount of polymorphism
detected, ability to detect small variation within species and reproducibility. It has been
widely used in bacteria, fungi, nematodes, plants and vertebrates. For example, AFLP has
been applied in fungi to detect intraspecific variation between isolates (Arenal *et al.*, 1999;
Kaplan et al., 1999), within a single lesion (Schnieder et al., 1998) and even between spores of
the same isolate (Rosendahl and Taylor, 1997). In addition, AFLP has been used to classify
isolates (Wang *et al.*, 1998), to monitor populations (Justesen and Hovmoller, 1999; Kaplan

et al., 1999) and to construct linkage maps (Van der Lee et al., 1997; Bonants *et al.*, 1998). It can be concluded from these reports that AFLP technique is very powerful in detecting genetic variation within a species and among closely related species.

In the present study, we applied AFLP analyses to determine the genetic variation among tomato powdery mildew isolates from Canada, The Czech Republic, France, Hungary, The Netherlands and the USA, and between tomato powdery mildew and other powdery mildew species. We also determined the size and the arrangement of conidial spores of these powdery mildew isolates, and compared the genetic variation with the morphological variation.

Materials and methods

Fungal isolates

All *O. lycopersici* isolates used are presented in Table 1 and Fig. 1 & 2. Five Dutch *O. lycopersici* (*Ol*) isolates (*Ol*-EZ, *Ol*-DR, *Ol*-gr, *Ol*-PV and *Ol*-RZ) were collected in 1996 from infected tomato plants in greenhouses in three regions at a distance of about 100 km from each other. *Ol*-DR and *Ol*-RZ were from a region in the west of The Netherlands at a distance of 20 km. *Ol*-gr and *Ol*-PV were from Wageningen at 3 km from each other and *Ol*-EZ was from Enkhuizen. To obtain sufficient amount of spores for DNA extraction, they were propagated separately on *Lycopersicon esculentum* cv Moneymaker in spore proof over-pressure growth chambers with a photoperiod of 16 hr, at 20±2 °C and 70±5% relative humidity (RH). Other isolates of tomato powdery mildew were obtained from Canada (*Ol*-Can, 1997), The Czech Republic (*Ol*-Cze, 1997), France (named locally as Et-1, 1998), Hungary (*Ol*-Hun, 1998) and Florida, USA (*Ol*-Flo, 1997), respectively. In addition, a Dutch isolate (*Ol*-tob) originating from *Ol*-DR but propagated on tobacco instead of tomato was included.

All other powdery mildew species used are also presented in Table 1 and Fig. 1 & 2. *Erysiphe pisi* originated from UK had been maintained on pea cv Finale since 1997 under isolated conditions as for the Dutch *O. lycopersici* isolates. Powdery mildews of *Brassica* (*Brassica rapa* = *B. campestris*), lettuce (*Lactuca sativa*) and potato (*Solanum tuberosum*) were collected from the respective naturally infected host plants in greenhouses, and lupine (*Lupinus mutabilis*) in the field of Wageningen University, The Netherlands. Begonia powdery mildew (*Begonia* sp.) was collected from an indoor window-sill begonia plant. Powdery mildews of knot-grass (*Polygonum aviculare*), bear's paw (*Heracleum mantegazzianum*), hemp nettle (*Galeopsis* sp.), plantain (*Plantago major*) and burdock (*Arctium lappa*) were collected from naturally grown plants in the surroundings of Wageningen, the Netherlands. Conidial spores of cucumber (*Cucumis sativus*) powdery mildew (*Sphaerotheca fuliginea*) were obtained in 1996 from the Lab of Phytopathology, Wageningen University, The Netherlands. Conidial spores of tobacco (*Nicotina tobaccum*) powdery mildew (*Erysiphe cichoracearum*) were obtained in 1998 from Plant Protection

Institute of the Hungarian Academy of Sciences, Budapest, Hungary. The conidial spores of the other powdery mildew species were collected during the summer of 1997. All spore samples were stored at –20 °C till DNA extraction.

Morphological measurement

The size of conidial spores, suspended in water, was measured by using a Nikon microscope (400x). Spore arrangement on infected leaves was checked *in planta* under microscope (100x) without any treatment.

DNA extraction

DNA was extracted directly from 20 mg of conidial spores of each isolate by using the cetyltrimethylammonium bromide (CTAB) procedure described by Chen *et al.*(1993) with a small modification: after RNA digestion, only one precipitation step was carried out.

AFLP analysis

The AFLP™ analysis was conducted as described by Vos *et al.*(1995). After digesting the fungal genomic DNA with the rare cutter *Eco*RI (G/AATTC) and the frequent cutter *Mse*I (T/TAA), the DNA fragments were ligated with the corresponding adapters to produce primary templates (Table 1). After ligation, pre-amplification was carried using primers E01 and M01 with one selective base (+1), to generate secondary templates. Secondary amplification (active PCR) was executed using nine primer combinations of primers with two (+2) or three (+3) selective bases: four +2/+2 (E22/M21, E22/M22, E22/M23 and E22/M24), two +2/+3 (E22/M50 and E22/M51) and three +3/+3 (E35/M48, E35/M50 and E35/M59) combinations (Table 1).

Data scoring and similarity analysis

AFLP markers were scored as presence or absence of an amplification products (bands). Similarity was analyzed with the UPGMA by using computer software Treecon (Van der Peer and De Wachter, 1997). Data of the measurement of the spore size were statistically processed by ONEWAY model and Duncan's Multiple Range Test (DMRT) to compare means using a computer software SSPS5.0.

Results

Size and arrangement of conidial spores

In order to determine to what extent the morphology of powdery mildews was diagnostic for classification, the size and arrangement of conidial spores were recorded (Table 2). Based on conidium arrangement (catenary or solitary), these powdery mildews were classified into two

groups: one with solitary conidial spores (Group 1, *Pseudoidium*) and another with catenary
conidial spores (Group 2, *Euoidium*). For the purpose of this paper, we refer to the tomato
powdery mildew isolates as Group1a, powdery mildews with solitary conidial spores but not
from tomato as Group1b and those with catenary conidial spores as Group 2. Therefore, the
powdery mildews were classified into three groups: 1a) tomato powdery mildew isolates -
Pseudoidium; 1b) other powdery mildew with solitary conidial spores - *Pseudoidium*; 2)
powdery mildew with catenary conidial spores - *Euoidium* (Table 2). Though there was a
tendency that conidial spores of Group 1b were longer than that of Groups 1a and 2, a great
variation in the size of conidial spores was observed within each group. The ratio of length
over width of Group 2 tended to be the smallest if compared with the other two groups, which
were very similar to each other. But there was almost no difference of this ratio between
lettuce powdery mildew in Group 2 and other species in Group 1 (Table 2). Therefore, the
size of conidial spores was not sufficiently diagnostic for distinguishing these powdery
mildew species.

Table 1. Sequences of AFLP primers and adapters

Primers/adapters		Sequences[1]
*Mse*I adapter		5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTC AT-5'
M00 (universal primer)		GATGAGTCCTGAG TAA
*Mse*I + 1 primer M01		M00 + A
*Mse*I + 2 primers	M21	M00 + CA
	M22	M00 + CC
	M23	M00 + CG
	M24	M00 + CT
*Mse*I + 3 primers	M48	M00 + CAC
	M50	M00 + CAT
	M51	M00 + CCA
	M59	M00 + CTA
	M62	M00 + CTT
*Eco*I adapter		5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGG TTAA-5'
E00 (universal primer)		GACTGCGTACC AATTC
*Eco*RI + 1 primer E01		E00 + A
*Eco*RI + 2 primer E22		E00 + AC
*Eco*RI + 3 primer E35		E00 + ACA

[1] DNA sequences are always from 5' to 3' orientation unless indicated otherwise.

Table 2. Size and arrangement of conidial spores of powdery mildews (mean of 100 conidia)

Powdery mildew isolates[1]	Origin[2]	Spore arrangement	Length (μm) [3]	Width (μm)	Length/width
Ol-PV (1a)	The Netherlands	Solitary	39.7 (4.49) ghi	20.1 (1.20) ghi	2.04 (0.42) e
Ol-DRg (1a)	The Netherlands	Solitary	38.0 (6.72) fg	19.7 (1.35) efg	1.92 (0.28) cde
Ol-DR (1a)	The Netherlands	Solitary	36.4 (5.72) cdef	19.5 (1.14) efg	1.84 (0.27) bcd
Ol-EZ (1a)	The Netherlands	Solitary	38.1 (4.49) fg	20.1 (0.40) ghi	1.90 (0.23) cde
Ol-RZ (1a)	The Netherlands	Solitary	38.7 (3.30) fgh	20.7 (1.04) ij	1.87 (0.18) bcde
Ol-gr (1a)	The Netherlands	Solitary	35.3 (4.71) bcde	20.3 (0.54) ghi	1.71 (0.23) b
Ol-Can (1a)	Canada (R. Cerkauskas)	Solitary[4]	36.9 (4.22) c ef	19.3 (1.63) ef	1.92 (0.25) de
Ol-Cze (1a)	Czech Republic (A. Lebeda)	Solitary	35.2 (4.31) d	19.3 (1.91) ef	1.83 (0.24) bcd
Ol-E24 (1a)	France (M. Bardin/P. Nicot)	Solitary	33.9 (5.53) ab	18.7 (1.56) d	1.83 (0.33) bcd
Ol-Flo (1a)	USA (J. Scott)	Solitary	35.8 (4.55) cde	19.7 (1.65) fh	1.81 (0.25) bc
E.pisi (1b)	UK (T. Carver)	Solitary	43.8 (4.45) j	17.2 (2.11) b	2.61 (0.58) f
Lupine (*Lupinus mutabilis*) (1b)	The Netherlands	Solitary	37.8 (3.83) efg	19.8 (0.48) efgh	1.90 (0.23) cde
Begonia (*Begonia* sp.) (1b)	The Netherlands (C. Anker)	Solitary	40.7 (6.18) hi	21.8 (1.70) k	1.89 (0.35) cde
Brassica (*Brassica compestris*) (1b)	The Netherlands	Solitary	41.7 (3.08) ij	14.7 (2.55) a	2.88 (0.47) g
Knot-grass (*Polygonum aviculare*) (1b)	The Netherlands	Solitary	46.4 (7.36) k	18.5 (1.20) cd	2.04 (0.42) f
Bear's paw (*Heracleum mantegazzianum*) (1b)	The Netherlands	Solitary	38.0 (3.06) fg	19.9 (0.55) fghi	1.91 (0.15) cde
Burdock (*Arctium lappa*) (2)	The Netherlands	Catenary	32.8 (4.48) ab	21.3 (1.68) jk	1.53 (0.24) a
Hemp nettle (*Galeopsis* sp.) (2)	The Netherlands	Catenary	34.8 (2.91) bcd	20.5 (2.14) g ij	1.70 (0.19) b
Lettuce (*Lactuca sativa*) (2)	The Netherlands	Catenary	32.1 (2.82) a	17.9 (1.79) bc	1.82 (0.29) bcd
Plantain(*Plantago major*) (2)	The Netherlands	Catenary	31.9 (3.54) a	18.9 (1.60) de	1.70 (0.22) b

1) In case the causal agent is not clear to the authors, only the host plant on which it was collected, is mentioned. Abbreviations: *Ol*-PV, *Ol*-DR, *Ol*-EZ, *Ol*-RZ are four Dutch isolates of *Oidium lycopersici* (*Ol*) on tomato maintained in growth chambers; *Ol*-DRg is the same as *Ol*-DR but propagated in a greenhouse (18-25 °C, 60-90% RH); *Ol*-Can, *Ol*-Cze, *Ol*-E24, *Ol*-Flo are isolates collected in Canada, Czech Republic, France and Florida respectively; *Ol*-gr: naturally occurring powdery mildew on tomato in a greenhouse (15-35 °C, 40-70% RH) in Wageningen. Figures in bracket indicate the grouping number.

2) Unless being indicated in the bracket, they were collected by the authors.

3) Figure in bracket is standard deviation. In each column different letters indicate a significant difference at 5% level according to Duncan's multiple range test.

4) Short chains of 3-5 spores were more often observed on tomato leaves infected with *Ol*-Can than with other *Ol* isolates.

AFLP fingerprints

Most molecular studies with fungi have utilized DNA extracted from mycelium. Because powdery mildews are obligate parasites and are difficult to grow on artificial media, DNA was extracted directly from conidial spores using the modified CTAB procedure (4). By using this protocol, an average of 100 ng (10-400 ng) of DNA was obtained from 20 mg of conidial spores. The obtained DNA was used for generating AFLP fingerprints to study the genetic differences among powdery mildew species.

In order to determine the reproducibility of the AFLP technique in generating fingerprints for powdery mildews, identical AFLP analyses were applied to DNA samples extracted on different days but from the same *Ol* isolates (*Ol*-PV and *Ol*-DR) and to DNA templates at different concentrations of the same DNA samples. No difference was observed among the fingerprints when using the DNA samples extracted on different days and the templates at different concentrations of the same DNA (not shown). This indicated that the AFLP technique was reproducible and thus suitable to measure genetic difference between powdery mildews. In addition, AFLP primers with different number of selective bases were compared on same set of DNA templates. In general, the more selective bases used, the fewer amplification products (bands) were observed, but the more polymorphism was detected. For example, fingerprints generated by using a +2/+2 primer combination consisted of more bands than using +3/+3 primer combination, and these fingerprints were identical for most *Ol* isolates except for *Ol*-CZ, *Ol*-tob and *Ol*-can (Fig. 1). When using a +3/+3 primer combination, fewer amplification products but more polymorphisms were obtained (Fig. 1B). In order to select the most informative AFLP markers, particularly among tomato powdery mildew isolates, we choose +3/+3 primer combinations to study the genetic variation among and between powdery mildew species.

The AFLP fingerprints of all powdery mildew species analyzed were very different (hardly any band in common) from each other (Fig. 1B). Thus, there was not any powdery mildew species which had similar AFLP fingerprints as the tomato powdery mildew isolates. This indicates the lack of relatedness between tomato powdery mildew and any other powdery mildew species. Moreover, these fingerprints also could not be classified according to the morphology groups of the fungi. This indicates that AFLP is too sensitive and hence is not useful for genetic similarity studies between powdery mildew species. However, as AFLPs were informative within *O. lycopersici*, further similarity analyses were focused on *Ol* isolates including pea powdery mildew as an out group.

For each of the three primer combinations E35/M48, E35/M50 and E35/M59, the number of AFLP amplification products (bands) scored for the *Ol* isolates was 46 (38-59), 64 (52-75) and 54 (48-64) respectively with a size between 80 and 500 bp. For *E. pisi* with the same primer combinations, the number of AFLP bands was 29, 46 and 39 respectively.
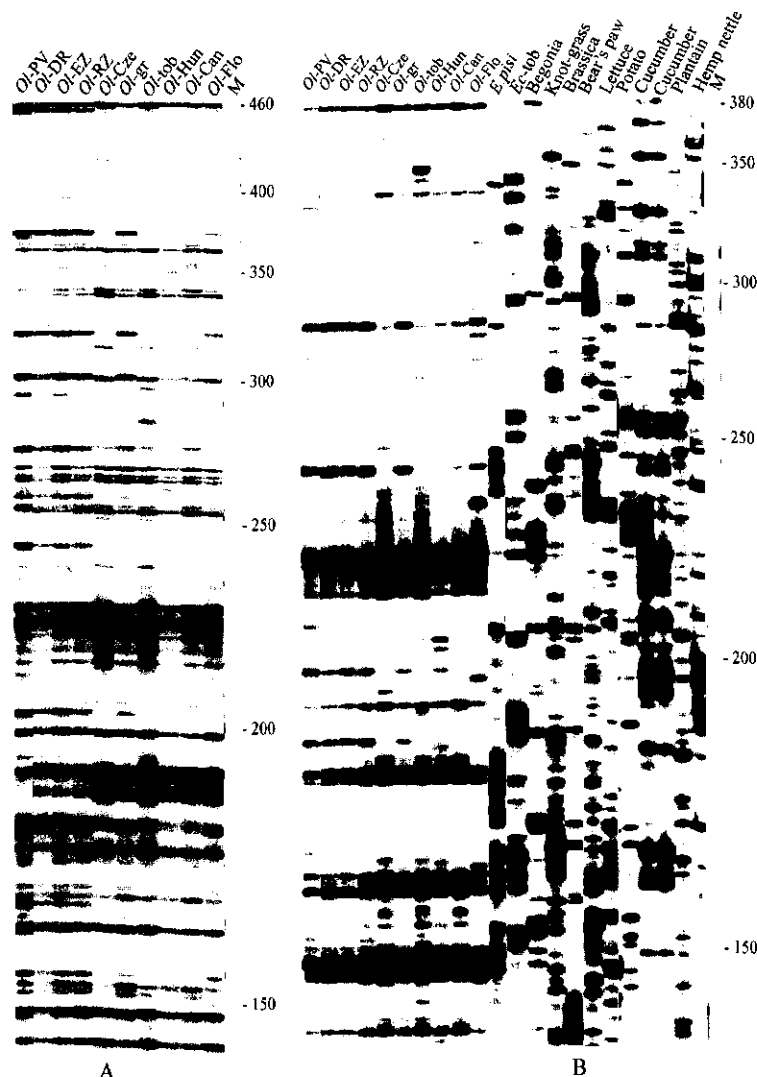
Fig. 1. AFLP fingerprints of powdery mildews generated with E22/M23 (+2/+2) (A) and E35/M48 (+3/+3) (B). M is a marker lane with 10 bp DNA size markers. Abbreviations: *Ol*-PV, *Ol*-DR, *Ol*-EZ, *Ol*-RZ are four Dutch isolates of *Oidium lycopersici* (*Ol*) on tomato maintained in growth chambers; *Ol*-DRg is the same as *Ol*-DR but propagated in a greenhouse (18-25 °C, 60-90% RH); *Ol*-Can, *Ol*-Cze, *Ol*-E24, *Ol*-Flo are isolates collected in Canada, Czech Republic, France and Florida respectively; *Ol*-gr: naturally occurring powdery mildew on tomato in a greenhouse (15-35 °C, 40-70% RH) in Wageningen (also see the legend of Table 2).
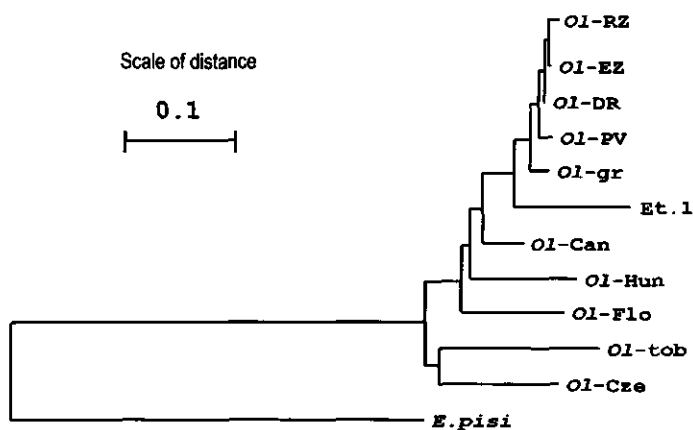
Fig. 2. Similarity analysis of pea powdery mildew (*E. pisi*), in comparison with lupine and pea powdery mildews. *Ol*-PV, *Ol*-DR, *Ol*-EZ, *Ol*-RZ are four Dutch isolates of *Oidium lycopersicum* (*Ol*) on tomato maintained in pollen-proof growth chambers (18-22 °C, 65-75% RH). *Ol*-Can, *Ol*-Cze, Et.1, *Ol*-Flo are isolates collected in Canada, Czech Republic, France and Florida respectively. *Ol*-gr is a spontaneously occurring powdery mildew on tomato in a greenhouse (15-35 °C, 40-70% RH) in Wageningen, the Netherlands. *Ol*-tob is *Ol*-DR but propagated on tobacco instead of tomato plants.

Similarity matrices generated with each of the three primer combinations were highly correlated. Consequently, these data were combined to determine the similarity among the *Ol* isolates. As a result, in total, 396 data points for both the *Ol* isolates and pea powdery mildew were processed to generate a dendrogram (Fig. 2).

In general, all *Ol* isolates were very similar to each other (Fig. 1 & 2). The Dutch *Ol* isolates, which did not show any polymorphism by using the +3/+3 primer combination E35/M48 (Fig. 1B), were more similar to each other than to those isolates from other parts of the world (Fig. 2). In addition, the fingerprints of *Ol*-tob, originated from *Ol*-DR but propagated on tobacco, was not identical to that of *Ol*-DR propagated on tomato (Fig. 1 & 2), while *Ol*-tob retained its pathogenicity to tomato (Huang, *et al*. 2000), suggesting that an isolate can consist of a population of different genotypes and the composition of this population change

upon propagation on a different host. In summary, the AFLP fingerprinting did not provide evidence for different sources of the origins of *Ol* isolates analyzed so far.

Discussion

The arrangement of conidial spores (i.e. either solitary or catenary) has been a key character in powdery mildew taxonomy. Because of this, powdery mildews have been classified as *Pseudoidium* type (with solitary conidial spores) or *Euoidium* type (with catenary conidial spores). However, as other morphological characters, spore arrangement is also highly variable. For instance, long chains of conidia of tomato powdery mildew were produced on eggplant and tobacco while almost no chain was produced on tomato (Fletcher and Smewin, 1988). This indicates an variable conidiophore morphology within tomato powdery mildew, and raises the question of its taxonomic position (Whipps and Helyer, 1994). Pseudo-chains of tomato powdery mildew were sometimes observed on tomato (e.g. Noordeloos and Loerakker, 1989). The powdery mildew species were extremely dissimilar in AFLP pattern. No similarity was detected within either *Pseudoidium* or *Euoidium* species groups. Apparently, AFLP is too sensitive in detecting genetic variation between powdery mildew species. Such poor overall correlation between AFLP fingerprints and conidiophores (solidary - *Pseudoidium* or catenary - *Euoidium*) can have two explanations: 1) conidiophores are highly variable (Fletcher and Smewin, 1988; Noordeloos and Loerakker, 1989; Wang *et al.*, 1998), and 2) the AFLP technique is too sensitive for genetic studies between species.

The present molecular analyses of *Ol* isolates are based on the fingerprints obtained using +3/+3 primer combinations, because +2/+2 primer combinations were not informative enough to distinguish the tomato powdery mildew isolates. However, this result was unexpected as +2/+2 primer combination should be preferred for small genome of organisms like *O. lycopersici* (Vos et al., 1995). For example, +2/+2 primer combinations were applied to assess the genetic variability of isolates of *E. nigrum* (Arenal et al., 1999), and to construct a linkage map of *P. infestans* (Van der Lee et al., 1997). Reports on using +3/+3 primer combinations in AFLP fingerprinting outside plant and animal kingdoms are scarce. Semblat *et al.* (1998), for instance, employed +3/+3 primer combinations to characterize root-knot nematode (*Meloidogyne* sp.) populations.

Though *Ol* is very similar to *Pseudoidium* type of powdery mildews in terms of conidial dimension and arrangement (Table 2), the AFLP fingerprints did not show any similarity between *Ol* and other powdery mildews analyzed (Fig. 1), including tobacco powdery mildew which was speculated as a candidate progenitor of *Ol* based on host range studies (Huang *et al.*, 2000). Therefore, still no conclusion can be drawn about the possible ancestor or origin of *Ol*.

The recent report of tomato powdery mildew of *Euoidium* type in Australia (Kiss *et al.*, 1999), a bio-assay of four field isolates (each from Czech Republic, Germany, England

and The Netherlands) (Lebeda and Mieslerová, 2000) and observations on the interaction of different resistance genes with tomato powdery mildew from the Mediterranean regions suggest the existence of races of the causal agents. However, because of the high variability in fungal morphology and environment, this speculation urgently requires verification by using molecular techniques like AFLP to compare the Australian *Euoidium* type of tomato powdery mildew with the Australian and the Mediterranean *Pseudoidium* type of tomato powdery mildew. Based on AFLP fingerprints and the resulting dendrogram (Fig 1 & 2), all *Ol* isolates analyzed in the present study are very similar to each other, particularly all the Dutch *Ol* isolates, suggesting that a single *Pseudoidium* species is present on tomatoes in Europe and North America. This is in agreement with the results of ITS (internal transcribed spacers) analyses obtained on tomato powdery mildew (Kiss *et al.*, 1999). The small difference among all *Ol* isolates may be due to minor changes in the genetic composition of these isolates resulting from a kind of adaptation to the host and/or environments as has been reported for *Heterodera schachtii* (Kaplan *et al.*, 1999). Such genetic variation has been detected by AFLP between spores of the same isolate of arbuscular mycorrhizal fungi (Rosendahl and Taylor, 1997) and even within a single lesion of *Septoria tritici* (teleomorph *Mycosphaerella graminicola*) in wheat (Schnieder et al., 1998). Therefore, tomato powdery mildew on the Northern Hemisphere might originate from one ancestor and has readily spread over the world, because tomato powdery mildew is air-borne and, thus, has a great potential for widespread, long-distance dispersal, resulting in great genetic uniformity across local populations.

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Chapter 5

Development of diagnostic PCR markers closely linked to the tomato powdery mildew resistance gene *Ol-1* on chromosome 6 of tomato[1]

Huang CC, Cui YY, Weng CR, Zabel P and Lindhout P

Chapter 5

Development of diagnostic PCR markers closely linked to the tomato powdery mildew resistance gene *Ol-1* on chromosome 6 of tomato

Huang CC[1], Cui YY[2,3], Weng CR[2], Zabel P[2] and Lindhout P[1]

[1]Laboratory of Plant Breeding & [2]Laboratory of Molecular Biology, The Graduate School of Experimental Plant Sciences, Wageningen Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands; [3]Department of Plant Pathology, College of Agriculture, Food & Natural resources, 108 Waters Hall, Plant Science Unit, University of Missouri-Columbia, Columbia, Missouri 65211, USA
Fax: +31-317-483457.
[*] To whom correspondence should be addressed.

Abstract

L. hirsutum Gl.1560 is one of the wild accessions which is resistant to *Oidium lycopersicum*, a frequently occurring tomato powdery mildew (Lindhout *et al.*, 1994). The resistance is largely controlled by an incompletely-dominant gene *Ol-1* near the *Aps-1* locus in the vicinity of the resistance genes *Mi* and *Cf-2/Cf-5* (Van der Beek *et al.*, 1994). Using a new F_2 population (N=150) segregating for resistance, the *Ol-1* gene was more accurately mapped between the RFLP markers TG153 and TG164. Furthermore, in saturating the *Ol-1* region with more molecular markers using bulked segregant analysis, five RAPDs were identified that were associated with the resistance. These RAPDs were then sequenced and converted into SCAR markers: SCAB01 and SCAF10 were *L. hirsutum* specific; SCAE16, SCAG11 and SCAK16 were *L. esculentum* specific. By linkage analysis a dense integrated map comprising RFLP and SCAR markers near *Ol-1* was obtained. This will facilitate a map-based cloning approach for *Ol-1* and marker-assisted selection for powdery mildew resistance in tomato breeding.

Key words: Resistance, Tomato powdery mildew, Tomato, Mapping, *Oidium lycopersicum*, RFLP, Sequence-characterised amplified region (SCAR)

Introduction

Tomato powdery mildew caused by the fungus *Oidium lycopersicum* has become a serious disease in the Northern Hemisphere, especially in protected tomato cultivation. At present, almost all tomato cultivars appear susceptible, except for some newly developed commercial hybrids including DRW 4061 (Nunnink, 1996), Belliro and Delito (De Ruiter Seeds catalogue 1998). Applying chemicals can control the disease but, nowadays, such an approach is undesirable in view of the growing need of an environmentally safe production. Moreover, as resistance genes to other pathogens have already been introgressed into tomato, *O. lycopersicum* remains as yet the only fungus to be controlled by fungicides in greenhouse in Northwest Europe.

Resistance sources to *O. lycopersicum* have been identified in some wild species, including *L. hirsutum* (G1.1257, G1.1290, G1.1560, G1.1606=CPRO742208, Lindhout *et al.*, 1994); LA1775, Kozik, 1993; PI247087, Laterrot and Moretti, 1993), *L. parviflorum* (G1.1601=CPRO731089) and *L. peruvianum* (LA2172) (Lindhout *et al.*, 1994). *L. hirsutum* G1.1560, among others, has been studied most extensively regarding inheritance of resistance (Van der Beek *et al.*, 1994), showing the resistance to be controlled by an incompletely-dominant gene, designated *Ol-1*, that maps on chromosome 6 near the *Aps-1* locus in the vicinity of the resistance genes *Mi* and *Cf-2/Cf-5* to *Meloidogyne spp.* and *Cladosporium fulvum*, respectively (Van der Beek *et al.*, 1994). Because of its monogenic and (incompletely-) dominant nature, *Ol-1* can easily be incorporated into modern cultivars by classical breeding in five to nine backcrosses. However, with the help of molecular markers, the same goal would be reached in two to three backcrosses. Therefore, marker-assisted selection (MAS) of *O. lycopersicum* (*Ol*) resistance would be of great help in developing new tomato cultivars carrying *Ol* resistance. As only a few linked markers have been identified so far, additional markers flanking the *Ol-1* region need to be developed to increase the efficiency of MAS. Such a saturated map should also facilitate map-based (positional) cloning of *Ol-1* in the near future.

In order to rapidly obtain markers linked to resistance genes for genetic analysis and for physically characterising the respective regions, Paran and Michelmore (1993) have developed the sequence-characterised amplified region (SCAR) marker that is most suitable for standard PCR analysis. They successfully derived eight SCARs from RAPDs linked to downy mildew resistance gene in lettuce, with three of them being codominant. Since then, SCAR analysis has been widely applied, for instance, to localise genes controlling disease resistance (e.g. Geffroy *et al.*, 1998 and Deng *et al.*, 1997), fruit quality (Fang *et al.*, 1997) and plant development (Jiang and Sink, 1997). It has also been employed in taxonomic studies of plants (Parent and Page, 1998; Bodenes *et al.*, 1996; Roose *et al.*, 1993) and fungi (Francis *et al.*, 1994; McDermott *et al.*, 1994). SCAR analysis has also become a useful technique in practical breeding of various crop species, for example,

banana (Damasco *et al.*, 1998), grapevine (Lahogue *et al.*, 1998), hemp (Mandolino *et al.*, 1999), kiwifruit (Harvey *et al.*, 1998) and orchid (Handa *et al.*, 1998).

In the present study, we analysed a new F_2 population (N=150) of Moneymaker × *L. hirsutum* G1.1560 to identify 15 additional RFLP markers which co-segregate with the resistance gene *Ol-1*. We also applied bulked segregant analysis (Michelmore *et al.*, 1991) with RAPDs as a bridge to develop SCARs (Paran and Michelmore, 1993) that will serve as convenient PCR markers in commercial breeding programmes. Two *L. hirsutum* specific and three *L. esculentum* specific SCAR markers were designed based on the sequences of the RAPDs which co-segregated with *Ol-1*. These SCARs will provide a major tool in rapidly detecting the resistance locus in practical breeding and future research.

Materials and methods

Plant and fungus materials

An F_2 population of 150 plants derived from an interspecific cross between individual plants of the susceptible *L. esculentum* cv Moneymaker and the resistant accession *L. hirsutum* G1.1560 (Lindhout *et al.*, 1994) was used for mapping the *Ol-1* gene. The F_2 plants as well as the F_1 and their parents were obtained from CPRO-DLO, Wageningen, The Netherlands. F_2 plants were selfed to generate F_3 lines.

The pathogenic fungus *O. lycopersicum*, which originated from infected commercial tomato plants (Lindhout *et al.*, 1994), was maintained on Moneymaker plants in a greenhouse compartment at 20 ± 3 °C with $70\pm15\%$ relative humidity.

Disease test

A disease test was performed by spraying 27-day-old tomato plants with a spore suspension of 2×10^4 conidia.ml^{-1}. The inoculum was prepared by washing conidial spores from the freshly sporulating leaves of heavily infected Moneymaker plants in tap water and used immediately. The experimental set-up of the disease test was according to a completely randomised block design with 15 blocks, each containing two plants of each parent, two F_1 plants and ten F_2 plants. The inoculated plants were grown in a greenhouse at 20 ± 3°C with 30-70% relative humidity.

The disease symptoms were evaluated at 10, 14, 17, 21, 24 and 28 days post inoculation (dpi). The evaluation was executed according to two categories of disease index (DI). DI 0-3 indicate the size of infected areas per inoculated leaf: 0 - no infection, 1 - less than 10% infected leaf area, 2 -

10-30% infected leaf area, 3 - more than 30% infected leaf area. DI a, b and c refer to the severity of infected leaf areas: a - faint mycelium visible often with only some yellow spots; b - obvious presence of mycelium but only with slight sporulation; c - abundant sporulation.

RFLP analysis

Total DNA was extracted from newly grown leaves without fungal infection and RFLP analysis was executed as described by Van der Beek *et al.* (1992). Fifteen RFLP markers were used: eight TG markers TG25, TG153, TG162, TG164, TG215, TG240, TG253 and TG298 (*Pst*I or *Eco*RI size-selected tomato genomic fragments; Miller and Tanksley, 1990; Tanksley *et al.*, 1992), two GP markers GP79 and GP164 (*Pst*I size-selected potato genomic fragments; Gebhardt *et al.*, 1989), three H markers H2C1, H8C4 and H9A11 (tomato genomic clones from a *HindIII* - library in plasmid *pUC*18; Klein-Lankhorst *et al.*, 1991a) and two cDNA clones, Adh-2 (Wisman *et al.*, 1991) and Aps-1 (Aarts *et al.*, 1991). The TG and GP markers were provided by S. D. Tanksley, Cornell University, New York, USA, and C. Gebhardt, Max Planck Institut für Züchtungsforschung, Köln, Germany, respectively. The cDNA clones were developed at the Laboratory of Molecular Biology, WAU, Wageningen, The Netherlands (Aarts *et al.*, 1991; Wisman *et al.*, 1991).

Identification of RAPD markers

Bulked segregant analysis (BSA; Michelmore *et al.*, 1991) was applied to identify RAPD markers associated with resistance to *O. lycopersicum*. Based on the disease test and RFLP analysis, seven resistant F_2 plants homozygous for *L. hirsutum* in the TG153 - TG164 interval (10.9 cM) spanning *Ol-1* and seven susceptible F_2 plants homozygous for *L. esculentum* in this interval were selected to constitute the resistant and susceptible pool, respectively. Three hundred arbitrary decamer primers (Operon) were used for RAPD analysis as described by Klein-Lankhorst (1991b). RAPDs associated with the resistance were used to identify the rest of the F_2 individuals.

Cloning and sequencing RAPD products

Diagnostic RAPD bands were excised from agarose gels, and the DNA was purified using a 'Gene Clean' kit (Bio 101 Inc). The purified DNA was reamplified using the same primer that generated the RAPD polymorphism. The reamplified products were resolved on a 1.5% agarose gel, excised from the gel, purified by 'Gene Clean' kit and blunt-end ligated into the *Sma*I site of pBluescript KS$^+$ vector. Before ligation, the linearized vector was tailed with `T' at 72 °C for two hours in 100 μl of a reaction mixture containing 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM dTTP, 2 μg vector DNA (digested with *Sma*I and purified with 'Gene

Clean' kit) and 1 unit Super*Taq*. The identity of the cloned RAPD products was verified by comparing the size of the digested plasmids using the corresponding enzymes, and by hybridizing the cloned fragments to Southern blots of resistant and susceptible pools. The plasmid DNA was isolated by alkali mini-preparation (Maniatis *et al.* 1989) and purified using QIAGEN-tip20 column (QIAGEN Inc). Sequencing was executed by Pharmacia Biotech Company.

Design of primers and analysis of SCAR markers

For each cloned RAPD amplification product, a pair of 21 to 25-mer oligonucleotide primers were designed to be used as SCAR primers, and synthesized by Pharmacia. Two SCAR primers, SCAE16 and SCAF10, were designed by extending the original 10-mer RAPD primer plus the next 14 nucleotides at the 3' end (Paran and Michelmore, 1993). The other three SCAR primers (SCAB01, SCAG11 and SCAK16) were designed by using the computer program 'Primer' based on their corresponding RAPD sequences. Amplification of genomic DNA of F_2 plants was executed in 50 µl of the same reaction mixture as applied in the RAPD reaction but with 100 ng of SCAR forward and reverse primers each. Each PCR consisted of 30 cycles of 1 min at 94 °C, 2 min at 60 °C (for SCAE16) and 2 min at 72 °C. The annealing temperature for SCAF10, SCAB01, SCAG11 and SCAK16 was 65 °C, 62 °C, 60 °C and 58 °C respectively. The amplified products were separated by electrophoresis in a 1.5% agarose gel.

Linkage analysis

Joinmap (Stam, 1993) was used to perform linkage analysis and to generate a genetic map, which was drawn by using Drawmap 2.0 (Van Ooijen, 1994).

Results

Disease test

A disease test on an F_2 of *L. esculentum* cv Moneymaker x *L. hirsutum* G1.1560 was performed to confirm the inheritance of resistance in *L. hirsutum* G1.1560. The resistance classification was carried out by using two categories of disease index (DI). DI 0-3 refers to the size of the infection areas on inoculated leaves, and index a-c to the severity of infection, respectively. As expected, all Moneymaker plants were scored as DI-3 (Fig. 1). Most individuals of the resistant parent were scored as DI-1, and a few as DI-2. Therefore, plants with a DI-3 were considered as susceptible, and plants with a DI of 0-2 as resistant. By using this index, most F_2 plants could be unambiguously identified as resistant or susceptible (Fig. 1). Segregation of resistance was in accordance with a

monogenic, dominant trait (Van der Beek *et al.*, 1994). Some F_1 plants were evaluated as susceptible, probably as a result from a cross between Moneymaker and a susceptible individual of *L. hirsutum* G1.1560, as has been reported previously (Lindhout *et al.*, 1994). In order to minimise misinterpretation of F_2 plants, the appearance of mycelia and sporulation was also taken into account. Thus, nearly all F_2 plants could be clearly classified as either resistant or susceptible.

Mapping of Ol-1 with RFLP markers

Though *Ol-1* has been mapped on chromosome 6 of tomato (Van der Beek *et al.*, 1994) using three RFLP markers (GP79, TG153 and TG178), the linkage map of the *Ol-1* region was still far from saturated. In order to add more marker to the map, the F_2 was therefore analysed by using 12 additional RFLP markers, which were well distributed on chromosome 6 (Tanksley *et al.*, 1992). All 15 markers showed a polymorphism between the two parents applying the restriction enzymes *EcoRI*, *HaeIII* and *TaqI*. Based on the segregation of the F_2, an RFLP map around the *Ol-1* region was constructed showing *Ol-1* to be located between TG153 and TG164 with an accuracy of about 3 cM (Fig. 2).

Identification of diagnostic RAPD markers

To saturate the TG153-TG164 interval encompassing *Ol-1*, BSA with RAPDs was performed on DNA from the resistant and susceptible pools as templates. With 300 decamer random primers, five diagnostic RAPDs were identified: two *L. hirsutum* specific, $OPAB01_{866}$ and $OPAF10_{464}$ with a length of 866 bp and 464 bp respectively and three *L. esculentum* specific, $OPAE16_{778}$, $OPAG11_{1400}$ and $OPAK16_{1200}$ of 778 bp, 1400 bp and 1200 bp in length, respectively. By analysing 27 recombinants in the TG153-TG164 interval, OPAB01, OPAE16, OPAF10, OPAK16 and OPAG11 were mapped near *Ol-1* (data not shown), confirming a tight linkage between these RAPDs and *Ol-1*.
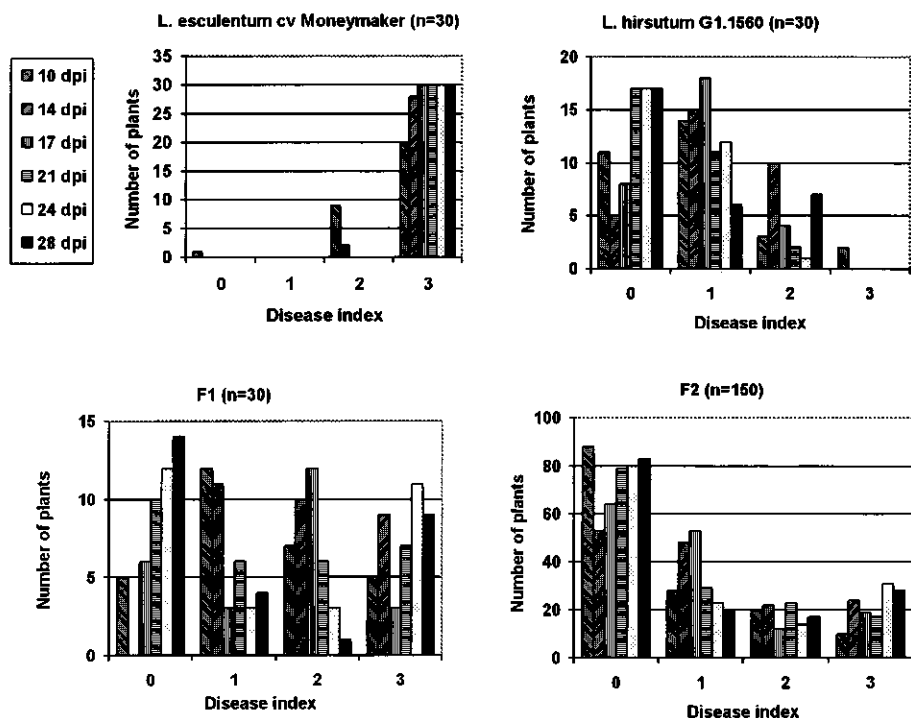
Cloning and sequencing of the diagnostic RAPD markers

Figure 1. Frequency distribution of resistance to *O. lycopersicum* infection in *L. hirsutum* G1.1560, *L. esculentum* cv Moneymaker and their F_1 and F_2 progenies as shown above the panels. The population size is indicated between brackets. The disease index (DI) was defined as: 0, no infection; 1: less than 10% foliar area affected; 2, 10-30% foliar area affected; 3, more than 30% foliar area affected. Evaluations were done at 10, 14, 17, 20, 24 and 28 days post inoculation (DPI).
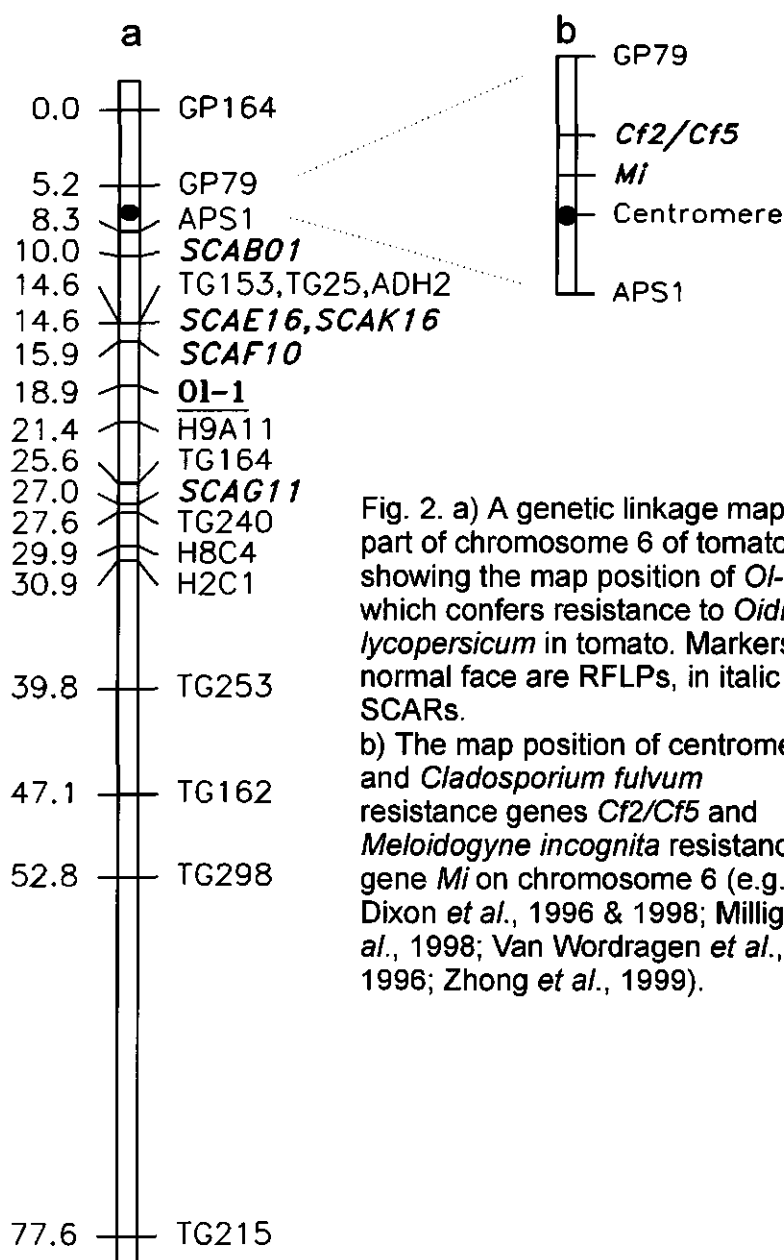
Fig. 2. a) A genetic linkage map of part of chromosome 6 of tomato, showing the map position of *Ol-1*, which confers resistance to *Oidium lycopersicum* in tomato. Markers in normal face are RFLPs, in italic are SCARs.
b) The map position of centromere and *Cladosporium fulvum* resistance genes *Cf2/Cf5* and *Meloidogyne incognita* resistance gene *Mi* on chromosome 6 (e.g. Dixon *et al.*, 1996 & 1998; Milligan *et al.*, 1998; Van Wordragen *et al.*, 1996; Zhong *et al.*, 1999).

Since SCAR markers are more reliable, reproducible and locus-specific than RAPD markers (Paran and Michelmore, 1993), the newly identified RAPDs were converted into SCARs. The amplified products $OPAF10_{464}$, $OPAB01_{866}$, $OPAE16_{778}$, $OPAG11_{1400}$ and $OPAK16_{1200}$ were extracted from the gel and cloned into the *Sma*I site of a pBluescript vector. Cloned fragments were shown to be derived from and identical to their corresponding RAPDs by Southern analysis. To determine whether each cloned fragment corresponded to multi-copy sequence family or to a single locus, Southern analysis was conducted with DNA of both parents following digestion with *Bam*HI, *Bgl*II, *Bst*NI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III and *Taq*I. $OPAF10_{464}$ and $OPAG11_{1400}$ appeared to represent members of a repeat family (not shown), the others were derived from a single locus. $OPAF10_{464}$, $OPAB01_{866}$ and $OPAE16_{778}$ were completely sequenced, while only two ends (500-600 bases) of $OPAG11_{1400}$ and $OPAK16_{1200}$ were analysed. At both ends, the terminal 10 bases corresponding to the original RAPD primers were recovered.

SCAR analysis

For SCAR analysis, a pair of primers for each cloned RAPD product was designed and synthesized as mentioned in Materials and Methods (Table 1). PCR reactions were performed, using genomic DNA of resistant and susceptible pools as templates. As expected, a unique band was detected in the susceptible pool when using *L. esculentum* specific SCAR primers, and *vice versa* (e.g. Fig. 3). The sizes of the SCAR amplification products were identical to those of the corresponding RAPD amplification products (data not shown). SCAR analysis for individual plants of resistant and susceptible pools was carried out to confirm the alleles of these marker loci. As expected, a susceptible allele was detected in the seven susceptible plants when using *L. esculentum* specific SCAR primers, and *vice versa* (Fig. 3). However, an *L. hirsutum* specific allele for SCAB01 was detected in one plant of the susceptible bulk (Fig. 3a), indicating the occurrence of recombination between *Ol-1* and SCAB01, or the heterozygousity between *Ol-1* and SCAB01. The absence of this *L. hirsutum* specific allele in the susceptible pool is probably due to the low relative concentration of the corresponding DNA (template) in that pool.

In order to map these SCARs, the whole F_2 population was screened for the presence or absence of corresponding polymorphic DNA bands by using each pair of the SCAR primers. All five SCARs identified only rare recombinants with *Ol-1*, indicating that they were tightly linked to this gene. The segregation data were used to construct an integrated genetic map including both RFLP and SCAR markers (Fig. 2).

Table 1. SCAR markers converted from RAPD markers closely linked to the *Ol-1* gene

SCAR	Primer	Sequence (5'→ 3')[*]	Annealing temperature (°C)[**]
SCAE16	OPAE16$_{778}$ forward	TCCGTGCTGAATGAAGATTCAAAC	60.0
	Reverse	TCCGTGCTGATAAAACTGTTAGAC	
SCAF10	OPAF10$_{464}$ forward	GGTTGGAGACGAATGGAAAGATGC	65.0
	reverse	GGTTGGAGACAATAGACTCGAGAT	
SCAB01	OPAB01$_{866}$ forward	GCTTCTAGATGCAGAAAGTTGGCG	62.0
	reverse	CGCCCATTCCCGCATATACAG	
SCAG11	OPAG11$_{1400}$ forward	TGGGATCACAGATTAACAAATGCG	60.0
	reverse	ATGTGTGCGATGAGAAACGTGG	
SCAK16	OPAK16$_{1200}$ forward	CAAACAAAGCAGTGGATTTTTTCG	58.0
	reverse	TAAAAGCCTTAGTGGGACAGGGC	

* Primers for SCAE16 and SCAF10 were designed by extending the original 10-mer RAPD primer (underlined) with 14 bases at the 3' end. Primers for SCAB01, SCAG11 and SCAK16 were designed by using computer program 'Primer'. The sequences of the original 10-mer RAPD primers OPAB01, OPAG11 and OPAK16 were CCGTCGGTAG, TTACGGTGGG and CTGCGTGCTC resp..
** The annealing temperature for RAPD was ±35 °C.

Discussion

In the present study we identified 15 RFLP markers and five SCAR markers closely linked to the powdery mildew resistance gene *Ol-1*, that was previously mapped on chromosome 6 of tomato (Van der Beek *et al.*, 1994). A genetic linkage map comprising these twenty markers was constructed showing *Ol-1* to be flanked by markers SCAF10 and H9A11. Since the centromere of chromosome 6 is located between GP79 and APS1 (Zhong *et al.*, 1999), *Ol-1* is apparently located on the long arm of chromosome 6 (Fig. 2), 10-13 cM distant from the recently cloned resistance genes *Mi* and *Cf2/Cf5* from the short arm of chromosome 6 (e.g. Van Wordragen *et al.*, 1996; Zhong *et al.*, 1999; Dixon *et al.*, 1996 & 1998; Milligan *et al.*, 1998). Resistance genes are often
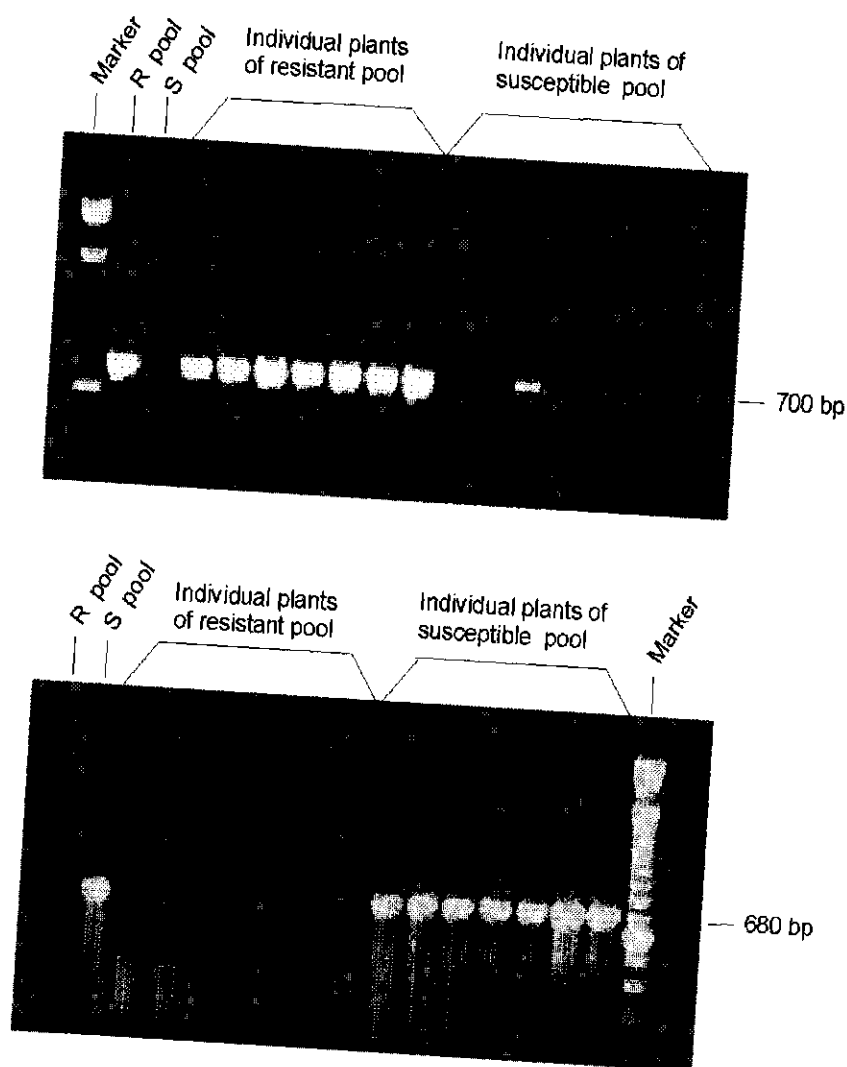
Figure 2. SCAR analysis of genomic DNA of individual plants of either the resistant or the susceptible pool using SCAR primers: a) *L. hirsutum* (resistant allele) specific SCAR primer SCAB01 (upper panel), b) *L. esculentum* (susceptible allele) specific SCAR primer SCAG11 (lower panel).

organised in clusters of homologous genes that may span from 36 kb upto several megabases in plant genomes (Meyers *et al.*, 1998; Tomas *et al.*, 1997). For instance, *Cf-4* and *Cf-9* are part of the so-called "Milky way" cluster that contains five very homologous genes and spans 36 kb in the tomato genome (Thomas *et al.*, 1997), while at least 24 *Dm* gene homologues span about 4 Mb in the lettuce genome (Meyers *et al.*, 1998). The distance between *Ol-1* and *Cf2/Cf5/Mi* suggests that these genes are part of a *Dm*-gene like cluster of more than 1 Mb. If so, *Ol-1* may be homologous to *Cf2/Cf5* or *Mi*. However, this is not very likely as *Cf2/Cf5* are not homologous to *Mi* and these genes are separated from *Ol-1* by the centromere.

The present map is based on an F_2 of *L. esculentum* x *L. hirsutum* and is similar to the genetic linkage map of *L. esculentum* x *L. pennellii* (Tanksley *et al.*, 1992). The distance between GP79 and TG215 in the present map is 72 cM (Fig. 2), and 89 cM in the Tanksley map (Tanksley *et al.*, 1992). However, some hitherto unresolvable markers (for example TG240 and TG298) became resolved in our map, probably as a result from the larger F_2 population applied.

By using bulked segregant analysis (BSA) with 300 RAPD primers, five RAPDs were identified, which were closely linked to *Ol-1*. This confirms the success of BSA for identifying RAPDs which are closely linked to a gene of interest (Michelmore *et al.*, 1991; Giovannoni *et al.*, 1991). Giovannoni *et al.* (1991) has suggested an optimal pool size of more than five but less than 10 individual plants for a target interval of 10 cM, because the probability for a plant to have maximally one double crossover in such a pool would then be less than 10%. In our study, an interval of 11 cM spanning the *Ol-1* gene between TG153 and TG164 was targeted, and seven plants were chosen for both pools. Among the five RAPDs identified, OPAF10$_{464}$ was a RAPD most closely linked to *Ol-1* (3 cM, shown as SCAF10 in Fig. 2).

Some disadvantages of the RAPD markers, including sensitivity to reaction conditions and amplification of multiple loci (Paran and Michelmore, 1993), can be overcome by converting RAPDs into SCAR markers. In the present study, all primer pairs deduced from the sequences of the RAPD amplification products indeed generated locus specific SCAR markers. SCAB01 and SCAF10 were *L. hirsutum* specific and SCAE16, SCAG11 and SCAK16 were *L. esculentum* specific. The five SCARs, especially SCAF10 and SCAG11, should be highly suited for commercial breeding programs as they are diagnostic for the introgression fragment containing the *Ol-1* gene and easy to handle as PCR markers. This would speed up the breeding programs for resistance to *O. lycopersicum* without disease tests or laborious RFLP analyses. However, since H9A11 is more closely linked to *Ol-1* than SCAG11, a corresponding SCAR marker of this RFLP marker would be desirable.

The genetic linkage map reported in this paper contains 20 markers in a region of about 80 cM,

i.e. one marker per 4 cM. This dense map thus provides an essential framework for any future map-based cloning of *Ol-1*, the more so considering the availability of YAC and BAC libraries and the AFLP technology to identify even more closely linked markers.

Acknowledgement

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Chapter 6

Characterization and mapping of resistance to *Oidium lycopersicum* in two *Lycopersicon hirsutum* accessions: Evidence for close linkage of two *Ol*-genes on chromosome 6 of tomato[1]

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Characterization and mapping of resistance to *Oidium lycopersicum* in two *Lycopersicon hirsutum* accessions: Evidence for close linkage of two *Ol*-genes on chromosome 6 of tomato

Cai-Cheng Huang[1], Petra M. Hoefs-van de Putte, Jantine G. Haanstra-van der Meer, Fien Meijer-Dekens, & Pim Lindhout[2]

The Graduate School of Experimental Plant Sciences, Laboratory of Plant Breeding,
Wageningen University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands.
[1] Present address: Novartis Seeds BV, P.O. Box 2, 1600 AA Enkhuizen, The Netherlands.
[2] To whom correspondence should be addressed. Tel. +31-317-483454, Fax: +31-317-483457;
e-mail: pim.lindhout@users.pv.wau.nl

Abstract

The cultivated tomato is susceptible to powdery mildew (*Oidium lycopersicum*). Several accessions of wild species are resistant. In the present study we described 1) the genetics and mapping of resistance to *O. lycopersicum* in G1.1290, one of the resistant accessions in *Lycopersicon hirsutum*, 2) fine mapping of *Ol-1* originated from *L. hirsutum* G1.1560, another resistant accession of *L. hirsutum*, and 3) tests of allelism for resistance in G1.1290 and G1.1560. First, the resistance in G1.1290 to *O. lycopersicum* was demonstrated to be controlled by an incompletely dominant gene, designated *Ol-3*. By using an advanced breeding line (ABL) containing introgression fragment(s) from G1.1290, *Ol-3* was found to be associated with some RFLP and SCAR markers on chromosome 6. By using these markers, *Ol-3* was mapped between markers TG25/SCAF10 and H9A11 on chromosome 6. Second, after testing some F_3 lines and their progenies from the cross between *L. esculentum* cv Moneymaker and *L. hirsutum* G1.1560, we provided more evidence for the map position of *Ol-1* to be between SCAF10 and H9A11, indicating that *Ol-1* and *Ol-3* were in the same chromosome region. Third, though allelism tests could not discriminate between *Ol-1* and *Ol-3*, some (indirect) evidence suggested that these two genes are not identical. They might represent functional genes of a cluster of *Ol*-homologues.

Key words: Tomato, *Lycopersicon hirsutum*, powdery mildew, resistance gene, allelism test

Introduction

Powdery mildew on tomato, caused by *Oidium lycopersicum* Cooke & Massee (Noordeloos & Loerakker, 1989), is the only fungal disease which has to be controlled by fungicides in protected tomato cultivation in Northwest Europe. Complete or high levels of resistance have been found in at least four *Lycopersicon* species, i.e. *L. esculentum* var. *cerasiforme*, *L. hirsutum*, *L. parviflorum* and *L. peruvianum* (Kozik, 1993; Laterrot & Moretti, 1993; Neshev, 1993; Lindhout *et al.*, 1994; Ciccarese *et al.*, 1998). The resistance in Gl.1560 to *O. lycopersicum* is controlled by an incompletely dominant gene, *Ol-1* which was mapped on chromosome 6 between RFLP markers GP79 and TG153, in the vicinity of the resistance genes *Mi* to *Meloidogyne spp.* and *Cf-2/Cf-5* to *Cladosporium fulvum* (Van der Beek *et al.*, 1994). More recent data confirmed the monogenic resistance in G1.1560, and showed a more accurate map position of *Ol-1* to be between SCAF10 and H9A11 (Huang *et al.*, 2000).

Resistance to *O. lycopersicum* seems to be widely distributed over the *Lycopersicon* species (see above). It is an intriguing question how the corresponding resistance genes have originated during evolution and how related these genes are. Clustering of disease resistance genes is quite common in plants (Michelmore & Meyers, 1998); examples are the *Cf*-cluster and the *Pto* cluster in tomato (Hammond-Kosack & Jones, 1997). Each of such clusters usually consists of homologues that have different specificities to different races of the corresponding pathogens. So far, only resistance of *L. hirsutum* G1.1560 has been characterized and the corresponding gene *Ol-1* has been mapped on chromosome 6 (Van der Beek *et al.*, 1994; Huang *et al.*, 2000). In order to find out whether the *O. lycopersicum* resistance genes (*Ol*-genes) are also organized in one or more clusters, we investigated the inheritance of resistance in *L. hirsutum* G1.1290 and mapped the resistance gene, designated *Ol-3*. In addition, we provided more evidence for the map position of *Ol-1*. Finally we performed allelism tests for *Ol-1* and *Ol-3* in order to assess their relationship.

Materials and methods

Plant and fungal materials

The resistant wild accessions *L. hirsutum* G1.1290 and G1.1560 (Lindhout *et al.*, 1994) were obtained from the Centre of Genetic Resources, Wageningen, The Netherlands. *L. esculentum* cv Moneymaker, as susceptible control, was maintained at the Laboratory of Plant Breeding, WAU, Wageningen, the Netherlands. Advanced breeding lines (ABLs), ABL1290.4 and ABL1560.2, derived from either G1.1290 or G1.1560, were obtained from commercial breeding programmes for resistance to *O. lycopersicum*. All the populations evaluated are briefly

described in Table 1. In addition, two other populations, H*Ol3* and H*Ol1*, heterozygous for *Ol-3* and *Ol-1* were generated by crossing Moneymaker with ABLs ABL1290.4 and ABL1560.2, respectively and used as heterozygous controls.

The stock of *O. lycopersicum* originated from infected commercial tomato plants (Lindhout *et al.*, 1994), and was maintained on cv Moneymaker plants in a growth chamber at 20±1 °C with 70±3% RH and 16-h day length.

Disease tests

Disease tests were carried out in eight experiments during 1994-1998. The experimental set-up was according to a completely randomized block design with two to 18 blocks (Table 1). Each block always contained susceptible and resistant control genotypes. All plants at the four true leaf stage were inoculated by spraying with a spore suspension at a concentration of 3-4×10^4 conidia.ml^{-1}. The inoculum was prepared by washing conidial spores from the freshly sporulating leaves of heavily infected Moneymaker plants in tap water and was used immediately. The inoculated plants were grown in a greenhouse at 20±3°C with 70±10% RH under natural light supplemented with artificial light to provide a photoperiod of 16 h.

Disease evaluation

The susceptibility or resistance was determined macroscopically by the degree of fungal sporulation. Depending on the development of the fungus, evaluations were performed per plant twice to five times from 7 to 29 days post inoculation (dpi), by using a disease index (DI) from 0 to 3, based on the degree of sporulation. The DI was defined as: 0: no sporulation; 1: slight sporulation, but less than 5% foliar area affected; 2: moderate sporulation, between 5 and 30% foliar area affected; 3: abundant sporulation, more than 30% foliar area affected. In addition, in order to evaluate each plant more precisely, DI from 0 to 4 was used in the allelism test (also Fig. 5 &6): 0 - no symptom; 1 - some mycelia without sporulation; 2 - moderate mycelia with poor sporulation; 3 - between 2 and 4; 4 - abundant mycelia with heavy sporulation.

Marker analysis

Total DNA was extracted from the frozen young leaves of most plant materials as described by Van der Beek *et al.* (1992), except for the ABLs in Experiment 2 from which DNA was isolated from a leaf-disc as described by Hong Wang *et al.* (1993). DNA (5 µg) was digested with the restriction enzymes *Eco*RI and *Hae*III (Life Technology), based on a pilot test of enzyme-probe combinations. RFLP analysis was performed as described by Van der Beek *et al.* (1992), and

Table 1. Summary of each experiment of disease tests

Experiment	Number of blocks	Aim of testing	Number of plants per block	Testing population(s)
1	18	inheritance of resistance in G1.1290	12-15: two or three plants of each parent, one or two of F_1 and nine or 10 of BC_1	BC_1: Moneymaker (MM)xF_1(MMxG1.1290)
2	10	resistance of F_3 lines derived from G1.1560	19-36: one plant of each parent, one or two plant(s) each of 17 F_3 lines	F_3 lines: selfings of the F_2 from F_1 (MMxG1.1560) (Huang *et al.*, 2000)
3	6	putative additional resistance gene (arg) or suppressor gene (sg)	23-27: 9-13 "F_2" plants, five each of Moneymaker and G1.1560, four of ABL1560.2	"F_2" for arg: MMxF_3 (*ol-1ol-1*), "F_2" for sg: F_3 (*Ol-1Ol-1*)xF_3(*Ol-1Ol-1*)[1]
4	2	fine mapping *Ol-1* by RFLP & SCAR	66: one cutting of each ABL, two seedlings of G1.1560, four cuttings and four seedlings of Moneymaker	ABLs derived from G1.1560
5	6	allelism between *Ol-1* and *Ol-3* using wild accessions (wa)	26-29: 22-23 plants of "BC_{1wa}", three of Moneymaker and two to three each of G1.1290 and G1.1560	"BC_{1wa}": MMx"F_{1wa}"(G1.1290xG1.1560)
6	6	allelism between *Ol-1* and *Ol-3* using ABLs (abl)	50-51: 32-33 plants of "BC_{1abl}", three each of Moneymaker, G1.1290, G1.1560, ABL1290.4, ABL1560.2 and "F_{1abl}"	"BC_{1abl}": MMx"F_{1abl}"(ABL1290.4xABL1560.2)
7	6	resistance of the selfing progenies from Exp.5	56-69: three to five plants each of 13 "$BC_{1wa}S_1$" lines, Moneymaker, G1.1290, G1.1560, HOl3 and HOl1[2]	"$BC_{1wa}S_1$" lines: selfing the most susceptible "BC_{1wa}" plants
8	6	resistance of the selfing progenies from Exp.6	48-49: three to five plants each of six "$BC_{1abl}S_1$" lines, Moneymaker, ABL1290.4, ABL1560.2, "F_{1abl}", HOl3 and HOl1	"$BC_{1abl}S_1$" lines: selfing the most susceptible "BC_{1abl}" plants

1) F_3(*ol-1ol-1*): (supposedly) resistant F_3 plant, an offspring of F_2 no.270, without introgression fragment (IF) from G1.1560; F_3(*Ol-1Ol-1*): (supposedly) susceptible F_3 plant, an offspring of F_2 no.101, with IF from G1.1560; F_3(*Ol-1Ol-1*) resistant F_3 plant with IF from G1.1560 (also see text).

2) HOl3 and HOl1, which were heterozygous for *Ol-1* and *Ol-3* were generated by crossing Moneymaker with ABL1290.4 and ABL1560.2, respectively.

SCAR analysis as by Paran & Michelmore (1993) and Huang *et al.* (2000). The four TG markers, TG153, TG25, TG164 and TG240, were provided by S. D. Tanksley, Cornell University, New York, USA and H9A11 was supplied by C. Gebhardt, Max Planck Institut für Züchtungsforschung, Cologne, Germany. The SCAR markers were from by P. Zabel, Laboratory of Molecular Biology, WAU, Wageningen, The Netherlands.

Linkage analysis

The mapping program JoinMap™ 2.0 (Stam & Van Ooijen, 1995) was used to estimate the map distances. Drawmap 2.0 (Van Ooijen, 1994) was employed to produce graphics of the maps.

Results

Inheritance and mapping of the resistance to O. lycopersicum in L. hirsutum G1.1290

The inheritance of resistance to *O. lycopersicum* was studied by applying a disease test on a BC_1 population of *L. esculentum* cv Moneymaker x *L. hirsutum* G1.1290. All plants were evaluated for the degree of sporulation expressed as disease index (DI) at scales from 0 to 3. The plants of resistant control G1.1290 either remained uninfected or were scored at most 1, while almost all plants of susceptible control Moneymaker were scored 3 (Fig. 1). All F_1 plants were scored 0-1, and hence were considered as resistant (Fig. 1), indicating that the resistance was dominant. The BC_1 segregated into 69 resistant and 80 susceptible plants, according to an 1:1 ratio ($X^2_{1 d.f.}$ = 0.81, P = 0.50-0.25), in accordance with one dominant resistance gene, which we designated as *Ol-3*. In addition, twenty-nine BC_1 plants could not be clearly classified because of their intermediate disease score (2). This might indicate the incomplete dominance of *Ol-3* or existence of minor gene effects. It might also be due to the influence of genetic background of *L. esculentum* on the expression levels of resistance as sometimes noticed in the ABL (also see Fig. 3, g).

As a first step towards mapping the *Ol-3* gene, a resistant advanced breeding line (ABL1290.4) derived from *L. hirsutum* G1.1290 was analyzed for the markers TG153, TG25, SCAF10 and TG164 on chromosome 6, which are closely linked to *Ol-1* (Huang *et al*, 2000). The resistance in this ABL was found to be associated with *L. hirsutum* alleles at these markers. Obviously, this ABL contained a fairly large introgression fragment (approximately 10 cM) between markers TG153 and TG164 (Fig. 2). This result at least indicated that *Ol-3* was located on chromosome 6, near *Ol-1*.
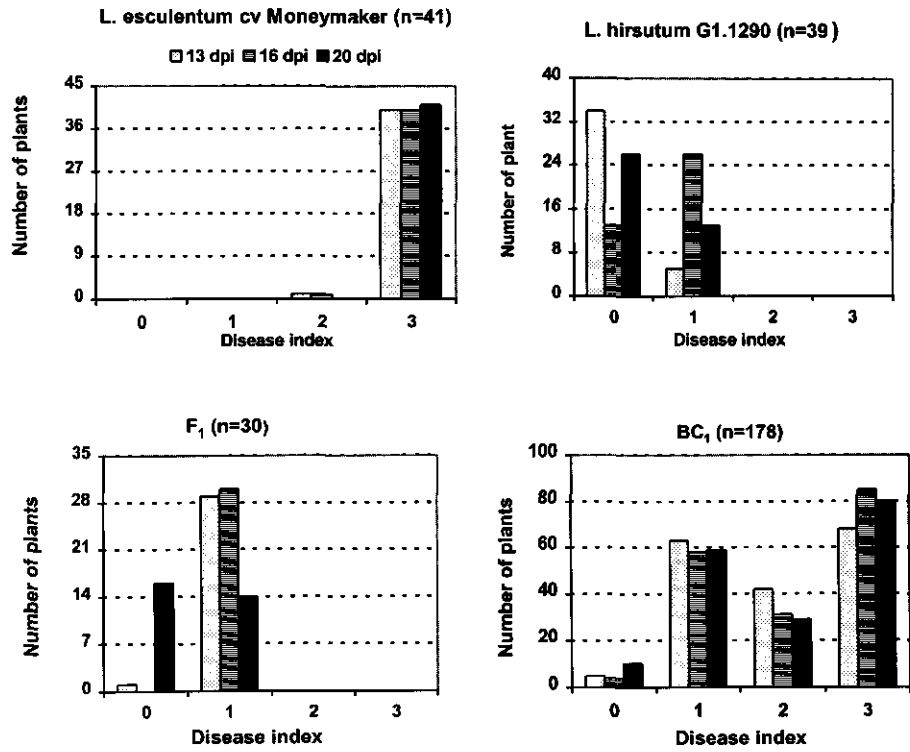
Fig. 1 Frequency distribution of resistance to *O. lycopersicum* infection in *L. hirsutum* G1.1290, *L. esculentum* cv Moneymaker and their F_1 and BC_1 progenies. The population size is indicated between brackets. The disease index (DI) was defined as: 0: no sporulation; 1: slight sporulation, but less than 5% foliar area affected; 2: moderate sporulation, between 5 and 30% foliar area affected; 3: abundant sporulation, more than 30% foliar area affected. Evaluations were done at 13, 16 and 20 days post inoculation (DPI).
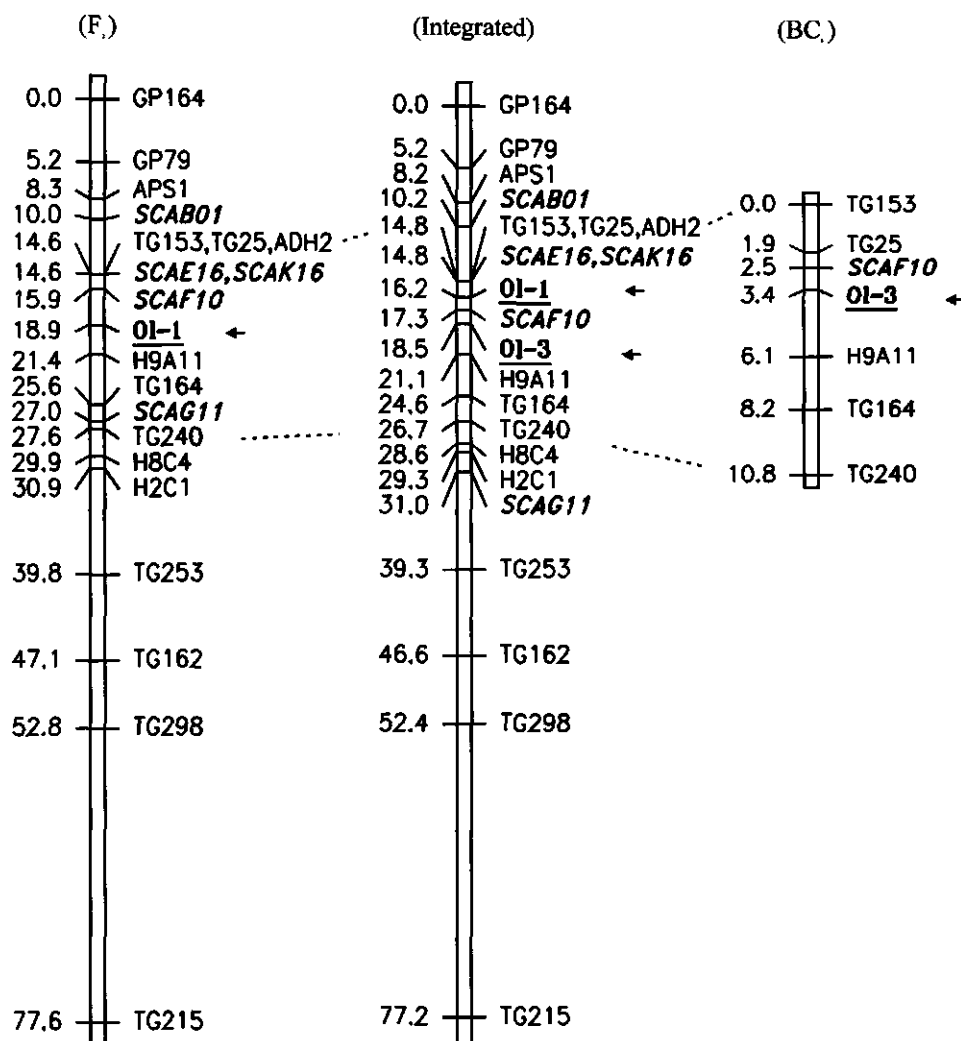
Fig. 2 Genetic linkage map of part of chromosome 6 of tomato, showing the position of the resistance genes *Ol-1* (*left*) and *Ol-3* (*right*) for tomato powdery mildew. Their relative position is shown in an integrated genetic map (*central*). *Ol-1* and *Ol-3* were mapped by using an F$_2$ of *L. esculentum* cv Moneymaker x *L. hirsutum* G1.1560 and a BC$_1$ of Moneymaker x *L. hirsutum* G1.1290, respectively. Markers in normal face are RFLPs, in bold are SCARs.

To map the gene *Ol-3*, RFLP analyses with chromosome 6 markers, were performed on 51 resistant (scored at most 1) and 58 susceptible (always scored 3) BC_1 plants. After testing 15 probe-enzyme combinations, 11 combinations generated sufficient polymorphisms between the parents Moneymaker and G1.1290 (data not shown). One combination, TG240 with *Hae*III, even showed a difference between the two *L. hirsutum* accessions G1.1290 and G1.1560, indicating a genetic difference between these two accessions. In the interval where *Ol-3* was mapped by using ABL1290.4, five probe-enzyme combinations (TG153, TG164 and TG240 with *Hae*III, and TG25 and H9A11 with *Eco*RI) and an *L. hirsutum* specific SCAR marker, SCAF10, were chosen for more precise mapping (Fig. 2). Based on the marker data and the disease resistance evaluation of the BC_1 plants, map distances between *Ol-3* and RFLPs as well as SCAR were estimated. This map was very reliable ($X^2=0.139$) and the order of the markers was the same as reported by Tanksley *et al.* (1992). The most likely map position of the resistance gene *Ol-3* was between TG25/SCAF10 and H9A11 (Fig. 2).

Fine mapping of Ol-1

Because *Ol-3* had previously been mapped in the same chromosome region as *Ol-1*, we wanted to investigate further the relationship of these two *Ol* genes. Though *Ol-1* had been mapped between SCAF10 and H9A11 in an F_2 (Huang *et al.*, 2000), the accuracy of mapping also allows a slightly different position. To improve the accuracy of the mapping of *Ol-1*, the F_3 progenies of 17 F_2 plants (Table 1) with ambiguous disease resistance evaluation, were genotyped for the SCAR markers and evaluated by a disease test to more accurately evaluate the resistance or susceptibility of these F_2 plants. By doing so, most of the F_2 plants that had been scored ambiguously could now be classified as either resistant or susceptible, which was confirmed by the SCAR-genotype. For two of the F_3 progenies from F_2 plants no.101 and no.270, however, the SCAR-genotype and the disease rating were contradictory.

The F_3 progeny of no.101 did carry the *Ol-1* resistance allele as judged by SCAR analysis, but was susceptible to *O. lycopersicum*. These plants might contain suppressor gene(s). In order to find a better explanation of such conflicting observations, they were crossed with resistant F_3 plants that did carry the *Ol-1* resistance allele. Then the "F_2" progeny of these crosses were subjected to a disease test. These were as resistant as the resistant parent G1.1560 (Fig. 3: a & b), indicating the absence of such putative suppressor gene(s) and the presence of the *Ol-1* resistance allele. Therefore, the corresponding F_2 plant no.101 was re-interpreted as resistant. In contrast, the F_3 progeny of no.270 did not contain the *Ol-1* allele as judged by SCAR analysis but was evaluated as resistant. These plants might contain additional gene(s) which also confer resistance to *O. lycopersicum*. Two of them were crossed with Moneymaker and the two derived "F_2" progenies were evaluated in a disease test. Both "F_2" populations tested were as

susceptible as Moneymaker (Fig. 3: c & d), indicating the absence of such putative additional gene(s) as well as the absence of the *Ol-1* allele. Therefore, the corresponding F_2 plant no.270 was re-interpreted as susceptible. After re-interpreting the two F_2 plants no.101 and no.270, *Ol-1* was still mapped between SCAF10 and H9A11, in the same region as *Ol-3* (Fig. 2).

In addition, 56 ABLs derived from *L. hirsutum* G1.1560 were used to determine the introgression fragment(s) around the *Ol-1* gene. They were subjected to RFLP and SCAR analyses with five RFLPs and four SCARs, which are tightly linked to *Ol-1*. The ABLs could be classified into eight classes according to the size of the introgression fragments. Six of them contained a large introgression fragment almost covering the whole region between SCAE16 and TG240 (not shown). The genotypes ABL1 and ABL2 showed smaller introgression fragments which indicated that *Ol-1* was indeed between SCAF10 and H9A11 (Fig. 4). This was in accordance with our earlier mapping study of *Ol-1* (Fig. 2).

Allelism test of Ol-3 and Ol-1

As stated above, *Ol-1* and *Ol-3* were mapped at similar positions on chromosome 6. Their relative distance was about 2 cM in an integrated map (Fig. 2), constructed by using both the *Ol-1* map and *Ol-3* map, suggesting that *Ol-1* and *Ol-3* are two different genes. However, because the integrated map was constructed by using linkage data from two different populations, F_2 and BC_1 respectively, the distance between *Ol-1* and *Ol-3* might be due to experimental error, as could the 4 cM shift of map position of SCAG11. In order to confirm the relative map position of *Ol-1* and *Ol-3*, allelism tests were conducted by using three-way test-cross progenies derived from wild accessions and ABLs respectively (Table 1).

Most of plants of the three-way-cross progeny "BC_{1wa}" (Moneymaker x "F_{1wa}"[G1.1290xG1.1560], also see Table 1) were resistant, but some plants might be considered as susceptible (Fig. 5). However, most plants of the selfed progenies ("$BC_{1wa}S_1$") from the most susceptible "BC_{1wa}" plants were resistant (Fig. 6a), indicating the absence of susceptible recombinants in the test-cross progenies ("BC_{1wa}"). Because no recombinant was found in the "BC_{1wa}" consisting of 135 plants, the distance between *Ol-1* and *Ol-3* was estimated to be smaller than 2.2 cM (P=0.05).

In order to test the allelism of *Ol-1* and *Ol-3* in a more *L. esculentum* background, ABL1290.4 and ABL1560.2 containing *Ol-3* and *Ol-1*, respectively, were used to make similar three-way-crosses as for the wild accessions. Similarly, most plants of the three-way-cross progeny "BC_{1abl}" (Moneymaker x "F_{1abl}"[G1.1290xG1.1560], also see Table 1) were resistant, but some plants might be considered as susceptible (Fig. 7). However, most plants of the selfed
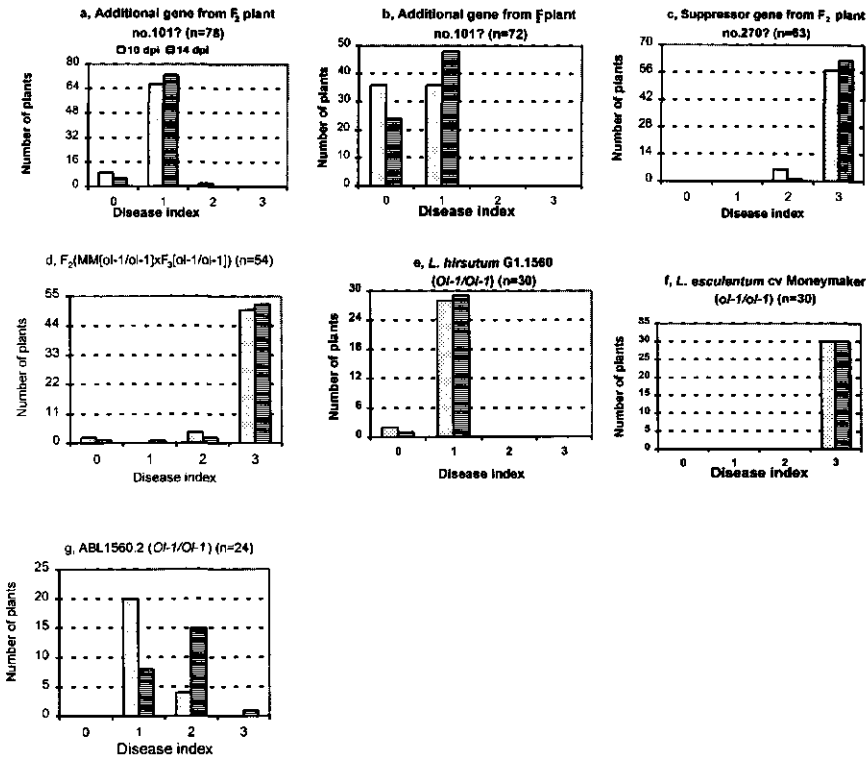
Fig. 3 Analysis of putative additional genes (a & b) or suppressor genes (c & d). *L. hirsutum*
G1.1560 (e) and the ABL (g) served as resistant control, while *L. esculentum* cv Moneymaker
(f) as susceptible control (see text). The experiment consisted of six blocks, each contained nine
to thirteen "F_2" plants, five each of Moneymaker and G1.1560, and four of ABL1560.2. The
disease index was defined as: 0, no symptom; 1, some mycelia without sporulation; 2, moderate
mycelia with poor sporulation; 3, abundant mycelia with heavy sporulation. Evaluations were
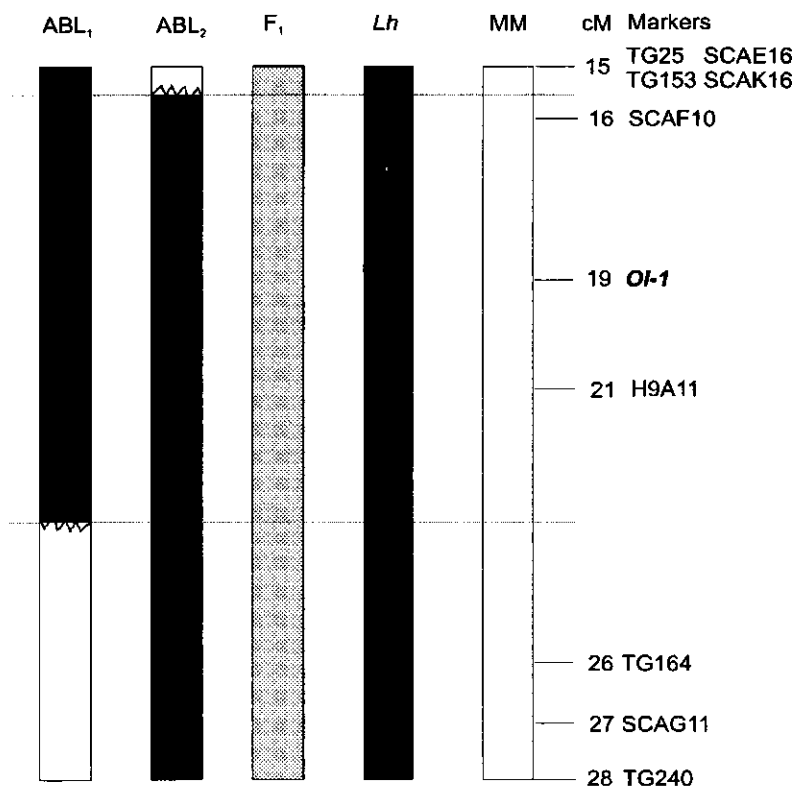done at 10 and 14 days post inoculation (DPI)

Fig. 4 Graphical map of part of chromosome 6, showing the map position of *Ol-1* between SCAF10 and H9A11. This map was constructed by using ABLs carrying the *Ol-1* resistance. Chromosome fragments in black represent *L. hirsutum* (*Lh*) loci, in white *L. esculentum* (MM) loci, in grey heterozygous loci. The relative map distance of each marker was based on the *Ol-1* map (Fig. 2).
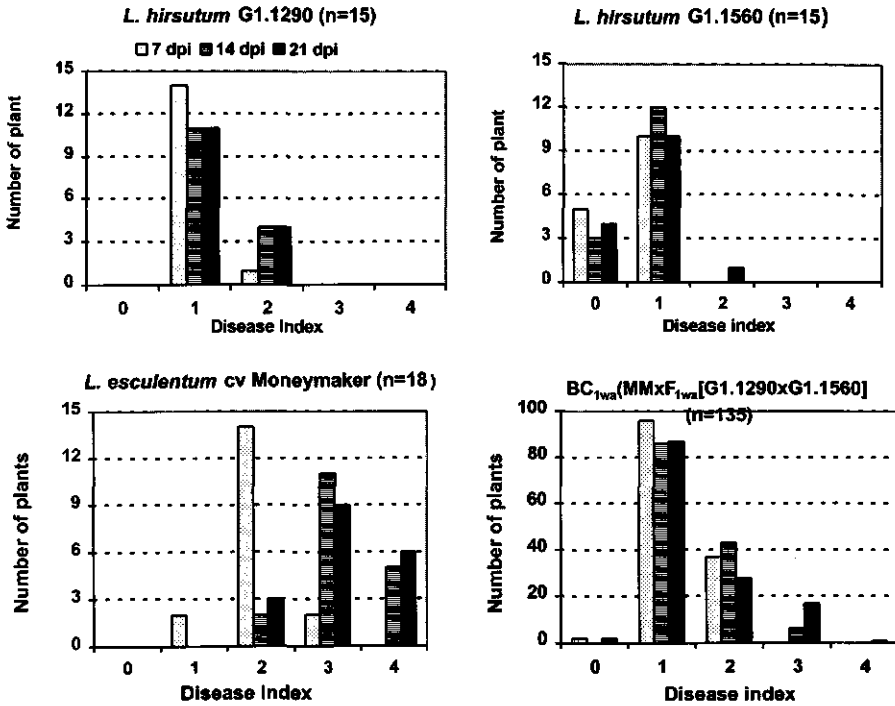
Fig. 5 Frequency distribution of resistance to *O. lycopersicum* infection in *L. hirsutum* G1.1290, *L. hirsutum* G1.1560, *L. esculentum* cv Moneymaker and their "BC$_{1wa}$" (MM×"F$_{1wa}$"[G1.1560×G1.1290]) progenies of the allelism test. The disease was scored as: 0, no symptom; 1, some mycelia without sporulation; 2, moderate mycelia with poor sporulation; 3, between 2 and 4; 4, abundant mycelia with heavy sporulation. Evaluations were done at 10, 14 and 21 days post inoculation (DPI).
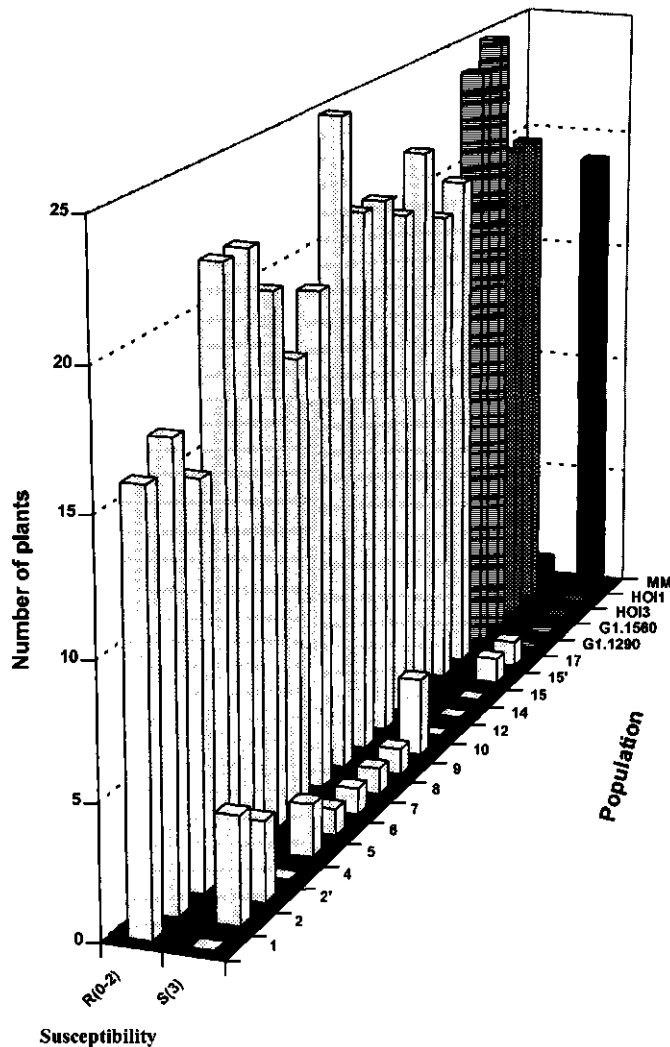
Allelism test of *Ol-1* and *Ol-3* using wild accessions

Fig. 6 Susceptibility of the "BC$_1$S$_1$" lines.

a) Frequency distribution of resistance to *O. lycopersicum* infection in the "BC$_{1wa}$S$_1$" lines (indicated by Arabic numbers, Lines 2' & 15' were repeats of Lines 2 & 15 respectively) at 28 days post inoculation (DPI). These lines were generated by self-pollinating the most susceptible "BC$_{1wa}$" plants. Here, "BC$_{1wa}$" was obtained by crossing cv Moneymaker (MM) with the progeny ("F$_{1wa}$") of the cross between *L. hirsutum* G1.1290 and *L. hirsutum* G1.1560. H*Ol3* and H*Ol1* are heterozygous for *Ol-3* and *Ol-1* respectively. For each genotype 17-23 plants were tested. Evaluations were done at 10, 14 and 28 dpi.
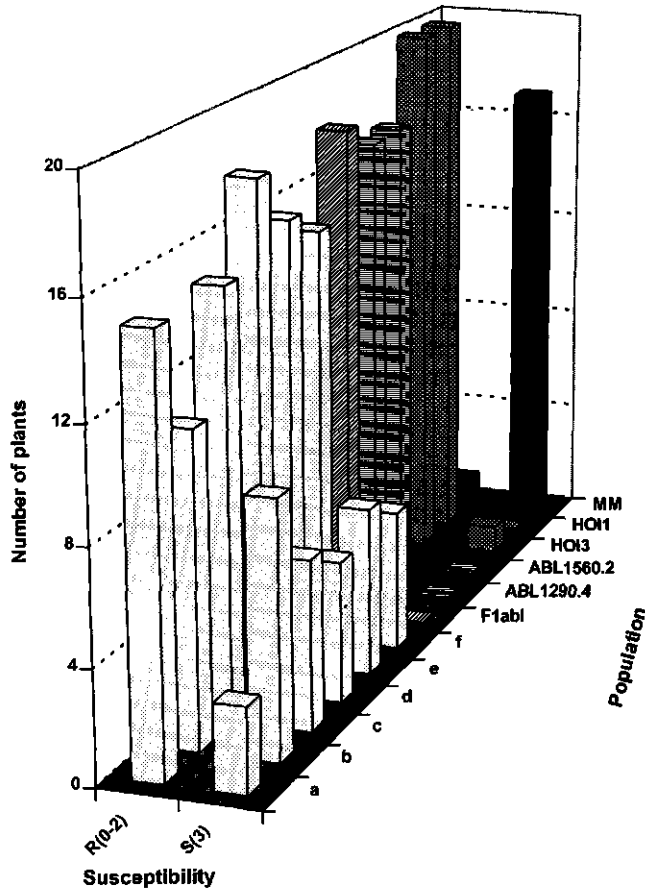
Allelism test of *Ol-1* and *Ol-3* using Advanced Breeding lines

Fig. 6 Susceptibility of the "BC$_1$S$_1$" lines.

b) Frequency distribution of resistance to *O. lycopersicum* infection in the "BC$_{1abl}$S$_1$" lines (indicated by *a-f*) at 21 days post inoculation (DPI). These lines were generated by self-pollinating the most susceptible "BC$_{1abl}$" plants. Here, "BC$_{1abl}$" was obtained by crossing cv Moneymaker with the progeny ("F$_{1abl}$") of the cross between two ABLs ABL1290.4 and ABL1560.2. For each genotype 17-23 plants were tested. Evaluations were done at 10, 14 and 21 dpi. The disease index (DI) was: 0: no sporulation; 1: slight sporulation, but less than 5% foliar area affected; 2: moderate sporulation, between 5 and 30% foliar area affected; 3: abundant sporulation, more than 30% foliar area affected.
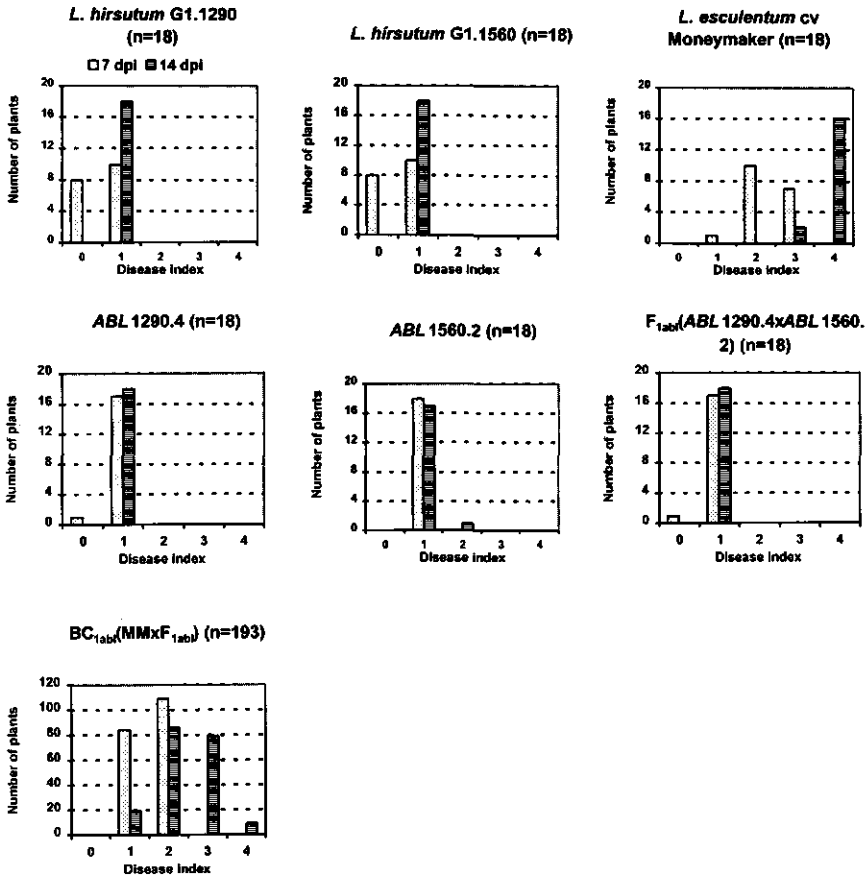
Fig. 7 Frequency distribution of resistance to *O. lycopersicum* infection in *L. hirsutum* G1.1290, *L. hirsutum* G1.1560, advanced breeding lines ABL1290.4 containing *Ol-3* and ABL1560.2 containing *Ol-1*, "F_{1abl}" of ABL1290.4 x ABL1560.2, *L. esculentum* cv Moneymaker (MM) and the "BC_{1abl}" (MM×"F_{1abl}"[ABL1290.4 x ABL1560.2]) populations. The DI scores were as defined in Fig. 5. Evaluations were done at 7 and 14 DPI.

progenies ("$BC_{1abl}S_1$") from the most susceptible "BC_{1abl}" plants were resistant (Fig. 6b), indicating the absence of susceptible recombinants in the test-cross progenies ("BC_{1abl}"). Compared with the resistant parental lines and the progeny of the putative susceptible plants from the experiment where the wild accessions were (Fig. 6a), the higher levels of susceptibility of the selfed plants from the putative susceptible BC_{1abl} plants, might be due to a higher perccentage of *L. esculentum* genome in the genetic background (e.g. Fig. 3g). Because no recombinant was found in the "BC_{1abl}" consisting of 193 plants, the distance between *Ol-1* and *Ol-3* was estimated to be smaller than 1.5 cM (P=0.05). This result was fully in agreement with that by using wild accessions.

Discussion

In the present study, we concluded that the resistance in *L. hirsutum* G1.1290 was controlled by one major gene *Ol-3*, designated *Ol-3*, that mapped between markers TG25/SCAF10 and H9A11 on chromosome 6 of tomato. The existence of some BC_1 plants which were ambiguous during disease evaluation, suggested that *Ol-3* was incompletely dominant. To map *Ol-3*, a BC_1 population was preferred over an F_2 as the larger *L. esculentum* background in BC_1 prevents negative effects of wild accession genes on the disease test, thus increasing the accuracy of disease evaluation. In the BC_1, there are only two genotypes, *Ol-3/ol-3* and *ol-3/ol-3*. The dominant *L. hirsutum* specific SCAR marker SCAF10 is informative to discriminate *ol-3* heterozygotes and homozygotes. However, the resistance allele(s) cannot be detected with the dominant *L. esculentum* specific SCAR markers such as SCAE16, SCAK16 and SCAG11, as they cannot discriminate the heterozygote resistant individuals and the homozygote susceptible individuals.

Though there might be ambiguity in disease evaluations, especially for segregating population, the disease test of the F_3 progenies and SCAR-genotyping showed that only two out of 17 F_2 plants, which were ambiguously evaluated (Huang *et al.*, 2000), had to be re-interpreted in the present study. Therefore, all the results of disease tests in the present or previous (e.g. Huang *et al.*, 2000) studies were reliable enough for interpreting inheritance of disease resistance. In addition, our investigation on the putative additional gene or suppressor gene strongly demonstrated the necessity of testing more than one generation, and the power of marker-genotyping in genetic studies.

The map positions of *Ol-1* and *Ol-3* were in the same region between SCAF10 and H9A11. By allelism test, *Ol-1* and *Ol-3* were demonstrated to be allelic or tightly linked. SCAF10 is the most closely linked marker and can be used, as a simple PCR marker, for indirect selection for either *Ol-1* or *Ol-3* resistances. However, pyramiding *Ol-1* and *Ol-3* in one tomato cultivar is

still not feasible.

The two tomato powdery mildew resistance genes identified so far, *Ol-1* and *Ol-3* may be in a cluster on chromosome 6. Disease resistance genes are commonly organized in complex loci or clusters (e.g. Hammond-Kosack & Jones, 1997; Michelmore & Meyers, 1998). The members of each cluster may originate from, genetically, similar accessions within a species, such as *Cf-2* and *Cf-5* from *L. pimpinellifolium* PI270254 and PI187002 respectively (Stevens & Rick, 1988). In contrast, they may also originate from very diverse species, like *Cf-1*, *Cf-4* and *Cf-9* (Kerr & Bailey, 1964; Jones *et al.*, 1993) from *L. esculentum*, *L. hirsutum* and *L. pimpinellifolium* respectively (Stevens & Rick, 1988).

Genetically, no difference between *Ol-1* and *Ol-3* has been found yet. Though *Ol-1* and *Ol-3* may be identical, they may also be homologues of a gene cluster. Morphologically, *L. hirsutum* G1.1290 differs from *L. hirsutum* G1.1560 by its broader leaves and by its higher level of necrosis under greenhouse conditions, especially at high humidity. Molecular analysis also demonstrated variation between the two accessions. For example, a polymorphism between *L. hirsutum* G1.1290 and G1.1560 was found for TG240. Large genetic variation between and within *L. hirsutum* accessions has also been reported by Miller & Tanksley (1990). Microscopically, G1.1290 plants showed a spreading hypersensitivity response (HR) (necrosis spread to the neighbouring cells of the haustorium-invaded cell) after inoculation with *O. lycopersicum*, while G1.1560 only showed single cell HR (necrosis confined to the haustorium-invaded cell), though this phenomenon of spreading HR in G1.1290 was not always manifest (Huang *et al.*, 1998). In addition, a lower level of resistance in ABLs carrying *Ol-3* than in ABLs containing *Ol-1*, was often observed (personal observations) especially at the seedling stage. However, it is still unknown whether such difference between G1.1560 and G1.1290 reflects the difference between the two powdery mildew resistance genes *Ol-1* and *Ol-3*. Therefore, the precise comparison of *Ol-1* and *Ol-3* awaits the sequence information after cloning the two genes.

Acknowledgement

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Chapter 7

Identification of QTLs for resistance to tomato powdery mildew (*Oidium lycopersici*) in *Lycopersicon parviflorum* G1.1601[1]

Huang CC, Meijer-Dekens F, Miedema D, Hoogendijk ECM, Dekker MCA, Niks RE, Lindhout P and Stam P

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[1] Submitted.

<div align="center">Chapter 7</div>

Identification of QTLs for resistance to tomato powdery mildew (*Oidium lycopersici*) in *Lycopersicon parviflorum* G1.1601

Huang CC[1], Meijer-Dekens F, Miedema D, Hoogendijk ECM, Dekker MCA, Niks RE, Lindhout P[2] and Stam P

Laboratory of Plant Breeding, The Graduate School of Experimental Plant Sciences, Wageningen University, PO Box 386, NL-6700 AJ Wageningen, Netherlands.
[1] Present address: Novartis Seeds BV, P.O. Box 2, 1600 AA Enkhuizen, The Netherlands.
[2] To whom correspondence should be addressed. Tel. +31-317-483454, Fax: +31-317-483457; e-mail: pim.lindhout@pv.dpw.wag-ur.nl

Abstract

Lycopersicon parviflorum G1.1601 is resistant to tomato powdery mildew (*Oidium lycopersici*). A disease test of an F_2 population from the interspecific cross between this resistant accession and the susceptible *L. esculentum* cv Moneymaker demonstrated that resistance in G1.1601 inherited quantitatively. To map the quantitative trait loci (QTLs) for the resistance to *O. lycopersici*, 104 F_2 plants were evaluated for the segregation of AFLP markers. A genetic map of 792 centimorgan (cM) was generated consisting of 259 AFLP markers. QTL mapping for resistance to *O. lycopersici* was first performed using the interval mapping method (IM), and QTLs were confirmed by multiple QTL mapping (MQM) with markers linked to the putative QTL as cofactors. Three QTLs for resistance were identified, one on Chromosome 12, two on still unassigned linkage groups. The identified QTLs showed clearly additive effects and explained in total 68% of the phenotypic variance. These results are discussed with repect to resistance mechanism and durability.

Keywords: polygenic resistance, tomato powdery mildew, *Oidium lycopersici*, mapping, QTL analysis.

Introduction

Tomato powdery mildew (*Oidium lycopersici* = *O. lycopersicum*) has become a globally important disease since 1986, when it was first reported in the Netherlands (Paternotte, 1988). Most modern tomato cultivars are susceptible. Resistance has been found in many *Lycopersicon* species (Kozik, 1993; Laterrot and Moretti, 1993; Neshev, 1993; Lindhout *et al.*, 1994a; Ciccarese *et al.*, 1998). Resistance in *L. hirsutum* G1.1290 and G1.1560 is controlled by incompletely dominant genes, *Ol-3* and *Ol-1*, respectively, which map in the same region between SCAF10 and H9A11 on Chromosome 6 and are not distinguishable yet (Van der Beek *et al.*, 1994; Huang *et al.*, 2000a; Huang *et al.*, 2000b). In most of the wild accessions evaluated, resistance is mainly associated with a hypersensitive response (HR), except for *L. parviflorum* G1.1601 in which the association of resistance with HR is not as strong as in other accessions (Huang *et al.*, 1998). This suggests that resistance in G1.1601 is at least partly due to a different mechanism than HR. In addition, earlier studies indicated that the inheritance of resistance in G1.1601 is polygenic or recessive (Pim Lindhout, unpublished).

Many agriculturally important traits that show continuous variation, are genetically complex and polygenic. By QTL (quantitative trait locus) mapping, these traits can be resolved into discrete Mendelian factors (e.g. Paterson *et al.*, 1988; Yamamoto *et al.*, 1998). In tomato, the first application of QTL mapping was to localize genes controlling fruit size, pH and soluble solids (Paterson *et al.*, 1988). Since then, QTLs have been identified for many morphological and horticultural traits (e.g. Lindhout *et al.*, 1994b; Monforte and Tanksley, 2000) and fruit quality (Bucheli *et al.*, 1999; Saliba-Colombani *et al.*, 1999). The successful cloning of *fw 2.2* and *Brix9-2-5*, two QTLs controlling respectively fruit weight and sugar content of tomato, indicates that a QTL may indeed correspond to a gene (minor allele) and differs from the major gene (major allele) by its smaller effect on a trait (Frary et al., 2000; Fridman *et al.*, 2000). QTL mapping has also been applied to identify genes controlling tolerance to abiotic factors like salt and chilling (e.g. Foolad and Chen, 1998) and resistance to biotic factors such as pathogenic bacteria (e.g. Danesh *et al.*, 1994; Van Heusden *et al.*, 1999) and insect pests (Maliepaard *et al.*, 1995; Mutschler *et al.*, 1996). However, application of QTL mapping to unravel the quantitative resistance to a fungal disease in tomato has not been reported yet.

All research on QTL mapping in tomato mentioned above, is based on genetic linkage maps consisting of either RAPD or RFLP markers. A limitation of RAPD markers is the poor reproducibility and non-locus specificity, and that of RFLP markers is the time and labour-consuming assay. In addition, these techniques are poorly informative for organisms with a low level of genetic variation. The development of AFLP^{TM} marker (Vos *et al.*, 1995)

allowed the construction of high density or saturated genetic maps also for species with limited variation. The distribution of AFLP markers in genetic linkage maps may depend on the combinations of restriction enzymes used. In barley and tomato, it has been found that the majority of the *EcoRI/MseI* markers are clustered in the heterochromatic regions around the centromere (e.g. Qi *et al.*, 1998; Haanstra *et al.*, 1999), likely due to the suppression of recombination in those regions (Haanstra *et al.*, 1999). In most genetic studies, especially QTL mapping, it is desirable to have markers evenly distributed over the genetic map. Therefore, AFLP markers based on the methylation sensitive restriction enzyme *PstI* are preferred because this enzyme recognizes restriction sites in non-methylated euchromatin (Gruenbaum *et al.*, 1981) in the distal parts of tomato chromosomes, where most functional genes and hence unique DNA is supposed to be present, while methylated DNA, likely present in the heterochromatin, is not recognized by *PstI*.

In the present study we investigated the inheritance of resistance in *L. parviflorum* G1.1601, by using an F_2 population which was evaluated for quantitative resistance to *O. lycopersici*. Subsequently, we constructed a genetic linkage map based on AFLP markers of which some were in common with an *L. esculentum* x *L. pennelli* AFLP map (Haanstra *et al.*, 1999) and were used as anchor markers to assign linkage groups to chromosomes. Furthermore, QTLs were identified and the quantitative effect of each QTL was assessed.

Materials and methods

Plant and fungus materials

An F_2 population of 209 plants derived from an interspecific cross between individual plants of the susceptible *L. esculentum* cv Moneymaker and the resistant accession *L. parviflorum* G1.1601 (Lindhout *et al.*, 1994b) was used to study the inheritance of resistance. All F_2 plants were selfed to produce F_3 lines for progeny testings, if needed.

The pathogenic fungus *O. lycopersici*, which originated from infected commercial tomato plants (Lindhout *et al.*, 1994b), was maintained on Moneymaker plants in a greenhouse compartment at 20 ± 3 °C with $70 \pm 15\%$ relative humidity (RH).

Disease test

A disease test was performed by inoculating one-month-old tomato plants with a suspension of 2×10^4 conidia.ml^{-1}. The inoculum was prepared by washing conidial spores from the freshly sporulating leaves of heavily infected Moneymaker plants in tap water and was used

immediately. The experiment was carried out according to a randomized block design with six blocks, each containing two plants of each parent and of the F_1, and 34-35 F_2 plants. The inoculated plants were grown in a greenhouse at 20±3°C with 30-70% RH.

The fungal growth was evaluated at 11, 14 and 19 days post inoculation (dpi), and was expressed as a disease index where 0 = no sporulation, 1 = slight sporulation, but less than 5% foliar area affected, 2 = moderate sporulation, 5-30% foliar area affected, 3 = abundant sporulation, more than 30% foliar area affected.

AFLP analysis

Total DNA was extracted from frozen young leaves as described by Van der Beek *et al.* (1992). Based on disease index (about equal numbers of plants per disease index class, if possible), the amount of DNA extracted per plant and the number of F_3 seeds obtained, 104 F_2 plants were selected and subjected to AFLP analysis. The AFLP procedure was performed as described by Vos *et al.* (1995). The genomic DNA was digested with restriction enzymes *Eco*RI (G/AATTC) or *Pst*I (TGCA/G) and *Mse*I (T/TAA), ligated to adapters, and a subset of DNA fragments was amplified using primers containing 16 adapter defined sequences with one additional arbitrary nucleotide to produce primary templates. Twenty-four primer combinations of primers with three (+3) selective bases (Table 1) were used in the second amplification (active PCR). They included 12 *Eco*RI/*Mse*I primer combinations: E32/M47, E32/M48, E32/M49, E32/M50, E32/M61, E35/M47, E35/M48, E35/M50, E35/M58, E35/M59, E35/M62 and E39/M50; and 12 *Pst*I/*Mse*I primer combinations: P11/M47, P11/M48, P11/M50, P11/M54, P11/M61, P11/M62, P14/M47, P14/M49, P14/M50, P14/M60, P14/M61 and P14/M62. All these primer combinations, except the underlined ones, were also used by Haanstra *et al.* (1999). The segregating AFLP markers were designated according to the primer combination used, the parent species from which they derive and the estimated fragment size (see Fig. 2).

CAPS analysis

CAPS primers CP60 and CT99 (Bendahmane *et al.*, 1997) were obtained from Dr. Rouppe van der Voort, Laboratory of Nematology, Wageningen University, Wageningen, the Netherlands. CAPS analysis was performed as described by Bendahmane *et al.* (1997) with minor modifications. The PCR conditions were: 94°C, 5 min, followed by 40 cycles of 30 s at 94°C, 30 s at 57 °C for CP60 (or 15 s at 94°C, 15 s at 62 °C for CT99) and 1 min at 72 °C, and then (after the last cycle) 7 min at 72 °C.

Table 1. Sequences of AFLP primers and adapters

Primers/adapters		Sequences[1]
*Mse*I adapter		5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTC AT-5'
M00 (universal primer)		GATGAGTCCTGAG TAA
*Mse*I + 1 primer M02		M00 + C
*Mse*I + 3 primers	M47	M00 + CAA
	M48	M00 + CAC
	M49	M00 + CAG
	M50	M00 + CAT
	M54	M00 + CCT
	M58	M00 + CGT
	M59	M00 + CTA
	M61	M00 + CTG
	M62	M00 + CTT
*Eco*I adapter		5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGG TTAA-5'
E00 (universal primer)		GACTGCGTACC AATTC
*Eco*RI + 1 primer E01		E00 + A
*Eco*RI + 3 primer	E32	E00 + AAC
	E35	E00 + ACA
	E39	E00 + AGA
*Pst*I adapter		5'-CTCGTAGACTGCGTACATGCA-3' 3'-CATCTAGACGCATGT-5'
P00 (universal primer)		GACTGCGTACATGCAG
*Pst*I + 1 primer P01		P00 + A
*Pst*I + 2 primer	P11	P00 + AA
	P14	P00 + AT

[1] DNA sequences are always from 5' to 3' orientation unless indicated otherwise.

Map construction and QTL mapping

JOINMAP (Stam and Van Ooijen, 1995) was used to facilitate linkage analysis and to generate a genetic map, which was drawn by using DRAWMAP 2.0 (Van Ooijen, 1994). Kosambi's mapping function (Kosambi, 1944) was applied to calculate map distances. Criteria were set for unreliable markers according to Haanstra *et al.* (1999).

QTL mapping was performed by using MapQTL (Maliepaard and Van Ooijen, 1996). The LOD threshold value for declaring a QTL was 3.0 in Interval Mapping. In the regions of the putative QTLs (LOD>3.0) the closely linked markers with the highest LOD value were taken as co-factors for running the multiple QTL mapping programme (MQM) to confirm the Interval Mapping.

Results

Inheritance of resistance to O. lycopersicum in L. parviflorum *G1.1601*

A disease test was performed on an F_2 population of *L. esculentum* cv Moneymaker x *L. parviflorum* G1.1601 to assess the inheritance pattern of resistance to *O. lycopersici*. All plants were evaluated for the degree of sporulation expressed as disease index (DI) at scales from 0 to 3. Plants of the resistant parent G1.1601 were either immune (DI=0) or showed weak mycelium growth (scored as 1), while all plants of the susceptible parent Moneymaker showed abundant sporulation (scored as 3, Fig. 1). The F_1 showed predominantly an intermediate DI of 1 or 2 and the F_2 plants were distributed over the DI classes 0 to 3 (Fig. 1). Thus no monogenic model for the inheritance of resistance could be deduced. This result indicates that the resistance to *O. lycopersici.* in G1.1601 is quantitatively inherited, and is likely to be controlled by more than one gene (QTL).

Development of AFLP markers

In order to identify QTLs for resistance to *O. lycopersicum* in G1.1601, a genetic linkage map was constructed. For map construction, molecular markers were efficiently generated by using the AFLP technique with 24 primer combinations, 17 of which have previously also been used by Haanstra *et al.* (1999) in constructing an *L. esculentum* x *L. pennellii* AFLP map. Because *Eco*RI/*Mse*I markers occurred more often in clusters than *Pst*I/*Mse*I markers (e.g. Qi *et al.*, 1998; Haanstra *et al.*, 1999), the latter were also included. All markers were scored dominantly. In total, 371 markers were obtained: 216 were *L. parviflorum* specific,
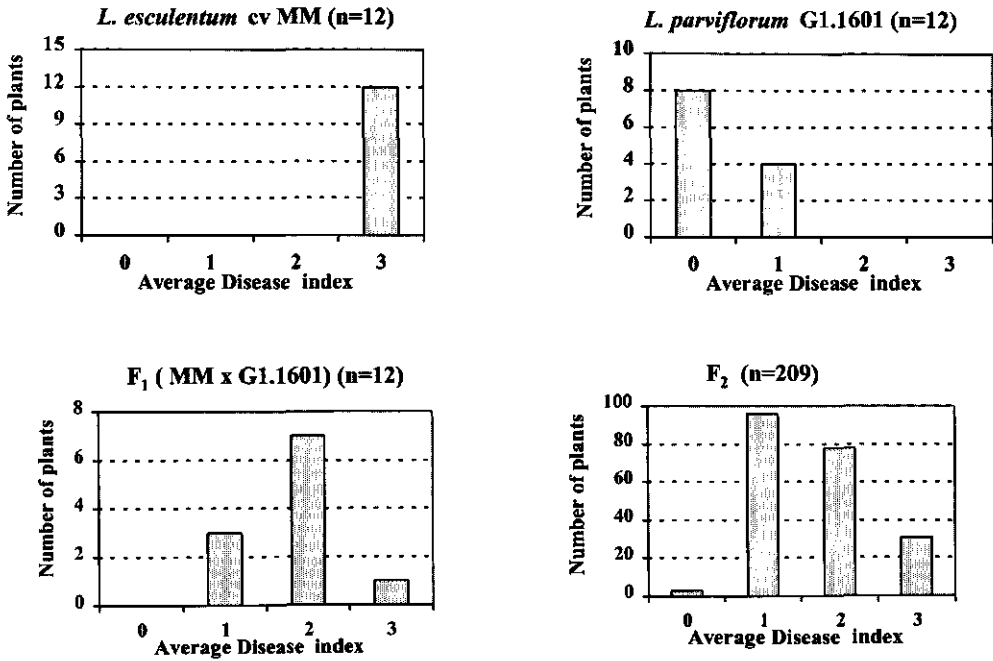
Fig. 1 Frequency distribution of *L. parviflorum* G1.1601, *L. esculentum* cv Moneymaker (MM) and their F_1 and F_2 progenies for resistance to *O. lycopersici* infection. The population size is indicated between brackets. The average disease index was the mean of disease indices evaluated at 11, 14 and 19 days post inoculation (dpi). The disease index (DI) was defined as: 0: no sporulation; 1: slight sporulation, but less than 5% foliar area affected; 2: moderate sporulation, between 5 and 30% foliar area affected; 3: abundant sporulation, more than 30% foliar area affected.

and 155 were *L. esculentum* specific among which 87 were in common with Haanstra's map. The average numbers of informative markers per *EcoRI/MseI* primer combination were 22, compared to nine markers identified per *PstI/MseI* primer combination. The *EcoRI/MseI* primer combinations had an average polymorphism rate of 43% compared to 22% of the *PstI/MseI* primer combinations.

Map construction

After generating the AFLP markers, a genetic linkage map was constructed by using JoinMap. Sixteen linkage groups were established at a LOD treshold for linkage of 4.5 to 6.5. After removing markers which caused a poor overall fit of the resulting map, 258 AFLP markers were used for the map, 41 of which were in common to Haanstra's map. These common markers were used as anchor markers to assign linkage groups to chromosomes, as comigrating AFLP bands within a species are generally allele specific (Rouppe van der Voort *et al.*, 1997; Qi *et al.*, 1998). Consequently, seven linkage groups could be assigned to chromosomes. All markers that were in one linkage group in Haanstra's map, were also in one linkage group in the present *L. esculentum* x *L. parviflorum* map. The remaining nine linkage groups, mainly consisting of *L. parviflorum* specific markers, remained unassigned because of lack of common markers. The resulting genetic map covered 790 cM of the tomato genome (not shown). In this map, clustering of markers occurred quite often, and many unassigned linkage groups consisted of only either *L. esculentum* or *L. parviflorum* specific markers. This is not unexpected since all markers specific to either one parent are in coupling phase, whereas marker pairs across parents are in repulsion phase. For the latter, significant linkage is much harder to assess in an F_2 (e.g. Maliepaard *et al.*, 1998). Therefore, the total map length of 790 cM probably is an over-estimate since some linkage groups in repulsion phase may actually belong to the same chromosome.

QTL mapping

By applying Interval Mapping (IM), three QTLs for resistance to *O. lycopersici* in G1.1601 were identified (Table 2). One QTL mapped on Chromosome 12, the other two remained unassigned (Fig. 2, Table 2). This result was confirmed by using MQM using "peak markers" for the putative QTLs from IM as cofactors.

In order to increase the accuracy of mapping and QTL analysis, two co-dominant chromosome specific CAPS markers, CP60 and CT99, were used to confirm the map of Chromosome 12 and consequently the map position of the QTL on this chromosome (Fig. 2).
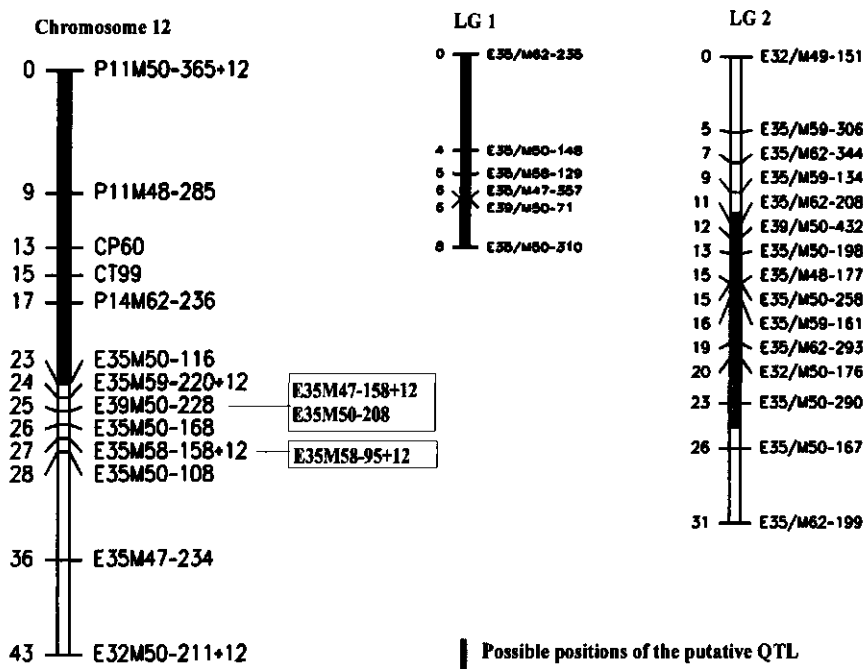
Fig. 2. Genetic linkage map of part of the tomato genome, showing the possible map positions of the putative QTLs. This map was constructed by using AFLP markers on 104 F_2 plants from the cross of *L. esculentum* cv Moneymaker × *L. parviflorum* G1.1601. Markers with a slash (/) are *L. parviflorum* specific, those with a plus (+) are *L. esculentum* specific and common to the *L. esculentum* x *L. pennellii* map (Haanstra *et al.*, 1999). The figures following the pluses (+) indicate the chromosomes on which these markers map in Haanstra's map. The markers in boxes were at the same position as the corresponding representative markers.

Table 2. The three QTLs associated with resistance to *O. lycopersici*, detected by interval mapping and confirmed by MQM mapping in an F_2 population of *L. esculentum* cv Moneymaker x *L. parviflorum* G1.1601.

Chromosome	Nearest marker	LOD value (IM)	Variation explained (%)	Additive effect on the DI
12	P14M62-236	4.2	19.0	-0.44
Unassigned LG1	E35/M50-310	4.5	28.7	-0.53
Unassigned LG2	E35/M50-198	3.2	20.2	-0.46

Table 3. Effects of QTLs on the level of resistance to *O. lycopersici*

Population		Genotype[1]	Number of resistance alleles[2]	Average disease index[3]		Number of plants[4]	
				Observed	Expected	Observed	Expected
Moneymaker		qqqqqq	0 (12/12)	3.0	3.0	12	12
G1.1601		QQQQQQ	6 (12/12)	0.3	0.1	12	12
F_1		QqQqQq	3 (12/12)	1.7	1.6	12	12
F_2	3 QTLs (3-6 alleles)	Q.Q.Q.	6 (1/64), 5 (6/64), 4 (12/64), 3 (8/64)	1.6	1.3	65	40
	2 QTLs (2-4 alleles)	Q.Q.qq	4 (3/64), 3 (12/64), 2 (12/64)	1.8	1.8	20	40
	1 QTLs (1-2 alleles)	Q.qqqq	2 (3/64), 1 (6/64)	2.6	2.5	9	14
	0 QTL	qqqqqq	0 (1/64)	2.2	3.0	2	2

[1] QQQQQQ: All three QTLs present and in homozygous form. qqqqqq: no QTL.

[2] Figures in brackets are theoretical frequencies of F_2 plants with a certain number of resistance alleles.

[3] The observed disease indexes are means of three evaluations at 11, 14 and 19 dpi. The evaluation was executed by using a disease index (DI) from 0 to 3. The DI was defined as: 0 = no sporulation; 1: slight sporulation, but less than 5% foliar area affected; 2 = moderate sporulation, between 5 and 30% foliar area affected; 3 = abundant sporulation, more than 30% foliar area affected. The expected DI was estimated assuming that each QTL allele had an average effect of -0.48 on the DI (Table 2).

[4] The expected number of plants for F_2 was calculated based on the theoretical frequencies in column 3.

The results of these markers confirmed the mapping of Chromosome 12 and the map position of this QTL.

Effects of the identified QTLs on the level of resistance

In order to study the QTL effects on the level of resistance, the 104 F_2 plants were grouped according to the numbers of resistance alleles (QTLs) they putatively contained. This was done by taking markers nearest to a QTL as indicators for the presence of QTL allele(s). An expected segregation ratio was calculated based on the theoretical frequencies of each possible genotype according to three independently segregating genes (Table 3). The resulting (observed) segregation ratio of the F_2 plants deviated significantly from the expected one ($X^2 = 23.34 > X^2_{(P=0.01)} = 11.35$), indicating a skewed segregation of markers nearest to the QTLs. Such skewness may also be due to the selection of 104 F_2 plants out of the 209 plants of the complete population.

All three QTLs have almost equal effect on the disease index (about -0.5). Together these QTLs explained 68% of the total phenotypic variation. The additive effects of each QTL were –0.44, –0.53 and –0.46 respectively (Table 2). Assuming the absence of epistasis, the difference between F_2 plants with zero and six resistance alleles could be –2.86. This approximately covers the difference between the parents, suggesting that most of the genetic variation is explained by these QTLs. As dominant AFLP markers were used, it was often impossible to determine whether one or two resistance allele(s) (heterozygote or homozygote) of each QTL were present. To estimate the expected DI for each F_2 class with the same minimum number of resistance alleles (Table 3), the expected frequency of heterozygous or homozygous loci within each class was calculated and the weighed mean DI determined. There was a remarkably good agreement between the expected and the observed DI per F_2 class. Only two F_2 plants did not contain any QTL. They were still slightly less susceptible than Moneymaker, suggesting the presence of additional minor QTL(s). Similarly, the chance to find an F_2 plant with six resistance alleles is also very low (1/64). Indeed, hardly any F_2 plant was as resistant as the resistant parent (Fig. 1). In conlusion, these QTLs had clear effects on the level of resistance (Fig. 4)

Discussion

The disease test on the F_2 population demonstrated that resistance to *O. lycopersici* in *L. parviflorum* G1.1601 inherits quantitatively. This suggests that the resistance of *L. parviflorum* G1.1601 is a different type of resistance to *O. lycopersici* than found in other wild *Lycopersicon* accessions, viz. the monogenic and dominant resistance in *L. hirsutum*
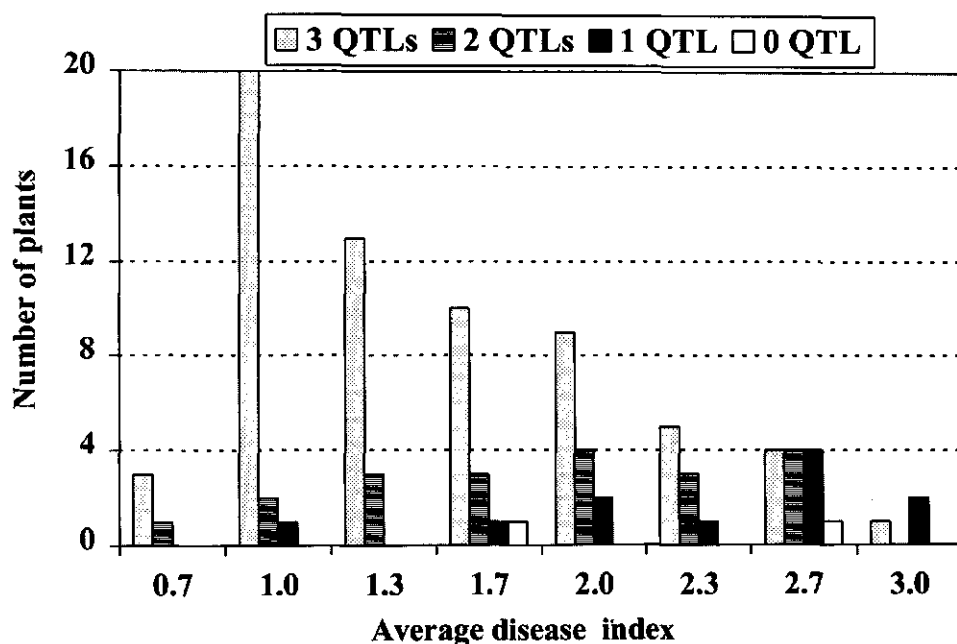
Fig. 3. Frequency distribution of the F_2 plants containing different numbers of QTLs (either homozygous or heterozygous) from *L. parviflorum* G1.1601, and their effect on the level of resistance to *O. lycopersici*. The average of disease index in the F_2 population was 1.7. The disease index is the average over three evaluations (see Table 3)

G1.1290 (Huang *et al.*, in press) and *L. hirsutum* G1.1560 (Van der Beek *et al.*, 1994; Huang *et al.*, 2000). Also the microscopic observation that the resistance in G1.1601 is less clearly associated with HR than that in G1.1290 and G1.1560 (Huang *et al.*, 1998), supports the hypothesis that the resistance in G1.1601 is of a different type than in the *L. hirsutum* accessions. This is the first report on identification of QTLs for quantitative resistance to a fungal pathogen in tomato.

In the present study, 371 AFLP markers were generated by using 24 primer combinations in the F_2 of *L. esculentum* x *L. parviflorum*, which was much fewer than 627 markers obtained by using 17 primer combinations in an F_2 of *L. esculentum* x *L. pennellii* (Haanstra *et al.*, 1999). Very likely, this is due to the lower degree of genetic variation between the two parents, because *L. esculentum* is more closely related to *L. parviflorum* than to *L. pennellii* (Rick, 1976; Taylor, 1986; Miller and Tanksley, 1990). The fact that out of the 279 *L. esculentum* specific markers in the *L. esculentum* x *L. pennellii* map of Haanstra *et al.* (1999), only 87 were polymorphic in our cross, may also be due to the difference in genetic distance between the parents used in these crosses. The relatively small number of common markers, of which only 41 were mapped in the present map, as well as the clustering of these markers resulted in the failure to assign nine linkage groups to chromosomes.

Similarly to the AFLP map of tomato (Haanstra *et al.*, 1999) and of other crop plants like barley (Qi *et al.*, 1998), clustering of AFLP markers was frequently observed in the present map, very likely due to the suppression of recombination in the heterochromatic regions (Haanstra *et al.*, 1999). However, in disagreement with Haanstra *et al.* (1999), many *PstI/MseI* markers on Chromosomes 2 and 11 in our map were also in clusters. Based on the recombination frequency between AFLP markers, our AFLP map was only 53% in length compared with Haanstra's map. This length difference between maps may have several causes. Firstly, because Haanstra *et al.* used more markers, one may expect a more complete genome coverage. Secondly, since these maps are based on two distinct interspecific crosses, the overall rate of recombination may be different. Thirdly, the difference could be an artifact resulting from scoring errors. Even small error rates in scoring of markers lead to significant overestimation of recombination frequencies. This overestimation increases as the true recombination between markers decreases (Lincoln and Lander, 1992). Thus, at equal error rates, a high density marker map will be more inflated than a sparse one. For example, there are 64 markers between E32M48-234 and E39M50-525 on Chromosome 4 in Haanstra's map but only 10 markers in our map; the distance between the two markers is 14 cM in Haanstra's map but only 5 cM in our map. Remarkably, P14M50-210 and P14M50-241 are 21-23 cM from E35M58-413 on Chromosome 2 in Haanstra's map, while they are all at the same

position in our map. It is clear that the present map is not complete yet. However, as the QTLs are clearly mapped on these linkage groups, future efforts will mainly be focused on fine mapping these QTLs using the F_3 lines rather than completing the map.

The fact that only a few F_2 plant were as resistant as the resistant parent G1.1601 (Fig. 1), was probably more due to the low chance of finding an F_2 plant with six resistance alleles (1/64) rather than due to additional QTLs that escaped our attention. Also, the total explained variance of almost 70% and the sum of the QTL effects that almost equals to the difference between the parents suggest that the principal QTLs are identified in this study. By using marker-assisted selection (MAS), the knowledge on the map positions of these QTLs will facilitate selection of the favourable QTL alleles for *O. lycopersici* resistance, even without knowing their chromosome locations. Since the resistance in G1.1601 appears to be of a different nature than that in the *L. hirsutum* accessions, and quantitative resistance is generally believed to be more durable than qualitative resistance, it would be of great interest to combine and incorporate these favourable QTL alleles into modern tomato cultivars so as to obtain a more durable resistance.

Acknowledgement

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Chapter 8

Resistance of *L. peruvianum* LA2172 to *O. lycopersici* is monogenic and dominant

Huang CC, Meijer-Dekens F and Lindhout P

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Resistance of *L. peruvianum* LA2172 to *O. lycopersici* is monogenic and dominant

Huang CC[1], Meijer-Dekens F and Lindhout P[2]

The Graduate School of Experimental Plant Sciences, Laboratory of Plant Breeding, Wageningen Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands.
[1]Current address: Novartis Seeds BV, P.O. Box 2, 1600 AA Enkhuizen, The Netherlands.
[2]To whom correspondence should be addressed. Tel. +31-317-483454, Fax: +31-317-483457; e-mail: pim.lindhout@pv.dpw.wau.nl

Abstract

A disease test on an F_2 population suggests that the resistance of *L. peruvianum* LA2172 to *O. lycopersici* is monogenic and dominant. The perspectives of using this resistance source in commercial breeding programs is discussed.

Keywords: powdery mildew, tomato, *Lycopersicon peruvianum*, inheritance, monogenic resistance, dominance

Introduction

Lycopersicon peruvianum is one of the wild species of tomato. With *L. chilense*, it forms the peruvianum-complex, which is isolated from the esculentum-complex (consisting of *L. cheesmanii, L. chmielewskii, L. esculentum, L. hirsutum, L. parviflorum* and *L. pennellii*) by severe crossing barriers (Taylor, 1986). *L. peruvianum* provides a vast reservoir of valuable genetic traits for tomato improvement. These traits include disease, pest and virus resistance, cold and salt tolerance, good attributes of fruit quality and keeping quality (Table 1). Among others, *L. peruvianum* LA2172 has been found to be almost immune to *Oidium lycopersici* (formerly named *O. lycopersicum*), the causal agent of the recent powdery mildew outbreaks in tomato (Lindhout *et al.*, 1994).

The crossing barriers have limited the genetic characterization of *L. peruvianum* and its exploitation in tomato breeding for bio-stress resistance and quality improvement. These barriers have been partially overcome by using *in vitro* techniques such as embryo rescue and ovule culture. In practical breeding, resistances to bacterial canker (Crino *et al.*, 1995), root-knot nematodes (Cap *et al.*, 1991 & 1993; Veremis and Roberts,1996), tobacco mosaic virus (Bonito, 1985), tomato spotted wilt virus and leaf miner (Segeren *et al.*, 1993) have been incorporated from *L. peruvianum* into *L. esculentum* by embryo rescue (also see Table 1). *L. peruvianum* LA1708 and LA2172 are 'Northern races' that are crossable to *L. esculentum* (Lindhout and Purimahua, 1988; Gradziel *et al.*,1993; Veremis and Roberts, 1996b). These two accessions have been often used as a bridge for crossing other *L. peruvianum* accessions to *L. esculentum* (e.g. Gradziel *et al.*,1993; Veremis and Roberts, 1996b).

A genetic map consisting of 73 RFLP markers has been constructed by using three backcross populations derived from an intraspecific cross between two accessions, LA2157 and LA2172, of *L. peruvianum* (Van Ooijen *et al.*, 1994). This map is very similar to the *L. esculentum /L. pennelli* map (Tanksley *et al.*, 1992) in both the order of markers and the lengths of the chromosomes. This *L. peruvianum* map facilitate future genetic studies in *L. peruvianum*. Later, a higher recombination frequency in the region of the short arm of Chromosome 1 proximal to the *Cf-4/Cf-9* gene cluster has been found in an *L. esculentum* x *L. peruvianum* map, compared with the *L. esculentum* x *L. pennellii* map (Bonnema *et al.*, 1997), indicating a higher mapping efficiency with *L. peruvianum*.

As most of the wild accessions evaluated, like *L. hirsutum* G1.1290 and G1.1560, resistance of LA2172 to *O. lycopersici* is associated with the hypersensitive response (HR) (Huang *et al.*, 1998). Resistance in the two *L. hirsutum* accessions G1.1290 and G1.1560 is controlled by incompletely dominant genes, *Ol-3* and *Ol-1*, respectively, which map in the

Table 1. Valuable traits of *L. peruvianum* accessions

Accession	Resistance or other traits	Corresponding gene	Chromosome	Population development	Reference
not reported	tomato spotted wilt virus	*Sw-5*	9	not reported	Stevens *et al.*, 1996
PI306811 *etc.*	tomato spotted wilt virus			*in vitro* germination	Duval *et al.*, 1993
PI128650	tobacco mosaic virus	*Tm-2ᵃ*	6	Conventional cross	Young *et al.*, 1988; Ganal and Tanksley, 1996
not reported	tobacco mosaic virus			Embryo rescue	Segeren *et al.*, 1993
EC104395	tomato yellow leaf curl virus	(three genes)		not reported	Vidavsky *et al.*, 1998
PI126946, PI128643, PI128650, PI128652	Bacterial speck (*Pseudomonas syringae* pv *tomato*)			Conventional cross	Stockinger and Walling, 1994
LA2157	Bacterial canker (*Clavibacter michiganensis* ssp. *michiganensis*)	3 QTLs	5, 7 & 9 resp.	Conventional cross	Van Heusden *et al.*, 1999
LA2172	**powdery mildew (*O. lycopersici*)**	**(monogenic)**		**Conventional cross**	**(present study)**
PI126944	Fusarium crown and root rot (*Fusarium oxysporum* f. sp. *radicis-lycopersici*)	*Frl 9*	9	not reported	Rowe and Farley, 1981; Vakalounakis *et al.* 1997
	black leaf mold (Pseudocercospora fuligena)			none	(Hartman and Wang, 1993)
not reported	*Phytophthora infestans*			none	Kiku *et al.*, 1979
	Erwinia chrysanthemi			none	Victoria *et al.*, 1982
not reported	Corky root rot (*Pyrenochaeta lycopersici*)	*py-1*	3	not reported	Doganlar *et al.* 1998
PI128657	Root-knot nematodes (*M. incognita*, *M. arenaria* and *M. javanica*)	*Mi*	6	not reported	Gilbert and McGuire, 1953
PI270435	Root-knot nematodes (*M. incognita*, *M. arenaria*, *M. javanica* and *M. hapla*)	*Mi-2*	6	Conventional cross	Cap *et al.*, 1993
PI126443	Root-knot nematodes (*M. javanica* and *M. incognita*)	*Mi-3*	12	Conventional cross	Yaghoobi *et al.*, 1995
LA1708	Root-knot nematodes (*Meloidogyne incognita*, *M. arenaria*) (heat stable)	*Mi-4*		Conventional cross	Veremis and Roberts, 1996b
not reported	Leaf miner (*Scrobipalpuloides absoluta* [*Scrobipalpula absoluta*])			Embryo rescue	Segeren *et al.*, 1993
LP1650	cold tolerance			Embryo rescue	Brüggemann *et al.*, 1996
not reported	fruit dry matter content (over 5.5%)			none	Petrescu and Wu, 1981
not reported	high content of dry matter, sugar, acids and ascorbic acid			none	Kiku *et al.*, 1979
not reported	keeping quality			none	Kiku *et al.*, 1979
not reported	Salt tolerance			none	Li *et al.*, 1988

same region between SCAF10 and H9A11 on Chromosome 6 (Van der Beek *et al.*, 1994; Huang *et al.*, 2000a; Huang *et al.*, 2000b). Resistance in another wild accession *L. parviflorum* G1.1601 is only weakly associated with HR. It has been found to be controlled by three QTLs (Huang *et al.*, submitted).

In order to facilitate the introduction of the resistance of LA2172 into modern tomato cultivars, we investigated in the present study the inheritance of resistance of LA2172 to *O. lycopersici*.

Materials and Methods

Plant and fungus materials

More than 100 artificial pollinations were made between individual plants of *L. esculentum* cv Moneymaker (female) and *L. peruvianum* LA2172 (male). Pollinations by two random plants of LA2172 yielded 17 and 20 fruits respectively. Six out of the 17 fruits contained in total 22 seeds from which nine F_1 plants were raised, and nine out of the 20 fruits contained a total of 29 seeds from which two F_1 plants were obtained. Because these F_1 plants were self-incompatible, "F_2" populations were obtained by cross-pollinations between individual F_1 plants. Finally, several "F_2" populations of reasonable size were established that were suitable for genetic studies. Based on the number of seeds, an "F_2" population (PV963035) of 194 plants, derived from the cross between two F_1 plants (PV94473 plant no.719 and PV94489 plant no.717) from different *L. peruvianum* plants was selected for the present study. The "F_2" plants were selfed to generate F_3 lines for further characterization of the resistance.

The pathogenic fungus *O. lycopersici*, which originated from infected commercial tomato plants (Lindhout *et al.*, 1994b), was maintained on Moneymaker plants in a greenhouse compartment at 20±3 °C with 70±15% relative humidity.

Disease test

A disease test was performed by inoculating one-month-old plants with conidial spores in a spore suspension of 2×10^4 conidia.ml^{-1}. The inoculum was prepared by washing conidial spores from the freshly sporulating leaves of heavily infected Moneymaker plants in tap water and was used immediately. The experiment was carried out according to a randomized complete block design with six blocks, each containing 32-33 F_2 plants, three LA2172 plants, five plants each of *L. esculentum* cv Moneymaker and an Advanced Breeding Line (ABL) containing the *Ol-1* gene. LA2172 and the ABL served as resistant control and Moneymaker

as susceptible control. The inoculated plants were grown in a greenhouse at 20±3°C with 30-70% RH.

The fungal growth was evaluated at 14, 17 and 21 days post inoculation (dpi), and was expressed as a disease index where 0 = no sporulation, 1 = slight sporulation, but less than 5% foliar area affected, 2 = moderate sporulation, 5-30% foliar area affected, 3 = abundant sporulation, more than 30% foliar area affected.

Results

The inheritance of resistance in *L. peruvianum* LA2172 was determined by a disease test of the "F$_2$" population derived from two different LA2172 grandparents crossed with the tomato cultivar Moneymaker. The level of resistance of the "F$_2$" plants was classified by using the disease index (DI) on a scale from 0 to 3. As expected, especially at the later stage of disease development, all Moneymaker plants were heavily infected and thus were scored as 3 (Fig. 1). All individuals of the resistant parent and the ABL were either immune or slightly infected and were scored as 0 or 1 respectively. Therefore, plants with a 3 were considered as susceptible, and plants with a DI of 0-1 as resistant. By using this index, most "F$_2$" plants (152) could be unambiguously identified as resistant or susceptible (Fig. 1). Some "F$_2$" plants (42) were scored as 2 so that their score does not allow them to be classified unambiguously as resistant or susceptible. The existence of the ambiguous "F$_2$" plants suggested that the resistance is incomplete in heterozygous plants. These "F$_2$" plants were considered as resistant, before further analysis of their progenies. Segregation of resistance (151 R : 43 S at 21 dpi) was in accordance with a monogenic, dominant trait. We designate the corresponding resistance gene as *Ol-4*.

Discussion

Interspecific crosses, especially between remote species, usually yield neither normal seed nor fertile F$_1$ due to incompatibility and early embryo abortion. In these cases, the interspecific hybrid plants can only be recovered by *in vitro* techniques such as embryo rescue and *in vitro* germination of immature seeds (e.g. Duval et al., 1993; Poysa, 1990; Segeren *et al.*, 1993). However, *in vitro* culture is labour intensive and requires special equipment and chemicals. In addition, often plants raised from *in vitro* rescued embryos are sterile. In the present study, we aimed at obtaining normal seeds by doing a large number of artificial pollinations, as we expected that normal seeds may give rise to normal plants with a higher chance for fertility. Indeed, in our study most F$_1$ plants were fertile, but self-incompatible.normal seeds were eventually obtained, though a big number of artificial pollinations were required. Exceptionally,
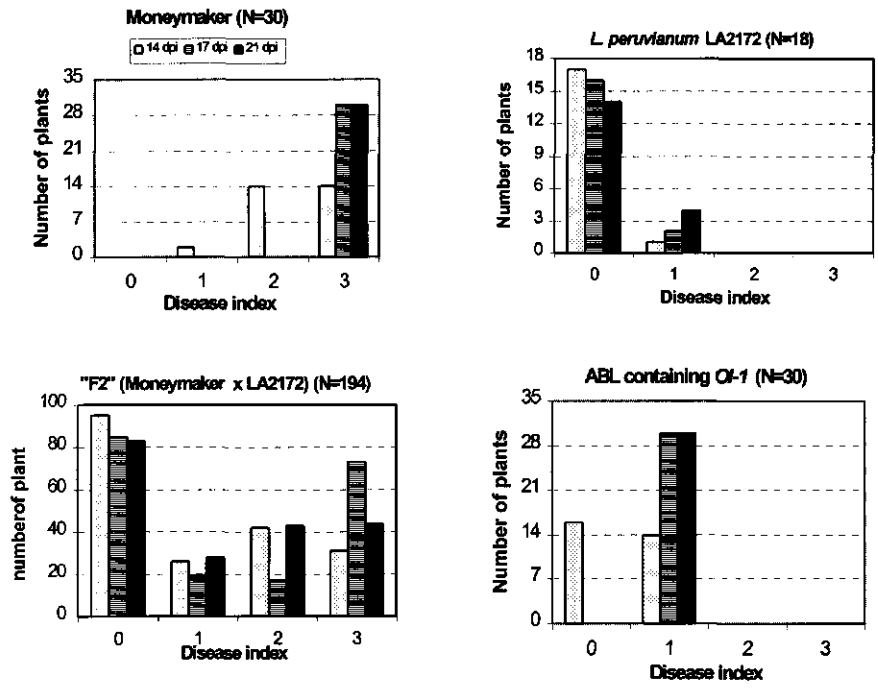
Fig. 1. Frequency distribution of resistance to *O. lycopersicum* infection on *L. esculentum* cv Moneymaker, *L. peruvianum* LA2172 and their "F₂" progenies, as well as on an *Ol-1* resistant ABL. The disease indexes were scored as: 0, no symptom; 1, some mycelia without sporulation; 2, moderate mycelia with poor sporulation; 3, abundant mycelia with heavy sporulation. Evaluations were done at 14, 17 and 21 days post inoculation (dpi).

an F_1 plant has been obtained via embryo rescue from the cross between *L. esculentum* and *L. peruvianum* LA2157 that was very fertile and self compatible (*e.g.* Van Heusden *et al.*, 1999; Van Ooijen *et al.*, 1994). In general, no matter how the F_1 progenies are generated from the cross between *L. esculentum* and *L. peruvianum*, the F_1 plants are mostly self-incompatible and the F_2 is often obtained by (half-) sib crosses (also see Duval *et al.*, 1993 and Segeren *et al.*, 1993). In the present study we also used this strategy to generate segregating "F_2" populations. These (partially) fertile "F_2" plants can then be used as a bridge to overcome the incompatibility barriers between *L. esculentum* and *L. peruvianum* in developing further advanced materials in breeding programs.

Evaluation of the *L. peruvianum* LA2172 derived F_2 progeny in a disease test suggests that resistance of LA2172 to *O. lycopersici* is monogenic and dominant. The resistance of LA2172 is characterized by highly restricted mycelium growth with trace sporulation. LA2172 can be another promising source of resistance to *O. lycopersici*, because the resistance is very likely controlled by a single gene which confers a high level of resistance.

The F_2 plants with ambiguous scoring (DI=2), need to be more accurately evaluated for their resistance/susceptibility. This can be done by testing their F_3 progenies. If F_3 progenies are uniformly resistant or susceptible, the corresponding F_2 plants must be homozygous for resistance or susceptibility, respectively. If F_3 progenies are segregating for resistance/susceptibility, the corresponding F_2 plants must be heterozygous for resistance. After doing so, the genetic basis of the *O. lycopersici* resistance can be more precisely deduced. Moreover, like *L. hirsutum* G1.1290 and G1.1560, the resistance in LA2172 is associated with HR, which may also support its hypothesized monogenic nature.

Mapping of this new resistance and comparing *Ol-4* with *Ol-1* and *Ol-3* will confirm the genetic basis of the resistance, and will facilitate pyrimading *Ol-4* with other *Ol*-genes or other resistances (Table 1) in a modern tomato cultivar for a more durable resistance to *O. lycopersici* or more environmentally friendly production.

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Chapter 9

General discussion

Since long, tomato (*Lycopersicon esculentum* Mill.) is one of the best studied crop plants, due to its easy crossability, clear genetics and economic importance (e.g. Rick, 1975). More recently, the small genome content (Arumuganathan and Earle, 1991), the high density molecular marker maps (Tanksley *et al.*, 1992; Haanstra *et al.*, 1999), the successful isolation of genes and the well developed transformation protocols have rendered tomato very suitable as a model organism for genetic and genomic studies.

Tomato powdery mildew caused by *Oidium lycopersici* Cooke & Mass. is a relatively new but nowadays worldwide disease of tomato. It is also the only disease which has to be controlled by using fungicides in protected tomato production in Northwestern Europe. All other important fungal diseases can readily be controlled by the use of resistant cultivars or sanitary measures. Therefore, with the increase of public concerns about the ecologically safe foods, it is urgent to develop new tomato cultivars containing resistance also to *O. lycopersici*. In the beginning of the present research, there was lack of knowledge of the pathogenecity of the fungus on tomato and other closely related species, of the genetic variation of the causal agent(s) worldwide, and of the inheritance of resistance in the resistant wild accessions. The development of resistant cultivars may benefit from insights on the interaction between tomato and *O. lycopersici*. The finding of resistant wild *Lycopersicon* accessions (e.g. Lindhout *et al.*, 1994), the development of some segregating populations derived from these accessions and the great interest of the industry provided good bases for the research presented in this thesis. The high density RFLP map (Tanksley *et al.*, 1992), the preliminary mapping of the *Ol-1* gene, and more recently, the AFLP map (Haanstra *et al.*, 1999) were also ingredients for support of this research. The results described in this thesis will not only facilitate the breeding of powdery mildew resistant tomato cultivars, but also stimulate scientific research on the function and structure of the *Ol*-genes.

Origin of *O. lycopersici*

The origin of *O. lycopersici* is still unknown. We hypothesized that *O. lycopersici* is a new form of another powdery mildew species which "jumped" from its host species to tomato by the acquisition of pathogenicity to the latter species, as has been documented in a number of plant-pathosystems (Savile, 1971; Baum and Savile, 1985; Storer *et al.*, 1994; Holst-Jensen *et al.*, 1997; Kaplan *et al.*, 1999). So, host range studies might provide clues to the possible origin(s) of the pathogen(s). Despite earlier reports on a wide host range, in the present study only

tobacco was identified as host species for *O. lycopersici* (Chapter 3). Consequently, tobacco
powdery mildew was considered as a candidate for the origin of *O. lycopersici*, because tobacco
was as susceptible as to the fungus tomato (Chapter 3). This hypothesis, however, was not
supported by marker analysis (Chapter 4). Still, tobacco powdery mildew may be an old
ancestor of tomato powdery mildew. Alternatively, another powdery mildew is the ancestor, but
it still escaped our attention.

Gene-for-gene interaction

The resistance to *O. lycopersici* in most wild resistant accessions and in the advanced breeding
lines is posthaustorial, and is clearly associated with a hypersensitive response (HR), though
much less HR was observed in one resistant accession *L. parviflorum* G1.1601. Such
combination of posthaustorial nature and HR usually indicates a race specific major gene
resistance (Heath 1981 & 1982). However, there are exceptions that resistance genes are not
related to HR but are race-specific, like the *Hm-1* gene in maize that detoxifies the HC-toxin of
Cochliobolus carbonum (Johal and Briggs, 1992) and the *mlo* gene in barley that is involved in
papillae formation, inhibiting the invagination of fungal hyphae of *Erysiphe graminis* f. sp.
hordei into barley cells (Büschges *et al.*, 1997). The resistance in *L. hirsutum* G1.1290 and
G1.1560, and in *L. peruvianum* LA2172 has been proven to be monogenic (Van der Beek *et al.*,
1994; Chapters 5, 6 & 8), and associated with HR. Most likely these resistance are race specific
and based on a gene-for-gene interaction. However, this hypothesis remains to be demonstrated.
Till now, there is no evidence that different races of *O. lycopersici* occur that have differential
interaction with tomato genotypes containing specific *Ol* genes (Chapter 4).

While many complete resistance genes are race-specific, it is conceivable that partial or
quantitative resistance genes (loci) might generally be race-nonspecific (also Young, 1996).
However, with QTL mapping by using molecular markers, more and more partial or
quantitative resistance genes (QTLs) are reported to be race-specific. For example, six out of 11
QTLs for resistance to *Phytophthora infestans* in potato showed specificity against just one race
(Leonards-Schippers *et al.*, 1994). Such race-specificity of QTLs have also been reported, for
instance, in the resistance to leaf rust (*Puccinia hordei*) in barley (Qi *et al.*, 1998) though now
withdrawn, to bacterial wilt (*Pseudomonas solanacearum*) in tomato (Danesh *et al.*, 1994) and
to cyst nematode (*Heterodera glycines*) in soybean (Concibido *et al.*, 1994). QTL analysis is
eventually a (multiple) linear analysis of markers and the interested traits. Its accuracy depends
on the genetic linkage maps used and the measurement of the traits. In most cases, QTL analysis
is a reliable approach to identify loci for quantitative traits. For example, the same major QTLs
for resistance to leaf rust have been identified in diferent populations (Qi *et al.*, 1998).
Remarkably, the QTL *fw 2.2* or *Brix9-2-5*, controlling respectively fruit weight and sugar

content of tomato, just corresponds to a gene (minor allele) and differs from the major gene (major allele) by its smaller effect on a trait (Frary et al., 2000; Fridman *et al.*, 2000). The three QTLs for resistance to *O. lycopersici* identified in the present research show clear effect on the level of resistance (Chapter 7). In order to exploit these QTLs in commercial breeding programs, it is necessary to develop more closely linked molecular markers to finemap these QTLs so as to facilitate marker assisted selection of these QTLs. Also the specificity of QTLs for resistance to *O. lycopersici* (Chapter 7) can only be tested when distinct isolates/races, that differ in their pathogenecities, are discovered.

Organization of *Ol*-genes

Over the last five years a number of plant disease resistance genes (R-genes) has been cloned. Remarkably, R-genes for diverse classes of pathogens like viruses, fungi and bacteria, cloned from a wide range of plants species (both mono- and dicots) share particular common motifs and domains (reviewed by Hammond-Kosack and Jones, 1997). A number of R-genes contain an N-terminal nucleotide binding site (NBS) domain and a number of leucine rich repeats (LRRs) at their C-terminus. These R-genes are either cytoplasmic or membrane anchored. One class of R-genes, e.g. the tomato *Cf*-genes, are membrane anchored glycoproteins with an extracytoplasmic domain mainly consisting of LRRs. Another class of R-genes consists of the tomato *Pto* gene, encodes a cytoplasmic serine threonine kinase and no LRR. The rice *Xa-21* gene, however, consists of both kinase and an LRR domain. Many homologs of both NBS and LRR domains were mapped in plant genomes. These homologs were often mapped on the same loci as race-specific R-genes (Kanazin *et al.*, 1996; Leister *et al.*, 1996 & 1998).

Many R-genes are organized in clusters. This is true for a number of tomato R-genes (*Cf*, *Pto* and *I-2*). Some R-genes to bacterial, fungal and viral pathogens are genetically linked. Such R-gene-rich regions are designated as major resistance complexes (MRCs) (Hammond-Kosack and Jones, 1997). The *Ol-1* and *Ol-3* genes map in such a MRC, since they are linked to the *Cladosporium fulvum* resistance genes *Cf-2/Cf-5*, and the root knot nematode (*Meloidogyne* spp.) resistance gene *Mi* and the aphid resistance gene *Meu-1* (= *Mi*) (Dixon *et al.*, 1995 & 1996; Kaloshian *et al.*, 1998; Rossi *et al.*, 1998). More information on the structure of the *Ol*-genes will be available within a few years, and contribute to our understanding of the evolution and function of R-genes.

Prospect in breeding for resistance to *O. lycopersici* in tomato

In the present research, monogenic and dominant resistance has been demonstrated in resistant accessions *L. hirsutum* G1.1290 and G1.1560 and *L. peruvianum* LA2172), and polygenic resistance

in *L. parviflorum* G1.1601. By using the strategy of marker-assisted selection (MAS), tomato breeders can apply molecular markers linked to the *Ol*-genes or QTLs for resistance to indirectly select for the resistant genotypes for both mono- and polygenic resistance (even without knowing the chromosome locations). Such indirect selection can usually be done at the early stage of plant development, and thus will improve the selection efficiency and reduce breeding costs. In addition, most, if not all, causal agents responsible for the recent outbreaks reported all over the world belong to one anamorph (Chapter 4; Kiss *et al.*, 1999). It is not unlikely that the resistance identified so far, or even incorporated into modern cultivars will be overcome by the fungus. The present study has shown that *Ol*-genes are available in the *Lycopersicon* genus that offer a genetic reseroir to the breeders who can exploit them when needed.

Future research

The present research has provided basic knowledge which can be applied to both practical breeding and fundamental research like map-based cloning of *Ol*-genes. However, many questions regarding to this plant pathosystem still remain. Is it possible to find the origin(s) of *O. lycopersici*, or searching for such origin is like looking for a needle in the sea (Chinese)/hay (Dutch)? Is the *L. esculentum*/*L. parviflorum* AFLP map accurate enough? To which chromosomes do the unassigned linkage groups belong (Chapter 7)? Do QTLs for resistance to *O. lycopersici* also give resistance to other powdery mildew species, such as *Laveillula taurica*? How does resistance in other resistant accessions inherit? To address to these questions, more *Ol*-genes or QTLs for resistance should be identified, mapped and eventually cloned.

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Summary

Since 1986, tomato powdery mildew (*O. lycopersici*) has become an important disease in tomato production around the world. All modern tomato cultivars were susceptible by the time the present project started. Both the *Pseudoidium* (*Oidium* with solitary conidia) and the *Euoidium* (*Oidium* with catenary conidia) anamorph have been repeatedly reported as causal agents of the recent powdery mildew epidemics. However, published data on the morphology of tomato powdery mildew were often conflicting which hampers exact identification of the causal agent. Based on the morphology of the asexual stage, the causal agent in the Netherlands and the UK has been identified as *Oidium lycopersicum* (recently renamed *Oidium lycopersici*), which was described in Australia for the first time in 1888 and has only been mentioned occasionally in the literature till 1986. The origin(s) of the organism(s) causing the recent outbreaks of tomato powdery mildew in the world is unknown. Resistance has been found in some accessions of several wild *Lycopersicon* species. Also, the mechanism of defence to *O. lycopersici* in *Lycopersicon* accessions was unkown. Only one of the resistant accessions had been studied for the inheritance of resistance.

The development of *O. lycopersici* on susceptible cv Moneymaker and the defence response to *O. lycopersici* in *Lycopersicon* accessions is described in Chapter 2. Spore germination and (primary) haustorium formation in resistant accessions were as frequent as on the susceptible *L. esculentum* cv Moneymaker. A high frequency of necrosis of epidermal cells in which a haustorium was formed appeared to be the major defence response, indicating that resistance to *O. lycopersici* in the *Lycopersicon* genus was predominantly associated with the hypersensitive reaction. However, the resistance in *L. parviflorum* was only weakly associated with hypersensitivity as compared with other resistant accessions, suggesting the existence of a different but still unknown resistance mechanism. In addition, evidence was provided that the level of resistance could depend on the genetic background and the plant developmental stage.

In order to determine the host range of *O. lycopersici* (Chapter 3), nine accessions of cucurbits, 10 of eight legume species, three of lettuce (*Lactuca sativa*) and 34 of 14 Solanaceae species were inoculated with a Dutch isolate of the tomato powdery mildew fungus. Thirty-six accessions were also subjected to histological observations in order to investigate the defence response. Macroscopically, no fungal growth was visible on sweet pepper (*Capsicum annuum*), lettuce, petunia (*Petunia* spp.) and most legume species (*Lupinus albus*, *L. luteus*, *L. mutabilis*, *Phaseolus vulgaris*, *Vicia faba*, *Vigna radiata*, *V. unguiculata*). Trace infection was occasionally observed on melon (*Cucumis melo*), cucumber (*C. sativus*), courgette (*Cucurbita pepo*), pea (*Pisum sativum*) and *Solanum dulcamara*. Eggplant (*S. melongena*), the cultivated

potato (*S. tuberosum*) and three wild potato species (*S. albicans, S. acaule* and *S. mochiquense*) were more heavily infected in comparison with melon, cucumber, courgette, pea and *S. dulcamara*, but the fungus did not reproduced sufficiently to be maintained on these plant species. All seven tobacco (*Nicotiana tabacum*) accessions were as susceptible to *O. lycopersici* as tomato (*Lycopersicon esculentum* cv Moneymaker), suggesting that tobacco was an alternative host. This host range differs from that of tomato powdery mildews reported from some other countries where milder criteria have been used to determine the host range (Chapter 3); these reports also vary among each other, indicating that host range studies can only be compared by using strict criteria and well designed experiments. Histologically, the defence response to *O. lycopersici* in all the plant species tested in this study was highly associated with a posthaustorial hypersensitive response.

The genetic variation among 11 field isolates of tomato powdery mildew collected worldwide was investigated by AFLP fingerprinting (Chapter 4). The AFLP fingerprints of tomato powdery mildew were compared with that of 12 other powdery mildew species to assess the possible phylogenetic origin of tomato powdery mildew. We also compared the molecular data with morphological data (size of spores and spore arrangement) for taxonomic purposes. Our results demonstrated that 1) tomato powdery mildew isolates are very similar to each other in morphology and AFLP fingerprint; and 2) tomato powdery mildew was genetically very different (hardly any AFLP band in common) from any other powdery mildew species. We did not find any correlation between AFLP fingerprints and size of spores or spore arrangement (*i.e.* solitary or catenary). We concluded that only one anamorph of powdery mildew has been responsible for the recent outbreaks.

L. hirsutum Gl.1560 is one of the resistant wild accessions. The resistance is known to be largely controlled by an incompletely-dominant gene *Ol-1* located near the *Aps-1* locus in the vicinity of the resistance genes *Mi* and *Cf-2/Cf-5* on tomato Chromosome 6. Using a newly developed F_2 population (N=150) segregating for resistance, the *Ol-1* gene was more accurately mapped between the RFLP markers TG153 and TG164 (Chapter 5). Furthermore, in saturating the *Ol-1* region with more molecular markers using bulked segregant analysis, five RAPDs were identified that were associated with the resistance. These RAPDs were sequenced and converted into SCAR markers: SCAB01 and SCAF10 were *L. hirsutum* specific; SCAE16, SCAG11 and SCAK16 were *L. esculentum* specific. By linkage analysis a dense integrated map comprising RFLP and SCAR markers near *Ol-1* was obtained. This will facilitate a map-based cloning approach for *Ol-1* and marker-assisted selection for powdery mildew resistance in tomato breeding.

   L. hirsutum G1.1290 is another resistant accession of the *Lycopersicon hirsutum* species. Chapter 6 describes 1) the genetics and mapping of resistance to *O. lycopersici* in G1.1290, 2) finemapping of *Ol-1* originating from *L. hirsutum* G1.1560, and 3) tests for allelism of resistance in G1.1290 and G1.1560. The resistance in G1.1290 to *O. lycopersici* was demonstrated to be controlled by an incompletely dominant gene, designated *Ol-3*, which was mapped between markers TG25/SCAF10 and H9A11 on Chromosome 6. By testing some F_3 lines and their progenies from the cross between *L. esculentum* cv Moneymaker and *L. hirsutum* G1.1560, we provided more evidence for the map position of *Ol-1* to be between SCAF10 and H9A11. This implies that *Ol-1* and *Ol-3* are in the same chromosome region. Allelism tests did not result in susceptible recombinants, indicating that *Ol-1* and *Ol-3* may be located on the same locus. There is, however, some (indirect) evidence from the observed differences in the infection process that these two genes are not identical (see Chapter 2). They might represent functional genes of a cluster of *Ol*-homologues.

   L. parviflorum G1.1601 is another resistant accession that was investigated. A disease test of an F_2 population of Moneymaker x G1.1601 showed that the resistance of G1.1601 was quantitative (Chapter 7). In order to map the quantitative trait loci (QTLs) for resistance to powdery mildew, a genetic map of 792 centimorgan (cM) in length consisting of 259 AFLP markers, was generated by using the same F_2 population. Three QTLs for resistance were identified, one of which mapped to Chromosome 12 whereas two reside on linkage groups that remained unassigned to chromosomes. Together they contributed approximately 63% to the total phenotypic variance. The identified QTLs clearly showed additive effects on the level of resistance without significant epistatic interaction.

   Resistance of *L. peruvianum* LA2172 to *O. lycopersici* is likely to be controlled by a single dominant gene, *Ol-4* (Chapter 8). This resistance gene awaits to be mapped.

Based on the experiments described in Chapters 2-8, the following conclusions are drawn:
1) Resistance to *O. lycopersici* in *Lycopersicon* species is mainly associated with hypersensitive response.
2) Tobacco may be an alternative host of *O. lycopersici*.
3) Tomato powdery mildew isolates from different continents are very similar to each other, suggesting a single origin of these (field) isolates.
4) Resistance in *L. hirsutum* G1.1560 as well as G1.1290 is monogenic and dominant. The two resistance genes *Ol-1* and *Ol-3* are both mapped between markers SCAF10 and H9A11, and are genetically not distinguishable from each other.
5) Resistance in G1.1601 is polygenic. Three QTLs for the resistance were identified, one on chromosome 12, two on linkage groups that remained unassigned.

6) Resistance in *L.peruvianum* LA2172 is also monogenic and dominant. The corresponding resistance gene is designated *Ol-4*.

Samenvatting

Sinds 1986 is de echte meeldauw van tomaat (*O. lycopersici*) een belangrijke ziekte geworden in tomatenteelten over de hele wereld. Bij de start van het huidige onderzoeksproject waren alle moderne tomatenrassen vatbaar. Zowel de *Pseudoidium* (*Oidium* met solitaire conidia) en de *Euoidium* (*Oidium* met conidia in rijtjes) anamorfen zijn herhaaldelijk gemeld als de veroorzakers van de recent opgetreden echte meeldauw epidemieën. Gepubliceerde gegevens over de morfologie van de echte meeldauw van tomaat zijn vaak tegenstrijdig, wat de eenduidige identificatie van de veroorzaker(s) verhindert. Gebaseerd op de morfologie van het asexuele stadium is de veroorzaker in Nederland en het Verenigd Koninkrijk geïdentificeerd als *Oidium lycopersicum* (recent hernoemd als *Oidium lycopersici*). Deze is voor het eerst in 1888 in Australie beschreven en is slechts af en toe in de literatuur van vóór 1986 vermeld. De oorsprong van het organisme, dat de recente uitbraak heeft veroorzaakt, is onbekend. In sommige herkomsten van wilde *Lycopersicon* soorten is resistentie aangetroffen. Het mechanisme van deze resistenties was nog onbekend. Slechts één van de resistente herkomsten was onderzocht op de overerving van resistentie.

De ontwikkeling van *O. lycopersici* op het vatbare ras 'Moneymaker' en de resistentiereactie van *O. lycopersici* in *Lycopersicon* herkomsten is beschreven in Hoofdstuk 2. De sporenkieming en de (primaire) haustoriavorming in resistente herkomsten was even frequent als op de vatbare *L. esculentum* cv Moneymaker. Een hoog-frequente necrose van de epidermis cellen waarin haustoria waren gevormd bleek de belangrijkste reactie te zijn, hetgeen aangeeft dat de resistentie tegen *O. lycopersici* in het *Lycopersicon* geslacht vooral geassocieerd is met de overgevoeligheidsreactie. De resistentie in *L. parviflorum* was echter minder uitgesproken geassocieerd met overgevoeligheid dan in andere resistente herkomsten, wat het bestaan van een ander maar nog onbekend resistentie mechanisme suggereert. Bovendien werden er aanwijzingen verkregen dat het niveau van resistentie afhankelijk kon zijn van de genetische achtergrond en het ontwikkelingsstadium van de plant.

Om de waardreeks van *O. lycopersici* te bepalen werden negen herkomsten van komkommerachtigen, tien van leguminosen, drie van sla (*Lactuca sativa*) en 34 van 14 soorten nachtschade geïnoculeerd met een Nederlands isolaat van de echte meeldauw schimmel van tomaat (Hoofdstuk 3). Zesendertig herkomsten werden ook aan een histologisch onderzoek onderworpen om de resistentiereactie te bestuderen. Macroscopisch was er geen schimmelgroei zichtbaar op paprika (*Capsicum annuum*), sla (*Lactuca sativa*), petunia (*Petunia* spp.) en de meeste groente soorten (*Lupinus albus, L. luteus, L. mutabilis, Phaseolus vulgaris, Vicia faba, Vigna radiata, V. unguiculata*). Een minimale infectie werd soms waargenomen op meloen

(*Cucumis melo*), komkommer (*C. sativus*), courgette (*Cucurbita pepo*), erwt (*Pisum sativum*) en bitterzoet (*Solanum dulcamara*). Aubergine (*S. melongena*), de cultuur aardappel (*S. tuberosum*) en drie wilde aardappel soorten (*S. albicans, S. acaule* en *S. mochiquense*) werden zwaarder aangetast in vergelijking met meloen, komkommer, courgette, erwt en bitterzoet, maar de schimmel reproduceerde niet voldoende om zich op deze soorten te handhaven. Alle zeven herkomsten van tabak (*Nicotiana tabacum*) waren even vatbaar voor *O. lycopersici* als tomaat cv Moneymaker. Dit suggereert dat tabak een alternatieve waard is. Deze waardreeks verschilt van die van echte meeldauw van tomaat welke in enkele andere landen gerapporteerd is, maar bij de laatste zijn mildere criteria gebruikt om de waardreeks te bepalen (Hoofdstuk 3). Deze meldingen spreken elkaar tegen, wat aangeeft dat waardreeksstudies alleen vergeleken kunnen worden wanneer heldere criteria en goed opgezette experimenten worden gebruikt. Uit histologisch onderzoek bleek dat de verdedigingsreactie tegen *O. Lycopersici* in alle getoetste planten soorten zeer sterk geassocieerd was met de overgevoeligheidsreactie. Dus zowel de waard- als de niet-waard- resistentie waren voornamelijk geassocieerd met overgevoeligheid.

De genetische variatie tussen elf veldisolaten van de echte meeldauw van tomaat, die wereldwijd verzameld waren, werd onderzocht met behulp van AFLP 'fingerprinting' (Hoofdstuk 4). De AFLP fingerprints van de echte meeldauw van tomaat werden vergeleken met die van twaalf andere soorten echte meeldauw om de mogelijke phylogenetische oorsprong van de echte meeldauw van tomaat te bepalen. De moleculaire gegevens werden met de morfologische gegevens (grootte en rangschikking van de sporen) vergeleken voor taxonomisch onderzoek. Onze resultaten toonden aan dat 1) isolaten van de echte meeldauw van tomaat morfologisch en qua AFLP fingerprint erg op elkaar lijken, en 2) de echte meeldauw van tomaat is genetisch sterk afwijkt (nauwelijks een gemeenschappelijke AFLP band) van enig andere soort echte meeldauw. We vonden geen enkele correlatie tussen de AFLP fingerprints en de grootte of rangschikking van de sporen (solitair of in een rijtje), hetgeen er op wijst dat de AFLP techniek niet geschikt is om verwantschappen tussen soorten te vinden, die is gebaseerd op de morfologie van de sporen. We concludeerden dat slechts één anamorf van de echte meeldauw verantwoordelijk is geweest voor de recente uitbraken.

L. hirsutum Gl.1560 is één van de resistente wilde herkomsten. De resistentie wordt vooral bepaald door een incompleet dominant gen *Ol-1*, dat - vlak bij het *Aps-1* locus in de nabijheid van de resistentie-genen *Mi* en *Cf-2/Cf-5* - op chromosoom 6 van tomaat ligt. Door gebruik te maken van een nieuw ontwikkelde F_2 populatie, die uitsplitst voor resistentie, werd het *Ol-1* gen nauwkeuriger in kaart gebracht tussen de RFLP merkers TG153 and TG164 (Hoofdstuk 5). Bovendien werden vijf met resistentie geassocieerde RAPD merkers geïdentificeerd door het *Ol-1* gebied te verzadigen met meer moleculaire merkers met behulp van de "Bulked Segregant Analysis". Deze RAPDs werden gesequenced en omgezet in SCAR merkers: SCAB01 en

SCAF10 waren specifiek voor *L. hirsutum* en SCAE16, SCAG11 en SCAK16 waren specifiek voor *L. esculentum*. Door middel van een koppelingsanalyse werd een dichte geïntegreerde kaart verkregen, die uit RFLP en SCAR merkers bestond. Dit zal de op een genetische kaart gebaseerde klonering van *Ol-1* en de merker gestuurde selectie van echte meeldauwresistentie in de tomatenveredeling vergemakkelijken.

L. hirsutum G1.1290 is een andere resistente herkomst van *Lycopersicon hirsutum*. Hoofdstuk 7 beschrijft 1) de genetica en kartering van de resistentie tegen *O. lycopersici* in G1.1290, 2) de fijnkartering van *Ol-1*, afkomstig uit *L. hirsutum* G1.1560 en 3) allelie toetsen voor resistentie in G1.1290 en in G1.1560. De *O. lycopersici* resistentie in G1.1290 bleek bepaald te worden door een incompleet dominant gen, aangeduid met *Ol-3*, dat gekarteerd werd tussen de merkers TG25/SCAF10 en H9A11 op chromosoom 6. Met behulp van toetsen van enkele F$_3$ lijntjes en de nakomelingen van de kruising van *L. esculentum* cv Moneymaker met *L. hirsutum* G1.1560 verkregen we meer aanwijzingen voor de kaartpositie van *Ol-1* tussen SCAF10 and H9A11. Dit betekent dat *Ol-1* en *Ol-3* in hetzelfde gebied op chromosoom 6 liggen. In allelie toetsen werd geen vatbare recombinant aangetoond, wat inhoudt dat *Ol-1* en *Ol-3* op hetzelfde locus kunnen liggen. Op grond van de waargenomen verschillen in het infectieproces van beide ouders van *Ol-1* en *Ol-3* lijken deze twee genen niet identiek te zijn (zie Hoofdstuk 2). Ze zouden functionele genen in een cluster van *Ol*-homologen kunnen zijn.

L. parviflorum G1.1601 is een nog andere resistente herkomst die onderzocht werd. Een ziektetoets met een F$_2$ populatie van Moneymaker x G1.1601 toonde aan dat de resistentie van G1.1601 kwantitatief was (Hoofdstuk 7). Om de loci, die betrokken zijn bij de resistentie tegen echte meeldauw te karteren, werd van dezelfde F$_2$ populatie een genetische kaart gemaakt, die 792 centimorgan lang was en 259 AFLP merkers bevat. Drie resistentie QTLs werden geïdentificeerd, waarvan er één op chromosoom 12 werd gekarteerd en twee andere op koppelingsgroepen, die nog niet aan chromosomen konden worden toegewezen. Samen verklaarden ze ongeveer 63% van de totale fenotypische variantie. Deze QTLs vertoonden duidelijke additieve effecten op het niveau van resistentie zonder significante epistatische interacties.

De resistentie tegen *O. lycopersici* in *L. peruvianum* LA2172 berust waarschijnlijk op een enkel dominant gen, *Ol-4* (Hoofdstuk 8). Dit resistentiegen moet nog gekarteerd worden.

Op grond van de resultaten van de experimenten, die in de hoofdstukken 2 t/m 8 zijn beschreven, worden de volgende conclusies getrokken:
1) De resistentie tegen *O. lycopersici* in *Lycopersicon* soorten is vooral geassocieerd met overgevoeligheidsreactie;

2) Tabak kan een alternatieve waard voor *O. lycopersici* zijn;

3) Isolaten van echte meeldauw van tomaat, afkomstig van verschillende continenten, lijken genetisch sterk op elkaar, hetgeen één enkele oorsprong van deze (veld)isolaten suggereert;

4) De resistenties in *L. hirsutum* G1.1560 en G1.1290 zijn monogeen en dominant. De twee resistentie genen *Ol-1* en *Ol-3* zijn allebei gekarteerd tussen de merkers SCAF10 and H9A11 en zijn genetisch niet van elkaar te onderscheiden.

5) De resistentie in *L. parviflorum* G1.1601 is polygeen. Drie resistentie QTLs werden geïdentificeerd, één op chromosoom 12 en twee op koppelingsgroepen, die nog niet aan chromosomen konden worden toegekend.

6) De resistentie in *L. peruvianum* LA2172 is ook monogeen en dominant. Het corresponderende resistentiegen wordt aangeduid met *Ol-4*.

摘要

自 1986 年起, 番茄白粉病 (*Oidium lycopersici*) 已成为世界番茄生产中的一种重要病害. 在本项目开始时全部的番茄品种均感病. 假粉孢 (*Pseudoidium*, 即产单分生孢子的白粉病菌)和真粉孢(*Euoidium*, 即产链状分生孢子的白粉病菌)均反复地被报道为该病的病原菌. 然而, 由於所发表的有关番茄白粉病的形态相去甚远, 难於准确鉴定该病的病原菌. 根据其无性阶段的形态, 该病原菌在荷兰和英国被鉴定为 *Oidium lycopersicum* (后更正为 *Oidium lycopersici*), 此菌於 1888 在澳大利亚曾有过描述但直至 1986 年却鲜有报道. 导致目前世界番茄白粉病昌獗之病原菌的起源问题仍是个谜. 在番茄属(*Lycopersicon*)的多个种中发现一些抗病材料, 但抗病机理不明, 且对抗病性之遗传规则的研究仅局限於一份抗病材料.

第二章描述了 *O. lycopersici* 在番茄感病品种 Moneymaker 的生长发育过程以及各抗病材料抗 *O. lycopersici* 之抗病机理. 在抗病材料与感病品种 Moneymaker 上, 白粉病菌孢子的萌发和初始吸器的形成情况大致相同. 大多数有吸器形成其中的表皮细胞之坏死乃抗 *O. lycopersici* 之主要抗病机理, 说明番茄属(*Lycopersicon*)对 *O. lycopersici* 之抗性主要与过敏性反应有关. 但是, *L. parviflorum* 的过敏性反应不如其它抗病材料明显, 预示着一种不同而又未知之抗病机理的存在. 此外, 有证据显示, 抗病水平之高低可能与遗传背景及植物的发育阶段有关.

为了研究 *O. lycopersici* 的寄主范围(第三章), 我们用在荷兰采集的番茄白粉病菌菌株一共接种鉴定了葫芦科(9 份材料), 豆科(10 份), 莴苣 (*Lactuca sativa*, 3 份)和茄科 14 个种(34 份)作物的发病性. 其中的 36 份材料还用於组织学观察以研究这些材料的抗病机理. 宏观上, 在青椒 (*Capsicum annuum*), 莴苣, 矮牵牛(*Petunia sp.*)以及大多数豆科作物(*Lupinus albus, L. luteus, L. mutabilis, Phaseolus vulgaris, Vicia faba, Vigna radiata, V. unguiculata*)上未发现白粉病菌的生长迹象. 极少量的病症偶尔也曾出现在甜瓜(*Cucumis melo*), 黄瓜(*C. sativus*), 西葫芦(*Cucurbita pepo*), 豌豆(*Pisum sativum*)和 *Solanum dulcamara* 上. 而虽然茄子(*S. melongena*)和马铃薯(*S.*

tuberosum)及其三个野生种(*S. albicans*, *S. acaule* 和 *S. mochiquense*)的感病程度比甜瓜, 黄瓜, 西葫芦, 豌豆和 *Solanum dulcamara* 严重, 但该病原菌仍不能在这些植物上有效地繁衍, 因而也就无法保存在这些植物上. 全部受鉴定的七份烟草(*Nicotiana tabacum*)均与 Moneymaker 一样感病, 这暗示着烟草是 *O. lycopersici* 的替代寄主. 由於对寄主的严格定义(第三章), 本研究所显示的 *O. lycopersici* 的寄主范围不同於已报导的彼此各异的寄主范围, 说明只有在使用严格的寄主定义以及设计合理的试验的情况下才能对不同的寄主范围研究进行比较. 在组织学上, 本研究已鉴定的全部植物对 *O. lycopersici* 的抗病性主要表现为吸器诱导的过敏性反应.

用 AFLP 指纹法研究了收集自世界各地的 11 份番茄白粉病菌田间菌株的遗传多样性(第四章). 为了研究番茄白粉病可能的种族起原, (我们)将其 AFLP 指纹图谱与其它 12 种白粉病菌的指纹图谱进行比较. 基于分类学的需要, 并试图将这些病原菌的指纹图谱与其形态数据(分生孢子的大小及其排列情况)联系起来. 结果表明: 1) 番茄白粉病菌田间菌株彼此间形态及 AFLP 指纹图谱极为相似; 2) 在遗传上, 番茄白粉病与其它白粉病毫无相似之处(几乎没有任何一条共同的 AFLP 谱带). 此外, 在 AFLP 指纹图谱与分生孢子的大小及其排列情况(单孢或链孢)之间没有找到任何联系. 本章的结论是: 导致目前世界番茄白粉病昌獗之病原菌只有一种.

L. hirsutum Gl.1560 是抗病的野生材料之一, 其抗病性主要受一个位于番茄第六染色体在抗病基因 *Mi* 与 *Cf-2/Cf-5* 附近靠近 *Aps-1* 位点的非完全显性基因 *Ol-1* 控制. 利用一个新的 F_2 分离群体, *Ol-1* 被更精确地定位於 RFLP 标记 TG153 与 TG164 之间(第五章). 此外, 为了提高 *Ol-1* 区域的分子标记密度, 我们利用"集团分离分析法(BSA, bulked segregant analysis)"鉴定出与抗病性连锁的五个 RAPD 标记, 并对这些 RAPD 标记进行测序, 再将其转换为 SCAR 标记, 其中 SCAB01 和 SCAF10 源自 *L. hirsutum*, SCAE16, SCAG11 和 SCAK16 源自 *L. esculentum*. 通过连锁分析, 获得了 *Ol-1* 区域含 RFLP 和 SCAR 标记的高密度基因图谱. 该图谱将加快利用基因定位法克隆 *Ol-1* 以及"分子标记辅助育种"在番茄抗白粉病育种中的应用.

L. hirsutum Gl.1290 是 *L. hirsutum* 中另一份抗病的野生材料. 第六章描述: 1) Gl.1290 抗病性的遗传分析及其基因定位; 2) 源自 *L. hirsutum* G1.1560 的基因 *Ol-1* 的精确定位; 3) Gl.1290 和 G1.1560 抗病性的等位性测定. 研究表明, Gl.1290 对 *O. lycopersici* 的抗病性主要受控於一个非完全显性基因, 称之为 *Ol-3*, 定位於第六染色体在分子标记 TG25/SCAF10 与 H9A11 之间. 通过分析源自 Moneymaker 与 *L. hirsutum* G1.1560 杂交 F₃ 系及其子代, *Ol-1* 在基因图谱的位置被进一步确认在 SCAF10 与 H9A11 之间. 这就意味着 *Ol-1* 和 *Ol-3* 两基因均位於同一染色体区域; *Ol-1* 和 *Ol-3* 之间的等位性杂交测定也未发现感病的重组型个体, 说明这两个基因可能位於同一位点. 但是, 在感病过程中观察到的一些间接证据显示, 这两个基因不完全相同 (参见第二章). 它们也许代表着 *Ol* 同源基因群的两个功能基因.

L. parviflorum G1.1601 是研究的另一份抗病的野生材料. Moneymaker 与 G1.1601 杂交的 F₂ 群体之抗病鉴定表明, G1.1601 的抗病性是个数量性状 (第七章). 为了定位出这些抗白粉病的数量性状位点(QTLs), 利用同一 F₂ 群体构建了一个含有 259 个 AFLP 标记总长为 792 cM 的遗传图谱. 共鉴定出三个 QTLs, 其中之一位於第十二染色体, 另两个所在的连锁基因组的染色体属性尚未确定. 这三个 QTLs 总共控制了 G1.1601 抗病性总方差的 63% 左右, 它们对抗病水平有明显的加性效应而又不存在显著的上位互作.

L. peruvianum LA2172 对 *O. lycopersici* 的抗病性似乎也受控於一个显性基因, 称之为 *Ol-4* (第八章). 该抗病基因仍有待定位.

从二至八章所阐述的试验结果可以得出如下结论:
1) 番茄属 (*Lycopersicon*) 抗 *O. lycopersici* 的主要抗病机理是过敏性反应.
2) 烟草可能是 *O. lycopersici* 的替代寄主.
3) 世界各地番茄白粉病菌田间菌株彼此间形态及 AFLP 指纹图谱极为相似, 说明它们出自同一起源.
4) *L. hirsutum* G1.1560 及 G1.1290 对 *O. lycopersici* 的抗病性均受显性单基因控制, 相应的两个抗病基因 *Ol-1* 和 *Ol-3* 均定位於分子标记 SCAF10 与 H9A11 之间, 且在遗传上两者毫无区别.

5) *L. parviflorum* G1.1601 的抗病性受三个 QTLs 控制, 其中之一位於第十二染色体, 另两个所在的连锁基因组的染色体属性尚未确定.

6) *L. peruvianum* LA2172 对 *O. lycopersici* 的抗病性也受控於一个显性基因, 特称之为 *Ol-4*.

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Caicheng Huang
Wageningen, March 2001

Curriculum vitae

Cai-Cheng HUANG was born on 20 December 1962 in Puning City, Guangdong (Canton) Province, China. After completing his high school education at Puning Overseas Chinese School in 1980, he commenced a BSc study at the Department of Tropical Agriculture, South China University of Tropical Agriculture (SCUTA). In July 1984 he obtained his BSc Degree on tropical agriculture with emphasis on plant breeding, plant protection and farm management. The title of his BSc thesis was: "Chemical induction and cytogenetic identification of polyploids deriving from sex organs of rubber tree (*Hevea brasiliensis*)".

He has been employed as Research Assistant (1984-1989) and as Assistant Professor (from 1990 onwards) by the Institute of Tropical Horticulture, Chinese Academy of Tropical Agricultural Sciences (CATAS). During this period, he worked mainly as a cashew breeder, interpreter (Chinese-English) and a kind of coordinator for an EU project, "Hainan Cashew Development (NA 83/40)", in collaboration with Royal Tropical Institute (KIT), Amsterdam, The Netherlands. In 1988, as part of the EU project, he was selected and sent to participate in the International Course on Applied Plant Breeding at the International Agricultural Centre (IAC), Wageningen. He did a practical training in *in vitro* culture of cashew and other woody species at the former Department of Tropical Agricultural Sciences of Wageningen University (WU) and the former IBN-DLO (Institute for Forestry and Urban Ecology), Wageningen. He was also trained in statistics at the Department of Mathematics of WU, The Netherlands. In 1989-1990, on behaviour of his institute, he worked as Head of Changjiang County Cashew Research and Extension Station, Hainan. He also worked as a consultant to China Kanhua Group Ltd. (Hainan Changjiang Branch) for cashew production in China. Till 1990, he trained several technicians on cashew breeding. He also trained and supervised 35 persons on cashew budding (a kind of vegetative propagation), obtaining an over 90% success rate in the nursery (compared to only 40% in other countries) and a 70% success rate in field. He has become a candidate consultant of cashew production at the World Bank since early 1991.

Since 1991, he was invited to work as Director Assistant by the Director of the National Key Biotechnology Laboratory of Tropical Crops (NBLTC). This Laboratory is one of the six such national institutions in the Ministry of Agriculture of China and specially funded by the Ministry of Science and Technology of China (MSTC, formerly named the State Science and Technology Commission of China) and the Ministry of Planning of China. As a consequence, he reported, on behaviour of his Director, directly to the three Ministries. Meanwhile, he was involved in *in vitro* culture of tropical crops like banana, pineapple, coffee and papaya and had

working knowledge on genetic manipulation and transformation to develop virus resistance in papaya and banana, and bacterial resistance in cassava and *Bogostemum*. He was the Course Director of the International Course on Biotechnology of Tropical Crops in 1991 and 1992 (in English), organized by NBLTC and sponsored by the MSTC. He also prepared the same course for 1994.

He came to Wageningen University again in April 1994 to conduct his post-graduate studies. In August 1995 he fulfilled all the requirements and obtained his MSc Degree on Crop Sciences with a specialization in Crop Breeding. The title of his dissertation was: "Characterization of new resistance to *Fusarium oxysporum* f. sp. *lycopersici* in wild *Lycopesicon* species". In November 1995, he commenced a four-year PhD research project, which is described in this thesis. The project was co-sponsored by Dutch seed companies, and was performed in the Laboratory (formerly Department) of Plant Breeding, WU. During his post-graduate studies, he supervised several MSc students in research on resistance to *F. oxysporum* f.sp. *lycopersici* and to *Oidium lycopersici* in tomato. He was a Technical Assistant for the 27th – 29th International Courses on Applied Plant Breeding organized by IAC, Wageningen, the Netherlands. He supported WU to receive many delegations from China (both central and provincial governments) and international organizations such as UNDP (Beijing Delegation).

He has been a Member of the Editing Board of 'World Tropical Agriculture' (a Chinese Journal) during 1994-1998. He was one of the Organizing Committee Members and key interpreter of the 2nd Chinese (Inter)National Congress on Cassava in 1992. He was also one of the Organizing Committee Members of the China-EU Biotechnology Meeting in 1993. He was a Council Member of CATAS and SCUTA. He has been Member of the Chinese Society of Tropical Crop Sciences and Member of the Chinese Society of Agro-Biotechnology (including *in vitro* propagation Branch). He has been a Candidate member of New York Academy of Sciences since 1997. He attended many (inter)national congresses/symposia such as the 2nd International Meeting of Cassava Biotechnology Network (CBN), Bogor, Indonesia in 1994 and the Symposium on the Prospects of Agro-biotechnology in the 21st Century in China in 1999.

Since February 2000, he has been hired as a Scientist by the Biotechnology Department of Syngenta Seeds BV (formerly Novartis Seeds BV) in Enkhuizen, the Netherlands.

List of publications

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Cai-Cheng Huang (CC Huang)

How do plant species defend themselves against *Oidium lycopersici*?
– Mapping of monogenic and polygenic resistance in *Lycopersicon* species

Bibliographic Abstract: The thesis describes: 1)Resistance mechanism of tomato and some other crop plant species against *O. lycopersici*, a causal agent of the recent outbreaks of tomato powdery mildew; 2) Host range and genetic variation of the pathogen; 3) Inheritance analysis of resistance in wild tomato accessions including *L. hirsutum* G1.1560 and G1.1290, *L. parviflorum* G1.1601 and *L. peruvianum* LA2172; and 4) Mapping of monogenic (in G1.1560 and G1.1290) and polygenic (in G1.1601) resistance.

It has been found that: 1) Resistance to *O. lycopersici* in *Lycopersicon* species is mainly associated with hypersensitive response. 2) Tobacco may be an alternative host of *O. lycopersici*. 3) Tomato powdery mildew isolates from different continents are very similar to each other, suggesting a single origin of these (field) isolates. 4) Resistances in G1.1560 and G1.1290 are monogenic and dominant. The two resistance genes *Ol-1* and *Ol-3* are both mapped between markers SCAF10 and H9A11, and are genetically not distinguishable from each other. 5) Resistance in G1.1601 is polygenic. Three QTLs for the resistance have been identified. 6) Resistance in LA2172 is monogenic and dominant. The corresponding resistance gene is designated *Ol-4*.